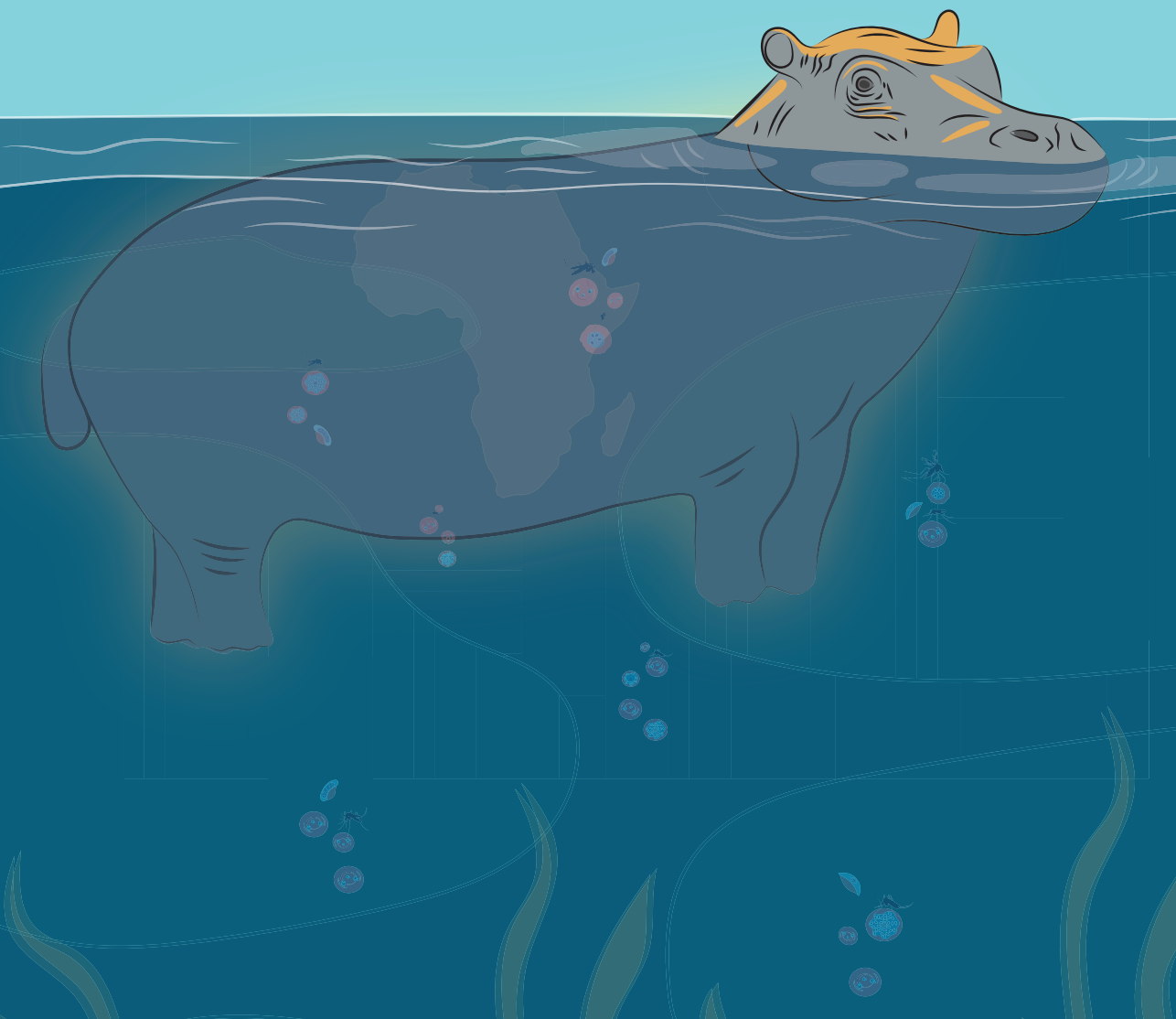
The ears of the hippopotamus:

Epidemiology and infectivity of asymptomatic *P. falciparum* and *P. vivax* malaria infections in Ethiopia



Elifaged Hailemeskel Beshah

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Radboud Dissertations Series

ISSN: 2950-2772 (Online); 2950-2780 (Print)

Published by RADBOUD UNIVERSITY PRESS
Postbus 9100, 6500 HA Nijmegen, The Netherlands
www.radbouduniversitypress.nl

Design: Proefschrift AIO | Katarzyna Kozak
Cover: Proefschrift AIO | Guntra Laivacuma
Printing: DPN Rikken/Pumbo

ISBN: 9789493296480
DOI: 10.54195/9789493296480
Free download at: www.boekenbestellen.nl/radboud-university-press/dissertations

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Proefschrift ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.M. Sanders,
volgens besluit van het college voor promoties
in het openbaar te verdedigen op

dinsdag 24 september 2024
om 12.30 uur precies

door

Elifaged Hailemeskel Beshah
geboren op 7 juli 1984
te Sekota (Ethiopië)

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Prof. dr. D. Yewhalaw (Jimma University, Ethiopië)

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Dissertation to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. dr. J.M. Sanders,
according to the decision of the Doctorate Board
to be defended in public on

Tuesday, September, 24, 2024
at 12.30 pm

by

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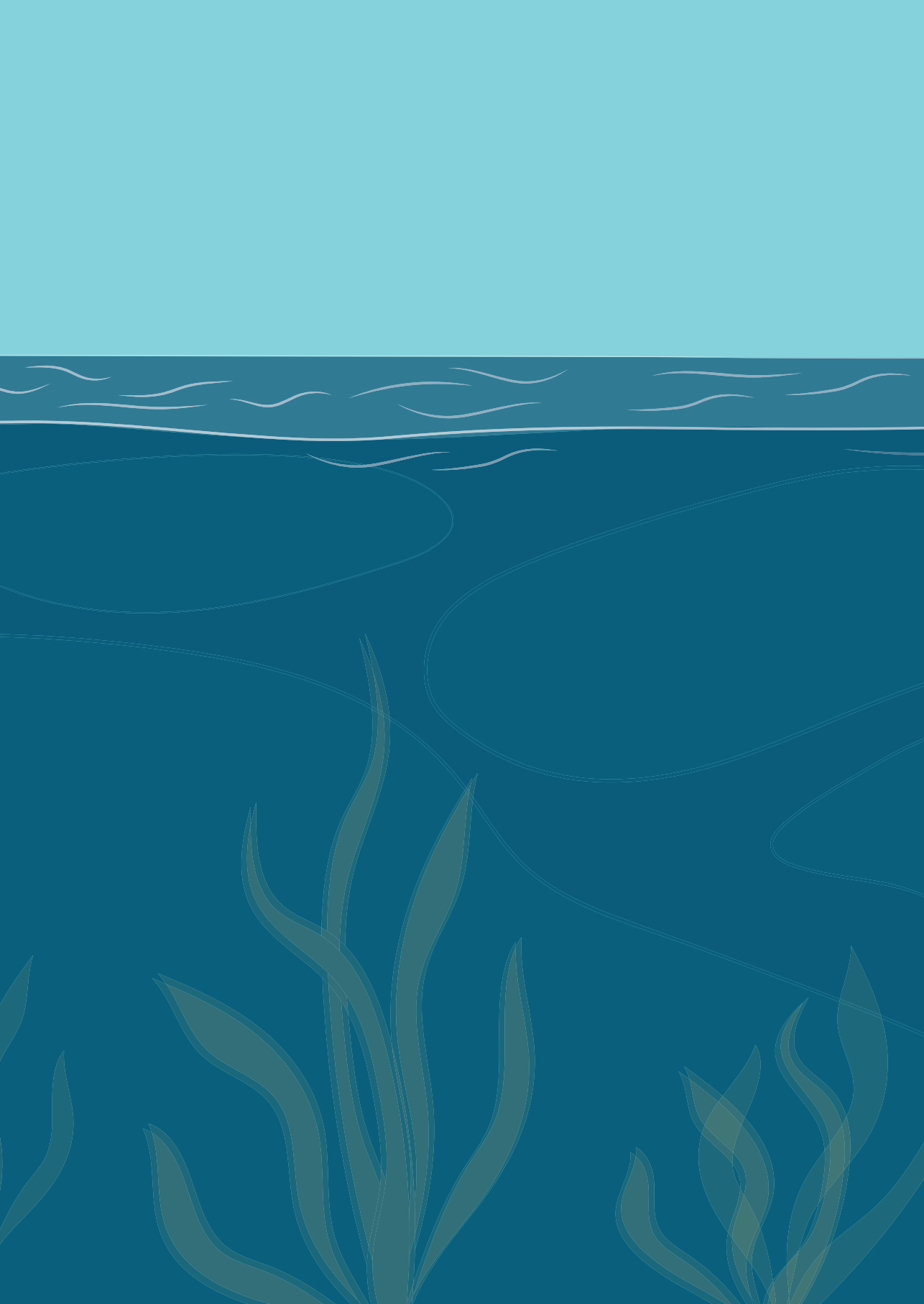

CHAPTER 1

General introduction

Malaria infection – the biology

Malaria is one of the oldest human diseases with significant mortality and morbidity in history, mainly affecting tropical and subtropical regions. Despite the progress made globally to avert malaria related morbidity and death since 2000, in 2022 there were an estimated 249 million cases with an increase of 5 million cases compared to 2021 [1]. The global malaria control and elimination efforts have been threatened by drug and insecticide resistance, humanitarian crises, resource constraints, climate change impacts and health service disruptions due to COVID-19 [1]. There are more than 120 *Plasmodium* species that infect a wide range of mammals, birds and reptiles. In humans, only six of these *Plasmodium* species (*Plasmodium falciparum, P. vivax, P. malariae, P. ovale wallikeri, P. ovale curtisi and P. knowlesi*) cause malaria [2]. Of these *Plasmodium* species, *P. falciparum* causes the most lethal form of the disease while *P. vivax* malaria is the most widespread form globally. *P. malariae* is common in the tropics and sub-tropics while *P. ovale curtisi* and *P. ovale wallikeri* are found in sub-Saharan Africa, Oceania and Asia [3]. The simian malaria parasite, *P. knowlesi*, is mainly reported in Southeast Asia [4].

As successful parasites, *Plasmodium* species that infect humans have a complex life cycle that switches from human to mosquito hosts with fine-tuned morphological developmental stages specialized to adapt to different environments and host cell types [5]. Infection in humans begins when a female *Anopheles* mosquito injects sporozoites, the motile and infective form of the parasite, into the skin during its bite for a blood meal (**Figure 1**). Within a few hours, these motile sporozoites migrate through skin tissues and invade hepatocytes in the liver [6]. Once inside the liver, parasites undergo schizogony, nuclear division without cell division, producing thousands of merozoites inside a parasitophorus vacuole membrane (PVM) [7, 8]. This liver stage development is an asymptomatic phase, producing up to 90,000 exoerythrocytic merozoites inside the PVM that can subsequently initiate the asexual erythrocytic phase that can cause symptomatic disease [8, 9]. The duration of pre-patency in the liver before merozoites appear in the blood may take 7-10 days depending on the parasite species [10]. For *P. vivax* and *P. ovale,* a proportion of the parasites will form latent liver stage forms called hypnozoites that remain dormant inside hepatocytes and can cause relapses weeks, months or even years after the initial infection [11, 12].

Following the release of the primary merozoites into the blood circulation, parasites start to invade red blood cells (RBCs) and develop into ring, trophozoite and schizont stages releasing 16-32 matured merozoites per RBC into the blood stream [13]. Each of the matured daughter merozoites begins to invade new RBCs to maintain the asexual blood stage development; this wave repeats itself every 48 hours on average, causing periodic fever and clinical symptoms of malaria. The invasion of RBCs and tropism for subsets of RBCs is species specific, determining the degree of virulence of the parasite. *P. vivax* has a strong preference to immature reticulocytes [14] while *P. falciparum* invades all stages of RBCs [15]. In their asexual phase in the bloodstream, *P. falciparum* trophozoites and schizonts sequester to the microvascular endothelium of various organs, contributing to severe complications such as cerebral malaria and placental malaria during pregnancy [16, 17].

During the exponential phase of asexual blood stage development, a portion of the parasites start sexual commitment. This process results in the formation of male and female gametocytes. In *P. falciparum*, immature gametocytes (stage I-IV) sequester away from the circulation and become mature gametocytes (stage V) in the bone marrow and spleen [18, 19]. The maturation of gametocytes from asexual stage precursors takes about 8-12 days for *P. falciparum* [20] while it takes 2-3 days for *P. vivax* [21, 22]. Whilst tissue sequestration is also important for *P. vivax* gametocytes [21, 23], it is less well studied. Mature gametocytes stay in the peripheral blood stream for an estimated mean circulation time of 3.5-6.5 days to be taken up by blood feeding mosquitoes to complete their lifecycle [24, 25]. To establish the infection in the mosquito midgut, a female *Anopheles* mosquito must take a minimum of one male and one female gametocyte in the 2-3μL that makes up an average blood meal [26].

Once these mature gametocytes are ingested by the female *Anopheles* mosquito, they become activated as male and female gametes that fuse to form a zygote inside the mosquito's midgut. Briefly, activated male gametocytes divide into eight motile flagellated microgametes and a single female gametocyte gives rise to a round shaped macrogamete [27]. The eight male microgametes can exfallagelate though only one fertilizes a single female macrogamete forming a diploid zygote [28]. The zygote undergoes meiosis and develops into a motile ookinete that penetrates the mosquito midgut wall and rests at the basal lamina of the midgut where it rounds up to transform into an oocyst [27]. Within the round oocyst, the parasite divides asexually and forms thousands of sporozoites in a process known as sporogony. When these oocysts rupture, sporozoites

migrate and invade the salivary glands of the infected mosquito where they can be inoculated back to a human host during its blood meal (**Figure** 1).

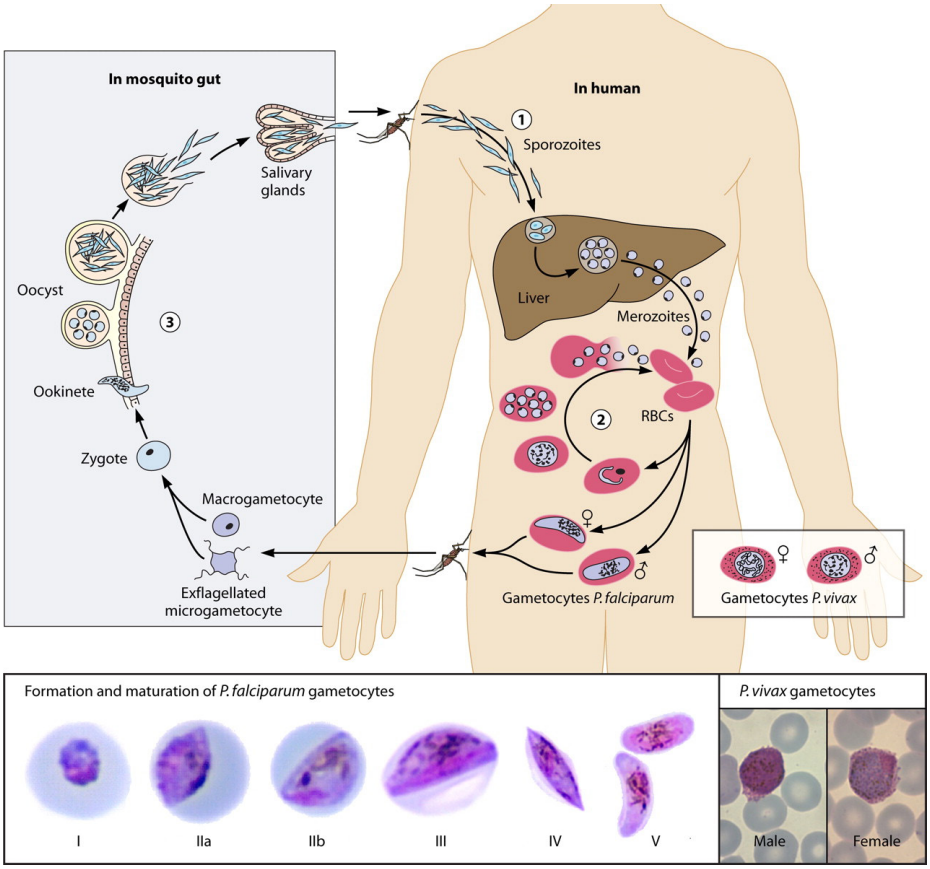

Figure 1. The malaria life cycle: both in human and mosquito gut (Top) and gametocyte stages of *P. falciparum* (Stage I- v) and male and female gametocytes of *P. vivax* (Bottom panel). Adapted with permission from Bousema T, Drakeley C. Clin Microbiol Rev. 2011; 24(2):377-410.

Malaria infection – clinical disease and asymptomatic carriage

The consequences of malaria infection can range from asymptomatic infections that do not elicit symptoms to uncomplicated (mild) symptomatic infections or severe (sometimes life-threatening) cases. The complexity of disease manifestations emanates from the underlying interplay between host, parasite

and environmental factors [29]. Infected individuals tend to develop protection against clinical malaria either by anti-parasite immune responses that result in reduced parasite density or by anti-disease immunity whereby (severe) manifestations of clinical symptoms are prevented [30]. The risk of developing clinical malaria symptoms declines with increasing age and cumulative exposure [31]. The development of immunity is at least partially clone dependent; the acquisition of new parasite clones increases the risk of developing symptomatic infections via increasing parasite density as the immune system is naïve to the new clones [32, 33].

In endemic settings, more than 75% of the infections that are detected in community surveys are asymptomatic infections [34]. Although there is no formal definition of asymptomatic malaria infection, it is often defined as infection with any malaria parasite density without concurrent fever (axillary temperature ≤ 37.5°C) or acute malaria symptoms [35]. Asymptomatic infections can be categorized as either submicroscopic infections that are detectable by molecular methods but not by microscopy/RDT or as asymptomatic microscopic infections that can be detected by microscopy or RDT [36]. Future efforts of malaria elimination would benefit from an improved understanding of asymptomatic malaria infections and their public health importance.

Malaria in Ethiopia: the context towards elimination

Ethiopia is one of the few African countries that managed to continue their successful efforts to control malaria towards country-wide elimination. In Ethiopia, more than 68% of the land mass is conducive for malaria transmission with about 60% of the population living in areas at risk of malaria. Five *Plasmodium* species can cause malaria in Ethiopia: *P. falciparum, P. vivax, P. malariae, P. ovale wallikeri, P. ovale curtisi* [37, 38]. *P. falciparum* and *P. vivax* are co-existing in many parts of the malaria endemic Ethiopian sites, contributing to approximately 70% and 30% of all infections, respectively [39]. Ethiopia is responsible for ~ 9% of the *P. vivax* global burden and has the highest *P. vivax* burden in Africa [40].

Malaria transmission is seasonal in most of the endemic sites except in the areas near to the southwestern borders of the country where transmission is perennial [37]. The major transmission season is from September to December following the main rainy season in June to August. In some districts, there is

also a minor transmission season from April to June following the short rainy season of February to March [39]. The major malaria vector in Ethiopia is *An. arabiensis* with *An. pharoensis*, *An. funestus* and *An. nili* having secondary roles in transmission [39]. Recently, *An. stephensi* has been emerging as an important vector that transmits malaria in urban and peri-urban areas Ethiopia [41, 42].

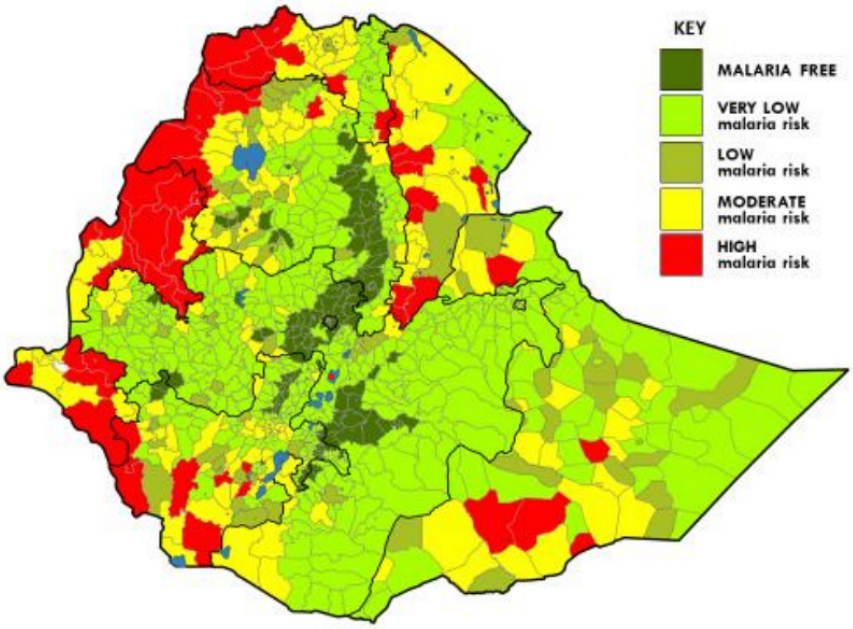

Figure 2. Map of Malaria Strata in Ethiopia (©2020). Source: FMOH/ NMSP 2021-2025 [43].

Since 2000, Ethiopia has successfully reduced malaria incidence and mortality rates by about 40% in 2020. However, in 2022, Ethiopia was one of the countries that contributed 1.3 million malaria cases to the global malaria burden [1]. Despite this fact, the country has planned to achieve nationwide malaria elimination by 2030 [43]. For this purpose, the new national malaria strategic plan (NMSP) of 2021-2025 has stratified the country based on annual parasite incidence (API) per 1000 people as malaria free (API=0), very low (API>0 & ≤5), low (API>5 &<10), moderate (API≥10& <50) and high (API≥50) transmission settings (**Figure 2**, [43]). The NMSP aims to achieve significant malaria burden reduction in high and moderate transmission settings, eliminate the disease in low transmission districts and prevent reintroduction into areas reporting zero indigenous malaria cases [43]. The plan includes effective surveillance and monitoring systems to detect and respond to epidemics, and test, treat and track individual cases or foci of malaria transmission with appropriate actions.

The main intervention approaches include the distribution of long-lasting insecticidal nets (LLINS), indoor residual spraying (IRS) and early diagnostic testing and prompt antimalaria treatment [43].

So far, Artemisinin combination therapy (ACT) in the form of Artemether-Lumefantrine (AL; Coartem®) or Artesunate-Amodiaquine has been the mainstay for treating uncomplicated *P. falciparum* malaria in sub-Saharan Africa. These ACTs were implemented following the widespread resistance to chloroquine (CQ) and Sulfadoxine-Pyrimethamine (SP). In Ethiopia, AL is being used as first-line treatment for uncomplicated *P. falciparum* after the withdrawal of CQ in 1998/9 followed by SP in 2004 [37], while CQ is still the first line treatment for *P. vivax* [43] where resistance is less of a concern. Recently, to support the malaria elimination program, the government of Ethiopia has implemented the use of single low dose Primaquine (PQ) for *P. falciparum* transmission prevention and a 14 day PQ dose for *P. vivax* radical cure [43].

Two challenges for malaria elimination are the emergence of insecticide resistant mosquitoes and drug resistant parasites. In Ethiopia, An. *arabiensis* is typically resistant to DDT, malathion, deltamethrine while it currently remains susceptible to pirimiphos methyl, bendiocarb and propoxur [43, 44]. Drug resistance for *P. falciparum* is linked with mutations and duplications in the genes encoding transporter and target proteins of the parasite [45]. Resistance to SP is conferred by the accumulation of mutations in *Pfdhfr* (N51I,C59R,S108N) and *Pfdhps* (A437G, K540E) genes encoding dihydrofolate reductase (dhfr) and dihydropteroate synthase(dhps) proteins involved in the folate biosynthesis pathway of the parasite [46]. CQ resistance for *P. falciparum* is primarily due to the *Pfcrt*-K76T mutation [47] which is further modulated by *Pfmdr1*-N86Y point mutation [48] encoding the transporter proteins of chloroquine-resistance transporter (*Pfcrt*) and multidrug resistance protein (Pfmdr1), respectively. Mutations in the propeller domain of the *P. falciparum* Kelch 13 (*pfKelch* 13) genes such as Y493H, R539T, I543T and C580Y have been linked to reduced sensitivity of *P. falciparum* to artemisinins [49]. An R622I mutation is appearing independently in countries in the Horn of Africa: Eritrea, Ethiopia, Somalia and Sudan though ACT is still effective in these countries [50].

Strikingly, 'old' malaria drugs may sometimes regain their clinical benefits. If there is a fitness cost for parasites associated with drug resistance, drug-sensitive parasites may return once drug pressure is reduced or removed. There are reports indicating that the shift from CQ to AL has resulted in re-appearance of *Pfcrt* wild-

type *P. falciparum* strains [51, 52]. However, the continued use of CQ for *P. vivax in P. falciparum* co-endemic settings can also sustain the selective drug resistance pressure to *P. falciparum* as misdiagnosis and thus mistreatment are common in co-endemic settings [53]. This could result in a sustained evolutionary pressure that favors mutant *P. falciparum* parasites, despite their lower fitness in the absence of drug pressure. Moreover, there might also be fixation of mutant strains due to selective drug pressure induced by mixed infections treatment. In this regard there are no data in Ethiopia as to how asymptomatic *P. falciparum* parasite carriers sustain drug resistant parasites and to what extent CQ treatment of *P. vivax* may affect selection of drug resistance markers of *P. falciparum*. Currently, the emergence of diagnostic resistance linked with histidine-rich proteins 2 and 3 (*Pfhrp2/3*) gene deletion is also reported in the Horn of Africa including Ethiopia [54]. Parasites lacking the proteins encoded by these genes may escape diagnosis based on rapid tests that detect HRP-2. This would further complicate accurate detection and treatment in such settings.

Asymptomatic malaria infections and parasite detections tools

Accurate detection of malaria parasites and estimating the parasite prevalence at population level forms a corner stone of successful surveillance. This was traditionally done using light microscopy that has the capacity of examining 0.025-0.063μL of whole blood per slide [55] and later by rapid diagnostic tests (RDTs) that use ~5 μL of blood. There is an increased awareness that more sensitive detection tools are needed since parasites may circulate at lower parasite densities. Historically, the most common target used for molecular diagnosis of malaria is the small subunit 18S of the ribosomal RNA gene (18S rRNA, 5-8 copies/genome depending on the parasite strain), due to its sensitivity and specificity conserved across all species [56, 57]. The volume of blood analyzed and copy number of the target molecular marker are major determinants of detection limit and sensitivity of molecular assays [58].

Most often, DNA is extracted from about 5-100μL of whole blood to target the 18S rRNA gene for nested PCR (with an approximate detection limit of 10 parasite gene copies/sample) [56] and quantitative PCR (estimated sensitivity in the range of 0.01-0.1 parasites/μL when using 100 μL blood samples as input material [59]). By concentrating parasite DNA template from larger volumes of blood (e.g. 1mL of whole blood) during extraction, the sensitivity of 18S rRNA

DNA based qPCR can be improved resulting in a reported detection limit of 0.022 parasites/μL of blood. This ultra-sensitive PCR method is 50 times sensitive compared to the standard PCR methods that uses dried blood spot samples with parasite DNA template extracted from about 5 μL of whole blood [60].

The use of ultra-sensitive PCR assays that target multiple copy sequences such as telomere-associated repetitive element 2 (TARE-2) with about 250 copies/genome and var gen acidic terminal sequence (varATS) with 59 copies/genome of *P. falciparum* have been shown to be ~10 times more sensitive than that of 18S rRNA qPCR, improving the detection limit to 0.03-0.15 parasites/μL when using 100μL of whole blood [61]. Pv-mtCOX1-ultrasensitive-qPCR targeting cytochrome oxidase 1 gene (20 copies/genome) has been designed for low density *P. vivax* as well [62]. The sensitivity of the 18S rDNA-based PCR can also be enhanced by detecting and quantifying the highly abundant 18S rRNA transcripts using reverse-transcriptase-quantitative PCR (qRT-PCR). However, RNA extraction and qRT-PCR require advanced lab facilities and, because of transcript abundance, assays may be more prone to contamination as compared to DNA based PCR assays [58, 63]. Furthermore, to improve the time consumed by conventional nested PCR and potential risk of contamination in samples from co-endemic settings, a multiplex qPCR assay has been developed with simultaneous detection of the five human malaria parasites in a single sample with a detection limit of 1-6 parasites/μL of blood and species specific sensitivity ranging from 89.5%-100% [64].

Currently, several quantitative PCR methods have improved the detection and quantification of parasites over microscopy, mainly via increasing the volume of examined blood compared to microscopy. However, major limitations of these qPCR assays are: 1) the requirement for standard curves generated either from plasmids containing the target sequence or cultured parasites counted using microscopy [65]; 2) challenges in relating gene copies to parasite densities for *P. vivax* where schizonts from peripheral blood might contain 16-24 genomes [58, 66]; 3) The efficiency of DNA extraction to harvest the templates and 4) PCR efficiency across variable template concentrations [65]. Recently, improved and absolute quantification of malaria parasites has been achieved using droplet digital PCR (ddPCR) [65]; this assay does not require standard curves.

On the other hand, even the more sensitive nucleic acid-based assays will not detect inaccessible ultra-low parasite densities at a single time point [67]. In the case of *P. vivax* and *P. ovale*, the liver stage hypnozoites that can give rise

to subsequent blood-stage parasitemia are undetectable using nucleic acid-based assays on peripheral blood material [68]. One solution to this is to detect infections indirectly by detecting antibodies that are produced in response to antigen exposure. For *P. falciparum,* it is well-established that serological profiles can uncover past malaria exposure [69] and even differentiate between recent and cumulative exposure [70]. Therefore, antibodies against malaria parasite antigens are considered to be sensitive and lower cost tools to measure current, recent and historic exposure at population level; for some purposes they may be preferred over nucleic acid-based assays [67]. For *P. vivax* hypnozoites, *P. vivax* blood stage specific antibodies that can capture the primary infections that could have happened 1-9 months ago can be used as serological markers for hypnozoite carriage [71] and guide treatment of infected individuals who, at the moment of sampling, are free of blood-stage parasites.

Understanding asymptomatic malaria infections: their detectability

In light of the global motivation to eliminate malaria, the use of highly sensitive molecular tools made it possible to appreciate the widespread asymptomatic malaria burden that is present well below the detection limit of routine diagnostic approaches [72-74]. The increased sensitivity of molecular methods comes at a considerable financial and logistical cost. The practicalities and relevance of the detection of asymptomatic malaria for malaria elimination in endemic countries therefore needs to be carefully examined. Importantly, the prevalence of asymptomatic malaria infection is heterogeneous across different settings and populations infected [75-77]. Also, the average density of infections differs and, as a consequence, the proportion of infections that is submicroscopic can vary. This proportion of infections that is submicroscopic is estimated to be only 20% in high transmission settings (i.e where community malaria prevalence by microscopy is ≥75%) while it is can be 70-80% in low endemic settings with community malaria parasite prevalences <10% [26]. The sparse infections that are present in low endemic settings are thus of lower density and harder to detect by conventional diagnostics. This heterogeneity in the proportion of infections that is submicroscopic can be partly explained by the history of transmission intensity and the age structure of the population at risk [31]. A meta-analysis and systematic review conducted on cross-sectional malaria surveys indicated that on average, the detectability of asymptomatic malaria using microscopy was lower for adults (>15 years old) as compared to

infants (0-5 years old) and older children (5-15 years old) [78]. The explanation was that parasite densities are generally lower in older populations that, as a consequence of repeated exposure, have acquired immunity that controls infections better. A trend of having lower parasite densities was also observed in repeatedly exposed individuals living in areas of high entomological inoculation rate (EIR >5) as compared to less-exposed individuals [79]. It can also be hypothesized that as transmission intensity declines, less virulent parasites might not be exposed to high competition from more virulent strains. As a result, these less virulent parasites might persist at low-density and allow for future transmission after surviving early detection and antimalaria drugs [80].

On global scale, the relative importance of low-density submicroscopic malaria infection increases as transmission intensity declines [26, 72]. As transmission intensity is low, local transmission is believed to be sustained from hot spots [81] where low genetic diversity of parasites that circulate in the population will enable individuals to acquire immunity to the specific clones attributed to low density asymptomatic carriage [82, 83]. Moreover, in these settings, individuals receive fewer infectious mosquito bites while malaria infections tend to be older and persist for longer at lower parasitemia. This contrasts with areas of higher endemicity where the population experiences frequent infections and frequent superinfections [26, 34]. Because of repeated inocula and a higher likelihood of recent infections, parasite densities are on average higher in areas of higher endemicity compared to low transmission intensities [26].

A better understanding of the sources of heterogeneity of asymptomatic malaria can be achieved by characterizing infections with the same methodology across different settings [84]. In Ethiopia, it is not yet clear how much of the asymptomatic burden can be detected using conventional diagnostics and PCR assays in high, moderate and low transmission settings. This highlights the need to determine the value of different diagnostic approaches in high, moderate and low transmission settings for better quantification of potential transmission reservoirs [85].

In co-endemic settings, differences in the biology of *P. vivax* and *P. falciparum* can also influence the detectability of asymptomatic reservoirs. *P. vivax* has lower parasitemia compared to *P. falciparum* in natural infections [86, 87] mainly due to its strong preference to infect reticulocytes [88] and rapid acquisition of immunity [89]. PCR-based methods can detect on average 67%-70% of asymptomatic *P. vivax* infections [90, 91] and 49% of *P. falciparum*

infections [92] that were missed by microscopy/RDT in many endemic settings. Microscopy/RDT tends to miss a larger fraction of infections as transmission declines [92]. This highlights that in areas where *P. vivax* and *P. falciparum* are sympatric, the detection of low-density asymptomatic infections might require different diagnostic approaches for each species and/or deployment of more sensitive diagnostic tools than those currently available (i.e. more sensitive than microscopy or RDT).

Understanding asymptomatic malaria infections: their dynamics over time

Asymptomatic parasite densities among infected individuals can fluctuate over time. Infections with low-density parasites might have the following scenarios: 1) they can rise to higher parasite density as upcoming symptomatic infection [93], 2) they can be a consequence of residual parasites after anti-malarial treatment that are being cleared but may still circulate as detectable gene copies for several weeks [94, 95] and 3) they can be an older chronic infection that never reach microscopy detectable levels yet continue to replicate in the human blood stream [96]. These infections can also clear spontaneously [97]. *P. falciparum* parasites can persist circulating in the blood stream from several weeks up to 200 days [96] or even several years [98].

So far, many of the studies conducted to determine the prevalence of asymptomatic malaria infections and their contribution to onward transmission were based on cross-sectional surveys [99-101]. These studies were not able to capture the duration of infectiousness to mosquitoes in relation to parasite density fluctuations [72]. A longitudinal study conducted in a low transmission setting of Vietnam over a period of 24 months, followed 356 participants who were positive for *P. falciparum* and *P. vivax* using ultrasensitive qPCR and estimated that the median durations of *P. falciparum* and *P. vivax* infections are two and six months, respectively [93]. That study indicated the existence, persistence and parasite density oscillations with low parasite densities followed by higher density infections. However, two outstanding pressing questions were i) when do oscillating parasite densities become infectious to mosquitoes and ii) to what extent do these persisting asymptomatic infections remain infectious to mosquitoes before the onset of symptomatic infection? In this context, one can wonder whether gametocyte densities follow the same pattern as asexual parasite densities.

In endemic transmission settings, the duration of infections will greatly influence the reservoir of infection [84, 102]. Although previous studies indicated that asymptomatic infections during the dry season reduce the risk of developing clinical malaria in the ensuing wet season [103, 104], it is not fully understood for how long a chronic asymptomatic infection lasts and what proportion becomes febrile. Moreover, it is unclear what host, parasite and environmental factors are determining the duration of infections and their infectiousness to mosquitoes. A 2-year longitudinal study conducted in Malawi showed that asymptomatic *P. falciparum* infections were unlikely to become symptomatic while 92% of symptomatic episodes were due to newly acquired genotypes [105]. In light of this, there is a need for conclusive evidence on the duration of infections as well as the contribution of chronic infections to the infectious reservoir for malaria.

Understanding asymptomatic malaria infections: their consequences for the infected host

As described above, asymptomatic malaria is often defined as parasites at any density without concurrent fever. However, there are reports of other consequences of these infections in the absence of fever. For instance, inflammation [106], anemia and stunted growth in children [107] may occur. It is therefore not surprising that two recent expert reviews concluded that asymptomatic malaria infections are associated with maternal and neonatal mortality, co-infection with bacterial diseases, cognitive impairment and chronic anemia as a result of recurrent symptomatic higher density parasitemia [108, 109]. The term asymptomatic may thus be misleading and asymptomatic infections might better be considered as chronic infections that have relevant (but poorly understood) consequences for the infected host [108].

Depending on the density of parasitemia, symptomatic and microscopically detectable asymptomatic *P. vivax* infections may be associated with maternal anemia and low-birth weight [110, 111]. Low-density asymptomatic *P. falciparum* and *P. vivax* malaria infections were also found to be risk factors for anaemia [112]. Low level of hemoglobin was associated with high serum concentration of hepcidin (a hepatic peptide hormone involved in regulation of iron homeostasis) during *P. vivax* [113] and *P. falciparum* 'asymptomatic' infections [114]. High serum concentrations of hepcidin are linked with iron maldistribution [114] and reduced iron absorption [115]. Persistent subclinical

P. falciparum infections have been linked with low-grade haemolysis and raised levels of serum Haem oxygenase-1 enzyme (HO-1) and IL-10 [116]. In mouse models, the Haemoxygenase-1 and IL-10 have been shown to favor invading Non-Typhoidal Salmonella (NTS) bacterial infection during malaria infections [117, 118]. The clinical importance of this finding was further supported by the epidemiological overlap of NTS with malaria transmission in natural settings [119, 120]. In conclusion, although the severity of inflammation is lower compared to symptomatic infections, asymptomatic malaria infections are associated with systemic inflammation and this is seen for both *P. vivax* [113] and *P. falciparum* [114]. *P. falciparum* asymptomatic individuals also showed increased plasma levels of pro-inflammatory (tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin (IL)-6, IL-12p70, IL-17A, and granzyme B) and anti-inflammatory cytokines (IL-4, IL-7, IL-10 and IL-13) as compared to uninfected individuals [121]. Similarly, *P. vivax* asymptomatic infections have been associated with lower levels of pro-inflammatory cytokines (TNF-α, and INF-γ) and higher levels of IL-10 [122, 123] compared to symptomatic infections while systemic inflammation perturbation in 'asymptomatic infections' is higher than that observed in parasite-negative controls [124]. Compared to uninfected individuals from endemic sites, *P. falciparum* asymptomatic parasite carriers may have elevated level of inflammation markers such as von Willebrand factor, platelet factor-4 and lower levels of platelet counts and hemoglobin levels [106]. C-reactive protein (CRP), a known inflammatory marker and indicator of malaria parasitemia [125, 126], was shown moderately elevated in asymptomatic *P. falciparum* [127] and asymptomatic *P. vivax* infections compared to non-infected controls [128]. However, some studies reported contrasting results of no difference in median CRP level among asymptomatic *P. falciparum* versus healthy controls [129] as well as in asymptomatic *P. vivax* versus endemic controls [127].

There is an increasingly consistent picture that microscopically detectable asymptomatic infections are associated with low-grade symptoms. By comparison, it remains to be convincingly demonstrated whether submicroscopic infections are also associated with disadvantageous health consequences for the infected host. At the same time, one could argue that the most important implications of so-called asymptomatic infections for public health lie in their contribution to onward transmission. Several studies from Cambodia [130], Burkina Faso [131], Uganda [132] and Ethiopia [99] have assessed the potential of asymptomatic malaria reservoirs to infect mosquitoes. More detailed information is needed on the contribution of asymptomatic infections and their

detectability in the context of malaria elimination. In this regard, specific focus is needed on low-endemic settings targeted for elimination.

From infection potential of individuals to their opportunities to transmit malaria parasites

P. falciparum asymptomatic submicroscopic infections and asymptomatic microscopically patent infections have different likelihoods of infecting mosquitoes [36]. From statistical modeling conducted on cross-sectional datasets that used membrane feeding to assess the infectious reservoir, an estimated 20-50% of human to mosquito transmission is caused by submicroscopic infections in low transmission settings [26]. A recent meta-analysis that quantified detectability, duration and infectiousness of asymptomatic infections, estimated that submicroscopic infections were considerable less infective compared to microscopy or RDT positive infections (approximately 3-fold less infective [72]) but compensate for this by their high prevalence in communities [72]. Adding to this evidence, a recent 2-year longitudinal study conducted in an area of low transmission in Uganda showed that the relative contribution of asymptomatic microscopy-detected *P. falciparum* infections to transmission was 83.8% while 15.5% and 0.6% was due to submicroscopic infections and symptomatic infections, respectively. In this study, more than half (58.7%) of the infectious reservoir comprised children aged 5-15 years followed by younger children of less than 5 years (25.8%) and older than 16 years (15.6%) [133].

The infectiousness of individual infections depends on several factors. The most important of these factors is gametocyte density. All infections may produce gametocytes although the relationship between asexual parasite density, gametocyte density and mosquito infection rates are not straightforward [78]. In some settings, symptomatic individuals may carry higher gametocyte densities [130] while in others more gametocytes are detected in asymptomatic infections that were detectable by microscopy or RDT as compared to symptomatic infections or asymptomatic infections that can only be detected by PCR [134]. Importantly, these patterns may differ between *P. falciparum* and *P. vivax.* Overall, the density of gametocytes is related with mosquito infection rate; *P. falciparum* infections with >100 gametocytes/μL are capable of infecting more than 25% of mosquitoes while infection rates are much lower for lower gametocyte densities [135, 136]. The prediction of mosquito infection rates

can be improved by quantifying both male and female gametocytes in a given potential transmission reservoir [137].

For *P. vivax,* a very different picture emerges. Symptomatic cases are more infectious to mosquitoes than asymptomatic infection carriers with mosquito infection rates being positively correlated with blood parasitemia [99, 138]. This is because the gametocytes appear early in the blood stream following the first microscopically observable asexual parasite wave [22]. In a low transmission setting of Ethiopia, population adjusted relative contributions to the infectious reservoir of symptomatic, asymptomatic microscopy detectable and sub-microscopic *P. vivax* infections were 8.0%, 76.2% and 15.8%, respectively [99]. However, it is currently unknown whether the specific detection or quantification of *P. vivax* gametocytes is needed to estimate transmission potential or whether (the strongly associated) asexual stages are equally informative. Given the stability of DNA to degradation, ease of accessibility for assessment and availability of high gene copy number of total parasitemia, determining the relationship between Pv18S DNA based PCR total parasitemia would benefit the prediction of infectiousness in field settings as compared to the less stable and easily degradable mRNA based Pvs25 gametocyte quantification. It is yet important to investigate how far the Pvs25 gametocyte marker density would improve the prediction of infectiousness to mosquitoes over the total parasitemia density as determined by 18S PCR and/or microscopy in *P. vivax* infections; these assessments may need to be repeated for different vector populations and for distinct epidemiological contexts.

Using mosquito feeding assays to directly quantify transmissibility

Accurate quantification of transmission potential and infectivity relies on the use of mosquito feeding assays [139]. Mosquito feeding assays are crucial tools to assess and quantify the infectious reservoir of malaria in a given community using laboratory reared primary vector mosquitoes [84, 100, 136]. For this purpose, direct skin feeding assays (DSF) and membrane feeding assay (MFA) are widely used to quantify natural infectiousness of gametocyte carriers [136]. The DSF is based on allowing laboratory reared uninfected mosquitoes to feed directly on the skin of the infected individual [101]. The MFA assay is conducted by feeding venous blood from gametocyte carrier individual to mosquitoes through a membrane via water-jacketed glass feeders that are kept between

37 and 38°C [136]. The DSF is claimed to result in higher mosquito infection rates compared to MFA as mosquitoes have direct access to gametocytes through human skin [140]. Both DSF and MFA involve examining mosquitoes after the blood-meal to determine if they have become infected as a consequence of the blood meal. For oocyst-level infections, this typically involves the dissection of mosquitoes around day 7-10 after the blood meal. Given some ethical aspects associated with DSF, the advantage of MFA over DSF are allowing direct quantification of gametocytes from the feeding blood and maximizing the number of mosquitoes per feeding moment providing more accurate prediction of mosquito infection rate [140].

In line with the benefits of MFA to study the infectious reservoirs in field settings, it is also important to consider the transmission efficiency of field parasite strains in relation to colony mosquitoes and local wild caught mosquitoes in a given epidemiological context. A recent study that compared permissiveness of age-matched wild and colony *Anopheles stephensi* mosquitoes using *P. vivax* infected patient blood has showed that wild mosquitoes were more susceptible to develop sporozoites as compared to laboratory reared colony mosquitoes [141]. Study outcomes with colony mosquitoes may thus not be immediately translatable to field mosquitoes. In addition, many successive generations of reproduction in caged laboratory conditions potentially results in adaptive changes of genotypic and physiological characteristics of colony mosquitoes [142, 143] that might influence the transmission of *Plasmodium* parasites. The mosquito-parasite relationship is also influenced by mosquito diet [144] and the midgut-microbiota [145] of the mosquitoes. Therefore, the comparative transmission efficiency of local parasite strains to local wild caught mosquitoes against colony mosquitoes must be assessed in field settings before investing to study the contribution of symptomatic and asymptomatic individuals using colony mosquitoes in membrane feeding assays.

In addition to the ability to directly confirm the infectiousness of gametocytes, mosquito feeding assays allow assessment of the transmission-modulating effects of, for example, serum factors. These serum factors may include antibodies against sexual stage antigens that may reduce transmission efficiency [146, 147]. For *P. falciparum,* it has been demonstrated that individuals who have antibodies to the gametocyte antigen Pfs48/45, for instance, have a threefold lower chance of infecting any mosquitoes and infect 6-fold fewer mosquitoes [146]. For *P. vivax* there have been no comprehensive assessments of the effects of anti-gametocyte immunity on transmission efficiency.

Aims and outline of the thesis

The main objective of this thesis is to comprehend the epidemiology and relevance of asymptomatic *P. falciparum* and *P. vivax* malaria in co-endemic settings of Ethiopia. It addresses three main topics: -

i. Assessing the epidemiology and detectability of asymptomatic malaria in Ethiopia
ii. Quantifying asymptomatic infection reservoirs and the public health relevance of asymptomatic malaria infections in a low transmission setting in Ethiopia
iii. Exploring the relevance of transmission reducing immunity against selected sexual stage *P. vivax* antigens for transmission efficiency.

Hence the following objectives were designed to address the above-mentioned topics:

- To assess the detectability and epidemiology of asymptomatic *P. falciparum* and *P. vivax* infections in high, moderate and low transmission settings in Ethiopia using routine diagnostic (Microcopy/ RDTs) and nested PCR methods (**Chapter** 2)
- To assess the prevalence of *Plasmodium falciparum Pfcrt-76* and *Pfmdr1* alleles of drug resistance markers in asymptomatic and symptomatic infections in settings with different levels of *Plasmodium vivax* co-endemicity (**Chapter** 3)
- To compare susceptibility and permissiveness of colony mosquitoes versus wild-primary vector mosquitoes (*An. arabiensis*) using parallel membrane feeding assays for the transmission of local *Plasmodium* species (**Chapter** 4)
- To investigate the duration and dynamics of asymptomatic *P. falciparum* and *P. vivax* infections and their role in sustaining onward transmission to mosquitoes in low endemic setting in Ethiopia (**Chapter** 5)
- To investigate infectiousness of *P. vivax* symptomatic infections to *An. arabiensis* mosquitoes and presence of transmission reducing immunity for selected *P. vivax* gametocyte antigens in Ethiopia (**Chapter** 6).

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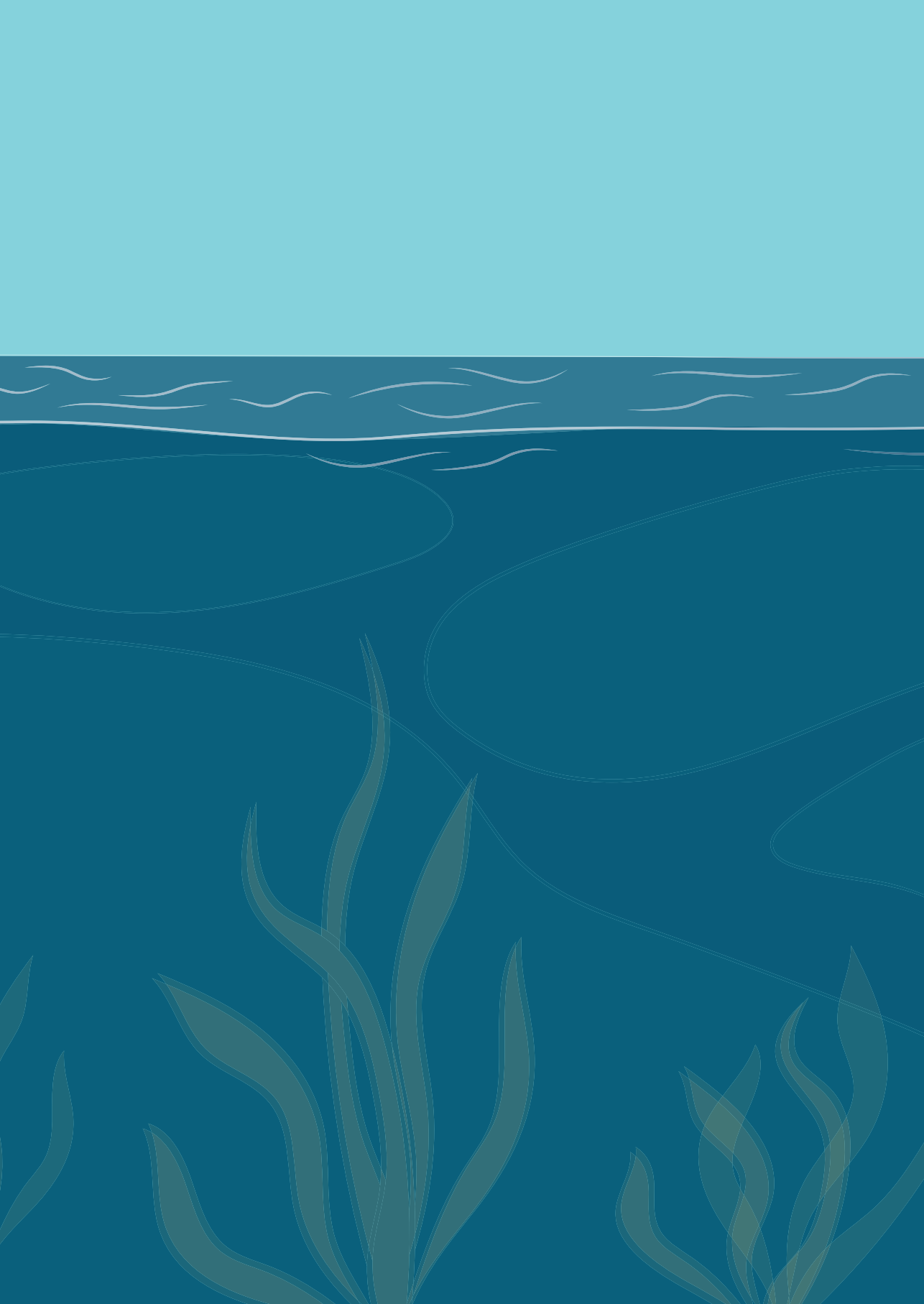

CHAPTER 2

The epidemiology and detectability of asymptomatic *Plasmodium vivax* and *Plasmodium falciparum* infections in low, moderate and high transmission settings in Ethiopia

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Malar J 20, 59 (2021). https://doi.org/10.1186/s12936-021-03587-4

Abstract

Background

As countries move to malaria elimination, detecting and targeting asymptomatic malaria infections might be needed. Here, the epidemiology and detectability of asymptomatic *Plasmodium falciparum* and *Plasmodium vivax* infections were investigated in different transmission settings in Ethiopia.

Methods

A total of 1093 dried blood spot (DBS) samples were collected from afebrile and apparently healthy individuals across ten study sites in Ethiopia from 2016 to 2020. Of these, 862 were from community and 231 from school based cross-sectional surveys. Malaria infection status was determined by microscopy or rapid diagnostics tests (RDT) and 18S rRNA-based nested PCR (nPCR). The annual parasite index (API) was used to classify endemicity as low (API>0 and<5), moderate (API ≥5 and <100) and high transmission (API≥100) and detectability of infections was assessed in these settings.

Results

In community surveys, the overall prevalence of asymptomatic *Plasmodium* infections by microscopy/RDT, nPCR and all methods combined was 12.2% (105/860), 21.6% (183/846) and 24.1% (208/862), respectively. The proportion of nPCR positive infections that was detectable by microscopy/RDT was 48.7% (73/150) for *P. falciparum* and 4.6% (2/44) for *P. vivax*. Compared to low transmission settings, the likelihood of detecting infections by microscopy/RDT was increased in moderate (Adjusted odds ratio [AOR]: 3.4; 95% confidence interval [95%CI] 1.6-7.2, P=0.002) and high endemic settings (AOR=5.1; 95%CI 2.6-9.9, P<0.001). After adjustment for site and correlation between observations from the same survey, the likelihood of detecting asymptomatic infections by microscopy/RDT (AOR per year increase = 0.95, 95% CI 0.9-1.0, P=0.013) declined with age.

Conclusion

Conventional diagnostics missed nearly half of the asymptomatic *Plasmodium* reservoir detected by nPCR. The detectability of infections was particularly low in older age groups and low transmission settings. These findings highlight the need for sensitive diagnostic tools to detect the entire parasite reservoir and potential infection transmitters.

Keywords: *Plasmodium* infection, Elimination, Asymptomatic, Transmission, nPCR, Detectability, Density distribution

Introduction

Following considerable successes in the control of malaria in the last two decades, progress plateaued or stalled in many settings in Africa [1]. Ethiopia runs a successful malaria control programme [2] that makes it one of the four countries (together with India, Rwanda, and Pakistan) that continues to maintain the declining trend in malaria burden [3]. As a result, the country is on track for a 40% reduction in incidence (together with Rwanda, Zambia, and Zimbabwe) and malaria mortality rates (together with Zambia) by 2020 [1]. To guide elimination efforts that currently targets 239 selected districts, the National Malaria Control Programme (NMCP) of Ethiopia stratified the country into four strata using district level annual parasite index (API) data from 2017 [4] as malaria-free (API, 0), low (API, 0–5), moderate (API, 5–100), and high (API, ≥100) [4]. Despite its value, the adopted stratification lacks granularity and is not able to capture relevant spatial and temporal heterogeneities in low endemic settings [5, 6]. The unique epidemiology of malaria transmission in Ethiopia; the presence of strictly seasonal transmission in some settings and perennial transmission elsewhere, as well as different levels of co-endemicity of *Plasmodium falciparum* and *Plasmodium vivax* [2], calls for the use of tailored approaches to characterize the epidemiology of malaria.

District level stratification that relies on malaria incidence data has limitations in settings where case numbers are extremely low. Incidence data are also sensitive to changes in care-seeking behaviour, rates of testing of suspected cases, and reporting completeness [7]. Screening approaches to determine the prevalence of (often asymptomatic) infections that are present in communities have great potential to define transmission intensity [8]. However, parasite prevalence estimates are greatly affected by parasite density distributions in communities that determine the detectability of infections by different diagnostics. Malaria elimination efforts may benefit from targeting all infections present in communities, irrespective of clinical presentation [9-11]. There is a growing body of evidence on the public health importance of asymptomatic malaria infections and their contribution to onwards malaria transmission in high [12, 13] and low transmission settings [13, 14]. Importantly, most asymptomatic infections detected in community surveys are of low parasite density and the proportion of all infections that are submicroscopic varies between settings [15]. Previous studies in Ethiopia detected a significant burden of asymptomatic *P. falciparum* and *P. vivax* infections [16-19]. These studies used different diagnostic techniques and sampling designs, making it

difficult to compare parasite prevalence estimates or diagnostic performance indicators across settings. The aim of the present study was to understand the epidemiology of asymptomatic *Plasmodium* infections in different settings in Ethiopia and their detectability by microscopy, rapid diagnostics test (RDT) and molecular methods.

Methods and materials

Study areas

The study was conducted in ten districts (woredas) encompassing different transmission settings (**Fig. 1**). Malaria transmission is highly heterogeneous in Ethiopia and transmission intensity varies spatially and temporally [20]. Study sites representing low (n = 2), moderate (n = 4), and high (n=4) transmission settings as per the national stratification were selected from five administrative regions (**Fig. 1**). Low transmission settings include Gomma and Babile districts from Oromia region. Moderate transmission settings include Bahir Dar Zuria and North Achefer districts from Amhara region and Arba Minch Zuria from the Southern region and Mao Komo from Benishangul region. High transmission districts were from Gambela (Lare and Abobo), Amhara (Jawi), and Benishangul (Meng) regions.

Study population and sample collection

Samples were collected in community and school-based cross-sectional surveys from 2016–2020. Specifically, community-based surveys were conducted at Abobo, Lare, Mao-komo, Meng, and Gomma districts in 2016, Babile district in 2018, and Arba Minch Zuria district in 2020. School based surveys were conducted at North Achefer, Bahir Dar Zuria, and Jawi districts in 2017. For the school-based surveys, students were randomly selected from elementary school students stratified by age as described before [21] following protocols developed by Brooker and colleagues [22].

Prior to recruitment of participants for community surveys, sensitization was undertaken by teams that involve study team members, village-based health extension workers, malaria focal person of the district, local administrators, and elderly. The study purpose, procedure, risk, and benefit were explained in local language. After this first step, volunteer community members were invited to join the study upon obtaining informed written consent and enrolled in the study on first come, first served basis.

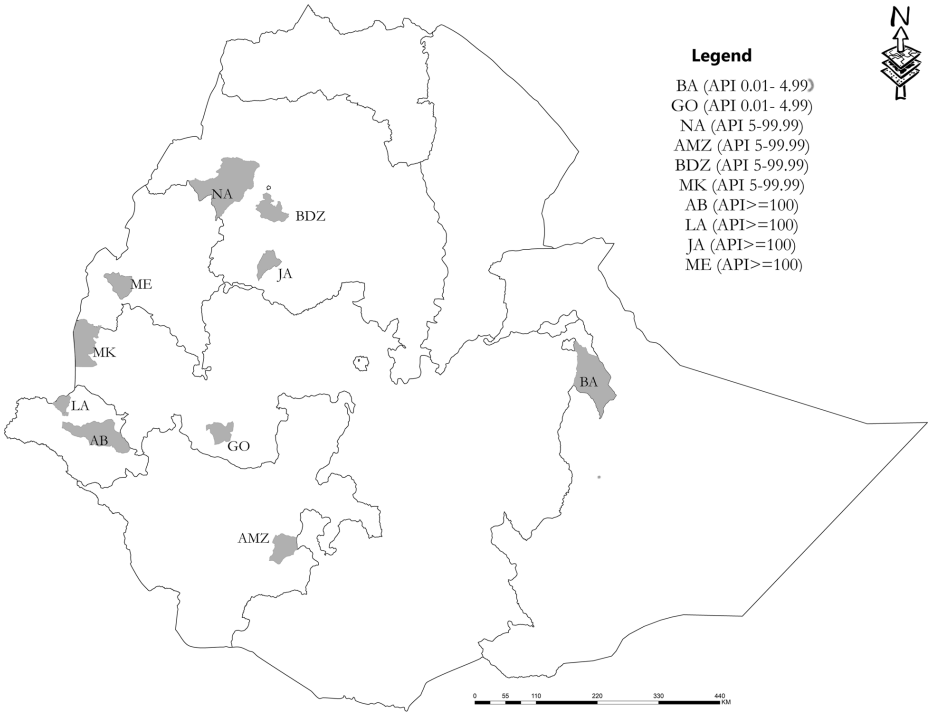

Fig. 1. Location of study sites and their Annual Parasite Index (API) as per the stratification by National Malaria Control Program based on 2017 data: BA = Babile, GO = Gomma, NA = North Achefer, AMZ = Arba Minch Zuria, BDZ = Bahir Dar Zuria, MK = Mao-komo, AB = Abobo, LA = Lare, JA = Jawi, ME = Meng

Finger prick blood samples (~300µL) collected from all participants were used to diagnose malaria using RDT (First Response® malaria Antigen pLDH/HRP2 P.f and Pan Combo Card Test, Premier Medical Corporation Ltd, Dist. Valsad, India) or thin and thick blood films, and to prepare dried blood spots (DBS) on 3MM Whatman filter papers (Whatman, Maidstone, UK). Malaria was diagnosed using RDT at Abobo, Lare, Mao-Komo, Meng, and Gomma districts whilst microscopy was used at the school surveys, Arba Minch Zuria and Babile districts. Detailed clinical and socio-demographic data were captured using a pretested semi-structured interview-based questionnaire. Axillary body

temperature was measured for all participants. If a participant was found febrile (axillary temperature ≥37.5°C) or reports history of fever in the past 48 hours, malaria status was checked using RDT and treated immediately when found positive following the national treatment guideline [23]. DBS were air dried, protected from direct sunlight, and enclosed in zip locked plastic bags individually with self-indicating silica gel (Loba Chemie, Mumbai, India). Samples were transported at ambient temperature and stored at -20˚C until further use. Giemsa-stained thick and thin smears were read independently by two experienced malaria microscopists. A third expert microscopist was consulted in case of discordant results. Thick smear slides were declared negative if no parasites were detected after observing 100 fields under oil immersion (100X magnification).

Species specific detection of Plasmodium parasites by 18S rRNA based nested polymerase chain reaction

Genomic DNA was extracted from 6mm diameter DBS punches using Chelex-Saponin extraction method [24]. In brief, DNA was eluted after an overnight lysis in 0.5% saponin (SIGMA)/PBS (SIGMA) buffer and washing step followed by boiling at 97 ˚C in 150 µL of 6% Chelex (Bio Rad) in DNase/RNase free water (SIGMA). From the final eluate, 80 µL was transferred into a new plate and stored at -20°C until further use. *Plasmodium* species identification was done by nested polymerase chain reaction (nPCR) that targeted the small subunit 18S rRNA gene as described before [25]. A positive control (for *P. falciparum* NF54 culture from Radboudumc, Nijmegen, The Netherlands; for *P. vivax* the malaria reference laboratory positive controls from the London School of Hygiene and Tropical Medicine, London, UK) and negative controls (PCR grade water) were run in every reaction plate. Amplified products were visualized using UV transilluminator (Bio Rad, USA) after electrophoresis using 2% agarose gels (SIGMA, ALDRICH) stained with Ethidium Bromide (Promega, Madison, USA).

Statistical analysis

For the school surveys, sample size was calculated based on protocols by Brooker and colleagues [22] for the original study that aimed at assessing longitudinal evaluation of parasite prevalence in school children [21]. For this study, 70.0% (231/330) of the students were successfully sampled. For the community surveys, an overall prevalence of 6.8% asymptomatic *Plasmodium* infections was expected based on previous observations [17, 19, 26-35] with

a precision of 5%. Based on previous experience, a minimum of 75 samples for the school surveys and 114 for the community samples was targeted across the study sites [21]. Data was double entered into excel, compiled, checked for consistency, and analysed using Stata version 15 (Stata corporation; College Station, TX, USA) and GraphPad Prism 5.3 (GraphPad Software Inc., CA, USA). Proportions were compared between categories using Fisher's exact test and Pearson's chi-squared test where it was appropriate. Equality tests on unmatched data such as age between school and community surveys were tested by two-sample Wilcoxon rank-sum (Mann-Whitney) test. Generalized Estimating Equation (GEE) was used to allow parameter estimates and standard errors adjusted for clustering across the study sites; exchangeable correlation matrix and robust standard errors were used. Sample characteristics such as age, gender, and transmission intensity were tested in the model for their association with infection prevalence and roles as potential confounders. A 5% level of significance was considered in all cases.

Results

Characteristics of study participants

A total of 1093 individuals, 231 from school (3 schools; 75–80 per school survey) and 862 from community surveys (7 surveys; 114–161 per study site) participated in the study. None of the participants was febrile at the time of sampling. Female participants constituted 43.5% (372/855) of community and 51.8% (118/228) of school surveys (P= 0.026). The overall median age of the participants was 16 years (Interquartile range [IQR]: 11–35). As expected, participants from the school surveys were younger (median age, 12; IQR, 11–14) than community surveys (median age, 23; IQR, 10–38; P < 0.001). Results are presented separately for community and school surveys, focusing on community surveys for the main comparisons (Table 1). Within the community surveys, participants from low (median age, 30; IQR, 18–45; n=232) and moderate (median age, 30; IQR, 12–42; n = 272) endemic settings were older than participants from high endemic settings (median age, 13; IQR, 8-28; n = 318; P < 0.001).

Table 1. Community-based prevalence of asymptomatic *Plasmodium* infection using nPCR and microscopy/RDT

Attributes	Category	Parasite prevalence by nPCR, % (n/N) [95% CI]	*P-value*	Parasite prevalence by microscopy/RDT, % (n/N) [95% CI]	*P-value*
Gender	Male	22.5 (106/472) [18.9-26.5]	0.442	13.5(65/483) [10.7-16.8]	0.232
	Female	20.2 (74/367) [16.4-24.6]		10.7(40/372) [7.9-14.3]	
Age group (years)	≤5	21.5 (14/65) [13.1-33.3]	0.008	10.6 (7/66) [5.1-20.7]	<0.001
	5-15	26.7 (62/232) [21.4-32.8]		20.7 (50/241) [16.1-26.3]	
	≥15	16.9 (87/513) [13.9-20.5]		5.6 (29/515) [3.9-7.9]	
Study sites (n/N)					
High transmission	Lare	46.1 (47/102) [36.6-55.8]	<0.001	35.9 (41/114) [27.6-45.2]	<0.001
	Abobo	34.2 (39/114) [26.1-43.4]		23.7 (28/118) [16.9-32.3]	
	Meng	17.6 (21/119) [11.8-25.6]		10.1 (12/119) [5.8-16.9]	
Moderate transmission	Mamo-Komo	29.3 (34/116) [21.7-38.3]		16.4 (19/116) [10.7-24.3]	
	Arba Minch zuria	12.4 (20/161) [8.1-18.5]		1.8 (3/161) [0.6-5.6]	
Low transmission	Babile	15.4 (18/117) [9.9-23.2]		1.7 (2/117) [0.4-6.6]	
	Gomma	3.4 (4/117) [1.3-8.8]		0.0 (0/115 [NA]	
Transmission intensity	High	31.9 (107/335) [27.1-37.1]	<0.001	23.1(81/351) [18.9-27.8]	<0.001
	Moderate	19.5 (54/277) [15.2-24.6]		7.9 (22/277) [5.3-11.8]	
	Low	9.4 (22/234) [6.3-13.9]		0.9 (2/232) [0.2-3.4]	
Overall Prevalence (n/N)	-------	21.6 (183/846) [18.9-24.5]		12.2 (105/860) [10.1-14.6]	

Note: Age was missed for 40 samples. Gender was missed for seven samples. CI= confidence interval, API= Annual Parasite Index /1000 people.

Prevalence of asymptomatic malaria infection across the study sites

In the community surveys, the overall prevalence of asymptomatic *Plasmodium* infections was 12.2% (105/860) by microscopy/RDT and 21.6% (183/846) by nPCR (**Table 1**); 24.1% (208/862) of participants were parasite positive by either nPCR and/or microscopy/RDT. When considering infecting *Plasmodium* species by nPCR, 16.4% (139/846) of samples were *P. falciparum* positive; 3.7% (31/846) were *P. vivax* and 1.5% (13/846) were mixed *P. vivax* and *P. falciparum*. Although the school surveys were from high and moderate transmission sites, there was overall lower *Plasmodium* infection prevalence in the school surveys than in the community surveys as measured by all methods combined (11.3% vs 24.1%; $\chi^2_{=}$ 17.9, P < 0.001)

Among the school surveys, the overall prevalence of asymptomatic malaria was 0.4% (1/231) by microscopy/RDT whilst 11.3% (26/231) were parasite positive either by nPCR or both methods combined. Of these nPCR positive malaria infections from the school surveys, 2.6% (6/231) were due to *P. falciparum*, 5.2% (12/231) were due to *P. vivax,* and 3.5% (8/231) were due to mixed *P. falciparum* and *P. vivax* species infections (Additional file 1: Table S1, Additional file 2: Table S2).

Across the community surveys, in high transmission settings, nPCR-based prevalence of malaria infection ranged from 17.6% (21/119) at Meng to 46.1% (47/102) at Lare district. In the moderate transmission sites, the nPCR-based prevalence was 29.3% (34/116) at Mao-Komo and 12.4% (20/161) at Arbaminch Zuria district. In low transmission sites, the overall nPCR infection prevalence was 9.4% (22/234). The overall microscopy/RDT based prevalence was 23.1% (81/351), 7.9% (22/277), and 0.9% (2/232), in high, moderate, and low transmission settings, respectively (Table 1).

Among the community samples, the prevalence of *Plasmodium* infections detected by all methods combined was substantially higher for the high transmission settings (36.7%, 129/351; 95% CI 31.9–41.9; P<0.001) compared to moderate (20.6%, 57/277; 95% CI 16.2–25.8) and low transmission settings (9.4%, 22/234; 95% CI 6.3–13.9). Moreover, the burden of asymptomatic *Plasmodium* infection was higher in the 5–15 age groups as measured by microscopy/RDT (20.7%, 50/241, P < 0.001) and nPCR (26.7%, 62/232, P = 0.008) (Table 1) as compared to under-five children and adults older than 15 years (Table 1).

Detectability of asymptomatic Plasmodium infections in different endemicities

Among community samples, microscopy/RDT detected 44.2% (80/181) of nPCR detected *Plasmodium* infections (Agreement=86.9%, κ=0.526, **Table 2**). All, but 8 RDT positive *P. falciparum* and 1 microscopy positive *P. vivax* sample, were also nPCR positive (**Additional file 2: Table S2**). The likelihood that *Plasmodium* infected individuals (i.e. individuals who were parasite positive by any diagnostic method) were detected by RDT was increased for individuals living in higher transmission settings (AOR= 5.1, 95% CI 2.6–9.9, P < 0.001) and individuals living in moderate transmission (AOR=3.4, 95% CI 1.6-7.2, P = 0.002) compared to low transmission settings (**Additional file 3: Table 3; Fig. 2**).

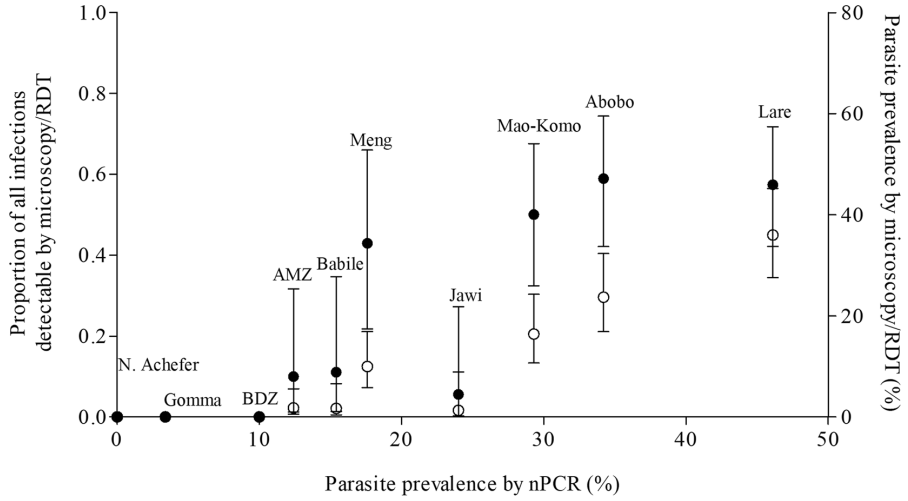

Fig. 2. Community and school- based surveys asymptomatic malaria infection prevalence and detectability using nPCR and microscopy/RDT: black circles represent parasite prevalence by nPCR (x-axis) and proportion all infections detected by microscopy/RDT (left y-axis); white circles indicate parasite prevalence by microscopy/RDT (right y-axis). School surveys were (N. Achefer) North Achefer, BDZ (Bahir Dar Zuria) and Jawi.

Age was an important predictor of asymptomatic malaria positivity by microscopy/RDT. After adjusting for site and correlation between observations from the same survey, a 5% decline in detection using microscopy/RDT was observed for every year increase of age from those that tested positive by all methods(AOR=0.95,95%CI0.9–1.0,P=0.013).

Table 2. Species specific *Plasmodium* parasite prevalence across the study sites using nPCR and microscopy/RDT from 2016-2020

Sample source and API	Study sites	Microscopy/RDT % [n/N]				nPCR % [n/N]				Proportion of infections detected by microscopy/RDT (%)		
		Pf	Pv	Mixed	Proportion (%) of Pv	Pf	Pv	Mixed	Proportion (%) of Pv	Any	Pf	Pv
Community-(API ≥100)	Lare*	33.3 [38/114]	0.0 [0/114]	2.6 [3/114]	7.3 [3/41]	41.2 [42/102]	0.9 [1/102]	3.9 [4/102]	10.6 [5/47]	57.4 [27/47]	56.5 [26/46]	0.0 [0/5]
	Abobo¥	17.8 [21/118]	0.0 [0/118]	5.9 [7/118]	25.0 [7/28]	28.1 [32/114]	3.5 [4/114]	2.6 [3/114]	17.9 [7/39]	58.9 [23/39]	57.1 [20/35]	14.3 [1/7]
	Meng	10.1 [12/119]	0.0 [0/119]	0.0 [0/119]	0.0 [0/12]	13.5 [16/119]	3.4 [4/119]	0.8 [1/119]	23.8 [5/21]	42.9 [9/21]	47.1 [8/17]	0.0 [0/5]
	Total	20.2 [71/351]	0.0 [0/351]	2.8 [10/351]	12.3 [10/81]	26.9 [90/335]	2.7 [9/335]	2.4 [8/335]	15.9 [17/107]	55.1 [59/107]	55.1 [54/98]	5.8 [1/17]
Community – (API≥5&<100)	Mao-komo	16.4 [19/116]	0.0 [0/116]	0.0 [0/116]	0.0 [0/19]	18.9 [22/116]	9.5 [11/116]	0.8 [1/116]	35.3 [12/34]	50.0 [17/34]	65.2 [15/23]	0.0 [0/12]
	AMZ	0.0 [0/161]	0.6 [1/161]	1.2 [2/161]	50.0 [3/6]	6.8 [11/161]	3.1 [5/161]	2.5 [4/161]	45.0 [9/20]	10.0 [2/20]	13.3 [2/15]	11.1 [1/9]
	Total	6.8 [19/277]	0.4 [1/277]	0.72 [2/277]	13.6 [3/22]	11.9 [33/277]	5.8 [16/277]	1.8 [5/277]	38.9 [21/54]	35.2 [19/54]	44.7 [17/38]	4.8 [1/21]
Low (API>0 &<5)	Gomma§	0.0 [0/115]	0.0 [0/115]	0.0 [0/115]	0.0 [0/115]	2.6 [3/117]	0.8 [1/117]	0.0 [0/117]	25.0 [1/4]	0.0 [0/2]	0.0 [0/1]	0.0 [0/1]
	Babile	1.7 [2/117]	0.0 [0/117]	0.0 [0/117]	0.0 [0/2]	11.1 [13/117]	4.3 [5/117]	0.0 [0/117]	27.8 [5/18]	15.4 [2/18]	15.4 [2/13]	0.0 [0/5]
	Total	0.9 [2/232]	0.0 [0/232	0.0 [0/232]	0.0 [0/2]	6.8 [16/234]	2.6 [6/234]	0.0 [0/234]	27.3 [6/22]	10.0 [2/20]	14.3 [2/14]	0.0 [0/6]
Community	Grand Total	10.7 [92/860]	0.1 [1/860]	1.4 [12/860]	12.4 [13/105]	16.4 [139/846]	3.7 [31/846]	1.5 [13/846]	24.0 [44/183]	44.2 [80/181]	48.7 [73/150]	4.6 [2/44]

Table 2. Continued

Sample source and API	Study sites	Microscopy/RDT % [n/N]				nPCR % [n/N]				Proportion of infections detected by microscopy/RDT (%)		
		Pf	Pv	Mixed	Proportion (%) of Pv	Pf	Pv	Mixed	Proportion (%) of Pv	Any	Pf	Pv
School (API ≥100)	Jawi	1.3 [1/75]	0.0 [0/75]	0.0 [0/75]	0.0 [0/1]	5.3 [4/75]	10.7 [8/75]	8.00 [6/75]	77.8 [14/18]	5.6 [1/18]	10.0 [1/10]	0.0 [0/14]
School (API≥5&<100)	BDZ	0.0 [0/80]	0.0 [0/80]	0.0 [0/80]	0.0 [0/80]	2.5 [2/80]	5.0 [4/80]	2.5 [2/80]	75.0 [6/8]	0.0 [0/8]	0.0 [0/4]	0.0 [0/6]
	N. Achefer	0.0 [0/76]	0.0 [0/76]	0.0 [0/76]	0.0 NA	0.0 [0/76]	0.0 [0/76]	0.0 NA	0.0 [0/76]	0.0 NA	0.0 NA	0.0 NA
School	Total	0.4 [1/231]	0.0 [0/231]	0.0 [0/231]	0.0 [0/1]	2.6 [6/231]	5.2 [12/231]	3.5 [8/231]	76.9 [20/26]	3.8 [1/26]	7.1 [1/14]	0.0 [0/20]

Note: AMZ= Arba Minch Zuria, BDZ= Bahir Dar Zuria, N. Achefer= North Achefer, Pf= *P. falciparum*, Pv= *P. vivax*, mixed= Pf+ Pv, Any= all species detected. Proportion of Pv is calculated together with mixed infections. API= annual parasite index /1000 people. * 12 DBS samples (11 Pf and 1 mixed infection positive by RDT) were missed from Lare for PCR. ¥ 4 DBS samples (3 Pf and 1 mixed infection positive by RDT) were missed from Abobo for PCR. § microscopy was not done for 2 samples that were Pf positive by PCR.

The parasite species composition and detectability varied between transmission settings (**Fig. 2**). Among the *Plasmodium* species detected in the community samples, the majority were attributable to *P. falciparum* (77.4%, 161/208) when all samples were combined. Of the nPCR detected *P. falciparum*-mono species infections (n=139) and mixed-species infections (n=13), microscopy/RDT successfully detected *P. falciparum* in 48.7% (73/150) of infections (**Table 2**). Of the nPCR detected *P. vivax*-mono infections (n = 31) and mixed-species infections (n = 13), microscopy/RDT successfully detected *P. vivax* in 4.6% (2/44) of infections (**Table 2**).

Discussion

This study describes the prevalence and detectability of asymptomatic *Plasmodium* infections in ten different transmission settings by nPCR and conventional diagnostics (i.e. microscopy/RDT). More asymptomatic infections were detected in high transmission settings by both methods. The detectability of asymptomatic *Plasmodium* infections using microscopy/RDT relative to nPCR increased as transmission intensity increases. As a result, most infections in low transmission settings were not detectable by microscopy/RDT.

In Ethiopia, several cross-sectional studies have documented asymptomatic parasite carriage using conventional and molecular methods [16-18, 33, 34]. The current multi-site study allowed an assessment of factors influencing the prevalence of infections as well as their detectability by microscopy/RDT. The prevalence of asymptomatic *Plasmodium* infections in the current study was in the same range as other reports from high [18, 34] and moderate [27] transmission settings in Ethiopia and elsewhere [17, 29, 36, 37].

Consistent with other studies [16, 38, 39], the current study observed that microscopy/RDT detected fewer asymptomatic infections as compared to PCR. The proportion of *Plasmodium* infections that was detectable by microscopy/RDT increased with increasing in transmission intensity. Whilst this trend has been reported in meta-analyses for *P. falciparum* [15, 36, 40], it is striking that this trend is also apparent in the current study within one country affected by both *P. falciparum* and *P. vivax*. Moreover, the effect size was comparatively large with approximately 5-fold higher detectability of infections in high endemic settings compared to low endemic settings. The trend of increasing detectability with increasing transmission intensity may be attributable to the fact that

asymptomatically infected individuals have higher average parasite densities in high transmission settings [15, 41]. Moreover, in low endemic settings individuals will receive fewer infectious bites with mosquitoes, due to the absence of super-infections, lower parasitaemia over the course of infection [9, 36]. Low genetic diversity of the parasite population in low transmission settings may also contribute to rapidly acquired immunity to the specific clones [42], further limiting parasite density. An impact of immunity on parasite density and the detectability of infections is also illustrated by the negative impact of increasing age on the detectability on infections in line with the current study [43].

Lower parasite densities in *P. vivax* compared to *P. falciparum* [44, 45] also results in a low detectability of *P. vivax* infections by microscopy/RDT. This low density in *P. vivax* is mainly attributable to the parasite's preference to infect reticulocytes [46, 47] that typically constitute less than 1% of the total erythrocyte population [48] and also to the early acquisition of immunity [47]. These findings have implications for estimates of the relative burden of *P. falciparum* and *P. vivax* infections. The introduction of sensitive molecular tools may thus improve the detection of *P. vivax* infections substantially. Since treatment strategies differ for *P. falciparum* and *P. vivax*, this is relevant for public health interventions.

Although RDT and microscopy were used separately in the study sites due to logistics reasons, the prevalence measured by conventional RDT and microscopy was assumed to be comparable [37]. Nine samples that were declared microscopy/RDT positive were negative by nPCR while seven samples that were detected *P. falciparum* positive by RDT were *P. vivax* positive by nPCR. False RDT positivity might be due to the presence of parasite antigens after adequate clearance of parasites which might explain the variation between RDT positivity and PCR negative detection among asymptomatic malaria infections [49, 50]. Hence, there is a possibility that RDT can be positive for lingering antigens of *P. falciparum* while missing the low-density *P. vivax* infection from the same patient.

Conclusion

Conventional diagnostics missed nearly half of the asymptomatic malaria reservoir detected by nPCR. Moreover, the detectability of asymptomatic *Plasmodium* infections in all endemic sites might reflect the long persistence of these infections from weeks up to months in high [51] as well as in low

transmission settings [52, 53] even in the presence of effective control and elimination interventions. As these infections can have relevance for onward malaria transmission [13-15], a detailed understanding of the distribution, detectability, and contribution to the infectious reservoir of asymptomatic infections will greatly improve our ability to target all relevant infections. The wide scale presence of low-density infections calls for more in-depth studies on understanding parasite density oscillations, their relevance for malaria symptoms, and onward transmission to mosquitoes.

Declarations

Ethical statement

The study protocol was approved by the Ethiopian National Research Ethics Review Committee (3.10/016/20), and the institutional ethics review boards of the Department of Biochemistry (Ref.No.SOM/DRERC/BCH005/2009) and the College of Natural Sciences (Ref.No. SOM/DRERC/BCH005/2009) at Addis Ababa University, and the Armauer Hansen Research Institute (P035/17, P024/17 and P032/18).

Availability of data and materials

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

Competing interests

The authors declared that they have no competing interests.

Funding

The study was supported by the Armauer Hansen Research Institute ((via its core funding from Norwegian Agency for Development Cooperation and Swedish International Development Cooperation); the Netherlands organization for international cooperation in higher education (Nuffic) [grant number NFP-PhD.14/150] to FGT; the European Research Council [ERC-2014-StG 639776] to TB; the Bill & Melinda Gates foundation [INDIE; OP P1173572] to TB, FGT and CD. The funders have no role in the in the design of the study, collection, analysis, interpretation of the data and in writing the manuscript.

Author's contributions

FGT, EH, EG, CD and TB conceived the study, contributed to data analysis and critically commented on the manuscript. EH analysed the data. EH and SKT drafted the manuscript. EH, SK, WC, GS and MK conducted the laboratory study. WC, MK, EH, SKT, AG, GS, TA collected blood samples. All authors read and approved the final manuscript.

Acknowledgements

We thank all the study participants for their willingness and local facilitators of the study sites for their support during the sample collection. We also thank the WHO certified microscopists (Tewabech Lema and Tsehay Orlando) at Adama Malaria Center for their support. We appreciate the regional and district health officers for their collaboration. The malaria team members and researchers at AHRI (Tizita Tsegaye, Tadele Emiru, Temesgen Tafesse, Mikiyas Gebremichael, Misgana Muluneh, Endashaw Esayas, Tsegaye Hailu, Haile Abera, Demekech Damte, Tiruwork Fanta, Senya Asfer, and Eyuel Asemahegn) played an important role in making the study successful. We are indebted to the drivers of AHRI for their support during the field sample collection.

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Supplementary information

Table S1. School-based prevalence of asymptomatic malaria in selected sites from different transmission settings using nPCR and microscopy/RDT from 2016- 2018, Ethiopia

Attributes	Category	Parasite prevalence by nPCR, % (n/N) [95% CI]	P-value	Parasite prevalence by microscopy/RDT, % (n/N) [95% CI]	P-value
		(N= 231)		(N= 231)	
Gender	Male	12.7(14/110) [7.6-20.4]	0. 544	0.0 (0/110) [NA]	0.333
	Female	10.2(12/118) [5.8-17.1]		0.85 (1/118) [0.01-5.8]	
Age group (years)	<5	-		-	
	5-15	10.2 (21/206) [6.7-15.2]	0.182	0.5 (1/206) [0.07-3.4]	0.755
	>15	20.0 (4/20) [7.5-43.6]		0.0 (0/20) [NA]	
Study sites (n/N)					
High transmission (API ≥100)	Jawi	24.0 (18/75) [15.62-35.01]		1.3 (1/75) [0.18-8.98]	
Moderate transmission (API≥5&<100)	BDZ	10.0 (8/80) [5.05-18.83]	<0.001	0.0 (0/80) [NA]	0.352
	N. Achefer	0.0 (0/76) [NA]		0.0 (0/76) [NA]	
Overall Prevalence (n/N)	-------	11.3 (26/231) [7.5-16.0]		0.4(1/231) [0.01-2.3]	

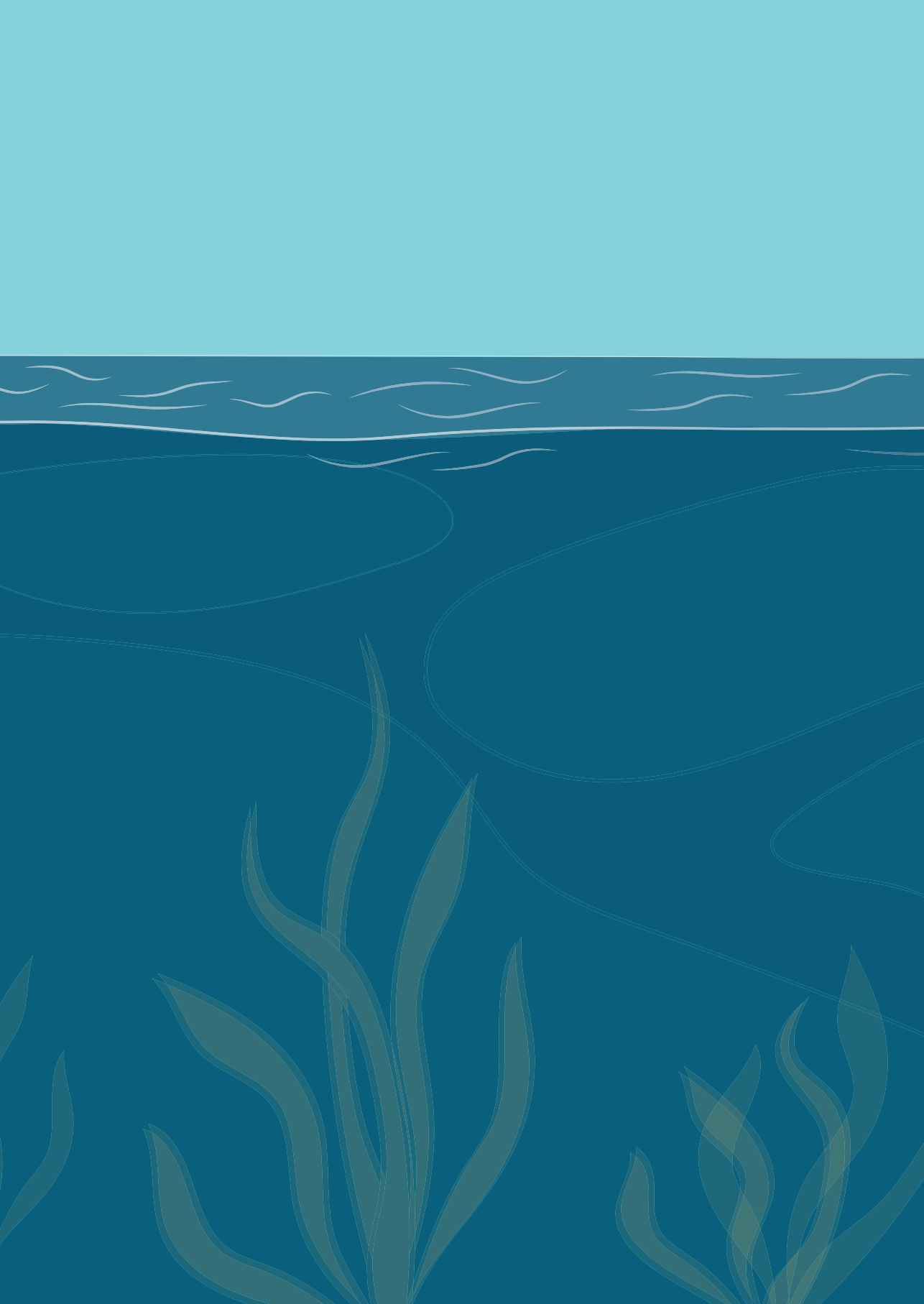
Table S2. Concordance of RDT and Microscopy detected samples compared to nPCR among the study participants, 2016-2020

		18S nPCR			
		P. falciparum		*P. vivax*	
		Positive	Negative	Positive	Negative
RDT	Positive	68	8	8	0
	Negative	52	302	20	302
Microscopy	Positive	4	0	1	1
	Negative	38	557	34	557

NB: the mixed species infections are added with the *P. falciparum* and *P. vivax* infections

Table S3. GEE model for association of malaria infection prevalence using all methods combined (nPCR and/or microscopy/RDT) among community survey samples with sample characteristics such as gender, age category, level of endemicity from 2016-2020, Ethiopia

Sample characteristics		Total malaria infection prevalence			
		COR (95% CI)	P- value	AOR (95% CI)	P-value
Gender	Female	1 (Ref)		1 (Ref)	-
	Male	1.2 (0.8-2.0)	0.385	-	-
Age as continuous variable (years)		0.95 (0.9-1.0)	0.001	0.95 (0.9-1.0)	0.013
Level of endemicity	Low (API >0 &<5)	1 (Ref)	-	1 (Ref)	-
	Moderate (API ≥5 & <100)	5.5 (1.6-19.1)	0.008	3.4 (1.6-7.2)	0.002
	High (API≥100)	15.0 (9.0-24.1)	<0.001	5.1 (2.6-10.0)	<0.001



CHAPTER 3

Prevalence of *Plasmodium falciparum Pfcrt* and *Pfmdr1* alleles in settings with different levels of *Plasmodium vivax* co-endemicity in Ethiopia

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International Journal for Parasitology: Drugs and Drug Resistance 11: 8-12.

Abstract

Plasmodium falciparum and *P. vivax* co-exist at different endemicity levels across Ethiopia. For over two decades Artemether-Lumefantrine (AL) is the first line treatment for uncomplicated *P. falciparum,* while chloroquine (CQ) is still used to treat *P. vivax*. It is currently unclear whether a shift from CQ to AL for *P. falciparum* treatment has implications for AL efficacy and results in a reversal of mutations in genes associated to CQ resistance, given the high co-endemicity of the two species and the continued availability of CQ for the treatment of *P. vivax*. This study thus assessed the prevalence of *Pfcrt*-K76T and *Pfmdr1*-N86Y point mutations in *P. falciparum.* 18S RNA gene based nested PCR confirmed *P. falciparum* samples (N=183) collected through community and health facility targeted cross-sectional surveys from settings with varying *P. vivax* and *P. falciparum* endemicity were used. The proportion of *Plasmodium* infections that were *P. vivax* was 62.2% in Adama, 41.4% in Babile, 30.0% in Benishangul-Gumuz to 6.9% in Gambella. The *Pfcrt*-76T mutant haplotype was observed more from samples with higher endemicity of *P. vivax* as being 98.4% (61/62), 100% (31/31), 65.2% (15/23) and 41.5% (22/53) in samples from Adama, Babile, Benishangul-Gumuz and Gambella, respectively. However, a relatively higher proportion of *Pfmdr1*-N86 allele (77.3-100%) were maintained in all sites. The observed high level of the mutant *Pfcrt*-76T allele in *P. vivax* co-endemic sites might require that utilization of CQ needs to be re-evaluated in settings co-endemic for the two species. A country-wide assessment is recommended to clarify the implication of the observed level of variation in drug resistance markers on the efficacy of AL-based treatment against uncomplicated *P. falciparum* malaria.

Keywords: Drug resistance, *Pfmdr*, *Pfcrt*, Artemisinin resistance, Artemether-Lumefantrine, Ethiopia

Introduction

Development and spread of anti-malaria drug resistance continued to be a stumbling-block in the fight against malaria. *Plasmodium falciparum* developed resistance to most of the antimalarials over the past 60 years [1]. Resistance against Artemisinin combination therapies (ACT) [2-4] is reported in Thai-Cambodian border, a historical 'hotspot' for multi-drug resistance parasite evolution, emergence and spread. Although ACTs retain high efficacy in sub-Saharan African countries including Ethiopia [5, 6], recent reports are suggestive of the emergence of ACT tolerant *P. falciparum* in African settings [7].

One of the mechanisms for emergence of drug resistance in *Plasmodium* is acquisition of mutations and duplication in target and/or transporter genes. Artemether-Lumefantrine (AL) selects for wild-type codons at the *P. falciparum* chloroquine resistance transporter (*Pfcrt*-K76) and *P. falciparum* multidrug resistance 1 (*Pfmdr1*) alleles [8, 9] and its use is associated with increases in *Pfmdr1* gene copy number [10]. Variations were reported on the selective effects of different ACTs on single-nucleotide polymorphisms in *Pfcrt* and *Pfmdr1*. A recent systemic review of 397 surveys in 30 countries in Africa documented that AL and artesunate-amodiaquine (ASAQ) exert opposing selective pressures and parasites under the selection pressure of one ACT tend to be more sensitive to the other [11]. Higher proportion of *Pfmdr1*-86N and increases in its copy number have been reported in AL treated patients with parasite recrudescence [12]. It was noted that ASAQ selects the *Pfmdr1* mutant haplotypes, 86Y, Y184, and 1246Y [12]. Chloroquine is known to select the mutant *Pfcrt*-K76T and the wild type *Pfmdr1*-N86Y haplotypes [13]. Therefore, in areas where both *P. falciparum* and *P. vivax* are co-endemic the continued use of CQ might result in pronounced different selective pressure and, as a consequence, different drug resistance profiles in communities.

Unlike most of Africa, *P. falciparum* (60%) co-exists with *P. vivax* (40%) in most settings in Ethiopia. Driven by the widespread CQ resistant *P. falciparum*, Ethiopia changed its treatment policy for *P. falciparum* from CQ to sulphadoxine-pyrimethamine (SP) in 1998 [6, 14]; shortly after SP was replaced by AL as first-line therapy for uncomplicated falciparum malaria in 2004 [6, 15]. Throughout this period, CQ continued to be used as first line treatment for *P. vivax* [15]. This may have implications for selection of drug resistance marker genes. Here, we examined the prevalence of *Pfcrt*-K76T and *Pfmdr1*-N86Y alleles in

P. falciparum after two decades of AL implementation as first line treatment in areas with different *P. vivax* co-endemicity levels in Ethiopia.

Materials and Methods

Study sites and samples

Study sites were selected based on reported difference in *P. vivax* and *P. falciparum* co-endemicity levels [16]. Six Woredas (districts) representing different epidemiological settings from three regional administrative states (Gambella, Benishangul-Gumuz and Oromia) were included. Gambella region, with Abobo and Lare districts, is a *P. falciparum* dominated area (97% of all *Plasmodium* infections are *P. falciparum*) [17] with perennial transmission. Benishangul-Gumuz region, with Mao-Komo and Meng districts, is also *P. falciparum* dominated with relatively higher prevalence of *P. vivax* (10.1%) [18] and more seasonal transmission. The Oromia region study site districts of Babile and Adama are characterized by seasonal malaria transmission with co-endemicity for *P. falciparum* and *P. vivax* where *P. vivax* infections attributed to be 41.4% [19] in the first and 62.2% [20] in the later district, respectively (**Figure 1** and **Table 1**). Oromia regional state has wider variation in the *P. vivax* and *P. falciparum* infection proportion due to its huge land mass and sampling was done in separate years i.e. Adama in 2016 and Babile in 2017, hence we have made separate treatment of the data from the study sites.

Study participants were recruited in community and health facility based cross-sectional surveys. Community based cross-sectional surveys were conducted in Babile district from July to November 2017 and from October to December 2016 in all other sites. In Adama passively-detected clinical malaria infected patients (n=36) were recruited in addition to the community survey samples (n=33). Finger prick blood samples (~300μL) were collected from the study participants for malaria diagnosis using rapid diagnostic test (RDT) (First Response Malaria Ag (pLDH/HRP2, Combo RDT, Premier Medical Corporation Ltd., India) or microscopy and to prepare dried blood spots (DBS) on Whatman 3MM filter paper (Whatman, Maidstone, UK). Socio-demographic and malariometric data were captured using pre-tested structured questionnaire.

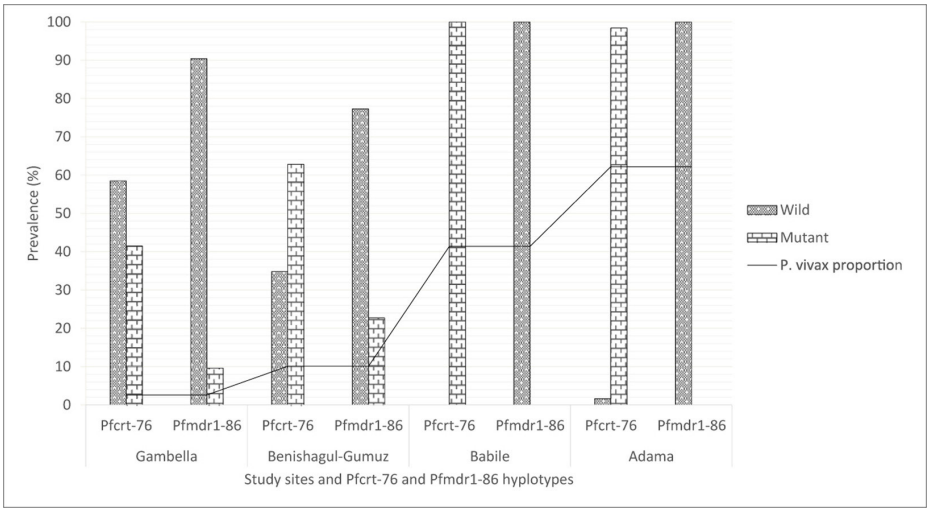

Fig. 1. The prevalence of Pfcrt-76K/T and Pfmdr1-86N/Y point mutations among samples from four study sites with different *P. vivax* co-endemicity level compared to *P. falciparum*. *Where; wild = wild type alleles Pfmdr1-86N and Pfcrt-76K and Mutant=mutant type alleles Pfmdr1-86Y and Pfcrt-76T*.

Detection of Pfcrt and Pfmdr1 polymorphisms

Study participants were diagnosed for malaria using thick blood smears and thin smears were used for species identification following Giemsa-staining by two experienced microscopists who were independent and blind for the participants' clinical status and RDT results. A third World health organization certified microscopist was consulted in case of discordant results. Blood film slides were considered negative if no parasite was detected after examining 100 microscopic fields [20].

Furthermore, DNA was extracted from a 6mm diameter punch of DBS using Chelex-Saponin extraction method as described elsewhere [21]; eluted DNA was stored at -20˚C until further use. Malaria species was confirmed with 18S based nested polymerase chain reaction (nPCR) as described elsewhere [22]. Samples that were confirmed to be *P. falciparum* mono-species infection using the 18S RNA nPCR [22] were further amplified for the *Pfcrt*-76 and *Pfmdr1*-86 genes using nPCR with outer (N1) and inner primer pairs (N2) (**Supplement 1**).

Table 1. Characteristics of study participants and frequency of point mutations *P. falciparum* chloroquine resistance transporter *(Pfcrt*-76) and multidrug resistance 1 (*Pfmdr1*-86) genes among samples from four sites in Ethiopia

Characteristics	Gambella N=55	Benishangul-Gumuz N=26	Babile N=33	Adama N=69	Total N=183	P-value
Age (years), Median (IQR)	13(8-18)	12(9-18)	15(8-35)	20(8-32)	14(8-25)	
Female sex, n(%)	24(43.6)	11(42.0)	13(39.4)	27(39.1)	75(41.0)	
Proportion of infections that are *P. vivax*, %	2.6[1]	10.1[2]	41.4[3]	62.2[4]		
Amplified samples for *Pfcrt*-76 codon, n	53	23	31	62	169	
Pfcrt-76K (wild type), %(n/N)	58.5(31/53)	34.8(8/23)	0.0(0/31)	1.6(1/62)	23.7(40/169)	
Pfcrt-76T (Mutant), %(n/N)	32.1(17/53)	47.8(11/23)	100.0(31/31)	91.9(57/62)	68.6(116/169)	
Pfcrt-76K/T (Mixed), %(n/N)	9.4(5/53)	17.4(4/23)	0.0(0/31)	4.0(6.45)	8.0(13/169)	<0.001
Amplified samples for *Pfmdr1*-86 codon, n	52	22	32	62	168	
Pfmdr1-86N (wild type), %(n/N)	90.4(47/52)	77.3(17/22)	100.0(32/32)	100.0(62/62)	94.1(158/168)	
Pfmdr1-86Y (Mutant), %(n/N)	3.9(2/52)	4.6(1/22)	0.0(0/32)	0.0(0/62)	1.8(3/168)	
Pfmdr1-86N/Y (mixed), %(n/N)	5.8(3/52)	18.2(4/22)	0.0(0/32)	0.0(0/62)	4.2(7/168)	<0.001

IQR, interquartile range; [1]*Tsegaye et al, Mal J 2014;* [2]*Geleta and Ketema Malar Res Treat 2016;* [3]*Keffale et al. TRSTMH 2019;* [4]*Tadesse et al. CID 2019*

The N2 products were assessed using restriction fragment length polymorphism (RFLP) to detect mutations at each locus [23, 24]. Restriction enzymes used and conditions of digestion were as described elsewhere [25]. For *Pfcrt*-76 codon, a known positive control of 3D7 (wild-type, *Pfcrt*-76K allele) and negative control of Dd2 (mutant) were included in each reaction (kindly provided by the Ethiopian Public Health Institute). Restriction enzyme digested products were visualized with UV transilluminator after electrophoresis using 2% Agarose gel (SIGMA-ALDRICH) with a no enzyme digest control run alongside positive controls, negative controls and test samples (Bio Rad, USA). Mixed haplotypes having both wild and mutant type were interpreted as combined band patterns of digested and undigested products identified per sample of DNA.

Ethics statement

The study protocol was approved by the National Research Ethical Review Committee (3.10|016\20), Institutional Ethical Review Board of the College of Natural Science at Addis Ababa University, and AHRI/ALERT (Ref.No.SOM/DRERC/BCH005/2009, PO24/17). Written informed consent was obtained from each adult and parent/legal guardians for children younger than 18 years. Symptomatic patients were treated as per the national treatment guideline [15].

Statistical analysis

Both laboratory and field data were double entered. The cleaned data was analyzed using STATA 13 (StataCorp, TX, USA). The difference in prevalence among study sites was compared using Fisher's exact test. Pairwise comparison, after Bonferroni correction, was run to test differences in distribution of the wild and mutant alleles between the study sites for both Pfcrt-76 and Pfmdr1-86 codons. A *P-value* ≤ 0.05 was considered statistically significant.

Results

Characteristics of the study participants

A total of 183 samples from 148 asymptomatic and 35 symptomatic participants with *P. falciparum* infection by 18S nPCR were included in the study. Out of the 183 samples, 169 (92.3 %) and 168 (91.8 %) were successfully amplified for *Pfcrt*-76 and *Pfmdr*1-86 codons, respectively. Adama was the only site where asymptomatic and symptomatic study participants were considered. There was no significant difference among wild type and mutant haplotypes of the *Pfcrt*-76 codons between the asymptomatic (7.4%, 2/27) and symptomatic (8.6%, 3/35;

P=0.125) samples of Adama hence considered together in the subsequent analysis. Only wild type form of *Pfmdr1*-86 was detected in both groups.

Prevalence of Pfcrt-76 haplotypes varies between study sites and *P. vivax* co-endemicity

Overall, the wild type *Pfcrt*-K76 haplotype was detected in 23.7% (40/169) and the mutant *Pfcrt*-76T in 76.3% (129/169) of samples across sites. We observed a statistically significant difference in the prevalence of *Pfcrt*-76 codons among the study sites (P<0.001): mutant (*Pfcrt*-76T) type was virtually fixed in Babile (100%, 31/31) and with near fixation at Adama (98.4%, 61/62) districts of Oromia region where *P. vivax* is highly endemic (41.4% and 62.2% of cases, respectively)[19, 20]. On the other hand, a higher prevalence of the wild type (*Pfcrt*-K76) codon was found in Abobo and Lare districts of Gambella (58.5%, 31/53) and in Mao-Komo and Meng districts of Benishangul-Gumuz (34.8%, 8/23) (**Table 1**). The proportion of infections that is *P. vivax* is very low (<10%) in these settings [17, 18] (**Table 1**). After Bonferroni correction, differences in the proportion of Pfcrt-76T among the study sites was observed (*P*=0.0342).

High prevalence of Pfmdr1-86 wild haplotype across the study sites

The overall prevalence of the mutant codon, *Pfmdr*1-86Y, was 5.9% (10/168). The wild type codon, *Pfmdr*1-N86, was found fixed (100%) in Babile and Adama districts with similar higher proportions in Abobo and Lare districts of Gambella (90.4%, 47/52) and Mao-Komo and Meng districts of Benishangul-Gumuz (77.3%, 17/22). The prevalence of the mutant type (*Pfmdr*1-86Y) was only 9.6 % (5/52) and 22.7 % (5/22) in the districts from Gambella and Benishangul-Gumuz, respectively. Moreover, the observed prevalence of the different haplotypes was significantly different among the study sites (P=0.001) (**Table 1**). No difference was observed in the proportion of Pfmdr1-N86 in between study sites after Bonferroni correction (*P*=1.0).

Discussion

The return of wild type alleles of CQ resistance marker genes has been reported across Africa following replacement of CQ by ACTs [11, 12, 26, 27]. The possible associations of these markers with ACT drug tolerance underline the need for continued surveillance for the success of malaria control program. In this study we assessed the prevalence of *Pfcrt*-K76T and *Pfmdr1*-N86Y codons from different *P. falciparum* and *P. vivax* co-endemicity settings in Ethiopia.

The current study has documented a higher frequency of *Pfcrt*-76T mutant haplotypes proportional to the endemicity level of *P. vivax* as being 98.4%, 100%, 65.2% and 41.5% in samples from Adama, Babile, Benishangul-Gumuz and Gambella, respectively. Lower proportion of parasites with the mutant alleles (*Pfcrt*-76T) was detected in samples from Gambella and Benishangul-Gumuz where *P. falciparum* is the dominant species. The nearly approaching fixation of *Pfcrt*-76T mutant haplotype in Adama and its fixation in Babile districts of Oromia region was in agreement with previous reports in Ethiopia [28, 29]. This might be partly due to the high prevalence of *P. vivax* co-endemicity that probably lead to the continued utilization and easy access of CQ in these areas. Similar findings associating resistance markers with CQ use have been reported elsewhere: persisting high levels of mutant *Pfcrt* haplotypes in infections from Brazil and, in contrast, the reappearance of wild type forms in Nigeria have been attributed to difference in selective pressure by CQ use [13]. Moreover, The slow reappearance of CQ sensitive parasites harboring *Pfcrt*-K76 haplotype in low *P. vivax* prevalence areas of Gambella and Benishangul-Gumuz corroborates observations from other African countries such as Malawi [30], Côte d'Ivoire [31], Zambia [32],Tanzania [33] and Kenya [34].

Higher proportion of the wild type allele, *Pfmdr1*-N86 allele (77.3-100%) was detected in all sites; fixed in Babile and Adama. This finding was in line with previous reports from different parts of Ethiopia [29, 35, 36]. Earlier studies conducted more than a decade ago indicated a non-negligible level of the mutant form in Ethiopia [37, 38]. This is probably due to the AL-regimen rolled out as first line treatment for *P. falciparum* infection across the study sites. In African settings, after decades of AL use, a rise in the "NFD" *Pfmdr1* haplotypes at codons-86,184 and 1246 have been observed with a parallel decline in the "YYY" haplotypes of these codons [11, 12, 39, 40]. These studies found that the "NFD" haplotypes were associated with the reduced sensitivity of the parasite to AL treatment.

The observed difference across the study sites is plausibly associated with antimalarial drug pressure as observed in other co-endemic settings. The re-emergence of genotypes such as *Pfcrt*-K76 and *Pfmdr*-1 codons-N86, 184F and D1246 after CQ withdrawal has been associated with selection of parasites with reduced sensitivity to AL [34, 41-43] which may eventually lead to resistance [8, 12, 44]. Moreover, in settings where *P. falciparum* and *P. vivax* are sympatric where mixed species infections are common, the detection of *P. falciparum* is frequent following treatment of *P. vivax* with CQ [45-47].

Thus, the low CQ level to which the *P. falciparum* will be exposed might favor selective pressure.

The strength of this study is that it considered samples from different levels of *P. vivax* and *P. falciparum* co-endemicity (with varied CQ pressure) and both symptomatic and asymptomatic individuals. It is less likely to have a shift in proportion of infections due to *P. vivax* in the two years of sampling (2016 and 2017); the over decade national malaria control program data summary (2001–2016), documented no considerable change in the proportion of infections due to *P. vivax* in Ethiopia [6]. The almost complete reversal of the wild haplotype, *Pfmdr1*-N86 codon, in all our sites reinforce the claim that such phenomenon might be due to ACT based treatment. Thus, we suggest parallel surveillance of drug resistance markers to monitor the effects of the antimalarial-drugs deployed in areas where *P. vivax* and *P. falciparum* are co-endemic. The similarity in the drug resistance profile among asymptomatic individuals and clinical patients is suggestive of asymptomatic infections could serve as hidden reservoirs. Therefore, our finding also underpin the importance of involving asymptomatic reservoirs in the evaluation of drug resistance especially in areas approaching elimination [48].

This study would have been more comprehensive if clonality of infections and copy number variations (CNVs) of target genes were assessed. We reported mixed haplotypes for *Pfcrt*-76 (8.0%, 13/169) and *Pfmdr1*-86 (4.2%, 7/168). As the study was based on PCR-RFLP method alone ascertaining whether any of the two haplotypes were coming from the same genome or multi-clonal infections was difficult; hence we reported the mixed haplotypes together with the mutant proportions. Also, *Pfmdr1*-86 CNV is implicated for the emergence of *P. falciparum* diminished susceptibility to anti-malarial drugs, such as mefloquine, AS-MQ and AL combinations [12, 40].

In conclusion, after two decades of the replacement of CQ with AL for the treatment of uncomplicated *falciparum* malaria in Ethiopia, there is slow but site-specific reversal of the *Pfcrt*-K76 haplotype while the *Pfmdr1*-N86 wild type was almost fixed across study sites, a preliminary finding that calls the need for monitoring and responding to emerging signs of drug resistance to preserve the efficacy of anti-malarial drugs. Thus, we recommend country-wide assessments to clarify the implication of the observed level of variation in drug resistance markers on the efficacy of AL-based treatment against uncomplicated *P. falciparum* malaria.

Acknowledgement

We would like to acknowledge the study participants, clinical staffs and local facilitators at the study sites. We would also like to acknowledge Ethiopian Public Health Institute (EPHI) for providing the reference DNA samples of *P. falciparum* from their previous IAEA project on malaria drug resistance markers.

Funding:

This work was supported by the Armauer Hansen Research Institute (core funding from Norad and Sida) and the Netherlands organization for international cooperation in higher education (Nuffic) [grant number NFP-PhD.14/150] to FGT.

Competing interest: none declared.

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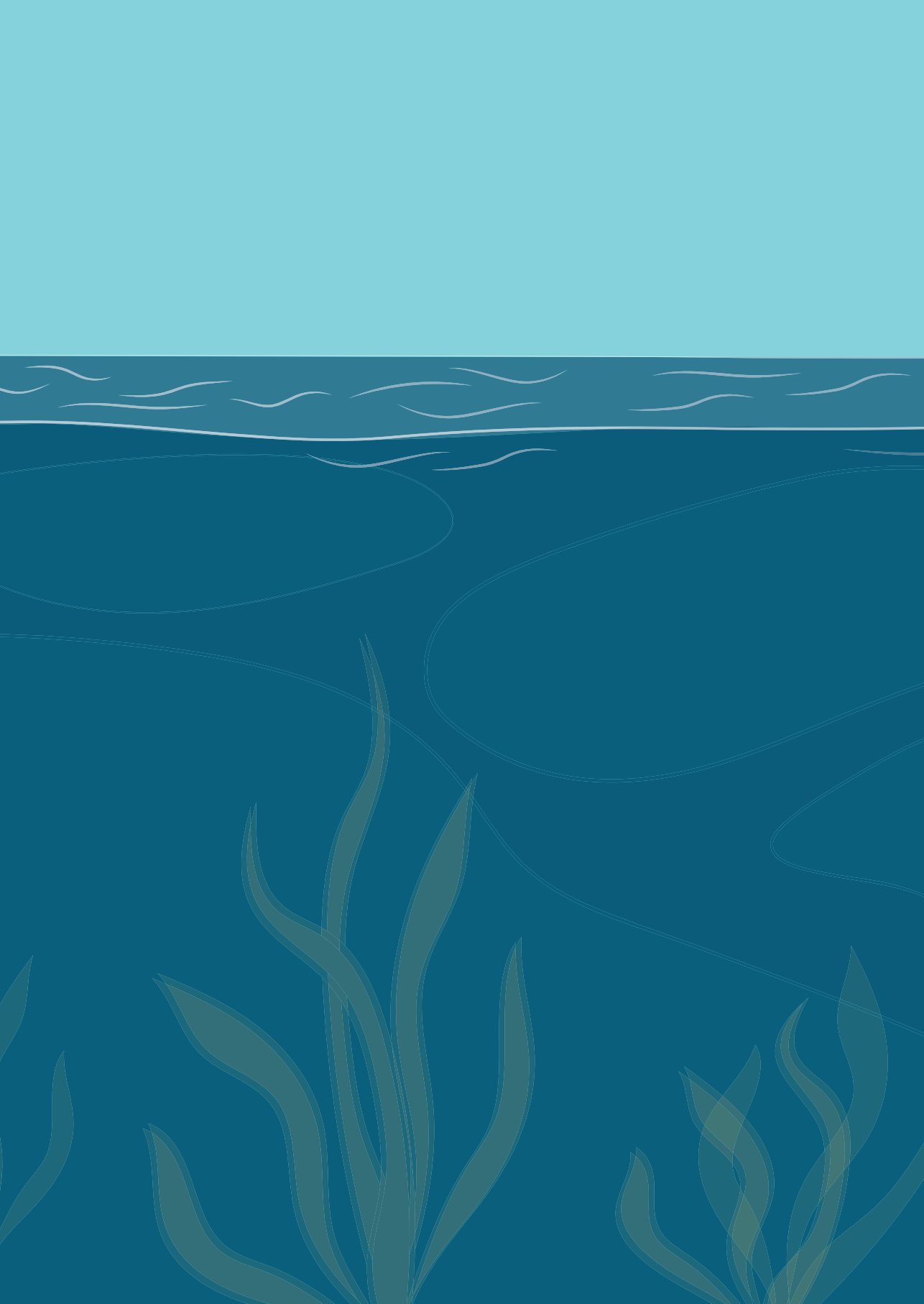
Supplementary information

Supplement 1. Primers used to amplify and detect Pfcrt-76 and Pfmdr1-86 genes

Target	Primer Name	Primer Sequence	Codon	PCR Cycling Condition
*Pf*CRT	N1FP-Pfcrt-76 N1RP-Pfcrt-76	OF: 5′CCGTTAATAATAAATACACGCAG-3′ NR: 5′GGATGTTACAAAACTATAGTTACC-3′	76	95°C for 10min (94°C- 30sec; 56°C-30sec; 62°C-1min)x35 cycles; 72°C for 10min.
	N2FP-Pfcrt-76 N2RP-Pfcrt-76	OF: 5′-TGTGCTCATGTGTTTAAACTT-3′ NR: 5′-CAAAACTATAGTTACCAATTTTG-3′	76	95°C for 10min (94°C- 30sec; 56°C-30sec; 65°C-1min)x30 cycles; 72°C for 10min.
*Pf*MDR-1	N1FP-Pfmdr86 N1RP-Pfmdr86	OF: 5′-AGGTTGAAAAAGAGTTGAAC-3′ NR: 5′-ATGACACCACAAACATAAAT-3′	86	95°C for 10min (94°C- 30sec; 55°C-30sec; 65°C-1min)x35 cycles; 72°C for 10min.
	N2FP-Pfmdr86 N2RP-Pfmdr86	OF: 5′-ACAAAAAGAGTACCGCTGAAT-3′ NR: 5′-AAACGCAAGTAATACATAAAGTC-3′	86	95°C for 10min (94°C-30sec; 55°C-30sec; 60°C-30sec; 65°C-1min)x30 cycles; 72°C for 10min.

NB: OF= outer forward, OR= outer reverse, NF= Nested forward, NR= Nested reverse



CHAPTER 4

Comparison of infectivity of *Plasmodium vivax* to wild-caught and laboratory-adapted (colonized) *Anopheles arabiensis* mosquitoes in Ethiopia

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Parasites Vectors (2020) 13:120 https://doi.org/10.1186/s13071-020-3998-2

Abstract

Background

Mosquito-feeding assays that assess transmission of Plasmodium from man-to-mosquito typically use laboratory mosquito colonies. The microbiome and genetic background of local mosquitoes may be different and influence Plasmodium transmission efficiency. In order to interpret transmission studies to the local epidemiology, it is therefore crucial to understand the relationship between infectivity in laboratory-adapted and local mosquitoes.

Methods

We assessed infectivity of *Plasmodium vivax*-infected patients from Adama, Ethiopia, using laboratory adapted (colony) and wild-caught (wild) mosquitoes raised from larval collections in paired feeding experiments. Feeding assays used 4–6 day-old female *Anopheles arabiensis* mosquitoes after starvation for 12 h (colony) and 18 h (wild). Oocyst development was assessed microscopically 7 days post-feeding. Wild mosquitoes were identified morphologically and confirmed by genotyping. Asexual parasites and gametocytes were quantified in donor blood by microscopy.

Results

In 36 paired experiments (25 *P. vivax* infections and 11 co-infections with *P. falciparum*), feeding efficiency was higher in colony (median: 62.5%; interquartile range, IQR: 47.0–79.0%) compared to wild mosquitoes (median: 27.8%; IQR: 17.0–38.0%; $Z = 5.02$; $P < 0.001$). *Plasmodium vivax* from infectious individuals (51.6%, 16/31) infected a median of 55.0% (IQR: 6.7–85.7%; range: 5.5–96.7%; $n = 14$) of the colony and 52.7% (IQR: 20.0–80.0%; range: 3.2–95.0%; $n = 14$) of the wild mosquitoes. A strong association ($\rho(16) = 0.819$; $P < 0.001$) was observed between the proportion of infected wild and colony mosquitoes. A positive association was detected between microscopically detected gametocytes and the proportion of infected colony ($\rho(31) = 0.452$; $P = 0.011$) and wild ($\rho(31) = 0.386$; $P = 0.032$) mosquitoes.

Conclusions

Infectivity assessments with colony and wild mosquitoes yielded similar infection results. This finding supports the use of colony mosquitoes for assessments of the infectious reservoir for malaria in this setting whilst acknowledging the importance of mosquito factors influencing sporogonic development of Plasmodium parasites.

Keywords: Wild mosquito, *Anopheles arabiensis*, *Plasmodium vivax*, Membrane-feeding, Infectivity, Relative permissiveness

Background

With the move towards malaria elimination and eradication, new tools and strategies to reduce onward transmission of *Plasmodium* infections, including transmission-blocking interventions (TBI), are considered highly beneficial [1, 2]. An increasing number of drug- and vaccine-based TBI are in the pipeline [3] and will require monitoring tools for efficacy. Additionally, it is considered highly beneficial to characterize the human infectious reservoir for malaria in low endemic settings approaching elimination, to better target and monitor TBI [4, 5]. Both TBI evaluation and infectious reservoir characterization require robust tools to measure human infectivity to mosquitoes. Mosquito-feeding assays can directly assess *Plasmodium* transmission from man-to-mosquitoes and play central role to estimate efficacy of TBI and the assessment of the infectious reservoir [6].

Mosquito-feeding assays allow mosquitoes to feed directly on skin of individuals (direct feeding) or on fresh human blood through an artificial membrane (membrane-feeding) [7], after which mosquito midguts are examined for parasite developmental stages (oocysts), the definitive proof that the mosquito became infected [6, 8]. Mosquito-feeding experiments are logistically demanding but increasingly used in field-based studies [5, 9-14]. Previous studies used mainly mosquitoes colonized in laboratories for several generations [5, 7, 9, 14-23]. Laboratory-adapted (colony) mosquitoes offer significant advantage over wild-caught (wild) mosquitoes in terms of logistics, ease of maintenance, flexibility of scaling-up and reproducibility of experiments [24]. However, colony mosquitoes may not fully reflect natural mosquito populations.

Maintenance of insects in artificial breeding conditions favors accumulation of traits that favor survival in the new environment, resulting in a change in genetic make-up over generations [25]. Parasite-mosquito combinations and their susceptibility to malaria infection are regulated at multiple steps during the development of the parasites [26] and numerous factors may modulate this interaction. These factors range from mosquito genetics [27, 28] and immune system [29] to parasite polymorphisms that allow evasion of the mosquito immune system [30]. Environmental factors such as midgut microbiota [31, 32], mosquito larval diet [33, 34], and temperature to support sporogony [35] are

also implicated. These findings emphasize that infectivity studies from colony mosquitoes might not represent the infectivity in natural settings and therefore, assessment of the relative permissiveness of colony and wild mosquitoes could assist in the interpretation of mosquito-feeding assays to the local context. In this study, the relative permissiveness to *Plasmodium vivax* infection of colony and wild *Anopheles arabiensis* mosquitoes was assessed in paired experiments.

Methods

Study site, immature mosquito stages collection and rearing

Data was collected from September 2018 to February 2019 in Adama, Ethiopia (formerly called Nazareth), a city located within the Great Rift Valley, with an average elevation of ~ 1624 meters above sea level. Extensive irrigation activities characterize the area surrounding Adama with an annual peak malaria transmission season occurring between September and November [5, 36]. Both *P. falciparum* and *P. vivax* are endemic; the latter contributes towards ~ 60% of the cases [5, 37].

Immature mosquito stages (larvae/pupae) were collected by standard dipping method from potential breeding sites located at ~ 35 km from the city, close to a hot spring resort (Sodere, 8°24′N, 39°23′E, at an altitude of 1360 meters above sea level) [38]. The breeding habitat is located at a publicly accessible site where there are temporary/permanent puddles made of rock pool or pools in a grassy area (**Additional file 1: Figure S1**) emanating from a natural hot spring sources which exist through- out the year and form a marshy area. The collected larvae, transported in plastic jars to the field laboratory, were maintained in plastic trays in the original water collected from the breeding sites and provided with fish food (Cichlid Sticks; Tetra, Maidenhead Aquatics, Leicester, UK). Pupae were picked in glass beakers containing sedimented water from the breeding sites and kept in cages until emergence to adults. Adult female *An. arabiensis* mosquitoes were identified morphologically using standard keys [39, 40]. Colony mosquitoes (> 800th generation) were reared to adulthood as described previously [5]. Mosquitoes of both sources were maintained at the same laboratory settings; developmental stages were reared using fish food (Cichlid Sticks, Tetra) and adult mosquitoes were maintained on sucrose solution (10%) at ambient conditions at temperatures of 26–30 °C and a relative humidity of 60–80% before and after feeding.

Membrane-feeding assays

Venous blood samples (5 ml) were collected after obtaining informed written consent from patients with micros- copy-confirmed *P. vivax* infection attending the Adama Malaria Clinic. Blood collected in lithium heparin tubes (Vacutainer; BD, Oxford, UK) was offered to colony and wild *An. arabiensis* mosquitoes in parallel using membrane-feeding apparatus as detailed previously [7]. Briefly, 5–6 day-old female mosquitoes were starved for 12 h (colony) and 18 h (wild) before feeding. This timing was decided upon following pilot experiments where aggressiveness was unfavorable for wild mosquitoes after 12 h starvation. We have observed a positive association between starvation time and feeding efficiency ($\rho_{(41)}$ = 0.352; P = 0.024); 18 h was considered appropriate for wild-caught mosquitoes with sufficient numbers of fully fed mosquitoes and minimal mortality. Feeding was performed in the dark for 25 min using water-jacketed glass-feeders (mini-feeder; Coelen Glastechniek, Arnemuiden, the Netherlands) that were covered with an artificial membrane (parafilm) and connected to a circulating water bath (Julabo GmbH; Seelbach, Germany) maintained at 38°C. Unfed and partially fed mosquitoes were removed from the holding cages, leaving fully-fed mosquitoes undisturbed. Fully-fed mosquitoes were maintained for 7 days under the same laboratory condition using 10% sucrose solution. At least 10 mosquitoes were dissected, and oocyst presence was assessed microscopically after staining with 1.0% mercurochrome (Sigma-Aldrich, Taufkirchen, Germany). This minimum number was mainly determined by the feeding efficiency and availability of wild mosquitoes. Asexual parasite and gametocyte densities were quantified in thick blood films, screening against 1000 leukocytes.

Mosquito genotyping

A representative set of wild and colony mosquitoes were genotyped using multiplex polymerase chain reaction targeting the intergenic spacer gene of the ribosomal DNA of all cryptic species in the *An. gambiae* complex as described previously [41], with a few modifications. All conditions, including primers, were as per the original protocol except that the $MgCl_2$ concentration was increased to 2mM and the amplification time (at 72°C) was raised to 40s. Two microliters of eluate of whole mosquito body crushed in phosphate buffer saline was run in a final reaction volume of 25 µL without prior DNA extraction. In every reaction round, negative (non-template and *An. stephensi* mosquitoes) and positive controls (*An. arabiensis* colony mosquitoes) were included.

Statistical analysis

All analyses were performed in STATA version 13 (Stata- Corp., TX, USA) and GraphPad Prism 5.3 (GraphPad Software Inc., CA, USA). Feeding efficiency (proportion of fully-fed mosquitoes) was compared in matched experiments using the Wilcoxon matched-pairs signed- rank test. Proportions were compared by Chi-square and Fischer's exact tests. Differences between median parasite densities between single-species infections and co-infections were assessed using Wilcoxon rank-sum test. The bias between wild and colony mosquitoes was compared using the Bland-Altman test. The correlation between mosquito infection prevalence and gametocyte density as continuous variable was determined by Spearman's rank correlation coefficient for colony and wild mosquitoes separately. Logistic regression was performed to compare infection status between colony and wild mosquitoes using individual mosquito data. A fixed effect for human participant was included thus taking into account the number of mosquito experiments and adjusting for correlations between mosquito observations from the same blood donor.

Results

A total of 36 matched membrane-feeding assays (MFA) with colony and wild mosquitoes were performed on blood samples from patients (25 *P. vivax* single- and 11 mixed-species infections with *P. falciparum*). The median age of the patients was 23.5 years (interquartile range, IQR: 18.0–29.5) and the majority of participants were male (72.2%, 26/36) (**Table 1**). A total of 1755 colony and 2303 wild mosquitoes were used for feeding experiments of which 1035 (59.0%) and 662 (28.7%) successfully took a blood meal, respectively. Feeding efficiency varied between colony (median: 62.5%; IQR: 47.0–79.0%) and wild (median: 27.8%; IQR: 17.0–38.0%; $Z = 5.02$; $P < 0.001$) mosquitoes (**Fig. 1a**). Of the total feeding experiments, 52.8% (19/36) infected at least one colony and/or wild mosquitoes. Of *P. vivax* single-species infected patients, 64.0% (16/25) were infectious to mosquitoes while 27.3% (3/11) of co-infected (*P. falciparum* + *P. vivax*) patients infected at least one mosquito (odds ratio, OR: 4.74; 95% confidence interval, CI: 1.0–22.5; $P = 0.04$). Parasite and gametocyte densities were highest in *P. vivax* infections (**Table 1**).

Table 1 . Comparison between *P. vivax* single species and mixed species infections with *P. falciparum*

	***P. vivax* single-species infections**[a]	***P. vivax* + *P. falciparum* co-infections**[a]	
N	**25**	**11**	**36**
Sex, % male (*n*/*N*)	68.0 (17/25)	81.8 (9/11)	72.2 (26/36)
Age, years	23 (20–29)	25 (12–30)	23.5 (18.0–29.5)
P. vivax asexual parasite density	10322.5 (2847.0–181,169.0)	5540.5 (1944.5–37,900.5)	8740.8 (2173.5–24,185.3)
P. vivax gametocyte density[b]	332.3 (133.0–605.5)	368.3 (228.5–623.8)	358.8 (133.0–605.5)
Feeding rate, colony mosquitoes	63.0 (47.0–80.0)	62.0 (44.0–78.0)	62.5 (47.0–79.0)
Feeding rate, wild mosquitoes	29.0 (16.0–38.0)	25.0 (18.0–38.2)	27.8 (17.0–38.0)

[a] All values indicated, except sex, are median (interquartile range)
[b] Indicated only among gametocyte carriers

After excluding 5 matched experiments for which fewer than 10 wild mosquitoes were available for dissection, there were 31 (21 *P. vivax* single- and 10 co-infections) successful matched feeding experiments with a minimum of 10 dissected mosquitoes for both feeding approaches. In total, 66.7% (14/21) of *P. vivax* single- species infected patients infected at least one mosquito. Two patients infected either colony or wild mosquitoes; one of them infecting only colony mosquitoes but not wild (5.8% infected mosquitoes, 4/69) and *vice versa* (6.7%, 1/15). Infectious individuals infected a median of 55.0% (IQR: 6.7 – 85.7; range: 5.5–96.7%; $n = 14$) colony and 52.7% (IQR: 20.0–80.0%; range: 3.2–95.0%; $n = 14$) wild mosquitoes (**Fig. 1b**). The two infectious co-infected patients infected either of the colony or wild mosquitoes; one infected 5.5% (1/18) colony and the other infected 3.2% (1/32) wild mosquitoes.

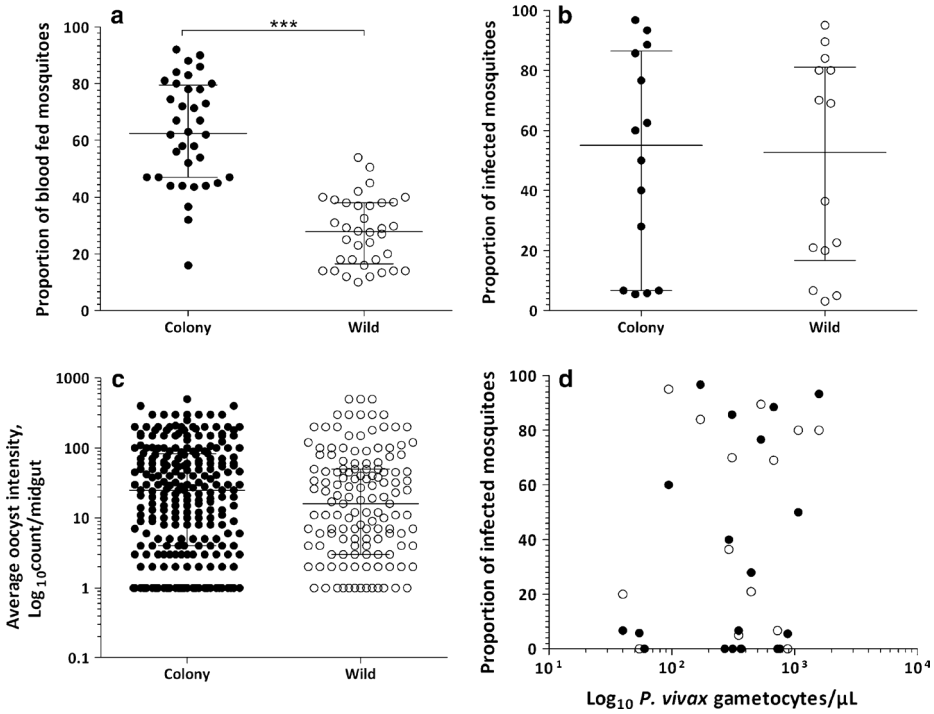

Fig. 1. Mosquito infection outcomes in matched colony and wild *An. arabiensis* membrane-feeding experiments. The proportion of mosquitoes that were fully-fed on the patient blood through the membrane feeder **(a)** with the resulting proportion of infected mosquitoes **(b)** are indicated together with the $\log_{10}$-transformed oocyst intensity per midgut of infected mosquito **(c)** for colony (filled dots) and wild-caught (unfilled dots) mosquitoes. **(d)** The association between the proportion of infected mosquitoes (Y-axis) and $\log_{10}$-transformed gametocyte densities/µl (X-axis) measured by microscopy for colony (filled dots) and wild-caught (unfilled dots) mosquitoes. Lines in **a**–**c** indicate median values and 25th and 75th percentiles. The asterisks in **a** indicate a statistically significant difference between the wild and colony mosquitoes ($P < 0.001$).

The median proportions of infected colony and wild mosquitoes were not different between the matched experiments (Z = 0.785; $P = 0.433$; **Fig. 1b**). A strong association was observed between the proportion of infected wild and colony mosquitoes ($\rho_{(16)} = 0.819$; $P < 0.001$; **Fig. 2a**). Overall, there was good agreement in the likelihood of becoming infected between wild and colony-reared mosquitoes. Estimation of infectivity to mosquitoes in MFAs showed no significant bias towards either mosquito source (average bias:– 4.79; 95% limits of agreement: – 40.79–31.22; $P = 0.381$; **Fig. 2b**).

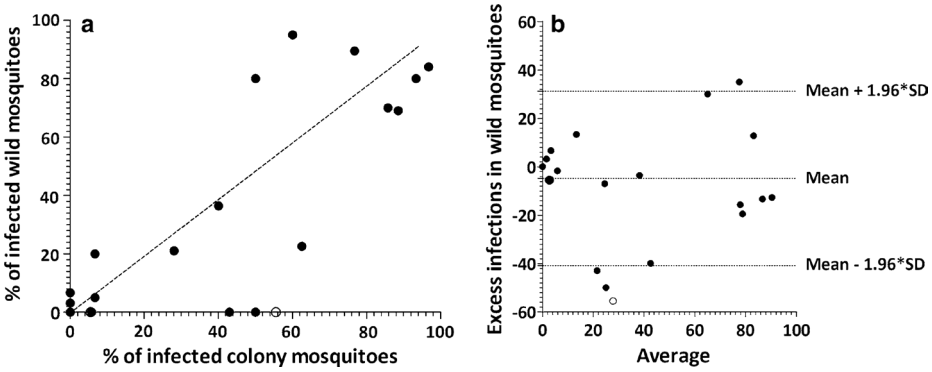

Fig. 2. Comparison of the proportion of infected colony *vs* wild mosquitoes. **A)** The proportion of infected wild mosquitoes (Y-axis) is plotted against colony mosquitoes (X-axis) for *P. vivax* single-species infections with at least 10 mosquitoes dissected. The dotted line is the line of perfect agreement. **B)** The differences between the proportion of infected colony and wild mosquitoes plotted against the averages of the two mosquito sources. The average of the proportion of infected colony and wild mosquitoes for each paired infection is indicated in the X-axis *vs* excess infections in wild mosquitoes (differences between proportions of infected wild mosquitoes *vs* colony mosquitoes) in the Y-axis. The limits of agreement are indicated as the mean difference (middle dotted line) and the 95% confidence interval of the limit of agreement (mean ± 1.96 SD of differences) with horizontal dotted lines. Unfilled dots indicate *P. falciparum* + *P. vivax* co-infections.

Similarly, there was no difference in the median number of oocysts (Z =209; P= 0.835; **Fig. 1c**) detected in infected midguts between the colony (median: 25.0 oocysts/midgut; IQR: 5.0–83.0) and wild (median: 20.5 oocysts/midgut; IQR: 5–47) mosquitoes. In an analysis of individual mosquito data adjusted for human participant, we observed a borderline significant lower proportion of infected wild mosquitoes (OR: 0.67; 95% CI: 0.45–1.00; P= 0.051); plausibly reflecting differences in the number of mosquito observations in experiments.

Microscopically detected parasite density (median: 8765.5; IQR: 2199.5–26158.0; n = 31) was not different between patients with *P. vivax* single-species infections and patients with co-infections ($U_{(31)}$ =–103.5; Z=–0.085; P = 0.933). Gametocytes were more frequently detected by microscopy in patients with *P. vivax* single-species infections (81.0%, 17/21) than in patients with *P. vivax* + *P. falciparum* co- infections (30.0%, 3/10; OR, 9.9; 95% CI, 1.75–56.30; P = 0.010) with a borderline significantly higher gametocyte density among gametocyte-positive *P. vivax* single-species infections compared to gametocyte-positive co-infections

($U_{(31)}$=– 149.5; Z = 1.902; P = 0.057). In co-infections, all microscopy- detected gametocytes were *P. vivax*. Microscopically detectable gametocyte carriers were more infectious than patients without microscopically detectable gametocytes to both colony (65.0%, 13/20 *vs* 9.1%, 1/11; OR: 18.6; 95% CI: 2.0–176.5; P = 0.002) and wild mosquitoes (60.0%, 12/20 *vs* 18.2%, 2/11; OR: 6.8; 95% CI: 1.1–39.8; P=0.021). The proportion of infected colony ($\rho_{(31)}$ = 0.452; P = 0.011) and wild ($\rho_{(31)}$ = 0.386; P = 0.032) mosquitoes associated positively with gametocyte density (Fig. 1d) but not with parasite density assessed by microscopy ($\rho_{(31)}$ = 0.044; P = 0.816) and ($\rho_{(31)}$ = 0.239; P = 0.195), respectively. Morphologically identified wild mosquitoes were confirmed to be *An. arabiensis* using species- specific PCR for the vast majority of tested mosquitoes (96.5%; 55/57).

Discussion

In recent years, there is increasing interest in transmission assays to evaluate TBI and assess the human infectious reservoir for malaria. More and more laboratories are establishing mosquito colonies to examine infectivity among natural infections [42]. Whilst established colonies offer some advantage in terms of feeding efficiency [43], it is generally assumed that locally relevant mosquitoes are important to allow inference to the local transmission situation. We evaluated the permissiveness of An*. arabiensis* mosquitoes raised from wild-collected larvae in comparison with colony mosquitoes maintained for over 800 generations in 36 paired MFA. Whilst mosquito feeding rates were markedly higher in colony mosquitoes, we found no evidence for epidemiologically meaningful differences in infection prevalence or infection burden between mosquito sources.

In our experiments, we encountered challenges with the aggressiveness of wild mosquitoes, exemplified by roughly two-fold lower feeding rates on the membrane for wild *versus* colony mosquitoes, which is not surprising given the selection over several hundred generations in the latter. Colony mosquitoes were maintained using rabbits as source of blood for generations in the present study. Fewer mosquito observations were available for wild mosquitoes on the day of dissection for some of the infections. This may have contributed to the borderline higher proportion of MFA resulting in at least one infected colony mosquito, simply reflecting the higher number of mosquito observations [7]. Despite this, we observed a similar proportion of infected mosquitoes among infectious feeds between colony and wild mosquitoes, in line with several

other studies [22, 44]. This holds true when mosquitoes were of the same [24] or different [44] species. The F1 progeny of wild-caught *An. funestus* compared with colonized *An. coluzzii* mosquitoes [44] and similarly, colonized *An. stephensi* mosquitoes compared with their field counterpart raised from wild-caught larvae and pupae [24] were equally susceptible, when the end point was oocyst detection in the midgut.

Importantly, oocyst density was high and similar between the colony and wild mosquitoes in our study in line with previous studies on *P. vivax* that used mosquitoes of different species [11, 12, 45]. Lower oocyst densities are typically observed in *P. falciparum* [46-48]. Earlier studies also examined sporozoite prevalence and load in feeding experiments; most reporting similar levels between colonized and wild mosquitoes [15]. Similar prevalence but higher sporozoite density (but only at higher sporozoite loads) was detected in the wild mosquitoes in one of the studies [24]. Given the strong association between oocyst prevalence and intensity [49] and the strong association between oocyst density and sporozoite densities [6, 8, 50], it seems intuitive that highly similar oocyst burden, as observed in our study, precludes large differences in sporozoite density.

Furthermore, variations in insectary and natural conditions that allow sporogony might potentially explain some of the differences observed [15, 24]. Mosquito innate immune responses can abrogate infections through melanization [51]. We have not observed any evidence for melanization in the present study. In addition, we also examined mosquito guts for pathogens that may influence parasite development such as microsporidia [43] and found no evidence for this. Future studies may nevertheless benefit from examining sporozoites, a limitation of the present study. Investigation of effects of environmental factors on sporogony with a specific focus on midgut microbiota that can influence transmission efficiency by stimulating the mosquito innate immune system and production of metabolites directly impairing parasite survival will also be informative [32]. In addition, mosquito blood-meal size, a poorly studied parameter that may be higher in colony and membrane-adapted mosquitoes, needs to be considered in future evaluations. We have reared wild collected and colony developmental stages to adults at the same laboratory conditions using the same larval food to minimize the chance this could contribute to a larger body size [52] and subsequently to higher oocyst prevalence and density as a function of larger volume of blood ingested (and therefore more gametocytes) [53, 54]. Future studies would benefit by including wing length measurement as an indication of mosquito body size.

To the best of our knowledge, our findings are the first of its kind with African *vivax* malaria which is commonly referred to as a major cause of malaria outside sub-Saharan Africa [55]. Ethiopia forms an exception with *vivax* malaria, contributing towards three-quarters of the global burden together with India and Pakistan [56]. One of the unique features of *P. vivax* is the earlier generation of gametocytes, i.e. within 3–4 days after the first appearance of asexual parasites [57]. As a result, most patients start infecting mosquitoes before the onset of symptoms [58]. Despite a limited number of studies reporting a lack of association between microscopically determined gametocyte density and infectivity to mosquitoes [59], a very strong association was observed in the likelihood of infectivity between gametocyte densities and both colony and wild mosquitoes in our study. This is concordant with previous reports that used colonized *An. dirus* [9] and *An. arabiensis* mosquitoes [5] as well as *An. stephensi* [60] and *An. darligi* wild mosquitoes [61] raised from wild-collected immature stages and F1 generations, respectively.

One relevant limitation of our study was the limited sample size, relying on 36 blood donors but a total of 1755 colony and 2303 wild mosquitoes were used for the feeding experiments. We can thus not rule-out subtle differences between colony and wild mosquitoes. It would, however, be questionable whether small differences would render colony mosquitoes less suitable for assessments of the human infectious reservoir or the evaluation of interventions.

Conclusions

The results of the present study indicate that colony mosquitoes perform at a similar level with mosquitoes caught from the wild that reflect the natural phenomenon, indicating colony mosquitoes can be used interchangeably. Our understanding of malaria transmission dynamics would benefit from similar studies in different settings with different vector and parasite species combinations, with a specific focus on mosquito determinants affecting sporogonic development.

Abbreviations

TBI: transmission-blocking interventions; MFA: membrane feeding assay.

Acknowledgements

We would like to acknowledge the study participants for their willingness to donate blood samples and the microscopists (Tewabech Lema and Tsehay Orlando) at Adama Malaria Center for their support. We would like to thank the malaria research team at Armauer Hansen Research Institute (Surafel K. Tebeje, Daniel Abebe Mekonnen, Tizita Tsegaye, Tadele Emiru, Tiruwork Fanta and Senya Asfer Sabir) and colleagues from Adama Regional Laboratory (Bayissa Bekele Binagdie, Henok Tadesse Taye and Teshome Bacha) for their continued support. Appreciation also goes to the regional and district health officers for their collaboration. The drivers from AHRI played an important role in making the study successful.

Authors' contributions

FGT, EG, HM, BP, CD and TB conceived the study, contributed to data analysis and critically commented on the manuscript. FGT, JB and TB analyzed the data. FGT drafted the manuscript. FGT, WC, TA, EH, DY and KL conducted the laboratory study. WC, TA, EH, AG, TT, EE, GS, DY and SWB collected the immature stages (larvae and pupae), reared adult mosquitoes, collected blood samples, performed the feeding experiments, dissected mosquitoes, and analyzed samples. All authors read and approved the final manuscript.

Funding

The study was supported by the Bill and Melinda Gates Foundation grant (INDIE OPP1173572) to FGT, TB and CD. The Armauer Hansen Research Insti tute (AHRI) has supported WC, TA, EH and EG through its Core funding from Sida and Norad.

Availability of data and materials

Data supporting the conclusions of this article are provided within the article and its additional file. Raw data will be made available upon request.

Ethics approval and consent to participate

The study protocol was reviewed and approved by the National Research Ethical Review Committee (3.10|016\20), Institutional Ethical Review Board of the College of Natural and Computational Sciences of Addis Ababa University, Addis Ababa, Ethiopia, the Observational/Interventions Research Ethics Committee of the London School of Hygiene and Tropical Medicine (15811), London, UK, and AHRI/ALERT Ethics Review Committee, Addis Ababa, Ethiopia (Ref.No.SOM/DRERC/BCH005/2009, PO24/17). All participants provided written informed consent; parent/legal guardians provided consent for participants younger than 18 years.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

Additional file 1:

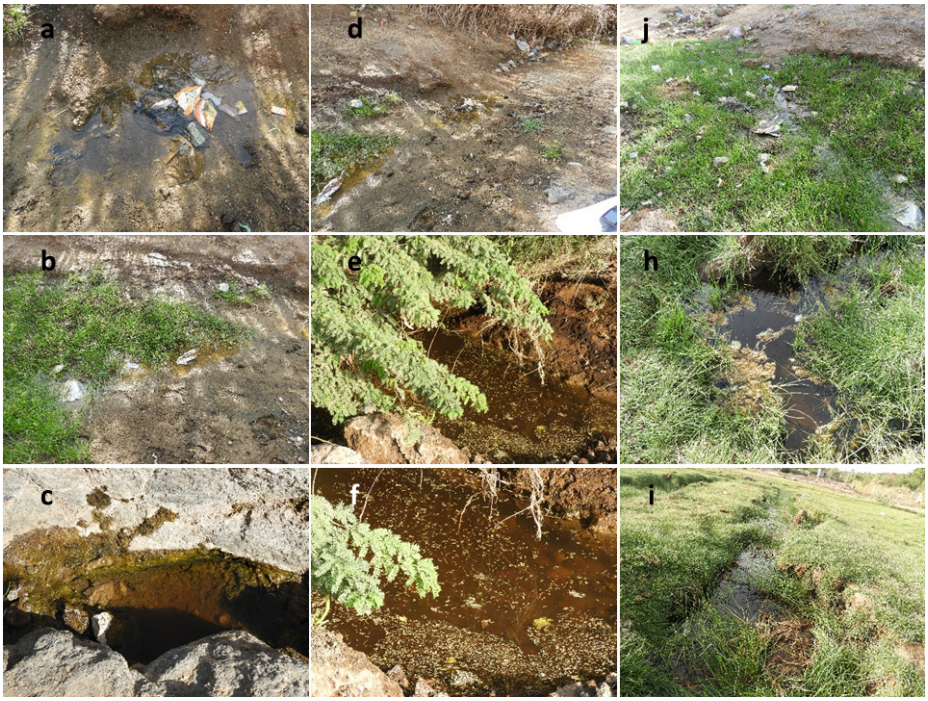
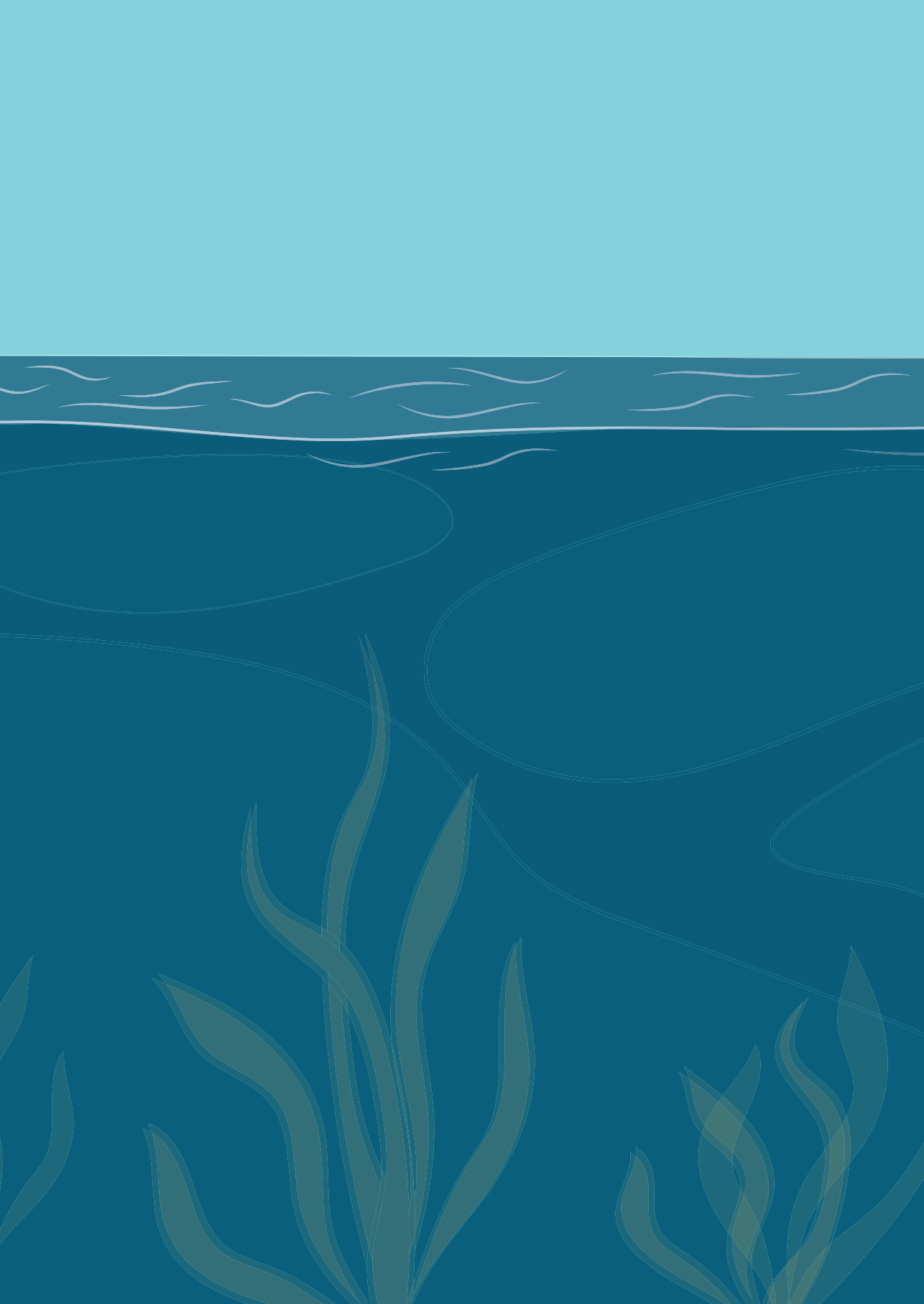


Figure S1. Larva and/or pupa collection habitats. Breeding habitats were mainly temporary/permanent puddles (**a–f**) or marshy (**g–h**) areas, following the streamline of a local hot spring. All the potential breeding habitats were not in use by people living close-by (within a radius of 300–500 m) and had no shading. Larvae were detected at all potential breeding sites with an average larval density of 19.5 larvae per dip. Pupae were detected at 4/9 sites where larvae were detected during a single visit. The median volume of the breeding habitat was 0.20 m3 (IQR: 0.08–0.57 m3; range: 0.004–7.50 m3).



CHAPTER 5

Dynamics of asymptomatic *Plasmodium falciparum* and *Plasmodium vivax* infections and their infectiousness to mosquitoes in a low transmission setting of Ethiopia: a longitudinal observational study

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International Journal of Infectious Diseases (https://doi.org/10.1016/j.ijid.2024.107010)

Abstract

Objective

A 15-month longitudinal study was conducted to determine the duration and infectivity of asymptomatic qPCR-detected *P. falciparum* and *P. vivax* infections in Ethiopia.

Method

Total parasite and gametocyte kinetics were determined by molecular methods; infectivity to *Anopheles arabiensis* mosquitoes by repeated membrane feeding assays. Infectivity results were contrasted with passively recruited symptomatic malaria cases.

Results

For *P. falciparum* and *P. vivax* infections detected at enrolment, median durations of infection were 37 days (95% confidence interval [CI], 15-93) and 60 days (95% CI, 18-213), respectively. *P. falciparum* and *P. vivax* parasite densities declined over the course of infections. From 47 feeding assays on 22 asymptomatic *P. falciparum* infections, 6.4% (3/47) were infectious and these infected 1.8% (29/1579) of mosquitoes. No transmission was observed in feeding assays on asymptomatic *P. vivax* mono-infections (0/56); one mixed-species infection was highly infectious. Among the symptomatic cases, 4.3% (2/47) of *P. falciparum* and 73.3% (53/86) of *P. vivax* patients were infectious to mosquitoes.

Conclusion

The majority of asymptomatic infections were of short duration and low parasite density. Only a minority of asymptomatic individuals were infectious to mosquitoes. This contrasts with earlier findings and is plausibly due to the low parasite densities in this population.

Keywords: asymptomatic, longitudinal, membrane feeding assay, infectiousness, transmission, *P. falciparum*, *P. vivax*

Introduction

Recent progress in malaria control has stalled in many endemic settings and gains are threatened by emerging biological, social and economic challenges [1]. Despite challenges, malaria control in Ethiopia remained on track with an aspirational target for elimination in selected low transmission settings by 2030 [2]. There is increased awareness of the widespread presence of *Plasmodium* infections that neither elicit acute symptoms nor treatment seeking behavior in low transmission settings [3-5]. Whether these asymptomatic infections form a hurdle for malaria elimination depends on their relative importance for sustaining malaria transmission [4, 6], which is in turn dependent on infection duration and the production of transmissible gametocytes [3]. The duration of asymptomatic infections varies from days [7, 8] to months or even years [9]. Notable differences were observed in infection dynamics and duration of infections between *P. falciparum* and *P. vivax* infections [10]. In natural infections, *P. vivax* has lower peripheral blood parasitemia density compared to *P. falciparum* [11, 12] due to its strong preference to infect reticulocytes [13] and rapid acquisition of immunity [14]. Uniquely, *P. vivax* has liver stage hypnozoites that can cause relapses within weeks to months attributed to over 80% of blood stage infections and sustaining transmission [15]. Both species show marked fluctuations in parasite and gametocyte densities over the course of infections [3, 6, 10].

Gametocytes are typically highly prevalent in asymptomatic infections and can be detected with high sensitivity by molecular methods that target gametocyte-specific mRNA transcripts [11, 16]. Gametocyte density is positively associated with the asexual parasite biomass [5, 11] but this association is weaker for *P. falciparum* where gametocytes develop over a prolonged 10-12 day period [17] than *P. vivax* where gametocytes appear within 2-3 days [11]. Importantly, there is considerable debate about the relative infectivity of asymptomatic infections and especially low-density asymptomatic infections as these predominate in malaria endemic populations. The likelihood of mosquito infection increases rapidly when densities in the human blood exceed ~5 gametocytes/μL for *P. falciparum* [18] or ~100 *Pvs25* gametocyte transcripts/μL for *P. vivax* [19]. A study in Africa estimated that >95% of all *P. falciparum* mosquito infections find their source in asymptomatic infections [6]; ~15% of mosquito infections may be attributed to submicroscopic parasite carriage in humans [4, 20], while a study from Southeast Asia reported that symptomatic infections with high gametocytemia are major drivers of transmission [21]. For *P. vivax*, studies on the human infectious reservoir give similarly contradicting

results on the transmission importance of asymptomatic versus symptomatic infections [4, 19, 21]. From a public-health perspective, it is important to understand the duration, detectability and transmission-potential of asymptomatic infections.

Here, we examined infection duration and gametocyte production in longitudinally monitored asymptomatic *P. falciparum* and *P. vivax* infections in a low-endemic setting where both species are co-endemic in Ethiopia. The transmissibility of infections to *Anopheles arabiensis* mosquitoes (a locally relevant vector) was investigated and contrasted with that of symptomatic malaria patients recruited from the same setting and period.

Methods and materials

Study area and participant selection

A longitudinal, observational study was conducted from September 2018 – March 2020 in Adama district, Ethiopia. The district is characterized by low and seasonal malaria transmission following the long and primary (mid-June to mid-September) and short and sporadic (February to May) rainy seasons [22]. Both *P. falciparum* and *P. vivax* are endemic in the district [4]. Following community meetings, inhabitants of the villages Batu Degaga, Hurufa Kurfa, Mermersa, Dibibisa, Dongore Furda and Guraja Furda were invited for screening for *P. falciparum* and *P. vivax* infections by 18S based quantitative PCR (qPCR). Individuals were eligible for participation if they were parasite positive, > 2 years old, permanent residents of the area, had no measured fever (axillary temperature < 37.4°C) or reported fever in the past 48 hours, and no known chronic illness and/or acute illness requiring immediate clinical care. Eligible participants or, in case of minors, parents/guardians provided written informed consent; additional assent was obtained for participants aged 12-17 years.

Participant recruitment, blood sampling and follow-up

Individuals who tested positive for either *P. falciparum* or *P. vivax* parasites were enrolled and followed twice weekly for 2.5 months and monthly for the subsequent 12 months. Finger prick blood samples (~0.3mL) were collected in microtainer EDTA tubes (K2E EDTA Vacutainers, BD) on all days except days 3, 18, 33, 48 and 63 when additional venous blood samples (5mL) were collected using Heparin tubes (Lithium Heparin Vacutainers, BD) for mosquito feeding assays (MFA). A separate population of symptomatic malaria cases

with measured fever (axillary temperature ≥ 37.5°C) or history of fever and microscopy-confirmed *P. vivax* and *P. falciparum* infections were passively recruited at Adama malaria control center. Symptomatic cases provided a single venous blood sample, after which they received treatment following the national malaria treatment guidelines [23].

Parasite quantification

Parasite and gametocyte prevalence was determined by microscopy by screening 100 microscopic fields. Parasite quantification was done by molecular methods following total nucleic acid extraction by MagNAPure LC automated extractor (Total Nucleic Acid Isolation Kit High Performance; Roche Applied Sciences, Indianapolis, IN, USA) from 100µL whole blood stored in RNAProtect (Qiagen cell reagent). *P. falciparum* and *P. vivax* parasites were quantified by quantitative PCR (qPCR) targeting the 18S small rRNA subunit gene [24, 25]. Gene copies were translated to parasite densities by assuming 5 and 3 gene copies per *P. falciparum* and *P. vivax* parasite, respectively. When assessing parasite clearance or the duration of infection, all qPCR positive signals were considered true positives, acknowledging that parasite densities may fluctuate around the threshold density for detection [6]. To classify as a new infection during follow-up and avoid over-interpreting very low qPCR signals, estimated parasite densities had to exceed 0.01 parasite/µL to be considered positive [5]. Gametocyte density was quantified in *P. falciparum* 18S positive samples by quantitative reverse transcriptase PCR (qRT-PCR) targeting the male *PfMGET* and female *CCp4* gametocyte mRNA transcripts [26]; *Pvs25* mRNA transcripts were quantified as transcripts/µL as an indicator of female *P. vivax* gametocyte density [16]. Primer and probe sequences are described elsewhere [6].

Mosquito infectivity assessment

MFA using locally reared *An. arabiensis* mosquitoes were performed as described previously [4]. MFA were conducted at enrolment for clinical malaria cases (prior to treatment), and on days 3, 18, 33, 48 and 63 for asymptomatic parasite carriers. Briefly, three aliquots of ~400µL whole blood collected in Lithium Heparin tubes (Lithium Heparin Vacutainers, BD) were fed to 3 cups with ~40 female mosquitoes (~120 mosquitoes per experiment) that were 4 - 7 days old, using water jacketed glass feeders maintained at 37°C using a circulating water bath. Fully fed mosquitoes were selected and maintained on 10% sucrose solution at 26°C + 2 and 60% + 10 humidity until dissection on day 7-10. Following staining in 1% mercurochrome, mosquito guts were examined by two microscopists to detect and quantify oocysts.

Statistical analysis

The association between parasite density and gametocyte density was determined on a log10 scale using Pearson's correlation. We estimated, using the Kaplan-Meier method, the proportion of individuals that remained infected over time. This is different from the observed proportions, in that the observed proportions exclude right-censored observations entirely after the time in which they are right-censored. These right-censored observations were infections that did not clear by the end of their follow-up. The gametocyte fraction was defined as the proportion of the total parasite biomass of parasites that was gametocyte for *P. falciparum* and the number of *Pvs25* mRNA copies per parasite for *P. vivax*. The trajectories of parasite density, gametocyte density, and gametocyte fraction over time were modelled using the generalized additive mixed effects models [17] where random intercepts were used for each individual to account for intra individual correlations. The slope, indicative of a decline in densities over time, was tested by running linear models with log10 parasite densities. Full details and mathematical description are described in [5]. R (v 4.2.2) and Rstudio (v 2023.03.1) with packages survival, survminer, and mgcv were used for the statistical analyses.

Results

A total of 2373 individuals were screened between September 2018 and March 2020. Microscopy detected *P. falciparum* in 0.47% (10/2107), *P. vivax* in 0.14% (3/2107) and mixed-species infections in 0.05% (1/2107) of the population. By qPCR, infection prevalence was 3.5% (83/2373) for *P. falciparum,* 4.0% (95/2373) for *P. vivax* and 0.08% (2/2373) for mixed-species infections. Seventy individuals with *P. falciparum* (n=35), *P. vivax* (n=34) or mixed-species infection (n=1) were enrolled into the longitudinal study; for the other 92 parasite positive individuals, follow-up was not possible due to COVID-19 lockdown measures. At enrolment, geometric mean parasite densities were 0.96 (95% confidence interval [CI], 0.33-2.75) parasites/μL for *P. falciparum* and 1.04 (95% CI, 0.54-1.99) parasites/μL for *P. vivax* mono-infections (**Table 1**).

Table 1. Baseline characteristics of study participants

Attributes	*P. falciparum*	*P. vivax*	Mixed (*P. falciparum* and *P. vivax*)
N	**35**	**34**	**1**
Sex, % male (n/N)	68.6 (24/35)	52.9 (18/34)	0.0 (0/1)
Age, median (IQR)	22 (12-40)	15 (9-28)	23
% microscopy positive	18.2 (6/33)	3.3 (1/30)	0.0 (0/1)
P. falciparum parasite density/µL, GM (95% CI)	0.96 (0.33-2.75)	-	1.54 (-)
P. vivax parasite density/ µL, GM (95% CI)	-	1.04 (0.54 -1.99)	1.74 (-)

IQR= interquartile range (25th - 75th percentile); GM = geometric mean; 95% CI=95% confidence interval.

Parasite persistence and densities during asymptomatic *P. falciparum* and *P. vivax* infections

The 70 participants in the longitudinal study contributed a total of 835 observations (median 12; interquartile range (IQR), 3-18 observations per individual) after enrollment. For 18 individuals (13 *P. falciparum* and 5 *P. vivax*), their infection was resolved immediately and no subsequent parasite positive samples were observed despite initially being parasite positive. 12 individuals (7 *P. falciparum* and 5 *P. vivax*) became symptomatic with the pre-symptomatic period ranging from 7-243 days for *P. falciparum* and 3-243 days for *P. vivax* and received treatment by the study team. At baseline, only one of the *P. vivax* infected individuals was microscopy positive; 18.2% (6/33) of individuals with submicroscopic qPCR-detected infections at enrolment became microscopy positive during follow-up. From all *P. vivax* infected individuals, 38.2% (13/34) had qPCR-detected *P. falciparum* infections at least once during follow-up (**Supplementary Table 1**). Among participants recruited with asymptomatic *P. falciparum* infections six were microscopy detectable at baseline. Of the remaining 29 qPCR-detected-submicroscopic infections at baseline, 31.0% (9/29) became microscopically detectable for *P. falciparum* during follow-up. Importantly, 14.2% (5/35) of individuals who were initially positive for *P. falciparum* later became positive for *P. vivax* on at least one time point during the follow-up (**Supplementary Table 1**).

When we only included individuals who were *P. falciparum* parasite positive at enrolment and accepted low qPCR signals as evidence for persisting infections, 21.7% of infections (95% CI, 10.3%- 46.0%) persisted for ≥3 months and the median duration of infection was 37 days (95% CI, 15-93 days) (**Figure 1**). When

we also included *P. falciparum* infections that were acquired during follow-up (giving a total of 54 *P. falciparum* infections), the median duration of infection was 18 days (95% CI, 11–49 days). For *P. vivax*, including only infections that were present at baseline resulted in 39.8% (95% CI, 25.5%-61.9%) of infections persisting for ≥3 months and an estimated median duration of blood-stage infection of 60 days (95% CI, 18-213) (**Figure 1**). Including all infections (n=43) gave a median infection duration of 45 days (95% CI, 22-123 days) (**Figure S1**). A log-rank test showed no statistically significant difference in the Kaplan-Meier curves when restricting to infections detected at baseline (P=0.23) or including all infections (P=0.12). Parasite densities at enrolment were variable and we observed faster clearance for infections with low parasite density at enrolment compared to infections with higher parasite densities, a pattern that was statistically significant for *P. falciparum* (P<0.0001) but not for *P. vivax* (P=0.24; **Figure S2**).

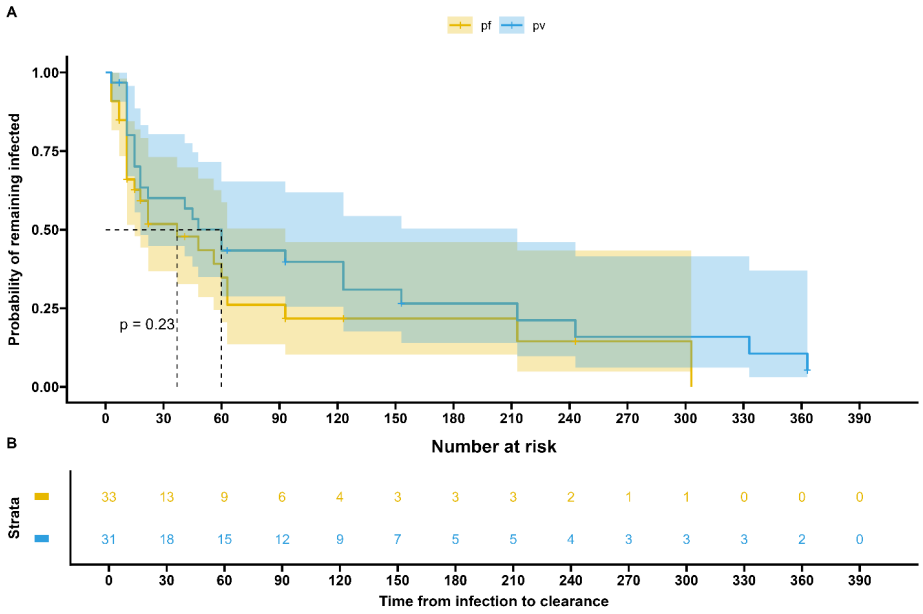

Figure 1. Kaplan-Meier plot showing the probability of remaining infected over time. Probability of remaining infected **(A)** is shown with numbers remaining infected **(B)**. Indicated on the X-axes are days since recruitment in the study. pf, *P. falciparum*; pv, *P. vivax*; n. censor, number censored. From a total of 33 *P. falciparum* infections that were present at baseline, 24 were observed to clear (9 right-censored); From a total of 31 *P. vivax* infections that were present at baseline, 25 were observed to clear (6 right-censored). A log-rank test showed no significant difference in the Kaplan-Meier curves (P=0.23).

P. falciparum gametocytes were detected by qRT-PCR in 62.0% (160/258) of *P. falciparum* qPCR positive samples; gametocyte prevalence by microscopy was only 10.5% (27/258) in this population. *P. falciparum* total parasite density was positively associated with concurrent gametocyte density (r=0.71, P<0.001; **Figure S3 A**). *P. vivax* gametocytes were detected by qRT-PCR in 75.0% (213/284) of qPCR positive samples and by microscopy in 1.1% (3/284) of this population. *P. vivax* parasitemia was strongly associated with concurrent Pvs25 transcript density (r=0.87, P<0.001; **Figure S3 B**).

The estimated geometric mean parasite density over follow-up was 1.21 parasites/µL (95% CI, 0.54-2.71) for *P. falciparum* and 0.19 parasites/µL (95% CI, 0.08-0.47) for *P. vivax* (P<0.001) (**Figure 2**).

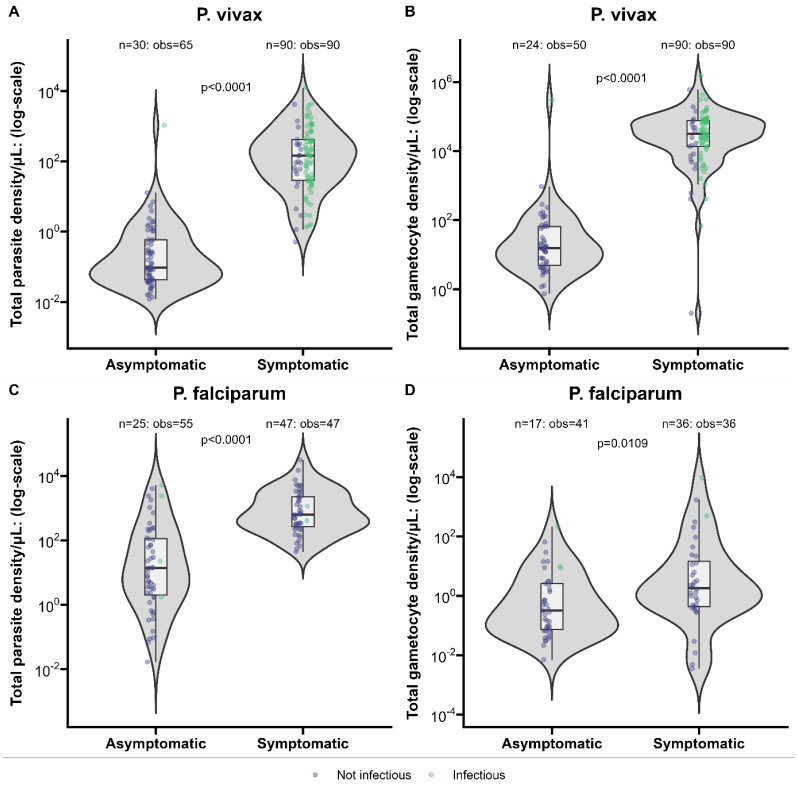

Figure 2. Distribution of parasite and gametocyte densities for infectious and non-infectious feeds. Violin plots showing the distribution of parasite densities and gametocyte densities by symptomatic status for *P. falciparum* and *P. vivax* infections. In each panel, n indicates the number of individuals and obs indicates the number of observations used in the violin plots. The boxplots indicate the quartiles of the densities by symptomatic status. Purple dots indicate observations that were not infectious while green dots indicate observations that were infectious i.e. resulted in at least one infected mosquito.

The estimated geometric mean gametocyte density over follow-up (amongst positive samples) was 0.13 gametocytes/µL (95% CI, 0.05-0.34) for *P. falciparum* and 30.68 (95% CI, 9.15-102.87) *Pvs25* mRNA transcripts/µL. In *P. falciparum* infections, parasite density (P<0.001), gametocyte density (P=0.001) and gametocyte fraction (P=0.018) all declined during follow-up. For *P. vivax* only parasite density was statistically significantly declining over time (P<0.001) while no statistically significant decline was observed for gametocyte transcript density (P=0.568) or the ratio of gametocyte transcripts over total parasite density (P=0.706) (**Figure 3**).

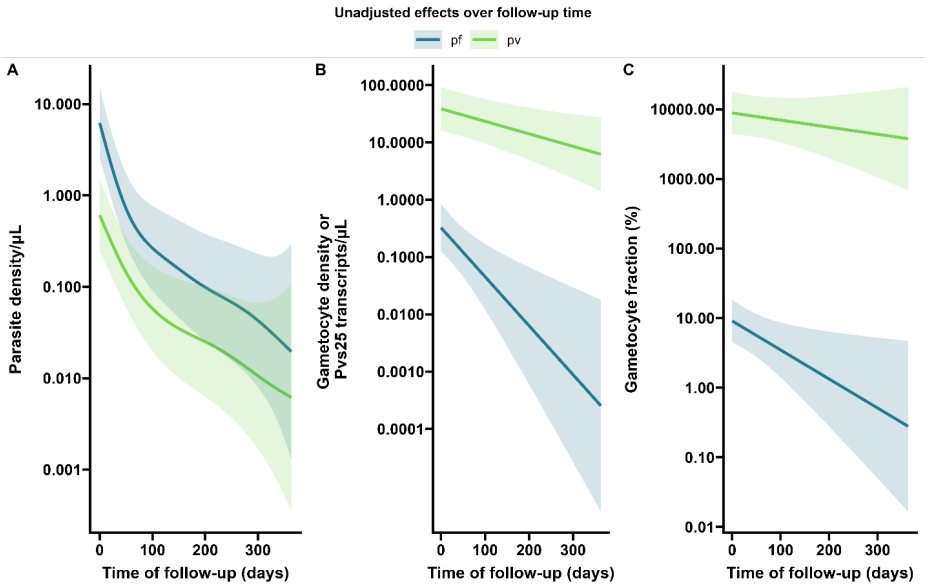

Figure 3. Estimated trends for parasite and gametocyte densities and fractions over time. Estimated trends for parasite density **(A)**, gametocyte density of *Pvs25* transcript density **(B)** and gametocyte fraction **(C)** over time. For parasite density 868 *P. falciparum* observations and 866 *P. vivax* observations from 70 individuals were used in the model. Of these observations, there were 258 *P. falciparum* parasite positive observations from 54 individuals and 284 *P. vivax* parasite positive observations from 43 individuals, together making up samples from 69 individuals. From these parasite positive observations, 224 *P. falciparum* and 253 *P. vivax* observations from 43 and 36 individuals, respectively had available gametocyte densities for the modelling of gametocyte density trajectories **(B)** and gametocyte fraction trajectories **(C)** over time. From these available gametocyte densities there were 160 *P. falciparum* gametocyte positive densities from 24 individuals and 216 *P. vivax* gametocyte positive densities from 28 individuals. Here we used a generalized additive mixed effects model with a negative binomial distribution to model trajectories of parasite density and gametocyte density and gametocyte fraction (all on natural log (ln) scale) over time while accounting for correlation between measures from the same individual through subject-specific random effects.

Infectivity of asymptomatic and symptomatic infections to mosquitoes

When including only those experiments that were conducted at time-points when parasites were detected in blood samples, a total of 114 membrane feeding experiments were performed on 56 asymptomatic participants. Three *P. falciparum*-infected individuals were infectious, each at one time-point only, and infected a total of 29 mosquitoes (1.8% of all dissected mosquitoes; **Table 2**). None of the asymptomatic individuals with *P. vivax* mono-infections infected mosquitoes while a single individual with a mixed infection infected 40 mosquitoes 83.3% (40/48) of this single feed. This person had 23.0 *P. falciparum* and 1066.7 *P. vivax* parasites per µL at the time of MFA; while no *P. falciparum* gametocytes were observed, the number of *P. vivax Pvs25* mRNA transcripts was estimated at 3200/µL.

Table 2. Mosquito feeding results from asymptomatic parasite carriers and symptomatic malaria cases.

	Experiments (donors)	Proportion infectious donors (n/N)	Proportion infectious feeds (n/N)	Proportion infected mosquitoes (n/N)
Asymptomatic infections				
P. falciparum	47 (22)	13.6 (3/22)	6.4 (3/47)	1.8 (29/1579)
P. vivax	56 (24)	0.0 (0/24)	0.0 (0/56)	0.0 (0/1800)
mixed species	11 (10)	10.0 (1/10)	9.1 (1/11)	12.2 (40/328)
Clinical malaria cases				
P. falciparum	47 (47)	4.3 (2/47)	4.3 (2/47)	2.7 (38/1389)
P. vivax	86 (86)	73.3 (63/86)	73.3 (63/86)	42.4 (1068/2519)
mixed species	4 (4)	100% (4/4)	100% (4/4)	32.8 (39/119)

Note: The number of experiments is identical to the number of donors for clinical malaria cases while asymptomatically infected individuals could contribute multiple observations. Of note: parasite status was determined at the moment of feeding and some individuals lost their infection prior to feeding. Mixed species = *P. falciparum* and *P. vivax* co-infection.

Infectious individuals were 7, 10, 13 and 42 years of age. Whilst three infectious individuals harbored submicroscopic infections at enrolment, at the time they infected mosquitoes 2 were microscopy positive for *P. falciparum*, 1 was microscopy positive for *P. vivax* and 1 was qPCR-positive but microscopy-negative for *P. falciparum*.

To compare infectivity in the cohort to that observed among clinically symptomatic individuals, 86 patients with clinical *P. vivax* malaria, 47 patients with clinical *P. falciparum* malaria and 4 patients who had mixed species infection were recruited for MFA (**Supplementary Table 2**). Fever was measured in 39.7% (52/131) of these symptomatic cases; others had reported fever. All *P. vivax* symptomatic cases (n=86) were gametocyte positive by qRT-PCR. Of these, 73.3% (63/86) resulted in mosquito infections with 42.4% (1068/2519) infected mosquitoes. Gametocyte positivity in *P. falciparum* symptomatic cases was 80.9% (38/47) by qRT-PCR, with 4.3% (2/47) infectious feeds and 2.7% (38/1389) of dissected mosquitoes being infected. All four mixed species infections were infectious to mosquitoes with 32.8% (39/119) of mosquitoes becoming infected (**Table 2**). Gametocyte densities were statistically significantly higher in symptomatic individuals for both species although numbers were very small for *P. falciparum,* and also higher in infectious feeds (**Figure 2**).

Discussion

We performed the first longitudinal assessment of parasite longevity, gametocyte production and infectivity in asymptomatic *P. vivax* and *P. falciparum* infections in a co-endemic setting in Africa. When restricting our analyses to those infections detected at enrolment, the median duration of infections was 37 days for *P. falciparum* and 60 days for *P. vivax*; new infections that were first detected during the follow-up period were of considerably shorter duration. Four of 70 asymptomatically infected individuals were infectious to mosquitoes at any time-point during follow-up.

Asymptomatic infections are increasingly considered as reservoirs for onward transmission that need to be targeted to accelerate malaria elimination efforts. However, for infections to contribute to transmission, their infection duration should be sufficiently long to produce gametocytes [5] and gametocyte densities should be sufficiently high to be transmissible [5, 6, 19, 21]. Infection duration is a major determinant of transmission potential, yet there is limited information on the duration of natural infections, especially for infections that are submicroscopic. In our study, most asymptomatic infections were submicroscopic at enrolment. A number of *P. falciparum* (31.0%, 9/29) and *P. vivax* (18.2%, 6/33) infections became microscopically detectable on at least one time-point during follow-up. Nevertheless, there was a general trend of declining parasite densities over time and majority of infections became undetectable

and were considered cleared early during follow-up. In our cohort, 22% of *P. falciparum* infections were persisting for at least 3 months and the median duration of infections already detected at baseline was 37 days. Infection clearance appeared to take longer for *P. vivax* with 40% persisting for at least 3 months and a median duration of infection of already detected at baseline of 60 days. Whilst very long infection durations have been reported for *P. falciparum* [9], many studies indicate that the majority of infections are of short duration. In Vietnam, median carriage times of 2 months for *P. falciparum* and 6 months for *P. vivax* were reported [10]; only 13% of *P. falciparum*-infected Cambodian adults carried these infections for ≥2 months, whereas 35% of *P. vivax* were of long duration [27]. The analysis of monthly blood samples in cohort studies indicated a median duration of blood-stage *P. falciparum* infection of 36 days in Papua New Guinea and 135 days in Thailand whilst the duration of *P. vivax* infections in these settings was in the range of 24-29 days [28]. Interestingly, a large proportion of individuals who were initially detected with either *P. vivax* or *P. falciparum* mono-infections later tested positive for the other species. These findings are in line with findings from Southeast Asia where both *Plasmodium* species were commonly observed in the same individuals [12, 27] suggesting that individuals at high risk of being infected by one species are also at elevated risk for other species. It should be noted that *P. falciparum* infections that were detected during follow-up were of considerably shorter duration and often detected only once. This is in line with findings from Uganda [5] and supported by earlier studies on spontaneous clearance of incident infections [29].

Given the very low gametocyte densities we observed during follow-up, it is unsurprising that only a minority of asymptomatic infections were infectious to mosquitoes. To put our mosquito findings in perspective, we also assessed infectivity of clinical cases that, as predicted, were highly infectious for *P. vivax* [4, 19]. Parasite and gametocyte densities were higher among clinical cases. Although this is commonly observed for *P. vivax* [4, 11, 19], *P. falciparum* gametocyte densities are sometimes lower in clinical cases compared to asymptomatic infections [6, 8]. The observed very low transmission from asymptomatic *P. vivax* infections is different from our previous estimates from the same setting where 32% of asymptomatic microscopy-positive infections were infectious to mosquitoes [4]. Of note, a single highly infectious asymptomatic individual who was co-infected with both *P. vivax* and *P. falciparum* probably infected mosquitoes with *P. vivax* since high gametocyte densities were observed for this species but no *P. falciparum* gametocytes were observed. Nevertheless, the overall pattern of low infectivity is striking. Since conducting the prior study in this setting, malaria

declined substantially following massive bed nets distribution, indoor residual spraying and the roll out of single dose primaquine since 2017 in addition to Artemether-Lumefantrine treatment [2] (**Figure S4**). In line with this, parasite densities among asymptomatic *P. vivax* infections were considerably lower with on average 0.19 *P. vivax* parasites/μL during asymptomatic infections in this study compared to ~22 parasites/μL reported previously [4]. We consider it likely that the lower average parasite density resulted in a lower gametocyte density that explains the considerably lower infectiousness of asymptomatic *P. vivax* infections [10, 30]. Whilst speculative, it is likely that some of the infections that we detected at baseline were older infections that were already past their peak parasite density, which occurs early in infections [5].

The strength of the current study was that we quantified infectiousness to mosquitoes and observe mosquito infections from both symptomatic and asymptomatic donors in the same setting. Extrapolating results from feeding experiments to population-wide transmission dynamics needs to be done with caution and requires adjustment for factors like mosquito exposure and biting preferences that we did not assess in this study [20]. While *An. arabiensis* is a principal vector in Ethiopia [31], other native (*An. coustani complex, An. gambiae s.l.* and *An. pharoensis*) [32] and invasive species (*An. stephensi*) [33] also contribute to transmission. Competence of these vectors may differ but were not examined in the current study.

Our study has several other limitations. Firstly, COVID-19 lockdown measures resulted in a smaller study population than initially anticipated. Secondly, we have no information on the duration of infection prior to enrolment, resulting in underestimates of the duration of infections [10]. Thirdly, we did not perform genotyping that would have allowed us to distinguish persisting and newly acquired clones. This study focused on the duration of blood-stage parasitemia. Fourthly, infection duration estimates are influenced by the frequency of sampling and diagnostic sensitivity [29]; our frequency of sampling after the first 10 weeks was only once per month, limiting the precision of longer infection durations. Our intensive monitoring during the first phase of the study makes it unlikely that unreported self-treatment for malaria played a relevant role in infection clearance.

In conclusion, in our longitudinally followed cohort of individuals living in an area where both *P. falciparum* and *P. vivax* are endemic, total parasite densities fluctuate but show a general decline in densities over time with the majority

of infections for both species being cleared within 2-3 months. Related to the low parasite and gametocyte densities, a very low proportion of asymptomatic infections were infectious to mosquitoes.

Acknowledgements
We are grateful for the study participants, the field research team including the microscopists at Adama (Tewabech Lema and Tsehay Orlando), the community facilitators at Adam and the drivers at AHRI for their support during sample collection and transportation. We are also thankful for the regional and district health officers for their collaboration during the study period.

Funding
The study was supported by the Armauer Hansen Research Institute via its core funding from the Norwegian Agency for Development Cooperation and Swedish International Development Cooperation; the Bill and Melinda Gates Foundation [INDIE; OP P1173572] to TB, FGT and CD. The work was further supported by an AMMODO Science Award, the Bill and Melinda Gates Foundation (INV-002098) to TB and a European Research Council Consolidator Grant [ERC-CoG 864180; QUANTUM] to TB as well as the Bill and Melinda Gates Foundation [ACHIDES; INV-005898 and EMAGEN; INV-035257] to FGT. Funders had no role in the study design, data collection, interpretation and write-up of the manuscript.

Potential conflict of interest
All authors declared that they have no conflict of interest.

Author contributions
FGT, TB, CD, EH and SKT conceived the study. EH and SKT drafted the manuscript. FGT, TB, CD, EG, BP and HM critically commented on the manuscript. EH, TB, SKT and JR analyzed the data. TA, AG, EH, SKT, WC, EE, TT, T.Tsegaye, TE were involved in mosquito membrane feeding assay. EH, TA, AG, EE, TT and SK were involved in mosquito rearing. WC, EH, SKT, GS conducted mosquito midgut dissection. EH, SKT, TE and T. Tsegaye, GS, HA and MG collected follow-up data and blood samples from community participants. EH, FGT, SKT, SWB and KJ conducted the molecular laboratory work.

Ethical approval
The study protocol was approved by ethics review committees of the Armauer Hansen Research Institute and ALERT Hospital (PO35/17 and PO32/18), the

London School of Hygiene and Tropical Medicine (15811), and the National Research Ethics Review Committee (SHE/S.M./14.4/708/19) of Ethiopia.

Data sharing

Data from this study can be obtained upon formal request from the corresponding authors.

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Supplementary information

Supplementary table 1. Summary of parasite species oscillation patterns during follow-up

Species at baseline by 18s qPCR	Changes in parasite status during follow-up	Number of participants % (n/N)
P. vivax(n=34)	From Pv-sub-microscopic infection to Pv-microscopy positive	18.2 (6/33)
	From Pv-infected to Pv-Pf mixed infection	26.5 (9/34)
	From Pv-infected to Pf infected	38.2 (13/34)
P. falciparum (n=35)	From Pf-sub-microscopic infection to Pf-microscopy positive	31.0 (9/29)
	From Pf-infected to Pf-Pv mixed infection	17.1 (6/35)
	From Pf-infected to Pv infected	14.2 (5/35)

Note: 1 *P. vivax* and 6 *P. falciparum* infections were microscopy detectable at baseline.
Pf= *P. falciparum*, Pv= *P. vivax.*

Supplementary table 2. Characteristics of clinical malaria study participants

Attributes	***P. falciparum***	***P. vivax***	Mixed ***(P. falciparum*** and ***P. vivax)***
N	47	86	4
Sex, % male (n/N)	79.6 (35/44)	78.8 (67/85)	50.0 (2/4)
Age, median (IQR)	28.5(21.5-39)	26 (19-32)	13.5(10.5-22.5)
P. falciparum parasite density/µL, GM (95% CI)	445.0 (228.2-867.6)		-
P. vivax parasite density/µL, GM (95% CI)	-	111.2 (71.2-173.7)	-

IQR=interquartile range (25^{th} -75^{th} percentile); GM = geometric mean; 95% CI=95% confidence interval.

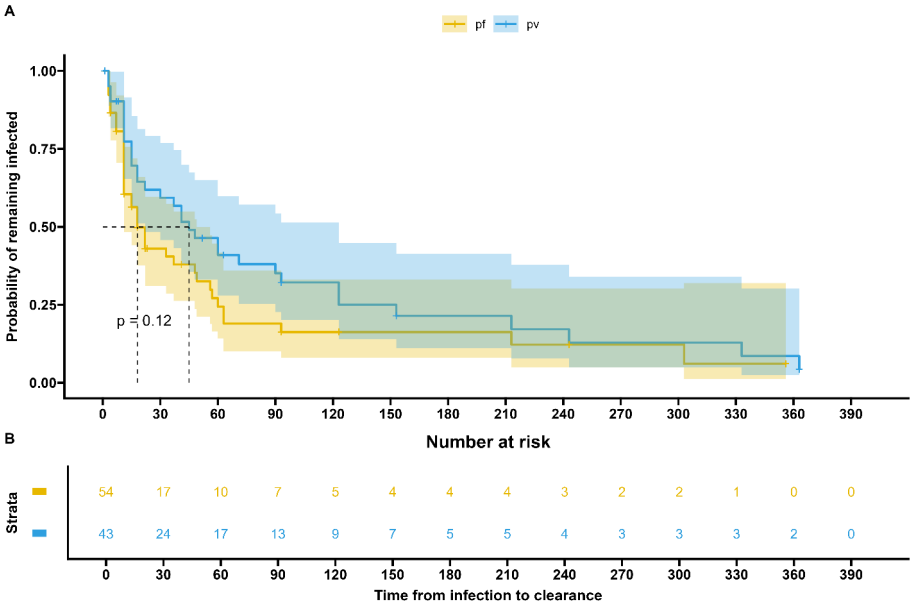
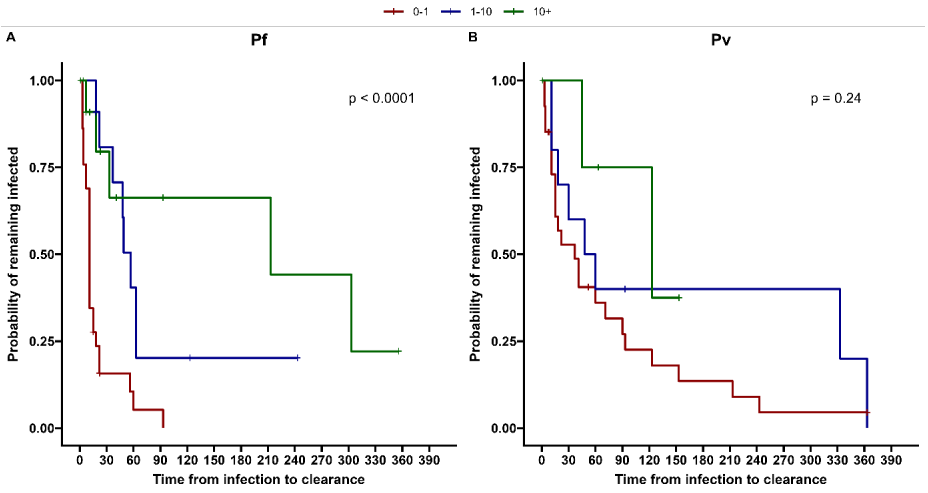


Figure S1. Kaplan-Meier plot showing the probability of remaining infected (i.e. not cleared) over the duration of follow-up. From the 54 pf infections, 33 were observed at baseline, and 40 were observed to clear (14 were right-censored) with a median time of 18 days (95% CI, 11 days – 49 days). From the 43 pv infections, 31 were observed at baseline, and 33 were observed to clear (10 were right-censored) with a median time of 45 days (95% CI, 22 days-123 days). A log-rank test showed no significant difference in the Kaplan-Meier curves (P=0.12).



For Pf:	Hazard ratio (95% CI, P-value)	
Reference group	(0, 1] vs ref	(1, 10] vs ref
(1,10]	4.0 (1.7 – 9.0, P=0.00010)	-
(10, 10000]	7.8 (2.6 – 24.0, P=0.0003)	2.0 (0.6 – 6.7, P=0.277)

For Pv:	Hazard ratio (95% CI, P-value)	
Reference group	(0, 1] vs ref	(1, 10] vs ref
(1,10]	1.6 (0.7 – 3.6, P=0.2690)	-
(10, 10000]	2.7 (0.6 – 11.6, P=0.1770)	1.7 (0.4 – 8.2, P=0.4990)

Figure S2. Kaplan Meier graphs showing that lower parasite density infections at baseline tend to have fastest parasite clearance. Here, persistence is indicated for three infection classes: <1 parasite/uL (red), 1-10 parasites/uL (blue) and >10 parasites/uL (green) for *P. falciparum* (left) and *P. vivax* (right) separately. Infections with the lower parasite density at baseline have the fastest parasite clearance although this is not stastically significant for *P. vivax* due to a small number of long-persisters in the population with the lowest parasite densities at baseline.

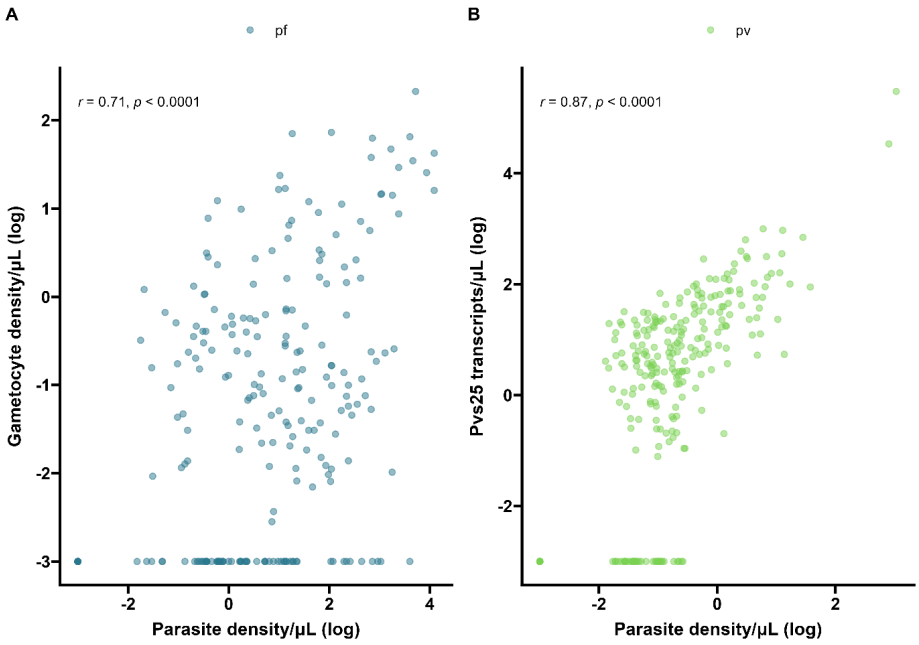


Figure S3. Association between parasite density and gametocyte density. Scatter plots for parasite density versus gametocyte density with Pearson's correlation coefficients on log10 transformed values and their corresponding p-values. These plots include 55 individuals with 465 follow-up pairs of parasite density and gametocyte density. For *P. falciparum,* 25 individuals contributed 160 pairs of parasite and gametocyte positive samples, and for *P. vivax* 29 individuals contributed 213 pairs of parasite positive and gametocyte positive samples.

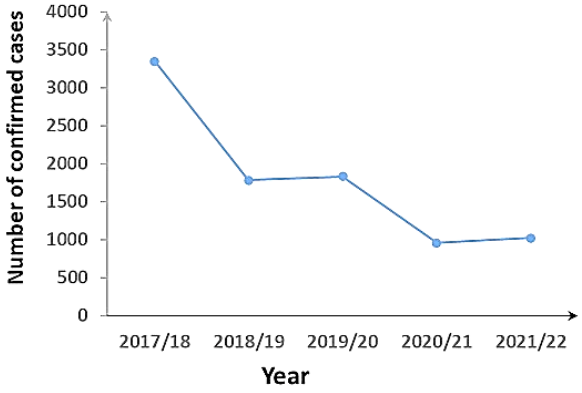
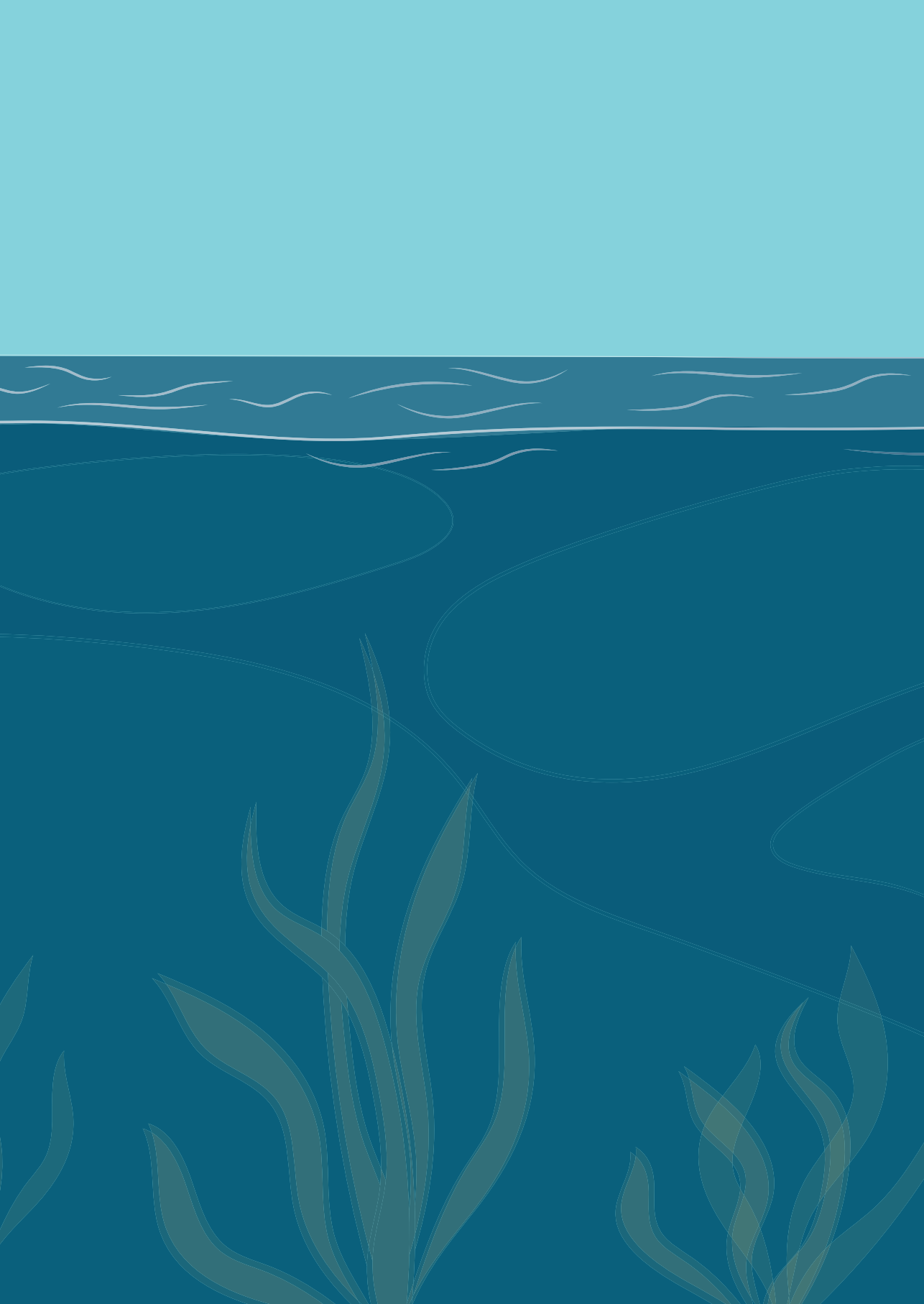


Figure S4. Trends of malaria transmission in the study area (Adama) from 2017-2022. The x-axis represents dates of the year and the y-axis represents the number of clinical malaria cases reported in routine data. The earlier study where asymptomatic infections were of relatively high parasite density took place in 2016, the current study in 2018-2020.



CHAPTER 6

Naturally acquired antibodies to gametocyte antigens are associated with reduced transmission of *Plasmodium vivax* gametocytes to *Anopheles arabiensis* mosquitoes

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Front. Cell. Infect. Microbiol. 12:1106369. doi: 10.3389/fcimb.2022.1106369

Abstract

Naturally acquired antibodies may reduce the transmission of *Plasmodium* gametocytes to mosquitoes. Here, we investigated associations between antibody prevalence and *P. vivax* infectivity to mosquitoes. A total of 368 microscopy confirmed *P. vivax* symptomatic patients were passively recruited from health centers in Ethiopia and supplemented with 56 observations from asymptomatic *P. vivax* parasite carriers. Direct membrane feeding assays (MFA) were performed to assess mosquito infectivity; for selected feeds these experiments were also performed after replacing autologous plasma with malaria naïve control serum (n=61). The prevalence of antibodies against 6 sexual stage antigens (Pvs47, Pvs48/45, Pvs230, PvsHAP2, Pvs25 and PvCelTOS) and an array of asexual antigens was determined by ELISA and multiplexed bead-based assays. Gametocyte (ρ= 0.42; p = 0.0001) and parasite (ρ= 0.21; p = 0.0001) densities were positively associated with mosquito infection rates. Antibodies against Pvs47, Pvs230 and Pvs25 were associated with 23 and 34% reductions in mosquito infection rates (p<0.0001), respectively. Individuals who showed evidence of transmission blockade in serum-replacement MFAs (n=8) were significantly more likely to have PvsHAP2 or Pvs47 antibodies. Further studies may demonstrate causality for the observed associations, improve our understanding of the natural transmission of *P. vivax* and support vaccine development.

Keywords: *Plasmodium vivax*, gametocyte, transmission, immunity, Anopheles, malaria, transmission-blocking, vaccine.

Introduction

The significant reduction in malaria burden in the first decade of this millennium has plateaued since 2015 and even reverted in some settings in recent years [1]. Current intervention strategies appear insufficient to achieve malaria elimination in the majority of endemic countries [2]. Whilst most malaria-attributed deaths are due to *Plasmodium falciparum*, *Plasmodium vivax* also has a high clinical burden with an estimated 4.5 million cases in 2020 [1]. *P. vivax* appears particularly hard to eliminate [3], principally due to its ability to form dormant liver stages that can result in relapsing infections unless treated with an effective radical cure. Another challenge in controlling and eliminating *P. vivax* infections is the rapid formation of gametocytes, the parasite life stage responsible for onward transmission to mosquitoes. Once taken up by blood-feeding *Anopheles* mosquitoes, gametocytes activate to become male and female gametes that fuse to form a motile ookinete. This ookinete penetrates the mosquito gut to develop into an oocyst that releases sporozoites that ultimately render the mosquito infectious upon its next bite. Whilst gametocyte maturation is a long process in *P. falciparum*, infectious *P. vivax* gametocytes appear in the bloodstream within 48 hours of blood stage infection [4]. The strong association between asexual parasite and gametocyte density [5] make clinical *P. vivax* malaria cases highly infectious to mosquitoes at the moment of clinical presentation [6, 7]. Given that *P. vivax* parasites are capable of infecting a wide variety of *Anopheles* mosquitoes [8, 9], it is unsurprising that *P. vivax* transmission is highly efficient in many settings.

Naturally acquired human immune responses can reduce or fully prevent the transmission of *Plasmodium* parasites from humans to mosquitoes (recently reviewed in [10]). For *P. vivax*, the first empirical evidence for naturally acquired transmission blocking activity (TBA) came from studies conducted in the 1980s and 1990s in Sri Lanka [11-13]. In these studies, investigators fed mosquitoes with blood from acutely infected Sri Lankan individuals. In paired feeding experiments, gametocyte infectivity was compared between a condition where the patient's autologous plasma was present in the bloodmeal versus a condition where this plasma was replaced with malaria naïve control serum. In most of these paired experiments, transmission was reduced in the presence of autologous plasma (indicative of plasma derived TBA) although in some experiments the autologous plasma had the opposite effect and thus enhanced transmission [12, 13]. In supporting experiments, indirect immunofluorescence tests showed that sera with TBA were more reactive to gamete surface antigens compared to sera without this functional activity. Moreover, purified

immunoglobulins from these suppressive sera blocked transmission when added to a gametocytaemic blood meal, confirming this TBA to be antibody-mediated [12]. Subsequent studies in Mexico [14], and Colombia [15] similarly demonstrated the existence of naturally acquired *P. vivax* TBA. However, the antibody specificity or strength of the associations remain largely undescribed. Several *P. vivax* gametocyte antigens have been developed as transmission blocking vaccine candidates. Antibody responses that are elicited by these proteins can reduce *P. vivax* transmission, as demonstrated by ookinete culture experiments or by mosquito feeding experiments where serum from vaccinated animals was added to gametocytes from naturally infected malaria-positive blood donors and offered to mosquitoes. These experiments have confirmed the potency of several pre-fertilization antigens including Pvs48/45 [16, 17], Pvs230 [18], Pvs47 [16] and PvHAP2 [19] and post-fertilization antigens Pvs25 and Pvs28 [20]. Lastly, *P. vivax* Cell-traversal protein for ookinetes and sporozoites (PvCelTOS) [21-23] was identified as potential inducer of TBA [24]. Recognition of these recombinant proteins has so far not been systematically studied in malaria-endemic populations or related to *P. vivax* transmission efficiency.

Here, we therefore investigated the relationship between the infectivity of *P. vivax* infections to locally reared *An. arabiensis* mosquitoes and naturally acquired antibodies against *P. vivax* gametocyte antigens in symptomatic and asymptomatic infections in Ethiopia.

Methods

Study design and population

This study was conducted in Adama district and Metehara town of Oromia Regional State and Arba Minch in Southern Region, Ethiopia. Adama district is characterized by low and seasonal malaria transmission that primarily occurs during the long (July to September) and short (May to June) rainy seasons [25]. Metehara town has low perennial malaria transmission with peaks in incidence from September to November and March to May. Arba Minch is characterized by moderately intense perennial malaria transmission. *P. falciparum* and *P. vivax* are co-endemic in all study sites where *Anopheles arabiensis* is a primary malaria vector [7, 26, 27].

Across sites, microscopy confirmed symptomatic *P. vivax* patients were recruited at health centers from December 2017 to March 2022 **(Table 1)**. Demographic

data was collected from patients or their guardians (if minors) at the moment of presentation. Patients were included in the study if they had microscopy-confirmed *P. vivax* infections, had symptoms suggestive of malaria (defined as axillary temperature ≥37.5°C or history of fever, alone or in combination with nausea, chills and headaches) and were ≥2 years of age. Patients were excluded if they had a chronic and/or an acute illness that required immediate clinical care or bleeding disorders. Hemoglobin concentration was measured by HemoCue photometer (HemoCue 201+, Angelholm, Sweden).

Table 1. Characteristics of study participants recruited between December 2017 and March 2022

Characteristics	Symptomatic	Asymptomatic
Age, median (IQR)	19 (13 - 28)	14 (9 - 21.5)
Asexual parasites/µl (microscopy), median (IQR)	4752.0 (1547.0 - 10706.0)	0.0 (0.0 - 0.0)
Gametocyte prevalence, microscopy (% (n/N))	36.4 (134/368)	0.0 (0.0/56)
Gametocyte prevalence (*Pvs25*), RT-qPCR (% (n/N))	76.1% (280/368)	80.4 (45/56)
Mosquito infectious individuals, % (n/N)	73.9 (272/368)	0.0 (0.0/56)*
Infected mosquitoes, % (n/N)	37.0 (4036/10,879)	0.0 (0.0/1,800)

*24 asymptomatic patients with 56 longitudinal data points were included.
Symptomatic (n= 368); Asymptomatic (n=24, with 56 longitudinal mosquito feeding moments); Total (n= 424). Abbreviations: IQR, inter quartile range
All data points selected for the asymptomatic individuals were *P. vivax* positive by RT-qPCR

Observations from clinical malaria cases were complemented with observations from asymptomatic infections. These comprised samples from qPCR confirmed *P. vivax* infections collected during the same study period from asymptomatic individuals who were enrolled in a longitudinal observational study on the dynamics of asymptomatic infections in Adama district (Hailemeskel, Tebeje *et al.* in preparation). Samples from these individuals were included in the current analysis to ensure coverage of a broad range of transmissible and non-transmissible gametocyte densities to allow more precise fitting of the associations between parasite and gametocyte densities and mosquito infection rates. For asymptomatic parasite carriers, afebrile community members were eligible if they were above the age of 2 years and positive for *P. vivax* parasites by qPCR, without symptoms suggestive of malaria in the past 48 hours and with no evidence of chronic and/or acute illness. Venous blood samples were collected

every 14 days for 2 and ½ months and used in mosquito feeding assays and for plasma collection. For the current study, we randomly selected samples from this cohort without the intention to describe (longitudinal) patterns in parasite carriage but to obtain an informative population of low parasite and gametocyte densities for curve-fitting.

Ethics statements

All participants or their guardians (if minor) provided written informed consents to participate in the study. The study protocol was approved by the Ethics Review Committees of the Armauer Hansen Research Institute (AHRI) (protocol number: PO32/18 and PO35/17), the Ethics committee of the London School of Hygiene and Tropical Medicine (LSHTM Ethics ref: 15811), and the National Research Ethics Review Committee (SHE/S.M./14.4/708/19) at the Ministry of Science and Higher Education of The Federal Democratic Republic of Ethiopia.

Study procedures

Blood sampling

Venous blood was collected from each participant for mosquito feeding, molecular assays and serology. Three mL of blood was collected in either EDTA (BD K2E EDTA Vacutainers) or serum tubes (BD Vacutainer Plus Serum) for nucleic acid collection and plasma/serum collection. An additional 2mL was collected in Heparin tubes (BD Lithium Heparin Vacutainers) for mosquito feeding experiments (to measure mosquito infectivity) since other anticoagulants interfere with transmission efficiency [28]. Plasma (1:1 in 0.05% NaAz) and serum were stored at -70°C for serological investigations.

Parasite quantification

Asexual parasites and gametocytes were detected and quantified by microscopy [29]. For quantification of gametocytes with reverse transcriptase quantitative PCR (RT-qPCR), total nucleic acids were extracted from 100µL whole blood stored in 500µL RNAProtect (Qiagen) and extracted by MagNAPure LC automatic extractor (Roche applied Sciences). RT-qPCR targeted *Pvs25* mRNA, an established marker of female gametocytes [7, 30]. All primer and probe sequences and combinations are included in supplementary file **(Table S3)**.

Infectivity assessment

Mosquito membrane feeding assays were performed as described previously [7, 31] to assess infectivity to locally reared *An. arabiensis* mosquitoes. Briefly, a heparinized whole blood sample was fed to ~ 40 (4 - 7 days old) female *An.*

arabiensis mosquitoes per cup (three cups with a total of 120 mosquitoes per experiment) using water jacketed glass feeders (0.3mL capacity) maintained on a water bath to keep the temperature in the feeders at 37°C. Mosquitoes were starved for ~12 hours prior to feeding. For the serum replacement mosquito feedings, heparinized whole blood (~833µL) was centrifuged at 1800 × g for 5 minutes in a pre-warmed (37°C) centrifuge to collect autologous plasma (~500µL). Pre-warmed (37°C) ~500µL naïve AB European serum (Sanquin, Nijmegen, the Netherlands) was used to replace the autologous plasma. Fully fed mosquitoes were maintained on 10% sucrose solution (g/mL) at 26° $\pm$ 2 room temperature and 60% $\pm$ 10 humidity post feeding. Mosquitoes (30 per experiment) were dissected on day 7 post feeding, and midguts were stained with 1% mercurochrome for microscopic detection and quantification of oocysts.

Serological analyses

Antibody levels against *P. vivax* antigens, Pvs25, Pvs48/45, Pvs47, Pvs230 and PvCelTOS were assessed by Enzyme Linked Immunosorbent Assay (ELISA). Pvs25, Pvs48/45, Pvs47 and Pvs230 were expressed using a wheat germ cell-free (WGCF) system (CellFree Sciences, Matsuyama, Japan) [32]; an additional Pvs230 was expressed in *Pichia pastoris* (Pvs230D1M [33]); and the sporozoite/ookinete antigen PvCelTOS was expressed in *E. coli* [22, 23, 34, 35]. ELISA plates were coated with 1µg/mL (in PBS) of antigen overnight at 4°C. After thorough washing with home-made PBS-Tween (0.05%), plates were blocked with 5% skimmed milk in 0.05% PBS-Tween for 1 hour at room temperature (RT). Plates were then washed. Diluted test samples/controls in 1% skimmed milk (in 0.05% PBS-Tween) were added to the plates and incubated for 2 hours at RT. After a washing step, Goat anti-human IgG-HRP (1:50,000) (Pierce 31412) in PBS-Tween (0.05%) was added and plates were incubated at RT for 2 hours. Following a final washing step, a colorizing substrate TMB (K-Blue substrate, Neogen, Sigma), was added and incubated for ~20 minutes. After ~20 minutes of incubation, a stopping solution of 0.2M Sulphuric acid (H_2SO_4, Merck KGaA cat 100731) was added to stop the reaction. Plates were immediately read by plate reader (Bio-Rad, iMark microplate reader) at 450 nm. OD values were used as measure of antibody density; antibody prevalence was assessed by a mixture model, taking the mean plus two standard deviations from the negative population as cut-off for positivity [36]. A more conservative approach with three standard deviations is sometimes used [37] but more appropriate for larger sample sizes. The availability of WGCF proteins was a limiting factor and not all samples could be processed by ELISA; samples with serum replacement observations were prioritized for a complete dataset.

Alongside ELISAs, we tested a panel of 7 *P. vivax* antigens from different life stages in a bead based multiplex immunoassay for comparison; one pre-erythrocytic stage (Circumsporozoite protein [VK210 CSP]), 5 asexual blood stage (Merozoite surface protein 1-19 [MSP1-19], Apical membrane antigen 1 [AMA1], Duffy binding protein [DBP RII], Reticulocyte binding protein [RBP 2b], and Erythrocyte binding protein [EBPII]), and one sexual stage (HAP2). Antibody responses were quantified using a Luminex MAGPIX© suspension bead array, as described previously [38]. Briefly, plasma/serum samples were assayed at a dilution of 1:400. Secondary antibody was an R-phycoerythrin conjugated goat anti-human IgG (Jackson Immuno Research, PA, USA; 109-116-098) diluted to 1:200. Data are presented as background adjusted median fluorescence intensities (MFI).

Data analyses

Statistical analyses were performed using STATA (version 14.2, StataCorp., TX, USA) and R (version 4.1.1). Proportions were compared using McNemar's test for paired observations, and Pearson $\chi 2$ test or Fisher exact test for independent observations. Spearman rank correlation coefficient (ρ) was used to assess associations between continuous variables. Continuous variables were presented as medians and interquartile ranges (IQRs). TBA was calculated as percent inhibition in oocyst density in naïve sera feeds compared to whole blood feeds [39]. A cut-off for antibody positivity was determined by first using a mixture model to distinguish two Gaussian distributions. The cut-off was determined as the estimated 97.5th percentile of the underlying Gaussian distribution for the negative antibodies (i.e. 2 standard deviations above the mean of the negative population). Logistic and linear mixed effects regression models were used for multivariate analyses. Analysis with $p<0.05$ were considered statistically significant.

Results

Participant characteristics

In total, 368 symptomatic patients with a median age of 19 years (interquartile range (IQR), 13 – 28 years) were enrolled in the study between December 2017 and March 2022 **(Table 1)**. Of these participants 47.8% (174/364) had an active fever and 0.6% (2/332) had hemoglobin levels <8g/dL. In these patients, the median asexual parasite density by microscopy was 4,752.0 parasites/μL (IQR, 1547.0 - 10706.0) and 36.4% (134/368) had microscopically detected gametocytes. By Pvs25 RT-qPCR, *P. vivax* gametocytes were detected in 76.1% (280/368) of samples with median *Pvs25* transcript numbers of 22245.9 copies/μL (IQR, 3770.4 - 70980.8). These data from clinical malaria patients, who typically harbor high parasite and gametocyte densities [40, 41], were supplemented with a total of 56 follow-up observations from 24 asymptomatic parasite carriers who were for the most part microscopy negative for malaria parasites but qPCR positive for *P. vivax* and thus carried very low density infections [42] **(Table 1)**. Asymptomatic parasite carriers had a median age of 14 years (IQR, 9 - 21.5). Microscopically quantified asexual parasites and molecularly quantified gametocyte densities differed markedly between symptomatic and asymptomatic populations **(Figure 1A)**. When combining observations from both symptomatic and asymptomatic populations, gametocyte density was positively associated with parasite density (**Figure 1B**,= 0.43; $p < 0.0001$).

Mosquito infection rates are associated with parasite and gametocyte density.

For all clinical patients and asymptomatic parasite carriers, whole blood was offered to locally reared *An. arabiensis* mosquitoes that were examined 7 days later for infection status. For 61 clinical patients recruited between March 2020 and March 2022, these mosquito feeding experiments were also conducted after replacing autologous plasma with malaria-naïve serum. Asexual parasite density was positively associated with the proportion of mosquitoes that became infected when feeding on whole blood of parasite carriers **(Figure 1C)**; this association was stronger for molecularly quantified gametocyte density (Figure 1D). Oocyst prevalence, or the proportion of infected mosquitoes, was strongly positively associated with mean oocyst density (= 0.63; $p < 0.0001$) **(Figure 2A)**.

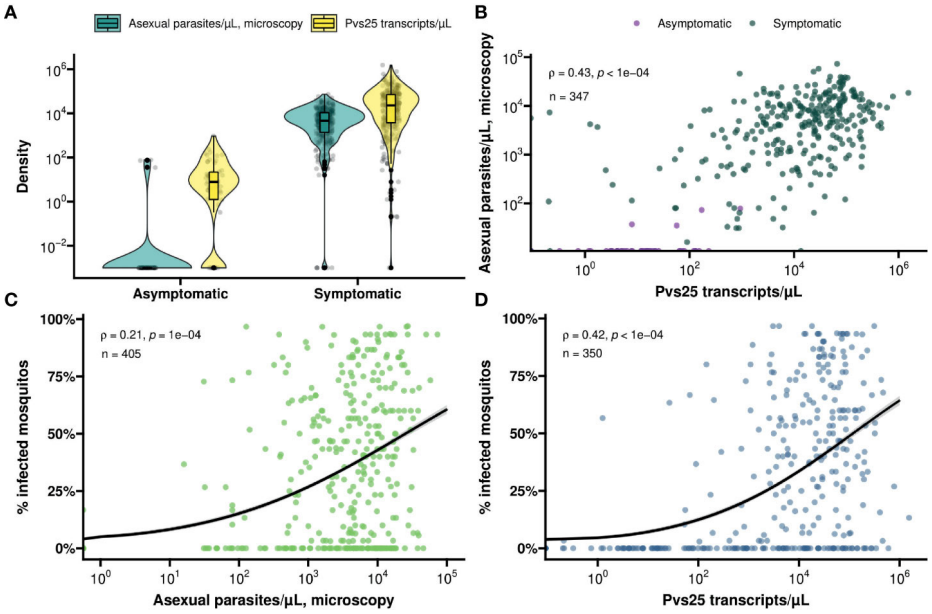

Figure 1. (A) Violin and box plot for the distribution of molecularly determined gametocyte density (yellow) and microscopically detected asexual parasite density (turquoise) by symptom status. **(B)** A scatter plot for the association between asexual parasite density and gametocyte density by symptom status (asymptomatic – violet, symptomatic – green). Spearman's ρ = 0.43 indicating a moderate positive monotonic association (p<0.0001). **(C)** Scatter plot showing the association between asexual parasite density and mosquito infectivity. Spearman's ρ = 0.21 indicating a weak positive monotonic association (p<0.0001). The black line shows the fit from a logistic regression model and the grey shading shows the 95% confidence interval. **(D)** Scatter plot showing the association between gametocyte density and mosquito infectivity. Spearman's ρ = 0.42 indicating a moderate positive monotonic association (p<0.0001). The black line shows the fit from a logistic regression model and the grey shading shows the 95% confidence interval.

Despite these statistically significant associations, many individuals with relatively high gametocyte densities did not infect mosquitoes. For the subset of participants whose blood was used in serum replacement experiments (n=61), we observed a strong positive association in mosquito infection rates between the whole blood and serum replacement feeds (**Figure 2B**; ρ = 0.92, p<0.0001). We observed higher mosquito infection rates for the control serum condition with a median increase of 13.2% (IQR, 0% - 25.0%) infected mosquitoes. We expressed the lower mosquito infection rates in the autologous plasma condition relative to that in the naïve serum condition as transmission blocking activity (TBA). This TBA ranged from -80% (enhancement) to 100% (complete blockade). For those showing higher infection rates in whole blood conditions (n=5),

this 'transmission enhancement' was mostly very weak (3.2 - 12.1%). Only 1 individual showed more than 80% enhancement with the proportion of infected mosquitoes being 15.6% for whole blood and 6.3% for serum replacement conditions. In contrast, 13.1% (8/61) of experiments showed greatly increased infection rates following serum replacement (>80% higher infection rates and thus TBA >80%). TBA estimates for five experiments were not interpretable since infection rates were zero in the whole blood and serum replacement conditions.

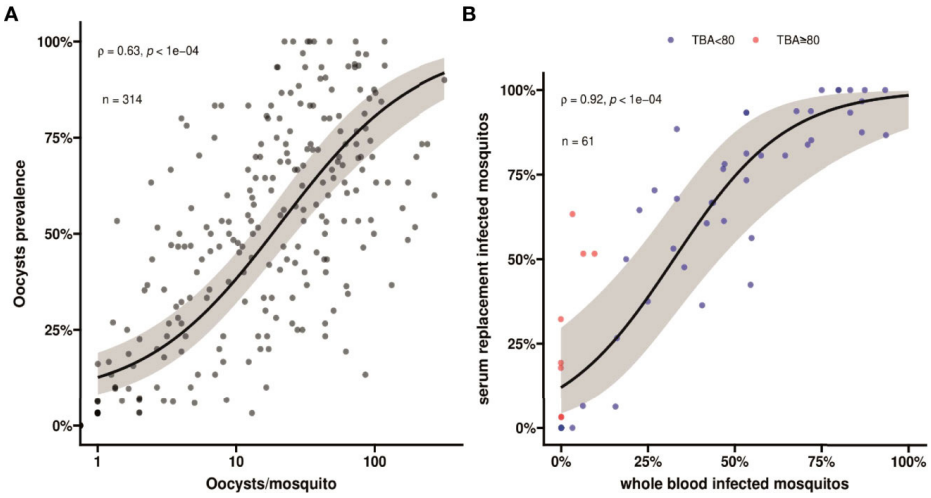

Figure 2. (A) A scatter plot showing association between oocyst prevalence, or mosquito infection rates, and mean oocyst per mosquito. Spearman's ρ =0.63 indicating a moderate positive monotonic association p<0.0001. The black line shows the fit from a logistic regression model and the grey shading shows the 95% confidence interval. **(B)** A scatter plot indicating the association in mosquito infection rates between whole blood and serum replacement feeds. Observations with TBA<80% are indicated in blue and TBA≥80% are indicated in red. Spearman's ρ = 0.92 indicates a strong positive monotonic association (p<0.0001). The black line shows the fit from a logistic regression model and the grey shading shows the 95% confidence interval. The plot also indicates that infectivity in the serum replacement condition (median 60.6%, IQR 6.3% - 85.2%) was consistently higher than in the whole blood condition (median 33.3%; IQR 0% - 57.6%) (p<0.0001). This value is different, but not contradicting, the comparison in the main text where we calculated the pairwise differences and report the median and IQR for the pairwise differences.

Transmission efficiency is associated with gametocyte immune responses

To test a possible impact of anti-gametocyte immune responses on transmission efficiency, we determined antibody responses to sexual stage antigens Pvs47, Pvs48/45, Pvs230 (two variants), PvCelTOS and Pvs25 by ELISA. These

assessments were done for 224 individuals, including all those participating in serum replacement mosquito feeding experiments. Due to antigen scarcity, not more samples were completed by ELISA. Additional antigens, including sexual-stage antigen PvHAP2, were measured by bead-based multiplex assay. Antibody positivity was defined based on a mixture model, assuming an antibody positive and antibody negative population for each antigen within the study population. The optical density values in ELISA, reflective of antibody densities, were strongly correlated between most of the antigens (**Figure 3**). For instance, responses to the two Pvs230 variants were strongly correlated ($\rho = 0.68$, $p<0.0001$), as were responses to Pvs230 and Pvs25 ($\rho = 0.63$, $p<0.0001$). Among individuals with microscopy-positive infections, antibody prevalence was weakly associated with a lower parasite density at the time of sampling for PvDPB-RII ($p = 0.06$) but not for any of the other antigens **(Table S1)**.

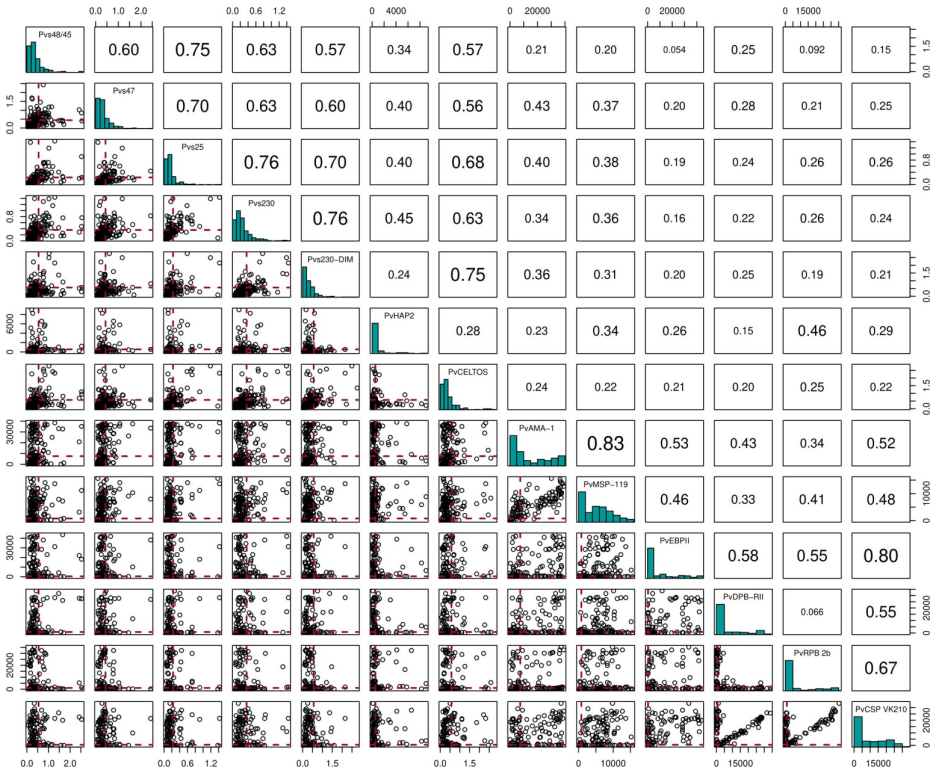

Figure 3. Correlation matrix plot for the 13 different antigens. Here the diagonal shows the histogram distribution for each of the antigens. The triangle above the diagonal shows the Spearman's Correlation Coefficient for every pair of antigens on the diagonal. The bottom triangle shows the scatter plots for every pair of antigens on the diagonal. Here, the red dashed lines indicate the cut-offs for antibody positivity.

Individuals with >80% TBA were more likely to be antibody positive for most gametocyte antigens **(Figure 4)**, although this difference only reached statistical significance for Pvs47 and PvHAP2 **(Table S2)**. We did not observe any evidence of associations with >80% TBA for any of the asexual antigens **(Figure 4, Table S2)**. We next examined whether antibody prevalence was also associated with transmission efficiency in the larger dataset where whole blood samples were offered to mosquitoes and gametocytes were quantified by molecular methods. We thus determined whether the proportion of infected mosquitoes for a given gametocyte density was lower in the presence of gametocyte antibodies. The presence of antibodies against Pvs47 was associated with a relative reduction in mosquito infection rates of 34% ($p<0.0001$). Similarly, Pvs230 and Pvs25 antibody prevalence were associated with statistically significant relative reductions of 23 and 34%, respectively; for Pvs48/45 antibodies a smaller and non-significant reduction was observed **(Figure 5)**. For PvCelTOS and asexual stage antigens, highly heterogeneous effects were observed. The presence of antibodies against CSP and RBP-2b was associated with 61% and 20% increases in mosquito infection rates, respectively; while antibodies against AMA-1 were associated with a 26% decrease in mosquito infection rates ($p<0.0001$) **(Figure S1)**.

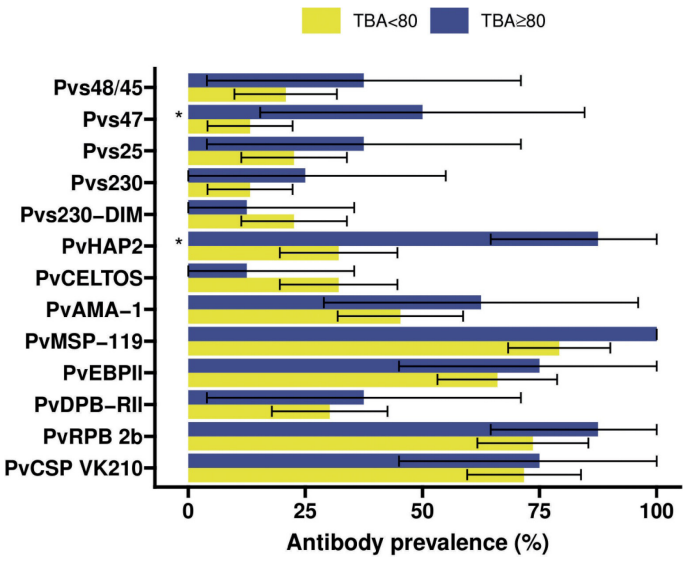

Figure 4. Antibody prevalence (Kim et al., 2011) for the 13 different antigens stratified for whether the transmission blocking activity (TBA) was at least 80% (blue) or less (yellow). 95% confidence intervals are shown by the black bars. Significant ($p<0.05$) differences in prevalence between TBA<80% and TBA≥80% are indicated by * where p-values were calculated from Fisher's exact tests.

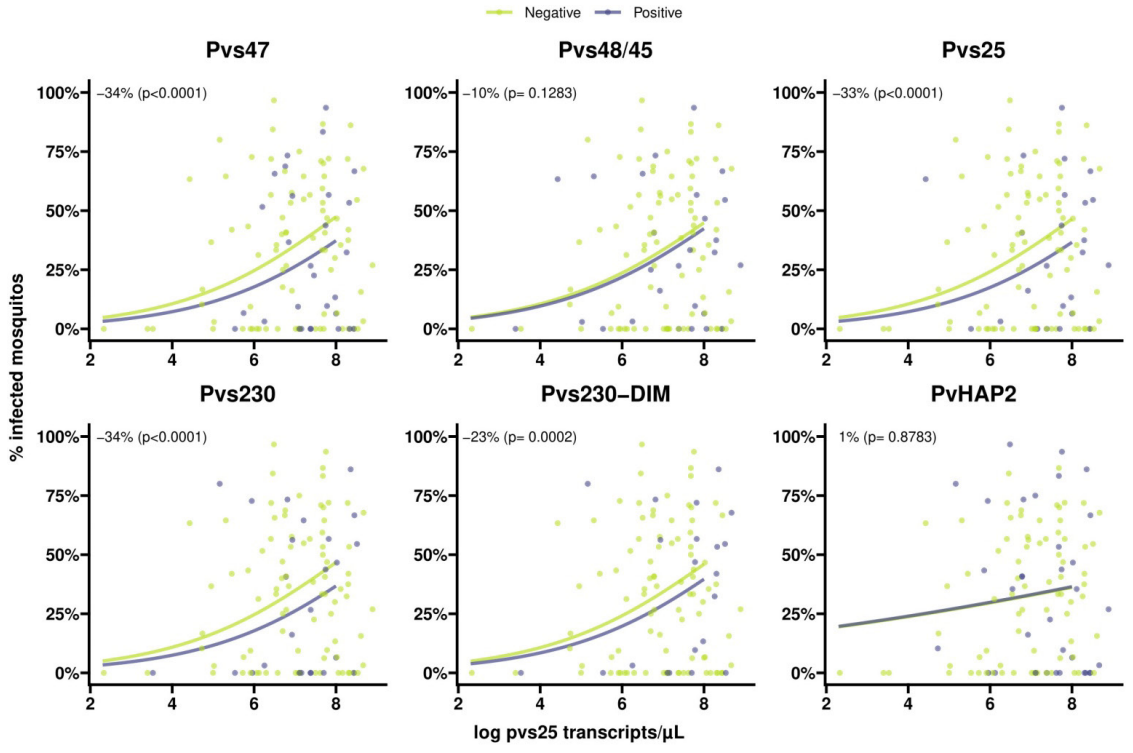

Figure 5. Scatter plots indicating the association between gametocyte density and antibody prevalence on mosquito infectivity for six different antigens. Positive antibodies are indicated in light blue and negative antibodies are indicated in green. The light blue and green lines shows the fit from logistic regression models for the association between gametocyte density and mosquito infectivity for positive and negative antibodies respectively. The numbers in the plot (estimated from logistic regression models) indicate the average difference in mosquito infectivity between positive and negative antibodies while accounting for gametocyte density, and the p-values indicate whether or not these differences are significantly different from 0%.

Discussion

Here, we described the association of antibody responses to a portfolio of gametocyte and non-gametocyte antigens with *P. vivax* transmission efficiency. We observed higher prevalence of antibodies against gametocyte and ookinete antigens Pvs47, Pvs48/45, Pvs230, PvHAP2 and Pvs25 among individuals with high levels of transmission blocking activity with statistically significant differences for Pvs47 and PvHAP2. No such association was found for antibodies against PvCelTOS and to a panel of asexual and sporozoite antigens. Antibody responses against Pv47 and Pvs25 were further associated with a lower per-gametocyte infectivity when whole blood samples were offered to mosquitoes.

The transmission of *P. vivax* parasites to mosquitoes is strongly associated with gametocyte density [7, 43, 44] that is in turn associated with asexual parasite density [7, 30, 44]. Whilst the positive association between mosquito infection rates and gametocyte density is also observed for *P. falciparum* [45, 46], for *P. vivax* this association is particularly strong and very high mosquito infection rates and infection intensities are commonly observed [7, 43, 44]. At present, *Pvs25* is the most widely used mRNA target to quantify circulating gametocytes although it may not accurately reflect gametocyte maturity or infectivity. These high mosquito infection rates and limited inter-person variation in per-gametocyte infectivity, suggest that host immune factors may play a relatively minor role in dictating transmission efficiency. Nevertheless, anti-gametocyte antibody responses have been observed for *P. vivax* and some of the most compelling evidence for immune-modulatory properties of serum factors comes from *P. vivax* infections [11-13] where antibodies may both reduce and enhance transmission (reviewed in [47]). In our cohort of patients with clinical *P. vivax* infections, 13.1% (8/61) individuals reduced transmission by more than 80%. We classified these individuals as individuals having high TBA. This arbitrary cut-off is commonly used in malaria transmission research although it is acknowledged that also lower levels of TBA may have a meaningful impact on transmission [48]. Too few individuals (n=5) enhanced transmission and the magnitude of transmission enhancement was very modest for most individuals. We concluded that this did not allow for meaningful analyses of immune responses associated with enhancement in our cohort. Also our number of transmission-blockers (n=8) was very modest but the effect was much larger with 80-100% reduction in the proportion of infected mosquitoes in whole blood compared to serum replacement conditions. We observed that the prevalence of antibodies against gametocyte antigens Pvs47 (OR=6.57; 95% CI: 1.33 – 32.48, p=0.0209) and

PvHAP2 (OR=14.82; 95% CI: 1.69 – 130.25, p=0.015) were significantly higher among individuals with this strong TBA (>80%) compared to those with lower levels of TBA. Whilst the small number of observations warn that these findings have to be interpreted with caution, it is striking that none of the asexual antigens was associated with TBA>80%, suggesting that cumulative prior exposure to *P. vivax* may not be predictive of functional transmission-reducing immunity.

We detected antibodies against the ookinete protein Pvs25 in ~25% of individuals which is in line with a previous study [49] where 19.2% of naturally infected patients had detectable Pvs25 antibodies. *P. falciparum* Pfs25 mRNA is translationally repressed [50] and no or very low levels of antibodies are detected in naturally exposed individuals [51-53]. In rodent models, Pys25, an ortholog of Pvs25 and Pfs25 in *P. yoelii*, protein expression was detected in gametocytes [54]. Our findings suggest that translational repression could be incomplete (leaky expression) in *P. vivax* similar as in *P. yoelii* [54]. Strikingly, the presence of Pvs25 antibodies was associated with lower transmission-efficiency in our broader dataset. Similar associations were observed for Pvs47, where antibody prevalence was associated with an estimated 34% reduction in mosquito infection rates, and Pvs230. PvCelTOS can be expressed on surfaces of both sporozoites and ookinetes [24], and we speculated that antibodies against PvCelTOS expressed on sporozoites could influence ookinete development, and thus transmission. However, in our cohort, we observed no association between PvCelTOS antibodies and either TBA or general transmission efficiency.

Our study has several limitations. First of all, our findings were primarily based on clinical malaria cases who are highly infectious in *P. vivax* [43, 44] and were therefore considered an informative population to examine changes in transmission efficiency. However, asymptomatic infections may also be relevant for the human infectious reservoir in some *P. vivax* endemic settings [7] and will have a different infection history and therefore plausibly a different antibody profile. Our findings can thus not be extrapolated to asymptomatic populations. We also used two platforms to quantify antibody responses. After initial attempts to couple antigens to beads for Luminex assays which were unsuccessful for all antigens except for HAP2, we used ELISA for all antigens other than HAP2. Most importantly, we describe epidemiological associations and our findings do not provide conclusive evidence for a causal role of anti-gametocyte antibodies in dictating *P. vivax* gametocyte infectivity. Whilst our findings for several antigens with an established role in gametocyte fertilization are biologically plausible and supported by pre-clinical studies where vaccine-

induced antibodies were causally associated with TRA and also broadly corroborate findings in *P. falciparum*, causality would require a different set of experiments. For *P. falciparum*, antibodies specific for Pfs48/45 and Pfs230 were purified from large-volume plasma samples and offered to mosquitoes in the presence of cultured gametocytes [52]. Similar experiments would allow demonstrating causality for antibodies against *P. vivax* gametocyte antigens but would have to rely on natural gametocyte donors since continuous culture of *P. vivax* has not been established. In addition, we did not collect the volumes of plasma (up to 9mL) that was used to demonstrate the functionality of antibodies against Pfs48/45 and Pfs230 in *P. falciparum* [52]. Furthermore, our study did not test these associations for different concentrations of antibodies or dilutions of autologous plasma. We also did not examine TBA after depleting plasma of antibodies using the recombinant proteins; this would have allowed us to explore TBA associated with antibodies to antigens other than the panel examined here or antibody-independent TBA. It is therefore important to interpret the described associations with caution, also demonstrated by positive and negative associations of mosquito infection rates with antibodies against several asexual antigens.

In conclusion, our mosquito feeding experiments on naturally infected Ethiopians provide evidence for a plausible role of antibodies against *P. vivax* sexual stage antigens in determining the transmission efficiency of *P. vivax* gametocytes to locally relevant *An. arabiensis* mosquitoes. These findings, that require confirmation in other populations, can help understand the natural transmission of *P. vivax* and support the development of transmission-blocking vaccine candidates.

Conflict of Interest

N.H.T. is listed as an inventor on patent US20190276506A1 related to CelTOS.

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

Author Contributions

Conceived, designed and supervised the study: TB, FGT. Wrote manuscript: SKT TB FGT. Edited manuscript: CD BW AV MJ WS IH WC EH NDS NHT DN[6]. Performed experiments: SKT WC EH AG TA DN[1] EE TE TT KT KL IH WS. Analysed data: JR TB SKT. Provided validated antigens: ET TT NDS NHT

Funding

This work was funded by an AMMODO Science Award to TB and the Gates Foundation (INV-002098; TB is further supported by a European Research Council Consolidator Grant (ERC-CoG 864180; QUANTUM). The work resulting in the production of PvCelTOS and Pvs230 antigens was funded by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health. WJRS is supported by a Wellcome trust fellowship (218676/Z/19/Z).

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Supplementary information

Table S1. Parasite density ratio between antibody positive and negative samples

Antigens	Density ratio	Lower CI	Upper CI	p-value
Pvs47	1.03376551	0.555177543	1.92491779	0.9167441
Pvs48/45	0.970252518	0.495599809	1.899496191	0.9298983
Pvs25	0.595658352	0.282660158	1.255248973	0.1749864
Pvs230	0.775207773	0.410002812	1.465714559	0.4344639
Pvs230-DIM	0.852875156	0.432412687	1.682180133	0.646683
PvCELTOS	1.0036775	0.523660802	1.923704275	0.9911899
PvAMA-1	0.890743838	0.423333609	1.874230081	0.761311
PvEBPII	0.816628842	0.388470056	1.716690015	0.5946083
PvHAP2	1.08067882	0.487100072	2.397590925	0.8491537
PvMSP-119	1.072617334	0.45735181	2.515586293	0.8723655
PvDPB-RII	0.488550347	0.236505021	1.009202429	0.0566313
PvRPB 2b	1.423115233	0.658281188	3.076583387	0.3725003
PvCSP VK210	0.737979624	0.34484648	1.579293855	0.4361784

Table S2. TBA of blocker and non-blocker sera

Antibody	Blocker (TBA>80%)	Non-blocker	OR (95% CI), p
Pvs47	50.0 (4/8)	13.2 (7/53)	6.57 (1.33, 32.48), p=0.0209
Pvs48/45	37.5 (3/8)	20.8 (11/53)	2.29 (0.47, 11.1), p=0.3031
Pvs25	37.5 (3/8)	22.6 (12/53)	2.05 (0.43, 9.85), p=0.3700
Pvs230	25.0 (2/8)	13.2 (7/53)	2.19 (0.37, 13.08), p=0.3898
Pvs230-DIM	12.5 (1/8)	22.6 (12/53)	0.49 (0.05, 4.37), p=0.5213
PvCELTOS	12.5 (1/8)	32.1 (17/53)	0.3 (0.03, 2.66), p=0.2809
PvHAP2	87.5 (7/8)	32.1 (17/53)	14.82 (1.69, 130.25), p=0.015
PvAMA-1	62.5 (5/8)	45.3 (24/53)	2.01 (0.44, 9.3), p=0.3699
PvEBPII	75.0 (6/8)	66.0 (35/53)	1.54 (0.28, 8.43), p=0.6168
PvMSP-119	100.0 (8/8)	79.2 (42/53)	8103773.78 (0, Infty), p=0.9894
PvDPB-RII	37.5 (3/8)	30.2 (16/53)	1.39 (0.3, 6.52), p=0.6782
PvRPB 2b	87.5 (7/8)	73.6 (39/53)	2.51 (0.28, 22.28), p=0.408
PvCSP VK210	75.0 (6/8)	71.7 (38/53)	1.18 (0.21, 6.54), p=0.8462

Table S3: Primers and Probes combinations

Primer type	P. falciparum (18S)	*P. vivax* (*Pvs25*)
Forward	5′-GTAATTGGAATGATAGGAATTTACAAGGT-3′	5′-ACA CTT GTG TGC TTG ATG TAT GTC-3′
Reverse	5′-TCAACTACGAACGTTTTAACTGCAAC-3′	5′-ACT TTG CCA ATA GCA CAT GAG CAA-3′
probe	5′-6FAM-AACAATTGGAGGGCAAG-MGBNFQ-3′	5′-FAM-TGC ATT GTT GAG TAC CTC TCG GAA-BHQ1-3′

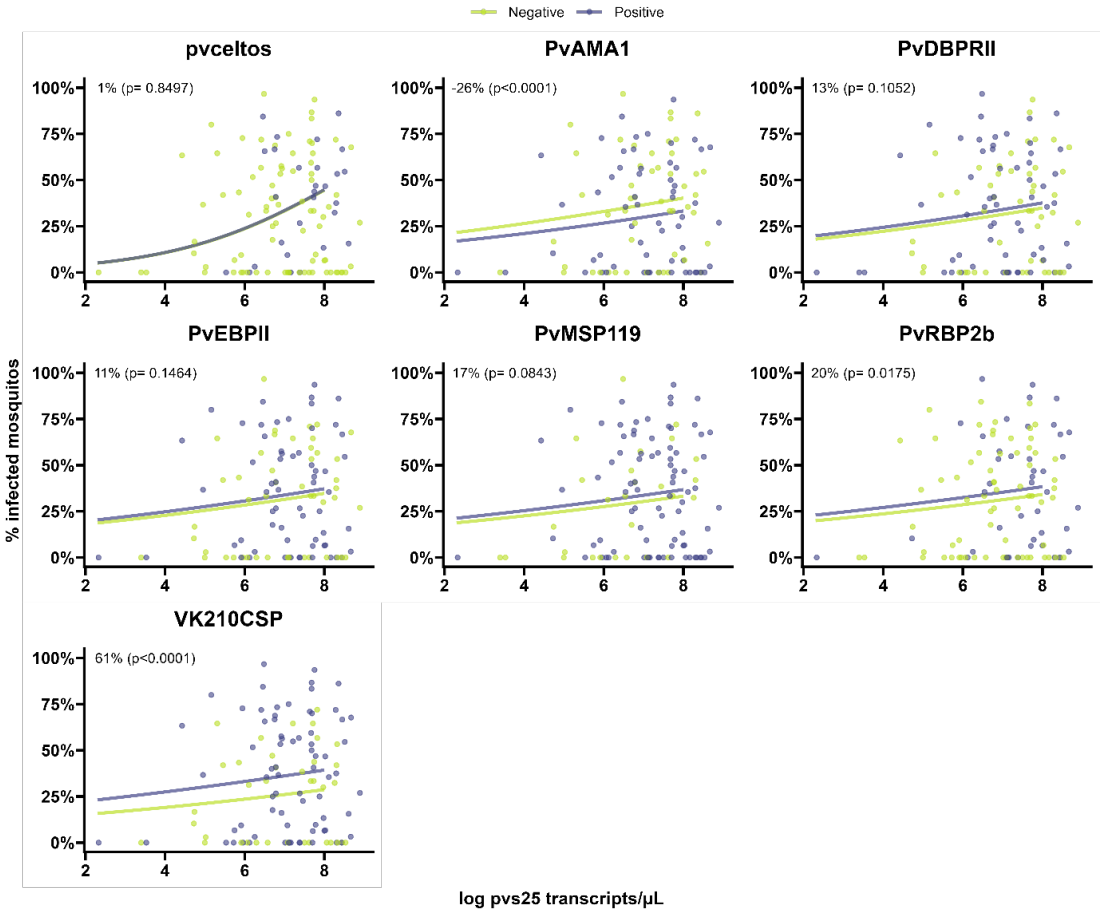
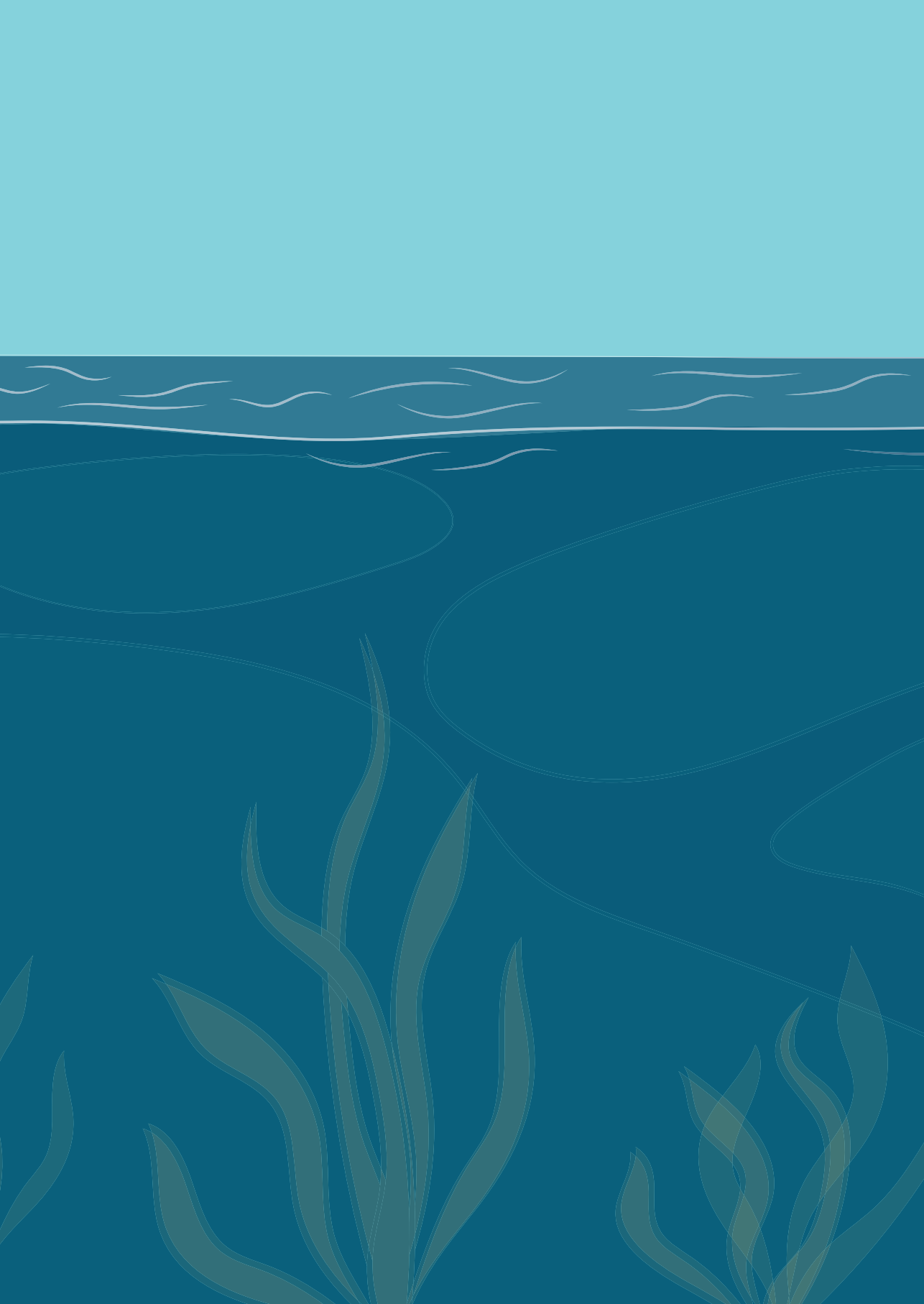


Figure S1. Scatter plots indicating the association between gametocyte density and antibody prevalence on mosquito infectivity for seven asexual stage antigens. Positive antibodies are indicated in light blue and negative antibodies are indicated in green. The light blue and green lines show the fit from logistic regression models for the association between gametocyte density and mosquito infectivity for positive and negative antibodies respectively. The numbers in the plot (estimated from logistic regression models) indicate the average difference in mosquito infectivity between positive and negative antibodies while accounting for gametocyte density and the p-values indicate whether or not these differences are significantly different from 0%.



CHAPTER 7

General discussion

Introduction

The work in this thesis has focused on the epidemiology and transmission dynamics of asymptomatic *P. falciparum* and *P. vivax* infections in Ethiopia. Firstly, we assessed the burden of asymptomatic malaria using routine diagnostic approaches (microscopy/RDT) and a sensitive molecular test (PCR) in low, moderate and high transmission settings of Ethiopia **(Chapter 2).** Secondly, we examined drug resistance profiles of *P. falciparum* asymptomatic infections by targeting known markers of resistance to chloroquine (namely, *Pfmdr1*-86 and *Pfcrt*-76) in areas with different levels of *P. vivax* co-endemicity that still use chloroquine for *P. vivax* treatment **(Chapter 3).** Thirdly, we compared the infectiousness of local *P. vivax* parasites to colony and wild-caught *Anopheles arabiensis* mosquitoes using direct membrane feeding assays (MFAs) in our field laboratory **(Chapter 4).** Fourthly, we conducted a 15-month longitudinal study to investigate the role of asymptomatic *P. vivax* and *P. falciparum* infections for onward transmission, as well as oscillations in parasite densities **(Chapter 5).** Finally, we assessed the presence of naturally acquired transmission reducing immunity among *P. vivax* symptomatic individuals against selected sexual stage antigens **(Chapter 6)**.

Part-1: Epidemiology and detectability of asymptomatic malaria in Ethiopia

Asymptomatic malaria infections: apparently silent yet with a noisy burden in all endemic settings

Given the advancement and use of more sensitive nucleic acid amplification techniques in the past decade [1, 2], the widespread presence of asymptomatic *Plasmodium* infections is increasingly appreciated across different endemic settings [3]. Perceived silent forms of the total malaria burden, the prevalence of these infections varies across settings and population segments [3-5]. In **chapter 2**, we determined the prevalence of asymptomatic *Plasmodium* infections and their detectability by comparing routine diagnostic approaches (Microscopy/RDT) with PCR in high, moderate, and low transmission settings of Ethiopia. Microscopy/RDT detected 44.2% of all asymptomatic infections that were positive by PCR, a finding in line with other studies where more than half of PCR detected asymptomatic infections were missed by RDT/microscopy [2, 6]. Most of the studies in Ethiopia are cross-sectional in design; used conventional RDT/microscopy to assess the prevalence of asymptomatic

malaria on community samples [7-12], school children [13, 14] and pregenant women (recently reviewed by [15]). Similar studies in Ethiopia that compared PCR and RDT/microscopy diagnosis reported that RDT/microscopy missed more than half of the infections that was deteceted by PCR [16-19].

Interestingly, the prevalence of asymptomatic *Plasmodium* infections was higher in 5-15 years of age groups (school age children) using both microscopy/RDT (20.7%) and PCR (26.7%) as compared to children below five years (10.6% by microscopy/RDT and 21.5% by PCR) and adults (>15 years old; 5.6% by microscopy/RDT and 16.9% by PCR). This relatively high burden of infection in school age children has been reported before [20-22] and may be partially explained by lower coverage with interventions like insecticide treated nets in this age group. Importantly, the detectability of these infections by microscopy/RDT was found to be lower in adults (>15 years age group) with an approximate 5% decline in detectability of infections for every year increase in age (Adjusted odds ratio= 0.95%, 95%CI: 0.9-1.0), measured across sites. Similar community-based studies also found that age is a determining factor in the detectability of asymptomatic infections where school age children are the most affected [23, 24]. The cumulative exposure (and thus age-) dependent acquisition of immunity that limits parasite densities explains why the detectability of infections decreases with increasing age. As indicated in **Chapter 2**, the overall proportion of asymptomatic infections detected by microscopy/RDT was 5-fold higher in high transmission compared to low transmission settings. Notably, the level of detectability by microscopy/RDT was lower as transmission intensity declines, as reported in other studies [24-26]. Several studies compared the detectability of asymptomatic *P. falciparum* infections using microscopy/RDT and PCR [2, 6, 27]. Relatively, few studies measured the prevalence of asymptomatic infections simultaneously using both microscopy/RDT and PCR in *P. vivax* and *P. falciparum* co-endemic settings [8, 16, 28, 29]. Among PCR detected asymptomatic infections, the detectability of asymptomatic *P. vivax* infections by microscopy/RDT was markedly lower compared to *P. falciparum*. Overall, 4.6 % of PCR-detected *P. vivax* infections were detectable by microscopy/RDT compared to 48.7% of PCR-detected *P. falciparum*. *P. vivax* has a much lower density of circulating peripheral parasitemia (<2%), explained by the strict preference of this parasite species to infect reticulocytes [30]. *P. vivax* might thus require a more sensitive diagnostic tools in co-endemic settings.

The detectability of asymptomatic *Plasmodium* infections depends on a multitude of factors. This includes the level [31] and history [32] of transmission, the variations in the methods of diagnosis [1, 6] as well as the parasite species at play. The detection and quantification of asymptomatic infections by molecular markers at large scale would not be cost-effective using nucleic acid-based methods, since these are not routinely accessible in many resource limited settings [33]. Given the required intensity of training, supplies and maintaining the competence to avoid misdiagnosis while using microscopy [34], RDTs might play an important role in clinical decision making [35]. However, in *P. falciparum* endemic countries, *P. falciparum* histidine rich protein 2 (*pfhrp2*) gene deletions that threaten the utility of the current HRP2 based-RDTs are being reported widely [36, 37]. In some settings up to 62% of clinical cases may be negative by RDT upon clinical presentation[38]. Accurate diagnosis and estimation of the burden would benefit from using field deployable more sensitive tools and next generation RDTs that can detect *P. falciparum* parasites with *pfhrp2* deletions. *P. vivax* infections have relatively low parasite densities compared to *P. falciparum* and are thus more likely to be missed by using microscopy and current RDTs [39]. For this species, the major challenge also comes from the hard to detect relapsing *P. vivax* hypnozoites [40]. Panels of serological markers of recent exposure are recommend as indirect measures of hypnozoite carriage and *P. vivax* asymptomatic burden [41, 42]. Moreover, as transmission declines the level of malaria heterogeneity increases and infections cluster in some locations and population segments [43, 44]. Importantly, RDT/microscopy-positive *P. falciparum* malaria infections are likely to be surrounded by sub-patent infection carriers [45]. This geographical clustering provides the opportunity for mass screening and treatment of asymptomatic infections around index cases. In addition, the relatively high burden of asymptomatic *Plasmodium* infections in school age children supports the notion of alternative malaria intervention approaches that could focus on school-age children to improve their health as well as reduction of onward transmission at community level [22, 46].

Asymptomatic Plasmodium parasites: linking the drug resistance profile of the parasite population

The first *P. falciparum* chloroquine (CQ) resistance was reported in South America [47] and Southeast Asia [48]. Decades later, CQ resistant *P. falciparum* parasites harboring *Pfcrt*-76T and the *Pfmdr1*-86Y, 1246Y haplotypes were found widespread across Africa [49, 50] which necessitated the change from CQ to sulphadoxine-pyrimethamine (SP). SP, in turn, was quickly replaced by artemisinin combination therapy (ACT) around the beginning of the millennium

due to the rapid emergence and spread of resistance to SP [51]. Although the first evidence of artemisinin resistance was reported in the Thai-Cambodia border region [52, 53] and recently in Africa [54], ACTs still remain highly effective as long as the non-artemisinin partner drug is effective. This is where the reports from this thesis become important. We determined the prevalence of mutations that are associated with partner drug efficacy. ACT partner drugs can have opposing selective pressures on parasite populations. CQ sensitive parasites that harbor *Pfmdr1*-N86 wild-types are less susceptible to lumefantrine [55, 56]. This is similar to amodiaquine (AQ) where the NFD haplotypes at positions 86, 184 and 1246 of the *Pfmdr*1 gene show reduced sensitivity to AL [57-59]. On the other hand, dihydroartemisini-piperaquine (DHA-PPQ) has an opposite effect of selecting parasites with *Pfmdr1* 86Y/Y184/Y1246, making it suitable choice for areas with AL tolerant or resistant parasites [60].

Several studies reported the return of CQ sensitive parasite harboring *Pfcrt*-K76 and *Pfmdr1*-N86 following CQ withdrawal [61-63]. Motivated by these reports on the reappearance of CQ sensitive parasites, we hypothesized that this may also occur in Ethiopia but may be dependent on the level of co-endemicity with *P. vivax*. Since CQ is still used for *P. vivax* treatment in co-endemic settings in Ethiopia, we hypothesized that this ongoing treatment may exert a selective pressure on *P. falciparum* parasites in these areas and slow down the reappearance of CQ sensitive parasites. To test this hypothesis, we screened known molecular markers of CQ resistance (*Pfcrt*-K76T and *Pfmdr1*-N86Y) in community samples of *P. falciparum* asymptomatic infections **(Chapter 2)** collected from previous cross-sectional studies [64-66]. We found a relatively high proportion of wild-type *Pfmdr1*-N86 (range:77%-100%) in all *P. vivax* and *P. falciparum* co-endemic settings, contrasting with earlier reports where the prevalence of mutant genotypes was still exceeding 80% in Ethiopia [67, 68]. The level of *Pfcrt*-76T mutant type was found still fixed in areas with higher *P. vivax* endemicity in our study (98-100%), whilst this proportion ranged from 42%-65% in settings of relatively low *P. vivax* endemicity **(Chapter 3).** Contrary to our finding on the fixation of *Pfcrt*-76T in areas with considerable *P. vivax* endemicity, a study from Southern and Eastern Ethiopia indicated the emergence of *Pfcrt*-K76 wild-type (84.1%) following CQ withdrawal [62]. Our study supports the re-appearance of CQ-sensitive *Pfmdr1*-N86 genotypes whilst the high frequency of the CQ-resistant *Pfcrt*-76T genotype, especially in *P. vivax* co-endemic settings, argues against the future utility of CQ for *P. falciparum* treatment in these areas. Most of the previous studies in Ethiopia were conducted on clinical isolates; our study **(Chapter 3)** in

asymptomatic infections is particularly relevant given the evidence that *Pfmdr1*-NFD haplotypes (SNPs linked with lumefantrine tolerant parasites) were observed more frequently in asymptomatic *P. falciparum* infections than symptomatic infections [69].

In *P. falciparum* endemic settings, there is a claim that AL-tolerant but artesunate-amodiaquine (AS-AQ) sensitive parasites would emerge in 7-10 years following AL introduction [70]. This has policy implications on cycling drugs with opposite selection pressure or use of multiple-first line drugs to slow down emergence of drug resistant parasites. However, in co-endemic settings this might be context specific and might benefit from careful interpretation. In our study, the fixation of *Pfcrt*-76T relatively high in *P. vivax* co-endemic settings calls for accurate diagnosis and treatment of malaria as CQ is the choice of treatment for *P. vivax*. It can be argued that in co-endemic settings with continued use of CQ for *P. vivax*, there is likely to be differential drug selective pressure on *P. falciparum* parasites. This may be due to misdiagnosis of *P. falciparum* infections as *P. vivax* cases exposing the *P. falciparum* parasites to CQ during treatment. Moreover, *P. falciparum* new infections can have a chance of residual CQ exposure due to the longer half-life of CQ [71] following *P. vivax* infection treatment. In this situation, *P. falciparum* infections would be acquired in a phase when low and ineffective CQ levels are present in the bloodstream, providing an environment that clearly allows for the selection of CQ-resistant *P. falciparum* parasites. Importantly, asymptomatic *P. falciparum* infections might be misdiagnosed during *P. vivax* symptomatic periods in co-infections and/or escaped as undetected low-density asymptomatic infections by microscopy/RDT **(Chapter 2)**.

Part-2: Asymptomatic malaria parasite transmission dynamics: when and for how long?

Is the measurement tool comparable and optimized for interpretation?

Direct membrane feeding assays that use colony mosquitoes that are maintained under controlled laboratory conditions are the main tools to study the infectiousness of human reservoirs to mosquitoes in natural settings [72, 73]. However, optimization of MFA for local primary vectors requires consideration of several parameters that can affect mosquito feeding rate such as starving conditions (with access to water or dry starvation) and duration of starving prior to feeding [74, 75], membrane type [74], type of feeders [76], mosquito age [77], the volume of blood in the feeder [74, 76], density of mosquitoes

during feeding [74, 77], water bath temperature [78] and feeding conditions in dark [79] versus light [74]. Moreover, the process of colonizing mosquitoes in a laboratory might result in undesirable adaptation and genetic inbreeding [80, 81] that will require careful interpretation when colony mosquitoes are used in transmission studies.

Before we embarked on a 15 month-study to investigate the infectiousness of asymptomatic malaria infections in our co-endemic setting of Adama, **(Chapter 5),** we assessed and compared the permissiveness of an established colony of *An. arabiensis* (>800th generations) relative to *An. arabiensis* mosquitoes raised from wild-collected larvae. This was done using paired feeding experiments on fresh blood from naturally infected *P. vivax* patients **(Chapter 4).** We found that blood feeding efficiency was higher in colony mosquitoes compared to wild mosquitoes. Of note, the starvation time was different between the two mosquito sources (12 hours for colony mosquitoes versus 18 hours for wild mosquitoes), based on observations of mosquito survival and aggressiveness. The duration of starvation for both wild and colony was still within the range of other studies that reported dry starvation (without access to water) of 5-24 hours [75, 79, 82]. We concluded that the difference in feeding rates is a consequence of differences in adaptation to membrane feeding.

Importantly, not all anopheles mosquitoes are equally susceptible and permissive to *P. falciparum* and *P. vivax* infections. The new world anophelines are believed to be evolutionary distant from the African counterparts and less efficient vectors [83]. The public health implications of this varying vector competency was indirectly demonstrated during the introduction of *An. arabeinsis* to Brazil in the 1930s when malaria prevalence and mortality increased [84]. A genetic basis for intra-species variation in susceptibility was demonstrated by the finding that *An. gambiae* mosquitoes carrying the homozygous 2La+^{a}/2La+^{a} inversion karyotype were associated with increased prevalence of infection and oocyst burden compared to 2La/2La homozygotes [85].

In our study **(Chapter 4)**, the proportion of *P. vivax* infected mosquitoes from colony mosquitoes (median: 55.0%, IQR: 6.7-85.7) was very similar to that of wild mosquitoes (median: 52.7%, IQR: 20.0=80.0%). However, the variation in *P. vivax* receptivity among species was noted in a study where higher proportion of *P. vivax* infection was recorded in colony mosquitoes of *An. aquasalis* compared to wild-collected mosquitoes of *An. darlingi*, *An. albitarsis s.l.*, *An. nuneztovari s.l.* and *An. triannulatus* s.l.

Notably, the extrinsic incubation period of the malaria parasites, the duration of the parasite's development from ingestion of gametocytes in a blood meal up to the development of invasive sporozoites in the salivary gland of the mosquito, can vary across vectors [86, 87]. *Plasmodium* infection establishment in mosquitoes is a function of several factors such as larval diet [88, 89], midgut microbiota [90, 91] as well as the innate immune system of the mosquito [92]. Room temperature fluctuation, especially at early infection establishment of the parasite (<12 hours post blood meal feeding) is also considered a determing factor of oocyst burden [93]. In our study **(Chapter 4)**, we found that the median oocyst load was comparable among the colony (25 oocyst/midgut: IQR=5.0-830) and wild mosquitoes (25.5 oocysts/midgut: IQR=5-47) similar to another study that compared *P. vivax* oocyst load among colony and wild *An. stephensi* [94]. Therefore, we showed that our colony mosquitoes can be readily used to assess parasite dynamic studies in our Adama cohort study **(Chapter 5)** and to assess prevalence of naturally acquired transmission reducing immunity against selected *P. vivax* gametocyte antigens at Arba Minch **(Chapter 6)**.

2.2. Transmission dynamics of naturally occurring asymptomatic *Plasmodium* infections: duration of infection and infectiousness to mosquitoes

Early in the 1950s, the duration of *Plasmodium* infections, as one of the fundamental biological questions, was estimated based on malariotherapy data [95, 96]. Whilst this allowed highly detailed estimates under controlled conditions, only a small number of *Plasmodium* isolates were used in such therapies that aimed to safely induce fever and therefore also focused on relatively benign parasite isolates. Doing the same studies with other parasite isolates in endemic settings is complicated due to the possibility of super-infection and limitations of parasite diagnostics in quantifying all circulating parasites. The recent advancements in molecular diagnostic tools allow better estimates of the duration of chronic asymptomatic infections in endemic settings. However, there is variation in study designs, transmission settings, and diagnostic approaches in determining the duration of asymptomatic malaria infections and their relevance in maintaining transmission in natural settings [97]. The median duration of asymptomatic *P. falciparum* infections has been estimated to be 6 months in high transmission settings [98, 99] and 2 months in low transmission settings [100, 101]. On the other hand, *Plasmodium vivax* may have an estimated average duration of six months [101]. To help better understand the epidemiology of *P. falciparum* and *P. vivax* infections, more data are needed to quantify (variation in) infection duration in different settings.

To address these gaps, we longitudinally evaluated the dynamics of low-density asymptomatic *P. vivax* and *P. falciparum* infections and their potential to infect *An. arabiensis* mosquitoes in a low endemic setting where both species are co-endemic **(Chapter 5)**. The rate of spontaneous infection clearance was relatively slow for *P. vivax*: 40% of *P. vivax* compared to 22% of *P. falciparum* infections persisted for at least 3 months with median durations of infection from baseline being 60 days (95% CI: 18-213) and 37 days (95% CI: 15-93), respectively **(Chapter 5)**. These estimates are similar to those observed in low transmission settings in Southeast Asia [100, 101] and have implications for the fraction of infections that can bridge the dry season in areas of seasonal malaria transmission [102, 103]: a large fraction of infections are cleared rapidly and are unlikely to sustain transmission. Notably, we observed that infections frequently alternated between the two species during the follow-up period with one species disappearing and the other (re)appearing. Specifically, 14.2% (5/35) of individuals who were initially positive for *P. falciparum* later became positive for *P. vivax* while 38.2% (13/34) of initially *P. vivax* mono-infections had qPCR-detected *P. falciparum* infections at least once during the follow-up **(Chapter 5)**. This phenomenon is common in co-endemic settings [39, 101], as *Plasmodium* infections can cluster spatially in a given population and/or within a single host [104] due to shared risk factors and co-transmission of different *Plasmodium* parasites by the same mosquito populations. Our findings raise several questions. It is unclear what the role of the host immune response in such settings with overlapping species is and whether co-infection with another species influences parasite fitness/infection duration of the alternate species. In addition, we don't know whether the existence of *P. falciparum* may trigger hypnozoite activation or perhaps attenuates it in co-endemic settings. Lastly, we do not fully understand the consequences of mixed-species infections for gametocyte development and transmission. A recent observation from a site with four sympatric *Plasmodium* species in Papua New Guinea revealed that *P. malariae* may increases its gametocyte and total parasite density when co-infection occurs with *P. falciparum* [104]. These observations might be important to guide vaccine designs or therapeutic interventions at cross-species level and underline the importance of targeting both species simultaneously for effective elimination.

In our cohort **(Chapter 5)**, 13 *P. falciparum* and five *P. vivax* infected individuals were able to resolve their infections immediately with no subsequent parasite positivity while seven *P. falciparum* and five *P. vivax* asymptomatic infections became symptomatic during the follow-up period. In addition, sub-microscopic

asymptomatic infections may clear or decline to parasite densities below the qPCR sensitivity level and/or increase in densities to microscopic detection levels [3]. Among our group of individuals who were *P. vivax*-positive at baseline (n=34), (97.0%, 33/34) were sub-microscopic infections of which 18.2% (6/33) were followed by microscopy detectable *P. vivax* mono-infections. Similarly, among the *P. falciparum* group (n=35), 81.8% were sub-microscopic at baseline while 31.0% (9/29) of these infections were followed by moments when *P. falciparum* was detectable by microscopy. These findings are in line with other findings presented in this thesis **(Chapter 2)** where most asymptomatic infections were sub-microscopic infections. In that chapter, **(Chapter 2)**, we further observed a lower average parasite density that was not detected by microscopy/RDT in low-endemic settings compared to high transmission settings. A meta-analysis concluded that a non-negligible proportion of low-density *P. falciparum* infections (with fluctuating parasite densities) can still be infectious to mosquitoes [3].

Although our study focused on the duration of infection carriage using 18S qPCR, genotyping of the detected infections would have improved estimates of parasite clearance at clonal level by capturing relapses from *P. vivax* hypnozoites [105] and/or subclinical tissue sequestration of *P. vivax* [106] as well as *P. falciparum* [107, 108]. Genotyping would also help to distinguish infections that apparently disappear from the blood stream but probably linger in very low concentrations before they recur after several weeks of parasite negativity by qPCR [20, 109].

To shed light on the infectivity of longitudinally monitored infections, a total of 114 membrane feeding experiments were conducted on 56 asymptomatic participant donors (22 *P. falciparum*, 24 *P. vivax* and 10 mixed). For *P. falciparum*, 6.4% (3/47) of the repeated feeds infected 1.8% (29/1579) of the dissected mosquitoes. Clinical *P. vivax* cases were more infectious than asymptomatic infections, as observed in our study **(Chapter 6)** as well as other studies [65, 110]. Strikingly, none of the asymptomatic *P. vivax* mono-infections appeared infectious (0/56) despite dissecting 1800 blood-fed mosquitoes. Only one mixed species infection was able to infect mosquitoes with high gametocyte and asexual stage parasitemia for *P. vivax* but with only asexual stage *P. falciparum* detectable using qPCR. It is thus likely that this single infectious case with a mixed *P. vivax/P. falciparum* infection, infected mosquitoes with *P.* vivax.

Table 1. Relative contribution of asymptomatic and symptomatic malaria infection reservoirs adjusted for population prevalence at Adam, Ethiopia in 2019/20.

Reservoir categories in a hypothetical population of 100,000 people	Prevalence in population	Percentage of infected mosquitoes from our data (number infected/ dissected)	Number of infected mosquitoes (assuming equal biting)	Percentage contribution
P. falciparum				
Clinical cases at this time point	18.25	2.70	0.50	0.05
Asymptomatic, microscopy+ infections at this time point	470.00	18.70	87.90	8.30
Asymptomatic, microscopy-, qPCR+ infections at this time point	3200.00	30.40	972.80	91.70
Total			1061.20	
P. vivax				
Clinical cases at this timepoint	19.63	42.40	8.30	6.70
Asymptomatic, microscopy+ infections at this time point	140.00	83.30	116.60	93.30
Asymptomatic, microscopy-, qPCR+ infections at this time point	3970.00	0.00	0.00	0.00
Total			124.90	

Note. This table is based on data from Adama district 2019/20 of *P. falciparum* clinical case=1318 and *P. vivax* clinical cases= 918. The district population was estimated to be 217,839 based on 2019 Ethiopian statistical agency [117]. The incidence rate was 6.05 and 4.20/per year/1000 people for *P. falciparum* and *P. vivax*. Based on the assumption that symptomatic untreated *P. falciparum* and *P. vivax* infections stays 11 days and 17 days respectively [118], the probability of a person having symptomatic infections of *P. falciparum* and *P. vivax* on a given day in our setting is 0.0001824 and 0.00019627, respectively. For a hypothetical 100,000 people, the probability of having symptomatic *P. falciparum* and *P. vivax* at a given time would thus be 18.25 and 19.63, respectively. Asymptomatic microscopy positive and submicroscopic infections for each species were extrapolated from our screening data where 0.47% and 3.20% for *P. falciparum* and 0.14% and 3.97% for *P. vivax*, respectively. These proportions (column 4) were adjusted for 100,000 people for each species in column two. Estimated number of infected mosquitoes for each category was also adjusted for the prevalence in 100,000 population (reservoir prevalence in 100,000 population (column two) x percentage of infected mosquitoes form the membrane feeding data(column3)/100). The percentage contribution was calculated as estimated number of infected mosquitoes (assuming equal biting)/ total number of infected mosquitoes infected x 100) in column five.

Although the potential infectiousness of low-density asymptomatic infections is smaller compared to high density infections [111-114], their relative contribution to transmission at population level might be compensated by their higher prevalence in natural settings [115, 116]. To put this in perspective in our study, we interpreted our findings on infectivity in the context of the plausible occurrence of different infection classes in our populations. If we assume a hypothetical population of 100,000 individuals in our low endemic setting, most of the contribution of the infectious reservoir to mosquitoes would be from submicroscopic *P. falciparum* infections (91.7%) and microscopy detectable asymptomatic *P. vivax* infections (93.3%) after adjustment for population prevalence **(Table 1)**. These findings obviously need to be interpreted with great caution, given the small number of infected mosquitoes in our population from an even smaller number of infectious donors. Nevertheless, they underline the importance of accurately capturing population frequencies and highlight that high infectivity of clinical malaria cases (as observed for *P. vivax*) does not necessarily translate into a large role in community transmission. During the study period **(Chapter 5)**, there was an overall decline of malaria incidence among the general population in the study site compared to an earlier assessment of the human infectious researvoir for malaria in the same setting [65].

As indicated in table 1 above, characterizing the infectious reservoir and contribution of human infectiousness to transmission is not sufficiently determined by comparing infectiousness of symptomatic and asymptomatic infections using MFA per se. Although such studies are highly informative regarding the transmission biology of the parasite and infection potential of reservoirs, their small sample size, the experimental nature of the membrane feeding using colony mosquitoes might not allow to capture the variation in human activity, vector competence and behavior, the parasite biology that might impact its transmission in uncontrolled natural settings [119]. Therefore, interpreting results from MFAs to actual transmission potential of individuals will require incorporating the human exposure to mosquito and the rate at which the individual reservoir is sampled by the mosquitoes together with other host and parasite factors of infectiousness in epidemiological settings. The variation in human exposure to mosquitoes and the biting preference of mosquitoes will probably influence transmission and the contribution of human infectiousness to mosquitoes [115].

Future longitudinal studies that aim to better understand transmission dynamics of low density asymptomatic infections in *P. falciparum* and *P. vivax* co-

endemic settings should consider:- 1) intensive sampling using more sensitive diagnostic tools coupled with advanced genotyping to track individual clones, 2) not recruiting individuals based on ongoing parasite carriage but capturing incident infections (for example by treating initial asymptomatic infections and following a cohort closely to capture incident infections), 3) address the impact of chronic asymptomatic infections on human health, particularly in areas of malnourishment, co-infections with other pathogens and limited access to health facilities. This could be relevant since there is paucity of data on the infectivity and public health importance of low-density asymptomatic infections in co-endemic settings. It further enables to guide appropriate malaria elimination interventions in co-endemic settings.

Part-3: Towards transmission blocking vaccine development: a promising approach for future interventions in co-endemic settings (Chapter-6)

P. vivax has the ability to produce gametocytes within 2-3 days after the first wave of asexual parasites [120]. Moreover, these gametocytes can infect a wide range of mosquitoes, making its transmission highly efficient [121]. This requires a practical distillation of novel tools to push the malaria elimination agenda, particularly in settings where this parasite is co-endemic with *P. falciparum*. A promising tool to support malaria elimination efforts would be to interrupt onward transmission using transmission blocking vaccines (TBVs). Since the first observations from 1958 and 1976 on reduced oocyst development in mosquito midguts that fed on chickens immunized with *P. gallinaceum* [122, 123], antigens of the sexual stages of malaria parasites (gametocytes, gametes, zygote and ookinetes) have been incriminated as potential targets for malaria transmission blocking vaccine development [124]. Transmission reducing immunity is thought to be mostly antibody mediated. Key advantages of TBV include that TBV antigens are less polymorphic compared to pre-erythrocytic (sporozoite and liver stage) or asexual blood stage antigens [125]. Compared to asexual stage vaccines, antibodies against TBV antigens also target fewer parasites at a biological bottleneck of the parasite lifecycle: whilst billions of *P. falciparum* asexual parasites can circulate in a single infected host, on average 2-5 oocysts per mosquito mid-gut are formed in natural infections [126]. In direct membrane feedings, about 80% of infected mosquitoes likely to have a mean of <5 *P. falciparum* oocysts per midgut [127] while this proportion may have 10-100 oocysts per midgut for *P. vivax* [110].

The most studied antigens that can elicit *P. falciparum* transmission-blocking antibodies are Pfs48/45, Pfs25, Pfs47, Pf230, and HAP2 [128-132]. Similarly, for *P. vivax,* Pvs48/45, Pvs230, Pvs47, PvHAP2 and Pvs25, Pvs28 and *P. vivax* Cell-traversal protein for ookinetes and sporozoites (PvCelTOS) orthologs of *P. falciparum* TBVs are being studied as potential TBV candidates [133-137]. The association between gametocyte (or total parasite) density and mosquito infection rate is very tight for *P. vivax* where a high percentage of mosquitoes is infected when feeding on blood of clinical cases with high parasite and gametocyte densities [110] **(Chapter 5)**. Given that the association between gametocyte density and mosquito infection rates appears stronger for *P. vivax* compared to *P. falciparum* [65], the role of naturally acquired transmission reducing immunity and its efficiency is not clear in *P. vivax*. At the same time, studies (including our own) typically observed that some individuals with high *P. vivax* gametocyte densities fail to infect mosquitoes, suggesting that serum factors might – like in *P. falciparum* – inhibit mosquito infections.

In **Chapter 6**, we have examined the association of anti-gametocyte immune response with transmission efficiency in natural *P. vivax* infections using 368 *P. vivax* passively recruited symptomatic cases and 56 samples from asymptomatic *P. vivax* parasite carriers who participated in our longitudinal study **(Chapter 5)**. MFA was performed to assess mosquito infectivity for all samples while the level of transmission reducing immunity was estimated for 61 samples by replacing the autologous plasma of infected donors with malaria naïve AB European control serum in paired feeding sessions. Six sexual stage antigens (Pvs47, Pvs48/45, Pvs230 (two variants), PvCelTOS, PvHAP2 and Pvs25) and five asexual stage antigens: Merozoite surface protein 1-19 [MSP1-19], Apical membrane antigen 1 [AMA1], Duffy binding protein [DBP RII], Reticulocyte binding protein [RBP 2b], Erythrocyte binding protein [EBPII]) were investigated by ELISA and multiplexed bead assay for antibody prevalence in association with *P. vivax* infectivity.

Our findings **(Chapter 6)** highlighted the presence of transmission reducing activity (TRA) associated with antibodies against the sexual stage antigens of Pvs47 (23% reduction in mosquito infection rate), Pvs230 and Pvs25 (both 34% mosquito infection rate reduction). Importantly, individuals with evidence of transmission blockade in paired feeding experiment (8 individuals whose autologous plasma inhibited oocyst formation by over 80% (TRA>80%)) were more likely to have antibodies against PvHAP2 (OR=14.82; 95% CI: 1.69 – 130.25, p=0.015) and Pvs47 (OR=6.57; 95% CI: 1.33 – 32.48, p=0.0209) compared to those

with lower levels of TRA. Studies elsewhere also reported an association of antibodies with significant TRA in MFA for PvHAP2 [135], Pvs48/45, Pvs47 [133] and Pvs230 [134]. Compared to these studies that were done using mouse antisera and small number of *P. vivax* cases [134, 135], our study **(Chapter 6)** was the first direct evidence using naturally infected individuals' sera and orthologous serum replacement to measure the transmission blockade/ reduction of antibodies directed against these *P. vivax* gametocyte antigens. Folllowing the publication of our study, work from Thailand indicated a weak association of antibody response against Pvs25, Pvs28 and Pvs230 with TRA of orthologous parasite strains [138]. That study further highlighted that naturally acquired TRA can be mediated by antibodies as well as other serum factors with heterogeneous response depending on *P. vivax* strain [138].

Importantly, an association of antibodies with TRA does not confirm their actual functional transmission reduction. It is theoretically possible that the responses we measured – whilst biologically plausible given their presence on the membrane surface of gametes – are not causally responsible but correlated with other antibody responses that functionally interfere with transmission. Since antibody responses to different antigens are typically correlated [129, 138], it is very difficult to formally prove causality in epidemiological studies. The reality of correlations with non-functional responses is illustrated in our data by the weak association of antibodies against AMA-1 with transmission; a 26% decrease in mosquito infection rates. AMA-1 is not expressed in gametocytes or gametes; the antigen in fact received a negative "Gametocyte score of -36.47" in a bioinformatics analysis of the gametocyte specificity of proteomic and transcriptomic data [139]. The (weak) correlation we observed with TRA therefore indicates that some serological associations may be misleading. In addition, there is evidence of TRA in the absence of antibodies against Pfs48/45 and Pfs230 in a given serum sample [129] suggestive of other causally important antibodies. Nevertheless, since antibody responses for some of the sexual stage antigens (PvCelTOS and Pvs230-DIM) were weak and none of the antibodies against the asexual stage antigens were associated with >TRA of 80% compared to less efficient TRA in our study **(Chapter 6)**; our findings are broadly in line with our biological understanding of transmission and transmission-relevant proteins. As such, our findings provide insights in the biological plausibility of antibody responses that can be orchestrated and work together for a better TRA [129]. We conclude that the observed association of antibodies against sexual stage *P. vivax* antigens with reduced transmission efficiency needs further investigation with a robust cohort study in endemic population that

ideally moves beyond statistical associations and may purify antibodies against specific antigens and offer these in the presence of infectious gametocytes to mosquitoes, as previously done successfully for *P. falciparum* [129].

We have demonstrated that the prevalence of antibodies against selected *P. vivax* sexual stage antigens in natural infections was associated with reduced transmission of gametocytes to locally reared *An. arabiensis* mosquitoes **(Chapter 6)**. However, to investigate the duration of TRA, confirm the functional role of antibodies and to discover novel and effective transmission-blocking antigens for *P. vivax*, longitudinal studies with intensive sample collection from *P. vivax* patients in natural settings would be required. The following study design could be considered: *Plasmodium vivax* symptomatic patients can be enrolled into field-based MFA serum replacement experiments using *An. arabiensis* colony mosquitoes. Preferably, individuals with TRA>80% can be followed by regular (e.g. monthly) venous blood sampling for peripheral blood mononuclear cells (PBMCs) isolation and immunophenotyping (single memory B cell screening and BCR germline sequencing). These cohort plasma samples can be used to track the duration, antibody kinetics and functionality of antibodies among those that are potential transmission blockers. Then, sexual stage proteins of *P. vivax* can be selected from existing proteomic and transcriptomic data sets as has been reported previously [139]. These proteins could be expressed using appropriate expression system such as the wheat germ cell free (WGCF) platform that can maintain high quality recombinant protein structure [140]. Protein microarray libraries baed on this expression can be used to assess the immune signatures of high TRA samples, using the cohort sera compared with known positive controls such as Pvs47 and PvHAP2 [129]. From these transmission blockers (ideally with persisting transmission blockade), potent human recombinant mAbs can be generated against the most promising *P. vivax* sexual stage proteins (e.g. those that exhibit TRA>80%) from their single specific–memory B-cells [141, 142]. Finally, the isolated potent human mAbs can be tested in the field using MFAs in natural settings for their TRA in comparison with antibody depleted and heat-in activated sera from the cohort transmission blockers sera, naïve European sera and whole blood using *An. arabiensis* colony mosquitoes. This allows assessment of functionality against genetically diverse parasites. The functional role of these antibodies against the sexual stage antigens can also be tested using natural gametocyte donors in Ethiopia or elsewhere. This can also be further replicated in a cohort of asymptomatic patients across different transmission settings to investigate the role of transmission reducing activity of these antigens and their mAbs in

this population **(Chapter 5).** This approach, whilst a major undertaking, will potentially be a major step forward in pushing *P. vivax* transmission blocking interventions to support malaria elimination in co-endemic settings.

Future perspectives and outstanding questions

The most pressing questions that will support our malaria elimination efforts are related to the large reservoir of asymptomatic malaria infections that cannot be readily detected by the current field deployable RDT and microscopy. These chronic asymptomatic malaria infections represent the majority of the infection burden and can be used as a source of valuable information to understand key host-parasite-vector interactions in natural settings. Some of the outstanding questions that remain to be answered following the knowledge generated in this thesis are:

1. What kinds of field-deployable sensitive diagnostics are feasible to target asymptomatic infections to harness malaria elimination in co-endemic settings? Do we need diagnostic approaches tailored to specific transmission settings as transmission approaches elimination?
2. What are the public health consequences of persisting low-density asymptomatic malaria infections in overlapping ecologies with other infections?
3. Does the environment (and changes in this environment such as climate change) shape vector competence and permissiveness towards the *Plasmodium* parasites? What host and parasite factors play a role in the duration and chronicity of asymptomatic infections?
4. What is the role of undetected ultra-low parasite densities that might sequestered as hypnozoites, spleen or bone marrow sequestered parasite biomass to onward transmission, infection duration and chronicity?
5. What is the longevity and functional basis of naturally acquired transmission blocking immunity for *P. vivax* gametocytes? Are there effective monoclonal antibodies that can target gametocytes or hypnozoites in the near future?

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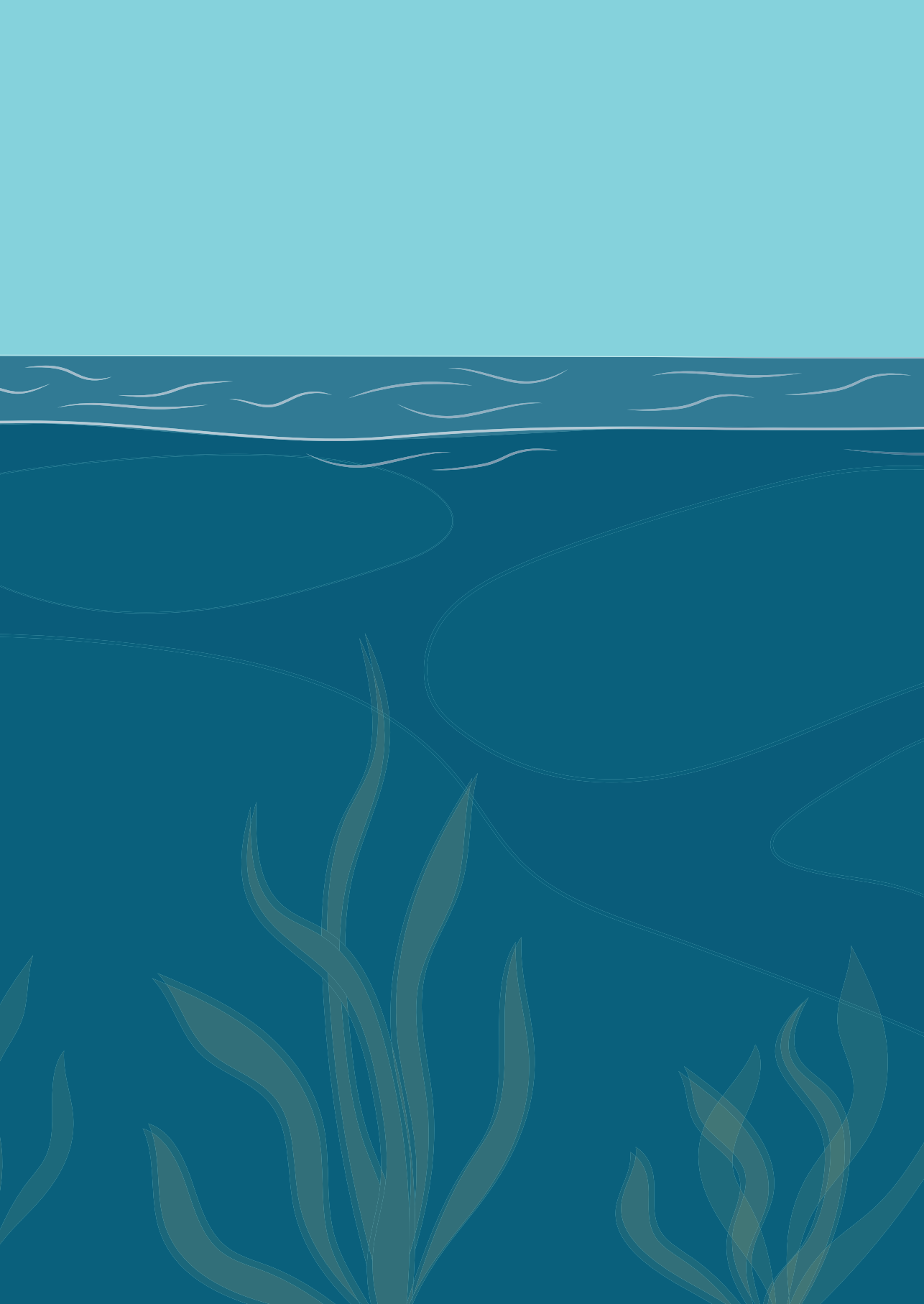


CHAPTER 8

Samenvatting
Summary

Samenvatting

"Als je denkt dat je te klein bent om een verschil te maken, heb je nog geen nacht doorgebracht met een mug" - Afrikaans spreekwoord.

Een van de grootste uitdagingen in de eliminatie van malaria is de aanwezigheid van personen met asymptomatische malaria-infectie die geen behandeling zoeken. Asymptomatische infecties komen veel voor in alle gebieden waar malaria endemisch is. Het is niet duidelijk hoe belangrijk deze asymptomatische infecties zijn voor de verdere verspreiding. Dit is relevant omdat het bepaalt hoe belangrijk het is ze te detecteren en gericht aan te pakken ter ondersteuning van malaria-eliminatie. In sub-Sahara Afrikaanse omgevingen zijn snelle diagnostische tests (sneltesten of Rapid Diagnostic Tests (RDT's)) en microscopie de meest toegankelijke diagnostische middelen voor malaria. Helaas zijn deze niet heel gevoelig voor het detecteren van alle asymptomatische malaria infecties omdat die vaak lage parasietconcentraties hebben. In Ethiopië hebben we de prevalentie en detecteerbaarheid van asymptomatische infecties met *P. falciparum* en *P. vivax* bepaald met behulp van microscopie/RDT in vergelijking met de moleculaire PCR techniek. Dit deden we in gebieden met hoge, matige en lage transmissie. We ontdekten dat deze infecties vaker voorkwamen bij kinderen in de schoolleeftijd (5-15 jaar) vergeleken met volwassenen. Daarnaast vonden we dat infecties minder vaak gedetecteerd werden naarmate de leeftijd van de geïnfecteerde persoon toeneemt of de transmissie-intensiteit in een gebied afneemt **(Hoofdstuk 2).** Belangrijk is dat microscopie en RDT meer dan de helft van deze infecties misten die detecteerbaar waren met PCR. In vergelijking met *P. falciparum* waren asymptomatische infecties met *P. vivax* minder vaak detecteerbaar met microscopie/RDT. In Ethiopië, waar malariaoverdracht seizoensgebonden en heterogeen is, en *P. vivax* en *P. falciparum* gelijktijdig voorkomen, is een betere aanpak nodig om asymptomatisch infecties op te sporen en te behandelen. Hiervoor is het van belang gevoeligere, in het veld inzetbare diagnostische instrumenten te gebruiken. Onze studie geeft aan dat het hierbij belangrijk is om oudere kinderen, zij die al naar school gaan, niet te vergeten omdat er veel infecties in die leeftijdsgroep voorkomen **(Hoofdstuk 2).**

Asymptomatische malaria-infecties kunnen ook een mogelijke bron zijn van parasieten met medicijnresistentie. Dit is zeker het geval als deze infecties langdurig zijn en er veel (suboptimaal) medicijngebruik is in de bevolking. Gezien het langdurige gebruik van chloroquine voor de behandeling van

P. vivax in Ethiopië, kan er selectiedruk zijn op *P. falciparum*-parasieten. Deze druk kan afhankelijk zijn van het niveau van *P. vivax* co-endemiciteit. Daarom hebben we het voorkomen van bekende moleculaire markers voor chloroquine medicijnresistentie (*Pfcrt*-K76T en *Pfmdr1*-N86Y) bepaald in onze bloedmonsters van asymptomatische parasietdragers **(Hoofdstuk 3)**. De *Pfcrt*-76T-mutatie in *P. falciparum* parasieten kwam veel voor in gebieden waar veel *vivax* malaria voorkwam (prevalentie 98.4-100%), terwijl het *Pfmdr1*-N86 (77,3-100%) wildtype fenotype werd waargenomen in alle endemische omgevingen na twee decennia van stopzetting van chloroquine voor de behandeling van ongecompliceerde *P. falciparum*-infectie. Dit geeft indirect epidemiologisch bewijs dat medicijndruk resulteert in meer resistente infecties, ook als die medicijndruk veroorzaakt wordt door behandeling van andere malariasoorten. Met het opkomende gevaar van artemisinine-lumefantrine-resistentie benadrukken onze bevindingen de noodzaak van contextspecifieke monitoring van patronen van medicijnresistentie en nauwkeurige behandeling en diagnose in co-endemische omgevingen.

Om beter inzicht te krijgen in de dynamiek van asymptomatische infecties met lage parasietdichtheid en hun bijdrage aan verdere transmissie, hebben we een longitudinale studie van 15 maanden uitgevoerd in een omgeving met lage malaria transmissie in Adama. In Adama zijn *P. falciparum* en *P. vivax* co-endemisch **(Hoofdstuk 5).** Hiervoor hebben we tussen september 2018 en maart 2020 2373 personen gescreend met behulp van kwantitatieve PCR. De prevalentie van infectie was 3,5% (83/2373) voor *P. falciparum* en 4,0% (95/2373) voor *P. vivax*, terwijl 0,08% (2/2373) van alle studiedeelnemers gemengde infecties hadden. Microscopie detecteerde *P. falciparum* bij 0,47% (10/2107), *P. vivax* bij 0,14% (3/2107) en infecties met gemengde soorten bij 0,05% (1/2017) van de bevolking. Vervolgens hebben we 70 individuen die positief waren voor *P. falciparum* (n=35), *P. vivax* (n=34) en gemengde infecties (n=1) lange tijd gevold. Hierbij hebben we tweemaal per week vingerprikmonsters genomen en daarnaast hun besmettelijkheid voor muggen bepaald door herhaalde membraanvoedingen in de eerste 10 weken van de studie. Daarna werden ze gedurende de daaropvolgende 12 maanden maandelijks gevolgd met vingerprikmonsters. Hun besmettelijkheid voor *Anopheles arabiensis* muggen die in Adama werden gekweekt gedurende meer dan 800 generaties, werd onderzocht met membraanvoedingstests. Belangrijk is dat voordat de longitudinale studie voor besmettelijkheidsbeoordeling begon, we de geschiktheid van deze muggenkolonie vergeleken met wilde muggen die hetzelfde bloedmaal kregen. We merkten op dat de bloedvoedingsefficiëntie

hoger was bij de kolonie-muggen. Deze koloniemuggen waren 'hongerig' na een kortere vastentijd (12 uur vs. 18 uur). Ondanks dit verschil in agressiviteit, leek er geen verschil te zijn voor de belangrijkste uitkomstmaat: de infectiegraad met *P. vivax*. Dit suggereert vergelijkbare vatbaarheid voor de lokale *Plasmodium*-parasieten **(Hoofdstuk 4)**.

Met deze bevinding voelden we ons gesteund in onze grotere studie met koloniemuggen. In de studieomgeving met lage transmissie intensiteit, waar de meeste studiedeelnemers aanvankelijk submicroscopische infecties hadden (97% van *P. vivax* en 82,8% van alle *P. falciparum* infecties), ontdekten we dat infecties in parasietdichtheid kunnen toenemen. Dit leidde tot microscopisch detecteerbaar infecties op tenminste een tijdspunt voor 18,2% van *P. vivax* en 38,2% van *P. falciparum* infecties. Belangrijk is dat een fractie van deze infecties symptomen kunnen veroorzaken (7 *P. falciparum* en 5 *P. vivax* infecties) maar ook spontaan kunnen verdwijnen uit de bloedcirculatie. De snelheid van spontane klaring van infecties was relatief langzaam voor *P. vivax.* Veertig procent van de *P. vivax*-infecties vergeleken met 22% van de *P. falciparum*-infecties duurde tenminste 3 maanden. De mediane infectieduur was 60 dagen voor *P. vivax* en 37 dagen voor *P. falciarpum* **(Hoofdstuk 5)**. Deze bevindingen wijzen op het potentieel dat chronische infecties het droge seizoen overbruggen en zo een bron vormen van nieuwe malaria tijdens het transmissieseizoen. Onze studie benadrukte ook het belang van gelijktijdige detectie van asymptomatische malaria infecties en behandeling van symptomatische gevallen om malaria terug te dringen in zo'n omgeving. Ook bleek dat veel proefpersonen zowel met *P. vivax* als *P. falciparum* besmet te zijn, in deze deelnemers wisselden de soorten elkaar af, waarbij de ene soort verdween en de andere verscheen bij hetzelfde individu. We ontdekten dat voor asymptomatische *P. falciparum*-infecties, 6,4% van de herhaalde voedingen 1,8% van de ontlede muggen infecteerden. In tegenstelling bleek geen van de asymptomatische *P. vivax*-infecties besmettelijk, behalve een gemengde infectie die gametocyten positief was voor *P. vivax* volgens onze moleculaire techniek. Aan de andere kant waren klinische *P. vivax*-gevallen besmettelijker dan asymptomatische P. vivax-infecties. Slechts één submicroscopische *P. falciparum*-infectie was besmettelijk voor muggen. De relatieve bijdrage van deze infecties aan verdere transmissie wordt echter ook bepaald door hun hoge prevalentie in de bevolking. Ook kunnen factoren zoals grotere aantrekkelijkheid voor muggenbeten, gastheerimmuniteit, leeftijd, gastheergrootte en onbekende gastheer- en parasietfactoren een rol spelen **(Hoofdstuk 5)**. Al met al wijst onze studie op het belang asymptomatische infecties te betrekken bij malariacontrole.

In onze studie konden sommige individuen met een hoge dichtheid aan *P. falciparum*- of *P. vivax*-gametocyten geen muggen infecteren, wat suggereert dat iets in het bloed de besmetting van muggen remt. Om dit beter te begrijpen hebben de de immuunrespons tegen gametocyten onderzocht in relatie tot muggeninfectiviteit. We hebben dit specifiek gedaan bij *P. vivax* waarvoor relatief weinig bekend is. Bij deze substudie hebben we gebruik hebben gemaakt van 368 klinische en 56 asymptomatische gevallen uit onze cohort **(Hoofdstuk 5** en **Hoofdstuk 6)**. Onze bevindingen toonden een sterke associatie van transmissieremmende activiteit met antilichamen tegen de gametocyt antigenen van Pvs47, Pvs230 en Pvs25. Dit resultaat werd bevestigd in experimenten waar we het plasma van patiënten vervingen door controleserum **(Hoofdstuk 6)**. Ook in die experimenten hadden individuen met efficiënte transmissieblokkade meer kans om antilichamen te hebben tegen de seksuele stadium-antigenen van Pvs47 en PvHAP2 in vergelijking met degenen met lagere transmissieremmende activiteit. Toch zijn we voorzichtig met onze conclusies: statistische verbanden bewijzen geen oorzakelijk verband. Het is mogelijk dat deze antistoffen transmissie kunnen blokkeren in samenwerking met andere antistoffen of dat er nog onbekende antistoffen zijn die ook een rol spelen en veel voorkomen in deelnemers met een hogere immuniteit. Onze studie benadrukte wel duidelijk de mogelijke rol van antistoffen tegen *P. vivax* seksuele stadium-antigenen bij het bepalen van de transmissie-efficiëntie van *P. vivax* gametocyten. In vervolgonderzoek zouden we de functionele rol van de transmissiebeperkende immuniteit en de levensduur ervan in de endemische bevolking willen bevestigen.

In de studies in dit proefschrift hebben we bewijs verzameld dat de aanpak van malaria-eliminatie kan ondersteunen. We richtten ons op gebieden in Ethiopië waar *P. vivax* en *P. falciparum* co-endemisch zijn maar zien toepassing in andere regionen waar beide malariasoorten ook voorkomen. Asymptomatische infecties kunnen langdurig zijn in gebieden waar eliminatie nagestreefd wordt en sterk geografisch of demografisch geclusterd zijn. Parasietdichtheden fluctueren in de tijd en bij niet alle besmette personen leiden infecties (ooit) tot symptomen. Soms klaren de infecties spontaan maar geregeld gaat hieraan een besmettelijke periode vooraf waarbij asymptomatische infecties een bron van verdere besmetting naar muggen zijn. Dit ondersteunt het idee van op maat gemaakte benaderingen met meer gevoelige diagnostische middelen die specifiek zijn. Dit werk heeft op unieke wijze de operationele uitdagingen aangepakt bij het detecteren en behandelen van asymptomatische malaria-infecties en hun potentieel voor verdere transmissie in *P. falciparum*- en *P. vivax*-co-endemische omgevingen.

Summary

"If you think you are too small to make a difference you have not spent a night with a mosquito" African proverb

One of the biggest challenges in the elimination of malaria is the presence of individuals with asymptomatic malaria infection who do not seek treatment. Asymptomatic infections are common in all areas where malaria is endemic. It is not clear how important these asymptomatic infections are for further transmission. This is relevant because it determines the importance of detecting and targeting them specifically to support malaria elimination. In sub-Saharan African settings, rapid diagnostic tests (RDTs) and microscopy are the most accessible diagnostic tools for malaria. Unfortunately, these are not very sensitive in detecting all asymptomatic malaria infections because they often have low parasite concentrations. In Ethiopia, we determined the prevalence and detectability of asymptomatic infections with *P. falciparum* and *P. vivax* using microscopy/RDT compared to the molecular PCR technique. We did this in areas with high, moderate, and low transmission. We found that these infections were more common in school-aged children (5-15 years) compared to adults. Additionally, we found that infections were less frequently detected as the age of the infected person increased or the transmission intensity decreased in an area (**Chapter 2**). Importantly, microscopy and RDT missed more than half of these infections that were detectable with PCR. Compared to *P. falciparum*, asymptomatic infections with *P. vivax* were less frequently detectable with microscopy/RDT. In Ethiopia, where malaria transmission is seasonal and heterogeneous, and *P. vivax* and *P. falciparum* occur simultaneously, a better approach is needed to detect and treat asymptomatic infections. For this purpose, it is important to use more sensitive diagnostic tools that can be deployed in the field. Our study indicates that it is important not to forget older children, those who are already attending school, because many infections occur in that age group (**Chapter 2**).

Asymptomatic malaria infections can also be a potential source of parasites with drug resistance. This is certainly the case when these infections are prolonged and there is widespread (suboptimal) drug use in the population. Given the prolonged use of chloroquine for the treatment of *P. vivax* in Ethiopia, there may be selection pressure on *P. falciparum* parasites. This pressure may depend on the level of *P. vivax* co-endemicity. Therefore, we determined the prevalence of known molecular markers for chloroquine drug resistance

(*Pfcrt*-K76T and *Pfmdr1*-N86Y) in our blood samples from asymptomatic parasite carriers (**Chapter 3**). The *Pfcrt*-76T mutation in *P. falciparum* parasites was common in areas where *vivax* malaria was prevalent (prevalence 98.4-100%), while the *Pfmdr1*-N86 (77.3-100%) wild-type phenotype was observed in all endemic environments after two decades of chloroquine discontinuation for the treatment of uncomplicated *P. falciparum* infection. This provides indirect epidemiological evidence that drug pressure results in more resistant infections, even if that drug pressure is caused by treatment of other malaria species. With the emerging threat of artemisinin-lumefantrine resistance, our findings emphasize the need for context-specific monitoring of patterns of drug resistance and accurate treatment and diagnosis in co-endemic settings.

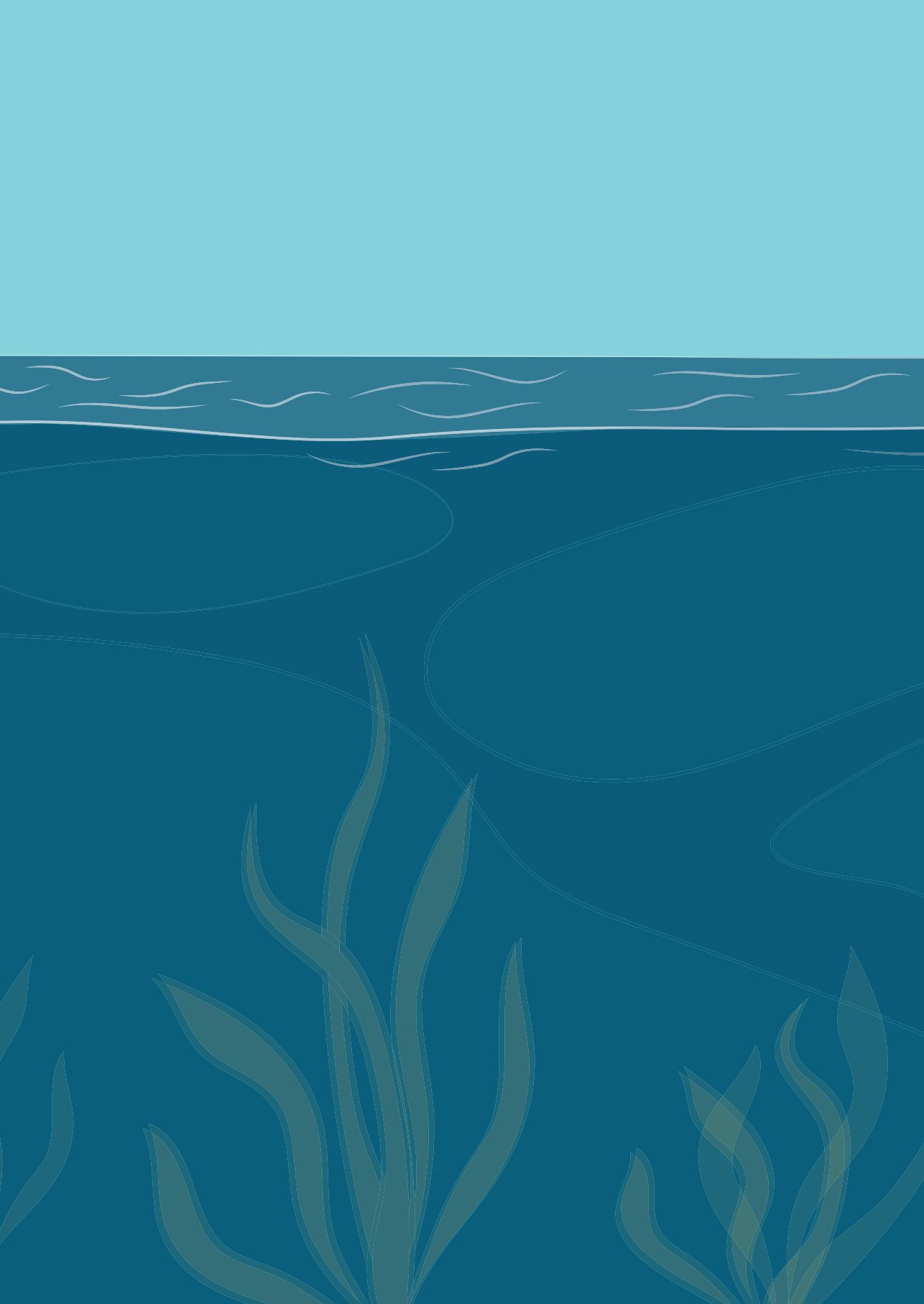
To gain a better understanding of the dynamics of asymptomatic infections with low parasite density and their contribution to further transmission, we conducted a longitudinal study over 15 months in a low malaria transmission setting in Adama. In Adama, *P. falciparum* and *P. vivax* are co-endemic (**Chapter 5**). Between September 2018 and March 2020, we screened 2373 individuals using quantitative PCR. The infection prevalence was 3.5% (83/2373) for *P. falciparum* and 4.0% (95/2373) for *P. vivax*, while 0.08% (2/2373) of all study participants had mixed infections. Microscopy detected *P. falciparum* in 0.47% (10/2107), *P. vivax* in 0.14% (3/2107), and mixed species infections in 0.05% (1/2017) of the population. Subsequently, we followed 70 individuals who tested positive for *P. falciparum* (n=35), *P. vivax* (n=34), and mixed infections (n=1) for a long period. We collected finger-prick samples twice a week and additionally determined their infectivity to mosquitoes by repeated membrane feeding during the first 10 weeks of the study. Then, they were followed monthly with finger-prick samples for the next 12 months. Their infectivity to Anopheles arabiensis mosquitoes cultured in Adama for over 800 generations was examined with direct membrane feeding assays. Importantly, before the longitudinal study for infectivity assessment began, we compared the suitability of these mosquito colonies with wild mosquitoes receiving the same blood meal during membrane feeding assay. We observed that the blood-feeding efficiency was higher in the colony mosquitoes. These colony mosquitoes showed shorter starvation time (12 hrs vs 18 hrs) before membrane feeding and less aggressive compared to wild mosquitoes during feeding. Despite this difference in aggressiveness, there seemed to be no difference for the primary outcome measure: the infection rate with *P. vivax*. This suggests similar susceptibility to local *Plasmodium* parasites (**Chapter 4**).

With this finding, we felt supported in our larger longitudinal study with the colony mosquitoes. In the study setting with low transmission intensity, where most study participants initially had submicroscopic infections (97% for *P. vivax* and 82.8% for all *P. falciparum* infections), we discovered that infections can increase in parasite density. This led to microscopically detectable infections at least at one time point for 18.2% of *P. vivax* and 38.2% of *P. falciparum* infections. Importantly, a fraction of these infections can cause symptoms (7 *P. falciparum* and 5 *P. vivax* infections) but can also spontaneously disappear from the bloodstream. The rate of spontaneous clearance of infections was relatively slow for *P. vivax*. Forty percent of *P. vivax* infections compared to 22% of *P. falciparum* infections lasted at least 3 months. The median duration of infection was 60 days for *P. vivax* and 37 days for *P. falciparum* (**Chapter 5**). These findings suggest the potential for chronic infections to bridge the dry season and thus serve as a source of new malaria infections during the transmission season. Our study also emphasized the importance of simultaneous detection of asymptomatic malaria infections and treatment of symptomatic cases to reduce malaria in such setting. It also emerged that many subjects were infected with both *P. vivax* and *P. falciparum*, with these species alternating in these participants, with one species disappearing and the other appearing in the same individual. We found that for asymptomatic *P. falciparum* infections, 6.4% of the repeated feedings infected 1.8% of the dissected mosquitoes. In contrast, none of the asymptomatic *P. vivax* infections were infectious, except for a mixed infection that was gametocyte-positive for *P. vivax* according to our RT-qPCR. On the other hand, clinical *P. vivax* cases were more infectious than asymptomatic *P. vivax* infections. Only one submicroscopic *P. falciparum* infection was infectious to mosquitoes. However, the relative contribution of these infections to further transmission is also determined by their high prevalence in the population. Factors such as greater attractiveness to mosquito bites, host immunity, age, host size, and unknown host and parasite factors may also play a role **(Chapter 5)**. Overall, our study highlights the importance of including asymptomatic infections in malaria control and elimination efforts.

In our study, some individuals with high densities of *P. falciparum* or *P. vivax* gametocytes were unable to infect mosquitoes, suggesting that something in the blood inhibits mosquito infection. To better understand this, we investigated the immune response against gametocytes in relation to mosquito infectivity. We specifically focused on *P. vivax*, for which relatively little is known. For this sub-study, we utilized 368 clinical and 56 asymptomatic cases from our cohort (**Chapter 5** and **Chapter 6**). Our findings showed a strong association of

transmission-blocking activity with antibodies against the gametocyte antigens of Pvs47, Pvs230, and Pvs25. This result was confirmed in experiments where we replaced patient plasma with control serum (**Chapter 6**). In those experiments as well, individuals with efficient transmission blockade were more likely to have antibodies against the sexual stage antigens of Pvs47 and PvHAP2 compared to those with lower transmission-blocking activity. However, we are cautious with our conclusions: statistical associations do not prove causality. It is possible that these antibodies may block transmission in collaboration with other antibodies or that there are still unknown antibodies that also play a role and are common in participants with higher immunity. Our study clearly emphasized the potential role of antibodies against *P. vivax* sexual stage antigens in determining the transmission efficiency of *P. vivax* gametocytes. In further research, we would like to confirm the functional role of transmission-restricting immunity and its duration in the endemic population.

In the studies in this thesis, we have gathered evidence that can support the approach to malaria elimination. We focused on areas in Ethiopia where *P. vivax* and *P. falciparum* are co-endemic but see applicability in other regions where both malaria species are also present. Asymptomatic infections can be prolonged in areas where elimination is pursued and can be strongly geographically or demographically clustered. Parasite densities fluctuate over time, and not all infected individuals develop symptoms (at any time). Sometimes infections clear spontaneously, but often precede an infectious period during which asymptomatic infections serve as a source of further mosquito infection. This supports the idea of tailored approaches with more sensitive diagnostic tools that are specific. This work has uniquely addressed the operational challenges in detecting and treating asymptomatic malaria infections and their potential for further transmission in *P. falciparum* and *P. vivax* co-endemic setting.



APPENDICES

Acknowledgements
Research data management
List of publications
Curriculum Vitae
PhD portfolio

Acknowledgements

I have come a long way in pursuit of my PhD, from sitting on rock chairs during elementary school in my village to delving into the sophisticated science of malaria where I find myself today. My inspiration has always been rooted in the belief that "Unless we see beyond the world we live in, we are not truly living." Throughout this remarkable PhD journey, I have encountered exceptional individuals whom I must sincerely acknowledge. Without their support, my success in completing this PhD would not have been possible.

Undoubtedly, the PhD journey presents a distinct set of opportunities and challenges. I initially secured a national PhD fellowship at Armauer Hansen Research Institute (AHRI) through Addis Ababa University. Expressing my interest in malaria research to Dr Endalamaw and Dr Fitsum marked the beginning of this remarkable journey. In 2017, the subsequent field activities and re-establishment of our insectary for membrane feeding assays in Adama laid the groundwork for years of intensive work at AHRI. The turning point came when I seized an exciting new opportunity: Dr Fitsum initiated the INDIE-1 project at Adama, coinciding with Prof Teun's visit to AHRI for the project. During my 20-minute presentation on asymptomatic malaria transmission dynamics and my proposed focus for my PhD study, I received an invitation to spend one month from May 5th to June 5th, 2019 in Teun's lab at Radboud University Medical Center. It was during this time that he suggested registering for my PhD at Radboud University Medical Center, marking a pivotal moment in the story of my doctoral pursuit once again.

Dear **Professor Teun**, I feel incredibly lucky to have your supervision of such a highly esteemed academic. Your steadfast belief in me has been instrumental in the success of this endeavor. Your consistent guidance and constructive feedback have greatly enhanced the quality of my work, serving as a profound source of inspiration. Amidst challenging times such as the COVID-19 pandemic and the local conflict in Ethiopia, your compassionate support was invaluable in keeping me motivated through it all. Your exceptional ability to nurture talent with patience is truly remarkable. Thank you!

Dear **Dr Fitsum**, you are an elder brother, a supervisor and cheerleader throughout my PhD journey. I just followed your foot-steps and happy that I am the first PhD graduate of your lab at AHRI and Teun's lab at Radboud University Medical Center, Nijmegen. You were the reason to work with Teun's team and get

his incredible supervision. Thank you indeed for your patience, and constructive criticism that has played a pivotal role in shaping my understanding of the subject matter and the depth of my research. Your supervision instilled in me the passion for continuous learning and improvement in any of my future endeavors. I am grateful for your guidance and support, which has been instrumental in the successful completion of my PhD journey.

Dear **Dr Endalamaw**, I truly value your dedicated leadership at AHRI during my PhD, which serves as the cornerstone of our team. Your unwavering support and openness to new ideas have made a significant impact on my professional growth. I also want to express gratitude for the valuable guidance you provided during challenging times. Additionally, I greatly appreciate the outstanding support not only from you but also from **Prof Beyene Petros** of Addis Ababa University when I was enrolling in my PhD program at Radboud University Medical Center.

Dear **Prof Beyene**, I am truly grateful for your invaluable supervision and guidance during my MSc. Your approval to pursue my PhD at Radboud University has led to an amazing experience. I deeply appreciate your unwavering support, even with a busy schedule filled with national and international commitments. Your insights have greatly contributed to my growth as a researcher.

Dear **Prof Hassen**, I greatly appreciate your invaluable assistance during my time at Adama and Addis Ababa University. Your guidance was crucial in navigating challenging scenarios in the field and working with diverse team members. Your support and patience were indispensable to the smooth progress of my PhD at AHRI in 2017.

Dear **Kjerstin**, I want to express my gratitude for your valuable guidance during my molecular assay lab work at Radboud University. Your expertise has taught me the importance of precision and attentiveness in the lab, drawing from your extensive experience in molecular assays. Furthermore, I continue to find great value in the structured and planned approach you introduced me to; focusing on one activity at a time has become an essential principle for my lab activities.

Dear **Prof Chris**, I truly appreciated the support and guidance you provided during my visit to your lab while I was concurrently taking the LSHTM advanced epidemiology course in 2019. Your insights and feedback have been invaluable to me.

Dear **Dr Quirijn**, I am grateful for your mentorship and insightful advice on my progress of the PhD track.

Dear **Dr Shehu**, thank you, your help and kindness was immense on my first day of arrival at Radboud University, in 2019.

Dear **Dr Fikadu**, I greatly appreciate our initial discussion prior to bringing the team to the Arba-Minch field site in 2020. Your support and humble approach have been instrumental in paving the way for future collaboration, leading to the successful establishment of a key site for the malaria team at Arba-Minch. Despite an unfortunate incident where my laptop was robbed by gangsters on my way home from the lab at around 8:00 AM, I am thankful for the support of the Arba-Minch community. On a positive note, I learned that road has been named after me as "**Elifaged's Road**" which serves as a reminder of gratitude amidst challenging times.

Our exceptional field team at Adama (**Temesgen Ashine, Tadele, Tizita, Abrham, Soria, Wakweya, Endashaw, Temesgen Tafesse, Haile, Mikiyas, Girma, Teshome**), your dedication is truly commendable. Whether it was in the challenging swampy fields of Sodere as we searched for larval habitats or during our community participant cohort study, your unwavering support has been invaluable. Having all of you here at AHRI truly makes us a "dream team." Thank you!

Thank you, my sincere community, the community of Adama, its leaders, the local facilitator, **Nigussie** and all the health workers for your kind support and commitment in participating in our study. Your home was mine; we shared the happiness, the sadness and the celebration of the holidays together.

I would like to extend my gratitude to our microscopists, **Tewabech Lema** and **Tsehay Orlando** at Adama Malaria Center for their excellent support and long years of service in the fight against malaria in Ethiopia.

Our team at AHRI lab, **Sinknesh, Melat, Migbaru and Eshetu**, thank you for your contribution and support. Working with you all was a big opportunity. Especially, **Sinknesh**, your role was very impressive for the success of our cohort study at Adama.

My PhD colleagues, **Daniel** and **Surafel**, I am very much grateful for your commitment and support in every success of my PhD objectives. Especially, Surafel and I shared most of our work together and that was real life experience; as "Iron sharpeneth iron; so, a man sharpeneth the countenance of his friend" Psalm 27:17. I hope we all have learned something meaningful in life.

My colleagues at Radboudumc, **Wouter Graumans**, **Chiara Andolina** and **Amanda Fabragarcia** you were so much supportive and caring, thank you for your kindness. And our senior colleagues, **Matthijs Jore, Katharine Collins**, thank you for your inspirational scientific achievements and supports when needed.

As an institution, AHRI has been paramount in facilitating my PhD journey. Heartfelt gratitude goes to **Dr. Alemseged Abdisa** and the esteemed **directors of AHRI** for their unwavering support throughout my project work. I would like to thank the AHRI knowledge management department staff, **Kiya**, **Minyahile** and **Dr Aklilu**, you were the reason to join my comprehensive meta-analysis and systematic review training of the Joanna Briggs Institute through your CEBHA+ project. I am equally indebted to the diligent drivers and all AHRI staff members whose assistance was invaluable in the attainment of my doctoral degree. Furthermore, my sincere appreciation extends to **Wollo University** for their steadfast support, with particular gratitude to the faculty members of the **biology department** whose encouragement bolstered me throughout my PhD journey.

To my dear family, your unwavering support and love have been instrumental in keeping me going during the challenging times of my PhD. **Mom**, your yearning to witness me achieve scholarly success has always been a driving force for me, despite the sorrowful loss of you in 2016. You embodied strength and inspiration to me. My dear wife, **Haregina**, words cannot express how grateful I am for your endless encouragement and steadfast support throughout my doctoral journey and thesis writing process. My cute daughter, **Nafiba**, you are truly the greatest blessing in my life; watching you grow with wisdom is a precious gift from our God, Lord Jesus Christ and his holy mother St. Mary.

Almighty God, "with your light, we see the light" (Psalm 39:6). The hippopotamus's ear may have been too shallow to foresee the full impact of malaria, but your light enables us to understand the unseen (asymptomatic malaria). Glory to you and your eternal kingdom, Amen!

Research data management

This thesis is based on the results of human studies, which were conducted in accordance with the principles of the Declaration of Helsinki. The institutional ethical review boards of Addis Ababa University (CNSDO/71/10/2017), Armauer Hansen Research Institute (AHRI) (P035/17), the National Research Ethics Review Committee of Ethiopia (Ref:310/150/2015 and SHE/S.M./14.4/708/19), and the London School of Hygiene and Tropical Medicine (LSHTM Ethics ref: 15811), gave approval to conduct these studies. The primary and secondary data obtained during this the PhD study at the Radboud University Medical Center (Radboudumc) have been captured and stored on the department of medical microbiology server: (H:) MMBdata$(\\umcfs083) and the knowledge management department (KMD) of the Armauer Hansen Research Institute (AHRI). The participant data for the analyses of the studies as presented in Chapters 2-4 is stored on the departments' H-drive and KMD of AHRI in STATA format (StataCorp., TX, USA). A member of the study team filled in the research form on paper. The paper data were stored in the KMD archive of AHRI. Socio-demographic and clinical data obtained during community surveys and assessment of patients were double entered into the computer by the use of Excel and cleaned by the KMD of AHRI. Data was verified for completeness and consistency with the source documents or ascribe source of error if any. Cleaned and de-linked data was merged with laboratory data obtained at AHRI and Radboudumc. For chapters (Chapters 5 and 6), validated and de-linked data was shared with collaborators and raw data have been deposited in the DRYAD data depository. For all studies, the privacy of the participants in this study is warranted by the use of encrypted and unique individual subject codes. Their codes correspond with the code on the participant booklet. The code was stored separately from the study data. Confidentiality of data was maintained; the paper forms were kept in a locked file cabinet at the KMD of AHRI and the databases have standard password systems for all onsite and offsite backups. The data will be saved for 10 years after termination of the study. Using these participant data in future research is only possible after a renewed permission by the respective ethics review committees as recorded in the informed consent. The datasets analyzed during these studies are available from the corresponding authors of the published chapters (2-6) on reasonable request.

List of publications

Included in this thesis

Hailemeskel E, Menberu T, Shumie G, Behaksra S, Chali W, Keffale M, Belachew M, Shitaye G, Mohammed H, Abebe D: Prevalence of *Plasmodium falciparum Pfcrt* and *Pfmdr1* alleles in settings with different levels of *Plasmodium vivax* co-endemicity in Ethiopia. International Journal for Parasitology: Drugs and Drug Resistance 2019, 11:8-12.

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Tebeje SK, Chali W, **Hailemeskel E**, Ramjith J, Gashaw A, Ashine T, Nebret D, Esayas E, Emiru T, Tsegaye T: Naturally acquired antibodies to gametocyte antigens are associated with reduced transmission of *Plasmodium vivax* gametocytes to *Anopheles arabiensis* mosquitoes. Frontiers in cellular and infection microbiology 2023, 12:1106369.

Elifaged Hailemeskel, Surafel K. Tebeje , Jordache Ramjith, Temesgen Ashine, Kjerstin Lanke, Sinknesh W. Behaksra, Tadele Emiru, Tizita Tsegaye, Abrham Gashaw, Soria Kedir, Wakweya Chalie, Endashaw Esayas, Temesgen Tafesse, Haile Abera, Mikiyas Gebremichael Bulto, Girma Shumie, Beyene Petros, Hassen Mamo, Chris Drakeley, Endalamaw Gadisa, Teun Bousema, Fitsum G. Tadesse: Dynamics of asymptomatic *Plasmodium falciparum* and *Plasmodium vivax* infections and their infectiousness to mosquitoes in a low transmission setting of Ethiopia: a longitudinal observational study. International Journal of Infectious Diseases 2024 (accepted).

Not included in this thesis

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Keffale M, Shumie G, Behaksra SW, Chali W, Hoogen LLvd, **Hailemeskel E**, Mekonnen D, Chanyalew M, Damte D, Fanta T: Serological evidence for a decline in malaria transmission following major scale-up of control efforts in a setting selected for *Plasmodium vivax* and *Plasmodium falciparum* malaria elimination in Babile district, Oromia, Ethiopia. *Transactions of The Royal Society of Tropical Medicine and Hygiene* 2019, **113:**305-311.

Tuasha N, **Hailemeskel E**, Erko B, Petros B: Comorbidity of intestinal helminthiases among malaria outpatients of Wondo Genet health centers, southern Ethiopia: implications for integrated control. BMC Infectious Diseases 2019, 19:1-8.

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Curriculum Vitae

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I have trained in biomedical sciences (MSc, Addis Ababa University) where I have gained knowledge in immunology, parasitology, molecular biology and medical entomology. Working as a lecturer and researcher at Wollo University, Ethiopia, I have acquired field-based research experience related with parasitology and malaria epidemiology. My research experience so far focused on malaria and neglected tropical diseases such as intestinal helminths. Most of my PhD work was at Armauer Hansen Research Institute (AHRI) and Radboud University Medical Center, Nijmegen where my study was focused on the epidemiology of asymptomatic malaria infections and their infectiousness to mosquitoes in Ethiopia. In my PhD we have shown the relevance of asymptomatic infections to public health and the practical challenges of these infections related with their detectability, duration of infections and their potential of onward transmission for both *P. falciparum* and *P. vivax* in co-endemic settings. In addition, we have shown the possible role of naturally acquired transmission reducing immunity against selected *P. vivax* sexual stage antigens. My PhD project was a collaboration effort of Radboud University Nijmegen (The Netherlands), London School of Hygiene and Tropical Medicine (UK), Addis Ababa University (Ethiopia), AHRI (Ethiopia) and Wollo University of Ethiopia. I have been teaching introductory courses of parasitology, immunology and molecular biology for BSc students since 2011 at Wollo University. Currently, I have joined as project coordinator working on a project in collaboration with Liverpool School of Tropical Medicine and AHRI on controlling emerging *Anopheles stephensi* invasion in Sudan and Ethiopia. My dream job and future focus is to work on developing transmsisison blocking interventions for malaria parasites.

Education

2017 – 2024 PhD in Parasitology, Department of Medical Microbiology, Radboud University, Nijmegen, The Netherlands and Armauer Hansen Research Institute in Ethiopia

2008 – 2011 **MSC in Biomedical sciences**, Addis Ababa University

2002 – 2006 **B.ED in Biology**, Bahir Dar University, Bahir Dar, Ethiopia

Experience

2023 – to date **Project coordinator** for "Controlling emergent *Anopheles stephensi* in Ethiopia and Sudan- (CEASE)" at Armauer Hansen Research Institute, Ethiopia.

2011– to date **Lecturer and researcher** at Wollo University, Ethiopia

2014 – 2018 **National trainer of entrepreneurship** for small and micro-enterprise holders at Entrepreneurship Development Center of Ethiopia and UNCTAD (United Nations Conference on Trade and Development), Addis Ababa, Ethiopia.

2006 – 2007 **Malaria and vector born diseases control junior expert**. Dehana woreda health office, Wollo, Ethiopia.

Hands on trainings and skills

i. Comprehensive systematic review and meta-analysis using JBI-SUMARI. **Year: 2022**. **Provider**: JBI, University of Adelaide, Adelaide, South Australia, Jimma University and Armauer Hansen Research Institute, Ethiopia.

ii. Techniques in Entomological monitoring and surveillance, insectary establishment for transmission biology related to malaria vectors and other vectors. **Year**: **2017-2024. Provider:** AHRI, Addis Ababa university and PMI-vector-link, Ethiopia.

iii. Grant development and management training.**Year**: **2022**. **Provider**: Armauer Hansen Research Institute in collaboration with Ministry of Health of Ethiopia.

iv. Advanced Epidemiological analysis using stata. **Year: 2019**: **Provider**: London School of Hygiene and Tropical Medicine.

v. Techniques in Immunology, Molecular biology and Parasitology. **Year: 2017**. **Provider:** Armauer Hansen Research Institute and Addis Ababa University.

vi. Experience on molecular and immunlogical assays related to transmsisision biology of malaria from human to mosquitoes-towards quantifying the infectious reserviour. **Year**: 2017-todate. **Provider**: Radboud University, Nijmegen, The Netherlands and Armauer Hansen Research Institute in Ethiopia.

Networks and recognitions:

Member of Ethiopian Teachers Association
Member of Wag Development Association (http://www.wagdev.org/)
Member of The Biological Society of Ethiopia (http://bsethiopia.org/)
Member of the John Snow Society of Epidemiology (https://johnsnowsociety.org/)
National certified trainer of Enterpreneurship at Enterpreneurship Develeopmet Institute (EDI) the former EDC, Ethiopia since 2014.

Participation in meetings, conference, and panel discussion

1. Malaria Research Project Dissemination and Malaria Molecular surveillance in the Horn of Africa (HAMMS) Project Kick of Conference from December,01-02,2023, Addis Ababa, Ethiopia; **oral presentation on**: Targeting Hidden Reservoirs: a longitudinal observational study in coendemic setting.
2. The 69th Annual meeting of the American Society of Tropical medicine and Hygiene (ASTMH), Novemeber 2020.
3. EMBL Conference: BioMalPar: XVI Biology and pathology of the malaria parasite-Visual EMBL conference from 18/05/2019-19/05/2019
4. The Tenth Ethiopian Malaria Research Network Symposium. MALARIA SURVEILLANCE IN THE CONTEXT OF MALARIA ELIMINATION from December 19-20, 2018, St. Paul Hospital Millennium Medical College; **oral presentation on**: "Prevalence of Pfcrt-76 and Pfmdr 1-86 alleles among asymptomatic *P.falciparum* malaria cases in different transmission settings in Ethiopia"
5. Inaguration of Ethiopian Society of Tropical and Infectious Diseases (ESTAIDs) Addis Ababa, Ethiopia, Decmber, 7-8, 2012; **oral Presentation on** "Plasmodium falciparum malaria related to sulfadoxinepyramithamine resistance-associated mutations"
6. International consultative workshop on Anti-malaria drug efficacy studies in Ethiopia held on Ethiopian Public Health Research Institute, Addis Ababa Ethiopia. July, 8-9, 2013; **oral presentation on** "*Plasmodium falciparum* malaria related to sulfadoxine-pyramithamine resistance-associated mutations"

Reviews

Journal articles: Tropical Medicine & International Health, Environmental Health Insights, Malaria Journal

PhD portfolio of Elifaged Hailemeskel Beshah

Graduate School: Radboud Institute for Health Sciences
Department: Medical Microbiology
PhD period: **05/05/2019 – 24/09/2024**
PhD Supervisor(s): **Prof. dr. T. Bousema, Prof. dr. Beyene Petros**
PhD Co-supervisor(s): **Dr. E. Gadisa, Dr. F.G.Tadesse**

Training activities	Hours
Courses	
• Advanced Course in Epidemiological Analysis (2019)	120.00
• RIHS PhD introduction course (2019)	21.00
• Techniques in Entomological monitoring and surveillance (2021)	72.00
• RIHS - Introduction course for PhD candidates (2021)	15.00
• Radboudumc - Scientific integrity (2022)	20.00
• Comprehensive Systematic Review Training Program (2022)	182.00
• Grant development and management training (2022)	40.00
Conferences	
Oral presentations:	
• 10th Ethiopian malaria research network symposium (2018)	7.50
• Malaria Research Project Dissemination and Malaria Molecular surveillance in the Horn of Africa (HAMMS) Project Kick off (2023)	7.50
Poster presentation	
• Poster presentation on Scientific Advisory Board (SAB) meeting at AHRI (2022)	7.50
Participation in conferences	
• EMBL Conference: BioMalPar: XVI Biology and pathology of the malaria parasite-Visual EMBL conference (2020)	16.00
• 69th Annual meeting of the American society of Tropical medicine and Hygiene (2020)	16.00
Other	
Reviewed articles for peer review journals	
• Malaria Journal, Tropical Medicine & International Health, Environmental Health Insights	7.50
• Weekly journal club at AHRI(1hr/week) (2019-2024)	176.00
• Weekly Journal club and group work meeting at Radboud and AHRI(1hr/week) (2019-2024)	220.00
Total	**928.00**

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university medical center

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Radboud University