PREVALENCE OF *PLASMODIUM VIVAX* IN DUFFY-NEGATIVE INDIVUALS ACROSS BROAD REGIONS OF ETHIOPIA

by

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ABSTRACT

EBONY LITTLE. Prevalence of *Plasmodium Vivax* in Duffy-Negative Individuals Across Broad Regions of Ethiopia
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Plasmodium parasites replicate asexually in the human host, and, in each replication cycle, a portion of the asexual stages develops into sexual gametocytes. The proportion of infections that carries gametocytes is a proxy for human-to-mosquito transmissibility. The documentation of *P. vivax* infections in Africa where the predominant population is Duffy-negative demonstrates the ability of *P. vivax* to replicate asexually in Duffy-negative hosts, causing malaria symptoms. However, it is unclear what proportion of *P. vivax* infections in Duffy-negatives carries gametocytes. This study aims to determine the prevalence of *P. vivax* in Duffy-negatives across broad regions of Ethiopia and characterize parasite stages including gametocytes among *P. vivax* infections. Of the 447 P. *vivax* confirmed samples collected from southwestern, northwestern, and eastern regions of Ethiopia, 17 were from Duffy-negatives with the highest number observed in the Amhara and Oromia. Among them, five (29.4%) were detected with gametocytes. There were 348 cases that were Duffy positives and 162 (46.5%) of them were detected with gametocytes, much higher than that in Duffy-negatives. A wide range of difference in parasitemia is observed among *P. vivax* samples in Duffy-negative individuals. A few infections in Amhara (north) and Oromia (southwestern) show high parasitemia comparable to Duffy-positives. In Oromia, mixed rings and trophozoites were most common in the *P. vivax* cases. This finding is the first to report the presence of gametocytes in *P. vivax* from Duffy-negatives,

suggestive of human-to-mosquito transmissibility. Although *P. vivax* infections in Duffy negatives are commonly associated with low parasitemia, these infections may represent hidden reservoirs that might contribute to transmission. A better understanding of *P. vivax* transmission biology and gametocyte function particularly in Duffy-negative populations would aid future treatment and management of vivax malaria in Africa.

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CHAPTER 1: INTRODUCTION

1.1 Malaria Distribution and Epidemiology Around the World

Malaria is caused by the eukaryotic *Plasmodium* parasites transmitted by *Anopheles* mosquitoes and infect several insect, vertebrate, and primate species. According to the WHO malaria report 2022, most malaria cases occur in sub-Saharan Africa based on a noticeable increase in prevalence and transmission in several African countries [1] with Africa having had the most reported [1] malaria cases within the last five years [2]. There are a total of 87 malaria-endemic countries worldwide [2], In 2021, 247 million cases were reported, with 2 million more cases than 2020. There was a radical decline in cases during 2000-2015, from 245 million to 230 million cases in 108 countries negatively impacted by malaria [1]. The steady incline since 2016 has been reported, with approximately 13 million more cases from 2019-2020 particularly during the pandemic [1]. There was no drastic difference for 2020 and 2021, with a predicted 13.4 million cases most likely contributing to COVID-19. The death rates from 2000 to 2015 and for 2019 were consistently reduced. However, there was an incline in 2020, with around 15.1 deaths per 100,000 individuals, before falling to 14.8 in the following year. In 2019, the death rate from malaria escalated from 57,000 to 625,000 in 2020. From 2019 to 2021, there were a massive 63,000 total deaths reported due to the inability to obtain proper malaria assistance during the pandemic [1], [3]. Globally, the death reports reported for over half of all the malaria fatality cases were from the United Republic of Tanzania (4.1%), and the Democratic Republic of Congo (12.6%), and Nigeria (3.9%) [1]. Nigeria (26.6%), the Democratic Republic of Congo (12.3%),

Mozambique (4.1%), and Uganda (5.1%) are four counties that reported nearly half of all malaria cases [1].

Yearly, there are about 619,000 malaria-related deaths and ~247 million cases reported globally [1]. Out of the five malaria *Plasmodium* species, *P. vivax* is the most widespread species [4],[5]. Nearly 262,000 cases and 728,000 deaths were caused by *P. vivax* malaria in Africa in 2021 [1]. Controlling and reducing malaria incidence and deaths is imperative to malaria-endemic regions. *P. vivax* is capable of remaining dormant in the liver [6], thus increasing survival probability accompanying symptoms that are debleating and furthermore fatal.

One of the factors that influences malaria prevalence and transmission is rainfall, particularly in these four African countries [7]. Weather patterns, such as temperature [8] change have an influence on the dissemination of malaria therefore has the capability of slowing or accelerating the disease's transmission frequency and mosquito survival [9], [10] Temperature variation directly impacts the parasite abilities to develop as it has been observed through previous studies that the optimal temperature for malaria *plasmodium* to develop successfully is approximately 20-30° C [10]. The Democratic Republic of the Congo (DRC) and Nigeria have the highest increase in malaria cases worldwide in 2020, with the majority of cases being *P. falciparum* [11]. Malaria control in DRC includes insecticide treated nets for bedding, artemisinin-based combination therapy, and use of rapid diagnostic tests widely in school-aged children [11] Although school-age children in the DRC are far less likely to contract severe malaria, they are nonetheless susceptible to symptomatic and asymptomatic infections. The

inclusion of school-aged children in national malaria control measures, such as malaria demographic health surveys (DHS), is urgently required. To enhance health and social equality benefits, plasmodium control and treatments should address all community members, notably school-age children [11].

In Nigeria, *P*. f*alciparum* prevalence is relatively low in adults and about 77% of the cases were in children under 5 years old [12]. Age, the presence of pesticides within one kilometer of home, travel to remote rural areas, health education on mosquito avoidance, and use of chemoprophylaxis are all significant factors that affect transmission [12]. Likewise, Mozambique has predominantly *P. falciparum* cases and these cases are mostly in pregnant women and children under 5 years old [12]. The high malaria burden in Mozambique is due to the lack of proper malaria prevention and treatment given a scarce number of health facilities in rural areas [12]. In Uganda, there is not sufficient resources and effort for implementing long-lasting insecticidal nets (LLIN) and indoor residual spraying (IRS) to reduce malaria transmission [13]. During 2013-2014, Uganda was one of the first countries to incorporate LLINs that in turn reduced the burden of malaria temporarily. Due to the discontinuation of IRS, malaria cases have resurged [13]. However, in high malaria burden countries, malaria cases are steadily reduced with the continued use of IRS and LLINs [14].

Compared to Africa, Southeast Asia region has nine countries that are malaria endemic in 2021 and has reported 5.4 million cases responsible for 2% of malaria reported cases worldwide [1]. In 2016, Sri Lanka was certified malaria-free. Timor-Leste, India was reported to have about

79% of malaria cases, with 40% being *P. vivax.* Malaria cases continued to reduce from 2000 to 2021, from 22.8 million to 5.4 million cases, with a reduction of about 74% in malaria fatalities. In 2021, India reported about 83% of deaths caused by malaria [1]. Timor-Leste, along with Bhutan, has reported no incidences of malaria deaths during 2013-2015, and in 2021. Likewise, Thailand reported no incidence of indigenous deaths, with only one death reported by Nepal. The WHO for the Eastern Mediterranean region has been consistently reduced since 2000 from 6.9 million to 6.2 million in 2015 [1]. However, in 2019- 2020 there was a rise in malaria following a drastic decline in 2021 at about 58% [2] , though some Middle East countries experienced a rise in malaria due to the floods in Pakistan recently [15].

In Western Pacific region, a 49% decline was reported with around 2.8 million- 1.4 million cases from 2000-2021, although death and reported rates seem to climb between 2019 and 2020 in Papua New Guinea [1]. *Plasmodium vivax* is the main cause of an upsurge in the cases observed between 2000 and 2021; however, preventive measures and proper treatment did reduce *P. falciparum* cases [16], [17]. Besides *P. vivax* and *P. falciparum*, there were 3575 *P. knowlesi* cases reported during 2021 [18]. There were as little as 10,000 cases reported in the Republic of Korea, Vietnam, the Lao People's Democratic Republic, Malaysia, and Vanuatu [1]. In South America, malaria cases declined from 2000-2021 from 60%-70% [19]; countries including Colombia, Brazil, and the Bolivarian Republic of Venezuela were responsible for 79% of cases due to the spread of *P. vivax* during 2021. There has been a rise of around 482,000 cases in 2017, 467,000 cases in 2019, and 205,000 cases in 2021 in the Bolivarian Republic of Venezuela [1]. In Venezuela there has been an increase in transmission due to the gold-mining

occurrence that created an upsurge in malaria since 2014 [20] along with the co-infection of COVID-19 [21].

1.2 Malaria Life Cycle and Symptoms

Five *Pasmodium* species are infectious to humans: *P. falciparum, P vivax, P. knowlesi, P. ovale,* and *P. malariae* [22]. The vector mosquitoes *Anopheles* spread the pathogen by biting an infected human mostly at night. After biting, *Plasmodium* sporozoites travel through the bloodstream to the liver. The pathogen then developed, going through the necessary morphology changes to become merozoites [23]. Once merozoites are developed, they release from the liver back into the bloodstream to infect red blood cells (RBC) [23]. Within the RBCs, the pathogen develops from ring stage trophozoites and then schizonts [24] before releasing the daughter parasites, merozoites, into the bloodstream again and invading other RBCs. Some schizonts emerging from the RBCs will convert into male gametocytes (microgametes) or female (macrogametes), re-entering the mosquito host for transmission [23]. Gametocytogenesis is the process of the sexual development that is influenced by epigenetic, ecological, and heritable variables. The parasite may spread via a range of habitats and situations attributable to the capacity of gametocytes to proliferate [25]. Early *P. vivax* gametocytes lack a vacuole, and are compressed, non-ameboid [26]. Osmiophilic bodies, or black pigment grains, are a cytoplasmic feature of gametocytes. The nucleus size of *P. vivax* male and female gametocytes can be used to identify them despite having identical morphologies [26]. Female gametocytes have a

substantially smaller nucleus compared to male gametocytes, which can take up as much as half of the pathogen. Male gametocytes are approximately 2-4 times more numerous than female ones based on microscopy. Humans' bone marrows are loaded with *P. vivax* gametocytes in addition to peripheral blood [26].

In the *Anopheles* mosquito, the gametocyte makes its way to the mosquito's midgut, where it develops and multiplies through the sporogonic cycle. The sporogonic cycle consists of the production of zygotes by the microgametes fusing with macrogametes [27]. Zygotes have whip-like elongations, "tails" that make them mobile, while evolving into ookinetes. They will develop into locusts in the cell on days 10-18 and then into a sporozoite [27]. The sporozoite will travel through the mosquito's body to reach the salivary gland. They will remain there until the female *Anopheles* bites a human and infests them with inoculated sporozoites [27].

Plasmodium vivax gametocytes can be detected by reverse transcription polymerase chain reaction (RT-PCR) by amplifying the RNA transcripts of genes expressed in the gametocytes. Quantitative RT-PCR of specific RNA transcripts offers high sensitivity in identifying gametocytes at remarkably low densities. In efforts of controlling and reducing *P. vivax,* there is a necessity in determining highly expressed P. *vivax* genes as biomarkers for gametocyte detection [25] and parasitemia [6]. After being inoculated with sporozoites by a mosquito bite and staying latent for up to 2 years, hypnozoites are triggered to start blood-stage infections [6]. When compared to those in tropical locations, parasite strains from temperate and subtropical regions often have shorter relapse intervals (8 months or longer) between the first infection and

relapse (around 3–6 weeks) [28] which could indicate the variation in gametocytes present among regions. As a result, the pattern of relapse differs from one place to another, and it is still unclear what exactly causes hypnozoite relapses as well as what causes this phenotypic heterogeneity [6]. Patients that are not permitted sufficient time to improve, and with numerous repeated episodes making them increasingly susceptible for more severe implications such as severe anemia and lethal hemolysis in glucose-6-phosphate (G6PD) lacking individuals [6].

There are other means of contracting malaria, one being congenital malaria, where an infected mother is pregnant and transmits malaria to the newborn during labor. This is a rare but accurate method of transmission [29]. Transfusion-transmitted malaria can occur when a patient is infected with malaria and donates blood. However, this is rare in the United States; there is currently no procedure/protocol for screening donated blood for malaria [30]. Airport malaria is caused by an infected local host getting bitten by an infected *Anopheles* mosquito that has made its way onto a plane, from a malaria-active region to most likely non or rarely known malaria [31]. Lastly, mosquito-borne malaria here in the US has been rare; however, the possible pathogen can become revenant solely due to the vector quantity and occurrence [32]. The weather plays a tremendous role in the effectiveness of malaria transmission and could potentially be transmitted all year depending upon the region [33]. This is a direct correspondence to the weather, giving the environment for the mosquito [34].

Malaria symptoms resemble flu as headache, chills, tiredness, diarrhea, vomiting, and nausea could happen once infected by the parasite. Jaundice accompanied by anemia would

occur and, if not promptly treated, could become more severe [35, 36]. Malaria can cause extreme illness and death, including severe anemia, which causes the red blood cell unable to carry enough oxygen through the entire body [37]. Malaria can also cause cerebral malaria, where small blood vessels are formed that can cause seizures and brain disruption, and/or coma [35, 37]. Antimalarial drugs are available as preventative measures and could reduce the risk of malaria by 90% [38]. Preventing transmission including the use of mosquito repellent or wearing clothing to cover the legs and arms at night can minimize the risk of *Anopheles* mosquito bites. Other measures such as insecticide-treated nets [39], intermittent preventive treatment in pregnant women and infants [40], indoor residual spray [41], targeting the vector mosquito's larvae with larvicides, and mass drug administration [42] also aid to reduce transmission in malaria-endemic regions [43]. Reported by the WHO. severe malaria accounts for 0.7% of p. vivax case with fewer case reported having suffered from cerebral, renal severe complications and respiratory issues at 0.5% [44] all resulting from p. *vivax* [45], [44].

1.3 Invasion Mechanisms Related to Duffy Blood Group

Plasmodium vivax infections have been documented across Africa, and these infections are reported more often among Duffy-negative African people [46] Plasmodium vivax Duffy-binding protein (DBP) is utilized during reticulocyte invasion. Prior to invasion, the Duffy-binding-like domain on DBP interacts with the Duffy antigen receptor for chemokines (DARC) in the host [23]. DARC serves as a receptor and antigen for pro-inflammation. The parasites gain

entrance into a red blood cell by reducing the cytotoxic chemokines [23]. With little to no DARC expression, Duffy negative hosts specifically in black individuals are thought to be resistant to *P. vivax* [47] The Duffy negative phenotype Fy (a-b-) directly corresponds with the mutation -67T-C mutation in both alleles of an individual [47]. About 95% of the population in West and Central Africa is Duffy negative, and thus explain the rarity of *P. vivax* in West/Central Africa [47]. Nevertheless, an increasing number of reports has shown that Duffy negative individuals can be infected by *P. vivax* [48].

This could reveal the potential of the parasite utilizing an alternative pathway(s) for RBC invasion [47]. Due to epidemiological and ethnic differences, prevalence of *P. vivax* in Duffy negative individuals could vary across Africa [49]. For example in Sudan, 16 Duffy negative and 53 Duffy positive were detected from 213 samples with a 24.4% infection rate among *P. vivax* patients; in Ethiopia, 9 Duffy negative and 107 Duffy positive were detected from 358 total samples with a 37.4% infection rate among *P. vivax* patients; in Botswana, 4 Duffy negative and 4 Duffy positive were detected from 176 total samples having 6.8% infection rate among *P. vivax* patients from Southern Africa [49]. Among a variety of regions reported Duffy-negative infected *P. vivax* individuals; In 2014 and in 2021, Cameroon reported 8 [49, 50], in 2020, Nigeria reported 5, in 2011 Angola reported 7 and 8 from Equatorial Guinea [51], and lastly in 2021 Dominican Republican of the Congo reported 14 [49]. This reveals *P. vivax* ability to evolve among a variety of regions/populations that are malaria burdened. Considering P. *vivax* can infect and adapt Duffy-negative individuals, it is possible that these infections can produce

gametocytes leading to transmission. The extent of transmission may vary by environmental and host factors [52].

From 2011-2015 it was noted that P. *falciparum* and P. *vivax* symptomatic and asymptomatic samples collected and was determined prevalence in Ethiopia by using long amplicon deep sequencing and aid in malaria plasmodium infection by molecular epidemiology [53] In 2016 Botswana P. *vivax* was identified and reported by PCR in 10 districts where the incidence of the parasite was rampant. Among the 10 districts, there were 1,614 cases from 2-13 years of age [54]. It was reported that many cases were around 5 years old with 8 years of age making up 75% and 3 years of age were 25% of the reported cases. It was reported of P. *vivax* having 12.7% noted as asymptomatic occurrence in the noted districts [54]. In 2017 there was an alarming upsurge in cases with accelerated transmission of p. *vivax* in southern Senegal reporting at 53% [53], and the climate fluctuation allows the mosquito to become more abundant as rainfall.

In 2018, Nigeria was reported to have p. *vivax* absent as the population is predominantly Duffy negative (this to includes Nigeria to the west and east) thus having protection form p. *vivax* [55] however, being P. *vivax* was deduced by observed surveillance that the parasite is in fact dispersed throughout Nigeria. It was reported of P. *vivax* being 4 out of a total of 256 (2years and older) amplified malaria parasite DNA samples cohesively having presented with febrile conditions around 38°C [55] with the confirmed P. *vivax* patients being Duffy negative. These findings were possibly the first to report the incidence of P. vivax in Nigeria, which could be due

to incomprehension/misapprehension of the blood slides or perhaps performing diagnose due to inadequate equipment supporting restricting detection [55] This overall, supported/permitted the population in 2018 to become a mix of Duffy negative and Duffy positive individuals within the population that in the past did report to be largely thought to be Duffy negative revealing increasing distribution on P. *vivax* in Africa. In 2020 there was a report of symptomatic Duffy negative P. *vivax* infected persons with confirmation via amplificon, sequencing of the events and comparisons to previously confirmed Duffy negative P. *vivax* samples from Nigeria [56] thus the incidence of the parasite widening parameters of infection in Nigeria now having a mixture of parasite target host. Supporting the evidence of P. *vivax* expansion as P. *vivax* can infect red blood cells that don not expression the DARC gene. This is a result of malaria epidemiological advances in spreading throughout Africa [56].

1.4 Transmission of *Plasmodium* by *Anopheles* Mosquitoes in Africa

Malaria transmission is determined by several factors including the quantity, diversity, compatibility to parasites, and resistance level of mosquitoes, the amount and infectivity of *Plasmodium* gametocytes present in an infection, climate, deforestation, and the extent of interventions in malaria-endemic regions [57] It is important to identify the untiring transmission factors involving the appearance of mosquitos in vast numbers thus drastically increasing P. *vivax* and P. *falciparum* emergence in Africa [57] Insecticide resistance has spread quickly across the nation since 2000, according to reports. The occurrence of insecticide resistance was

significant in both An. *funestus a*nd An. *gambiae* (s.l.). The substances most impacted by tolerance seemed to be DDT, permethrin, deltamethrin, and bendiocarb. Nearly all An. *gambiae* (s.l.) locations showed an increase in allelic between 2000 and 2017, with the L1014F allele being the most common. Resistance to DDT, pyrethroids, and bendiocarb was linked to many purification genes, including P450 monooxygenase [58]. The 119F-GSTe2 metabolic resistance marker and overexpression of P450 genes were primarily responsible for An. *funestus'* resistance to DDT and pyrethroids, while An. *funestus* that was resistant to dieldrin had the 296S-RDL mutation [58]. Consequently, it's been observed in Panama, in these low transmission circumstances, qRT-PCR will become very helpful to estimate the hidden reservoir of transmission throughout endemic regions and to assess the incidence of gametocyte carriage [59]. The bulk of the cases are pregnant women and young children as they remain exceptionally susceptible to P. *vivax* [44], thus elevating malaria morbidity and mortality in malaria burden regions in Africa.

By reducing the abundance of vectors can greatly reduce the extent of malaria transmission [60]. Transmission remains a fundamental factor regarding reducing and eliminating malaria, the spread is undeniably crucial to malaria endemic regions worldwide as targeting the infectious stages are crucial to eradication [27]. By focusing on transmission, this will influence development of newly found preventive measures as well as advanced control methods that currently exist today [27]. Deforestation plays a major role in the vector prevalence thus increasing malaria transmission [61]. Malaria with time has become divergent in humans as it continues to circulate within the parasite burden population within the vector as a result

becoming more easily spread with the presence more robust in individuals with gametocytemia [62]. It is considerably important to address deforestation giving rise to malaria spread as the aid in larvae, increase vector occurrence as well as increase the possibility infections to the human host.

It is important to note the impact of asymptomatic carriers which are infected individuals with no clinical malaria symptoms [63] as *P. vivax* has the capability to be dormant in the liver for a duration of weeks to years as hypnozoites [64]. *P. vivax* matures at a faster rate than *P. falciparum* gaining the ability to produce more gametocytes that allow higher risk of transmission [64] and as a result able to spread malaria rapidly without detection due to the individuals being untreated [65]. In the Brazilian Amazon, ~67% of 4,083 asymptomatic individuals were infected with *P. vivax* and among these infected individuals, about 53.4% contained gametocytes [66]. Malaria with time, has become divergent in humans as it continues to circulate within the parasite burden population within the vector as a result becoming more easily spread with the presence more robust in individuals with gametocytemia [62].

1.5 Existing Interventions Against *P. vivax*

Elimination of malaria requires in depth epidemiology knowledge/understanding as it has reduced asexual parasitemia thus having the ability to be undetectable in the liver [67], becoming easily transmitted. Focusing on the reduction of malaria prevalence in sub- Saharan Africa has incorporated control programmes geared toward tackling the growing number of mortality and

morbidity [67] by way of detection capabilities. Nationwide, the effort to reduce the presence, overall elimination of malaria is to understand malaria epidemiology in malaria-stricken regions and appropriately conjure effective methodology intended to the entire human species of malaria [67]. The quantity of malaria vector remains problematic as it aids the continued spread of the parasite. As malaria cases rise worldwide and sadly claim the life of countless malaria high incidence countries, prevention is crucial. For P. *vivax* clinical trials using the two most popular TBVs, Pfs25 and Pvs25, indicate that current compositions with conventional adjuvants will not consistently elicit high antibody titers for functional activity [68]. As a result, ongoing attempts are being made to improve the immunogenicity and durability of specified TBV antigens via antigen delivery systems, protein crosslinking, and adjuvant systems [68].

The development of medication resistance is an increasingly significant barrier to managing and eradicating malaria. To tackle medication resistance, it would be beneficial to develop reliable genetic markers and conduct routine checks for the existence of mutations in malaria-endemic regions [43]. The species and medication susceptibility of the parasites from the location of acquisition affect malaria prevention and therapy. For severe malaria, intravenous artesunate is the primary line of treatment [36] thus a form of prevention against malaria. This issue was focused on and addressed resulting in the released and effective single-dose 8-aminoquinoline tafenoquine (TFQ) that treats the hypnozoite stage of P. *vivax* with continued regiment required [69]. To avoid 8-aminoquinoline-induced hemolysis in individuals with underlying G6PD deficit, safe delivery is achievable when combined with the use of G6PD

diagnostics (G6PDd). According to study, TFQ shouldn't cause poor adherence and consequent clinical relapses [69].

The development of blood-stage P. vivax vaccines remains strongly inhibited by the delayed booster regimen of PvDBPII/ Matrix-MTM given to people who have never had malaria [70]. In 2022 it was reported that through trials, a vaccine that focused on P. *vivax* binding protein in conjunction with a mixture of recombinant viral vaccines (MVA vectors and ChAd63) would be a measure of protection for counties outside of Africa where the volunteers were Duffy positive, with no underlying health issues [70]. It was reported that a cumulation of medication resulted in an overall upsurge in antibody response and deduced PMR (parasite multiplication rate) thus successfully decreasing the growth rate of P. *vivax* parasite in the blood stage [70] which is undeniably promising for countries excluding Africa that are burden by P. *vivax.*

Vaccines developed come against the great possibility of limitation mainly due to the nearly nonexistent market due to technology, the lack of development with the current tech, and the need for developers to fight the pathogen. Currently, there are vaccines on the market going through clinical expansion. The WHO has recommended only one vaccine in 2020, GlaxoSmithKline Biologicals, RTS, S/AS01, in highly transmissible areas [71]. This vaccine has developed from about 1980 to the present, and 2015 concluded the trial for this medication. With technology and biology currently evolving, there are encouraging vaccines surrounding sporozoite vaccines [71]. Newley developed 2022 a vaccine called RTS, S has been identified as a potential vaccine geared towards children in endemic countries where malaria transmission is

excessively elevated [71]. The vaccine is a recombinant protein virus mimicking particle along with an adjuvant system that works by creating a robust immune response to the parasite, thus constructing a form of defense against the pathogen's infection [71] that should be routinely administered in conjunction with other vaccines to achieve appropriate protection [72].

Throughout the years trials have observed that the implantation of RTS, S/AS01 with standard routine vaccination for children and infants has been promising. In Sahel and surrounding areas, it has been reported that co-administration of RTS, S/AS01$_E$, amodiaquine and sulfadoxine-pyrimethamine deduced the occurrence of sever, uncomplicated malaria, as well as death [73]. It was reported in 2022 from a model study utilizing samples from Malawi, Kenya, and Ghana of reducing hospitalization due to severe malaria around 30%, malaria instance around 40%, and lastly when incorporated with insecticide treated bed netting were 90% beneficial for the children and infants that receive the vaccines [74]. It is important to briefly note, vaccine PfSPZ is a live attenuate, whole sporozoite geared toward P. *falciparum* that can defend against interventions contraction of malaria [75]. Newly, the R21 vaccine is geared towards transmission blocking focused on mRNA and the sexual stage of the parasite [75].

1.6 Thesis Overview

The distribution of *P. vivax* in Duffy-negatives across Ethiopia as well as the parasite stages of these infections remain largely unclear. The presence of gametocyte in symptomatic or asymptomatic individuals can lead to onward transmission in the communities. Knowledge of

gametocyte reservoirs and pathogenesis allows for prioritizing transmission blocking vaccines against P. vivax in Africa. This study aims to 1) compare the distribution of *P. vivax* in Duffy-negative across Ethiopia; 2) determine the different stages of *P. vivax* in Duffy-positive and Duffy-negative infections; and 3) examine demographic and clinical features of Duffy-negative *P. vivax* infections. These findings will advance current knowledge of vivax malaria distribution and transmission in Africa.

CHAPTER 2: MATERIAL AND METHODS

2.1 Study Site and Sample Collection

A total of 447 febrile patient samples from seven major regions of Ethiopia including Afar, Amhara, Benishangul/Gumuz, Gambella, Oromia, Sidama, and Southern Nations, Nationalities, and People's Region (SNNPR) (**Figure 1)** that range from high to low transmission settings were collected in 2020-2021. Afar Regional State in the northern-east part of the country (altitude 379 m; Lat, Long: 11.72654, 41.09440); Amhara Regional State in the northern part of the country (altitude 1268 m; Lat, Long: 10.01734, 39.91253); Gambella Regional State in the western part of the country (altitude 447 m; Lat, Long: 8.24810, 34.59071); Oromia Regional State in the eastern part of the country (altitude 959 m; Lat, Long: 8.89932, 39.91726); Sidama Regional State in the northern east part of the country (altitude 500 m; Lat, Long: 6.7372,38,4008); and the Southern Nation and Nationalities People Regional State in the southern part of the country (altitude 1200 m; Lat, Long: 6.02043, 37.56788). Finger-prick blood samples were collected to make thick and thin blood films for microscopic screening of *Plasmodium* parasites. Blood smears were stained for 10 minutes with 10% Giemsa staining solution (pH 7.2). The parasite species, the developmental stage of the parasites, the density of asexual parasites and sexual gametocytes were examined using Rapid diagnostic test (RDT).

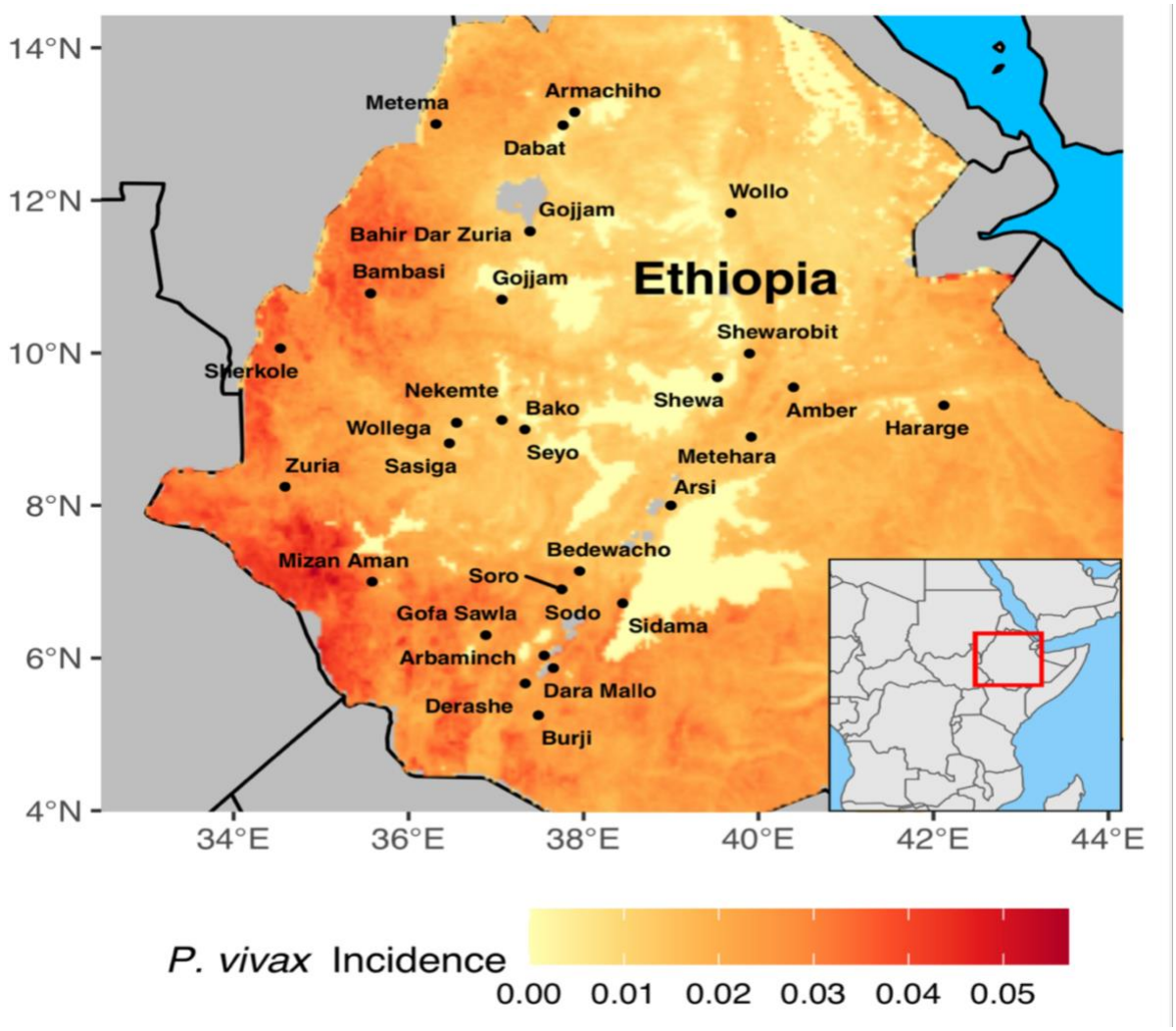


Figure 1. A map showing the study sites in Ethiopia with malaria incidence ranges from high in the western part to low in the eastern part of the country.

2.2 Molecular Screening of *Plasmodium vivax*

Parasite DNA was isolated from a dried blood spot using the Saponin/Chelex method [76]. Parasite gene copy number was estimated using qPCR, specifically the SYBR Green detection method [2,3] using published primers (forward: 5′-GAATTTTCTCTTCGGAGTTTATTCTTAGATTGC-3′; reverse: 5′GCCGCAAGCTCCACGCCTGGTGGTGC-3′) specific to *P. vivax* 18S rRNA, and primers (forward: 5′- TGG TAG CAC AAA TCC TTT AGG G-3′; reverse: 5′- TGG TAA TTG ACA TCC AAT CC-3′) specific to *P. vivax* cytochrome b (cytB) gene [77], [78]. Amplification was conducted in a 20 µl reaction mixture containing 2 µl of genomic DNA, 10 µl SYBR Green qPCR Master Mix (Thermo Scientific), and 0.5 uM primer.

The reactions were performed in QuantStudio Real-Time PCR Detection System (Thermo Fisher), with an initial denaturation at 95°C for 3 min, followed by 45 cycles at 94°C for 30 sec, 55°C for 30 sec, and 68°C for 1 min with a final 95°C for 10 sec. This was followed by a melting curve step of temperature ranging from 65°C to 95°C with 0.5°C increments to determine the melting temperature of each amplified product.

Each assay included positive controls of *P. vivax* Pakchong (MRA-342G) and Nicaragua (MRA-340G) isolates, in addition to negative controls, including uninfected samples and water. A standard curve was produced from a ten-fold dilution series of the *P. vivax* control plasmid to determine the efficiency and detection limit of the qPCR. Melting curve analyses were

performed for each amplified sample to confirm specific amplifications of the target sequence. The slope of the linear regression of threshold cycle number (*Ct*) versus log10 (gene copy number) was used to calculate amplification efficiency of each plate run based on internal standard controls. For the measure of reproducibility of the threshold cycle number, the mean *Ct* value and standard error was calculated from three independent assays of each sample. A cut-off threshold of 0.02 fluorescence units that robustly represented the threshold cycle at the log-linear phase of the amplification and above the background noise was set to determine *Ct* value for each assay. Samples yielding *Ct* values higher than 40 (as indicated in the negative controls) were considered negative for *Plasmodium* species. The amount of parasite density in a sample was quantified by converting the *Ct* values into gene copy number (GCN) using the follow equation: $GCN_{sample} = 2^{E\times(40-Ctsample)}$; where GCN stands for gene copy number, *Ct* for the threshold cycle of the sample, and E for amplification efficiency. The differences in the log-transformed parasite GCN between samples among the study sites were assessed for significance at the level of 0.05 by one-tailed t-tests. Variations in GCN among samples were presented as boxplots showing the median and interquartile range values.

2.3 Duffy Blood Group Genotyping

For all febrile patients, we first employed qPCR-based TaqMan assay to examine the point mutation (c.1-67T>C; rs2814778) in the GATA-1 transcription factor binding site of the *DARC* gene. The following primers (forward: 5'-GGCCTGAGGCTTGTGCAGGCAG-3'; reverse: 5'-CATACTCACCCTGTGCAGACAG-3') and dye-labeled probes (FAM-

CCTTGGCTCTTA[*C*]CTTGGAAGCACAGG-BHQ; HEX-CCTTGGCTCTTA[*T*]CTTGGAAGCACAGG-BHQ) were used. PCR reaction contained 5µl TaqMan Fast Advanced Master mix (Thermo), 1µl DNA template, and 0.5µl of each primer (10nM), and 0.5µl of each probe (10nM). The reactions were performed with an initial denaturation at 95°C for 2 min, followed by 45 cycles at 95°C for 3 sec and 58°C for 30 sec. A no-template control was used in each assay. The *Fy* genotypes were determined by the allelic discrimination plot based on the fluorescent signal emitted from the allele-specific probes. For *P. vivax* positive samples, a 1,100-bp fragment of the *DARC* gene was further amplified using previously published primers [4]. PCR reaction contained 20µl DreamTaq PCR Mastermix, 1µl DNA template, and 0.5µl each primer. PCR conditions were 94°C for 2-min, followed by 35 cycles of 94°C for 20s, 58°C for 30s, and 68°C for 60s, followed by a 4-min extension. PCR products were sequenced, and the chromatograms were visually inspected to determine and confirm the *Fy* genotypes based on the TaqMan assays.

2.4 Statical Analyses

SPSS version 21.0 was used for analyzing the socio-demographic information of the research participants using descriptive statistics. To test the association between malaria infection and factors including gender, age, ethnicity, and clinical symptoms, bivariate logistic regression was performed. The odds ratio and associated 95% confidence interval (CI) were computed to assess the strength of association. P values under 0.05 and 0.01 were considered as significant.

2.5 Ethics Statement

Scientific and ethical clearance was obtained from the institutional scientific and ethical review boards of Ethiopian Public Health Institute, Ethiopia and University of North Carolina, Charlotte, USA. Written informed consent/assent for study participation was obtained from all consenting heads of households, parents/guardians (for minors under 18 years old), and each individual who was willing to participate in the study.

CHAPTER 3: RESULTS

3.1 Socio-demographic characteristics of study participants

A total of 467 febrile cases were diagnosed for malaria in seven regional health facilities between April and November 2018. Socio-demographic data, finger prick blood samples (for DBS and blood film preparation) were obtained from 447 patients. The remaining case had excluded by exclusion criteria non-optimal blood specimens (insufficient samples), not willing to give consent, chronic patients where blood samples could not be taken. Thus, all analyses were performed based on 447 individuals. The age of the study participants was ranging from 0 to 70 years (**Table 1)**. The mean age was 20.82 and 269 (60.1%) of the respondents were males and 171 (38.2%) were females from febrile patients come and diagnosed for malaria during study period. *P. vivax* infections in Duffy-negatives distribute broadly across Ethiopia, especially in the northwestern and western regions (**Table 1**).

Table 1. Socio-Demographic characteristics of study participants across Ethiopia

Characteristics		**Participants n (%)**
Gender		
	Male	269 (60.1%)
	Female	171 (38.2%)
Age		
	<15	150 (33.5%)

≥16 and <45	273 (61.0%)
≥45 years	16 (3.5%)
Ethnicity	
Afar	5 (1.1%)
Amhara	107 (23.9%)
Benishangual/Gumuz	22 (4.9%)
Gambella	15 (3.3%)
Oromia	140 (31.3%)
Sidama	2 (0.4%)
SNNPR*	156 (34.8%)

* SNNPR = Southern Nations, Nationalities, and People's Region

3.2 Prevalence of P*lasmodium* infections and logistic regression analyses

Of the 447 study participants diagnosed for malaria, 415 (92.8%) had plasmodium infections, the prevalence of plasmodium infections in males was 92.9% and females 92.9%. The prevalence of P. vivax 365 (81.6%) and mixed infection 79 (17.6%). In males, the prevalence of P. vivax was 222 (82.5%), followed by mixed infection 47 (17.4%); whereas, in Females, the prevalence of P. vivax was 139(81.2%), followed by mixed infection 32(18.7%) observed. Although Plasmodium

infection was recorded in all age groups, a relatively higher Plasmodium infection prevalence 273(61.0%) was recorded among cases >15 - ≤45 age group, 150 (33.5%) <15 years and 16 (3.5%) >45 age groups (**Table 2**).

The variables which were considered for binary logistic regression model included gender, age, Duffy status, malaria status and symptoms (**Table 2**). The odds of having Plasmodium infection was 3.56 (95% CI: 1.11- 11.37, *P*=0.032) times higher among those who are Duffy positive, 1.36 (95% CI: 0.07- 24.57, *P*=0.834) who has fever and nearly 1.23(95% CI: 0.45-3.38, *P*=0.684) times higher among males and 0.86(95% CI: 0.29-2.46, *P*=0.777) times lower among those whose Age group <15 years old than age group between 16 to 45 years old. The result of one variable has shown significant association with Plasmodium infection (*P*< 0.05). All the other variables did not show significant association with Plasmodium infection (*P*> 0.05).

Table 2. Results of Bivariate and Multivariate Logistic Regression to Determine Main Predictors of *Plasmodium* Infection in Ethiopia

Parameter		**Number of samples**		***Duffy* -**	***Duffy* +**	
			Total infection	**Infection rate by 18s qPCR**	**Infection rate by 18s qPCR**	**Odds ratio (95% CI)**
Overall		447	415	16 (3.8%)	399 (96.1%)	3.56(1.11, 11.37) P=0.032*
		Negative		4(12.5%)	28(87.5%)	1
Gender						
	Female (%)	171 (38.2%)	159	7 (4.4%)	152 (95.5%)	1
	Male (%)	269 (60.1%)	250	9 (3.6%)	241 (96.3%)	1.23(0.45 to3.38) P=0.684

Age						
	<15 years (%)	150 (33.5%)	144	6 (4.1%)	138 (95.8%)	0.86(0.29, 2.46) P=0.777
	≥16 and <45 years (%)	273 (61.0%)	250	9 (3.6%)	241 (96.3%)	1
	≥45 years (%)	16 (3.5%)	14	1 (7.1%)	13 (92.8%)	0.48(0.06, 4.13) P=0.508
Symptoms						
Fever						
	Yes	398 (2.2)	369	12 (3.2%)	357 (96.7%)	1.36(0.07, 24.57) P=0.834
	No	10 (89.0)	10	0(0%)	10 (100%)	1
Headache						
	Yes	391 (87.4)	365	11(3)	354 (96.9)	2.5(0.29-20.64)P=0.402
	No	17 (3.8)	14	1(7.1)	13 (92.8)	1
Fatigue						
	Yes	257 (57.4)	238	7 (2.9)	231 (97)	1.2132(0.377, 3.897)P=0.745
	No	151 (33.7)	141	5 (3.5)	136 (96.4)	1
Muscle and Joint Pain						
	Yes	257 (57.7)	240	7 (2.9)	233 (97)	1.2420(0.3866, 3.9904) P=0.7159
	No	151 (33.7)	139	5 (3.5)	134 (96.4)	1
Chills						
	Yes	258 (57.7)	245	9 (3.6)	236 (96.3)	0.60(0.159, 2.257)P=0.45
	No	150 (33.5)	134	3 (2.2)	131 (97.7)	1
Sweating						
	Yes	218 (48.7)	208	6 (2.8)	202 (97.1)	1.224(0.387, 3.867)P=0.73
	No	190 (42.5)	171	6 (3.5)	165 (96.4)	1
Vomitting						
	Yes	177 (39.5)	163	7 (4.2)	156 (95.7)	0.531(0.165, 1.703)P=0.287
	No	230 (51.4)	215	5 (2.3)	210 (97.6)	

* and ** represent significance at level of 0.05 and 0.01, respectively.

3.3 Distribution of the Duffy genotypes across Ethiopia

Duffy-negatives distribute broadly across Ethiopia, especially in the northwestern and western regions **(Figure 1).** Except Afar, Gambella and Sidama all the other study site has Duffy negative plasmodium infected individuals. Gametocyte prevalence in Duffy-negative individuals is 29.4% (5 out of 17), lower than that in Duffy-positives samples 46.5% (162 out of 348). Gametocyte in Duffy-negatives were mostly found in the SNNPR and Amhara (40%) followed by Oromia (20%). The higher proportion of gametocyte in Duffy positive were observed in SNNPR, Amhara, Oromia and Gambella compared to those reported in Benishangual/Gumuz, Sidama, and Afar **(Table 3**).

Table 3. Distribution of the Duffy genotypes across Ethiopia

Region	Number of Samples	Duffy+		Duffy-	
		# of Pv	Pv with gametocytes (%)	# of Pv	Pv with gametocytes (%)
Afar	5	4	0	0	0
Amhara	107	94	56	5	2
Benishangual/Gumuz	22	19	5	3	0
Gambella	15	15	11	0	0
Oromia	140	107	20	5	1
Sidama	2	2	2	0	0
SNNPR*	156	107	68	4	2
Total	**447**	**348**	**162 (46.5%)**	**17**	**5 (29.4%)**

3.4 Asexual parasitemia and parasite stage comparisons

There was no significant parasitemia difference among the *P. vivax* samples retrieved from southwestern, southern, and eastern regions of Ethiopia, except for samples in Amhara, which is located in the northwest. Parasitemia in Duffy negatives widely vary among infections; relatively high parasitemia was observed in Oromia and Southern Nations, Nationalities & People's region.

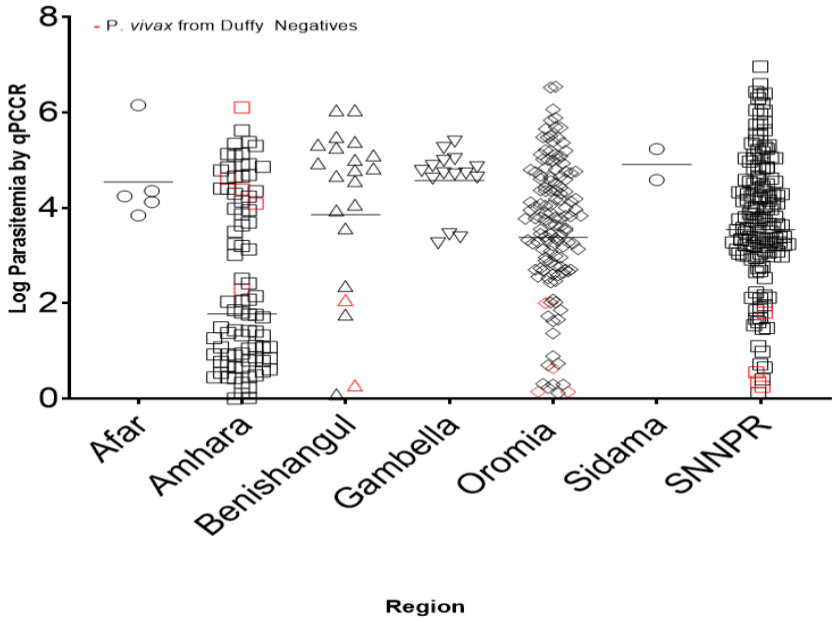
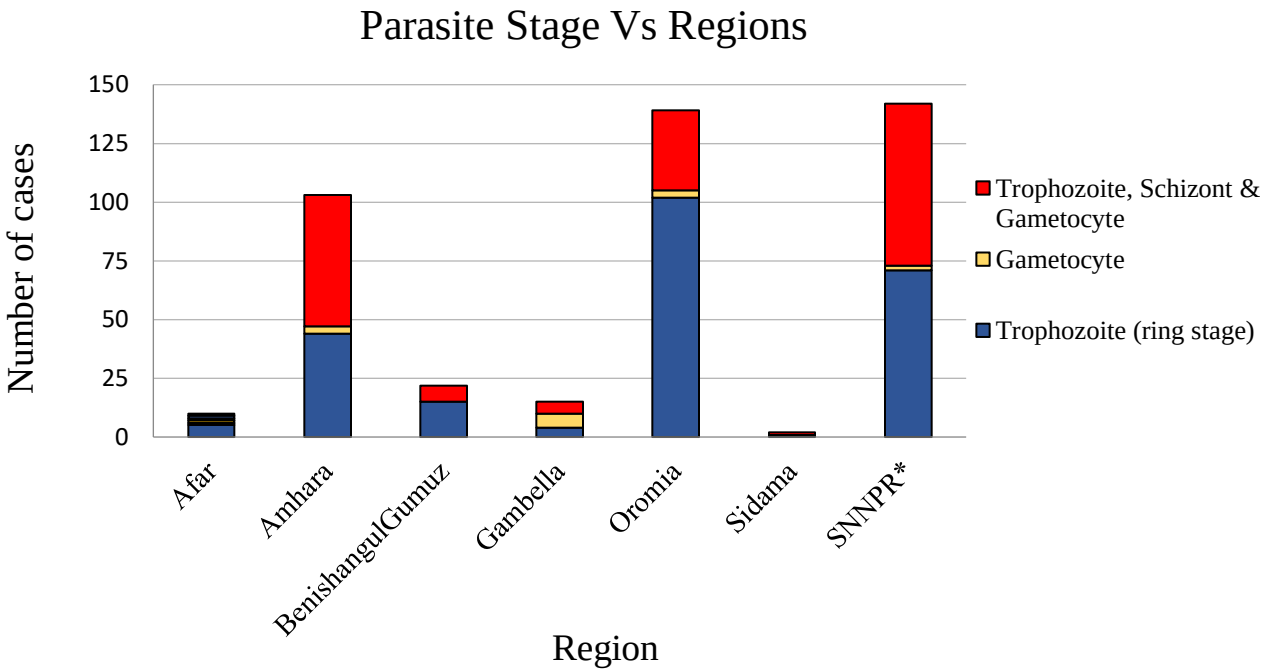


Figure 2. Log parasitemia comparison by region.

Most of the infections had mixed parasite stages, thus, the proportion of parasite stages vary among regions. SNNPR, there were 71 trophozoites reported following 56 of trophozoite, schizont, and gametocyte stage along with least reported being two gametocytes. Oromia reported 139 of P. vivax cases total that were confirmed to be 102 trophozoite following 34 of

trophozoite, schizont, and gametocyte stage along with least reported being 3 gametocytes. In Amhara region there were 56 of trophozoite, schizont, and gametocyte stage following 44



trophozoites along with least reported being 3 gametocytes. BenishangulGumuz, reported only two stages 15 trophozoites reported following 7 of trophozoite, schizont, and gametocyte. Afar, were reported only 5 trophozoites. Sidama reported two mixed stages trophozoite, schizont, and gametocyte stages. Collectively, trophozoite and mixed stages are predominantly found in each study site **(Figure 3).**

Figure 3. Parasitemia comparison by regions.

3.5 Sensitivity and Specificity Analysis of 18s rRNA and Cytochrome B

Using microscopy as a gold standard, the sensitivity and specificity of 18s rRNA was 92.8% and 0% respectively. The corresponding positive and negative predictive values were 100% and 0%, respectively. In detection of Plasmodium infection, there were discordant results occurred between microscopy, 18s rRNA qPCR and Cytochrome B, 444 (99.3%), 415 (92.8%) and 447 (100%) respectively observed. Of the total 32 negative by 18s rRNA, CytB qPCR detected additional 32 positive cases (**Table 4)**.

Table 4. Sensitivity and specificity of 18s rRNA against Cytochrome B, detection of *Plasmodium* infection in Ethiopia.

	CytB qPCR		
18s qPCR	**Positive n (%)**	**Negative n (%)**	**Total n (%)**
Positive n (%)	**415 (a) TP**	**0 (b) FP**	**415 (a+b)**
Negative n (%)	**32 (c) FN**	**0 (d) TN**	**32 (c+d)**
Total n (%)	**447 (a+c)**	**0 (b+d)**	**447 (a+b+c+d)**

Key = TP=True positive, TN=True negative, FN=False negative and FP=false positive

Sensitivity = True positive rate (a/(a+c)) =92.84%

Specificity =True negative rate (d/(b+d))=0%

Positive predictive value (PPV) = TP/ (TP+FP) =415/415 = 100%

Negative predictive value (NPV) =TN/ (TN+FN) =0/32= 0%

Accuracy of test TP+TN/All testes = 415/447 = 92.84%

Of the total 447 *Plasmodium* positive samples by cytB, microscopy detected only 444 (99.32%). With respect to each species, microscopy detected a total of 365 *P. vivax* cases while CytB qPCR detected 347 of the *P. vivax* cases. Moreover, only 79 of the mixed infections were detected by microscopy out of 100 mixed detected by CytB qPCR (**Table 5)**.

Table 5. Microscopy vs cytB qPCR for detection of *plasmodium* species in Ethiopia.

	CytB qPCR		
Microscope	**Pv n (%)**	**Mixed (Pv+Pf) n (%)**	**Total n (%)**
Pv n (%)	**278 (a) TP**	**85 (b) FP**	**363 (a+c)**
Mixed (Pv+Pf) n (%)	**64 (c) FN**	**15 (d) TN**	**79 (b+d)**
Total n (%)	**342 (a+b)**	**100 (c+d)**	**442 (a+b+c+d)**

Key = TP=True positive, TN=True negative, FN=False negative and FP=false positive

Sensitivity = True positive rate (a/(a+c)) =81.3%

Specificity =True negative rate (d/(b+d))=15%

Positive predictive value (PPV) = TP/ (TP+FP) =278/363 = 76.6%

Negative predictive value (NPV) =TN/ (TN+FN) =15/79= 18.98%

Accuracy of test TP+TN/All testes = 415/447 = 66.3%

CHAPTER 4: DISCUSSION AND CONCLUSION

4.1 Discussion

In areas where Duffy-negativity is prevalent, vivax malaria has been reported but it was unclear whether these infections can transmit among individuals. This study has revealed the prevalence and distribution of P. vivax from Duffy-negative individuals across broad regions of Ethiopia that represent a range of difference in demographic background, clinical symptoms as well as stage of infection. In the general population, Duffy negativity varied from 20–36% in East Africa to 84% in Southern Africa [49]. P. vivax prevalence in Duffy-negatives ranged from an average of 4% in southwestern Ethiopia and 9.2% in Sudan to 86% in Botswana. The prevalence of Duffy negative P. vivax infections (5.5%) in this study is consistent with prior studies. Compared to Duffy-positive infections, the averaged parasite density is much lower in Duffy-negative infections, consistent to previous study [49]. Nonetheless, a few Duffy negative *P. vivax* infections in Amhara were detected with relatively high parasitemia, suggestive of certain *P. vivax* strains can invade Duffy negative erythrocytes more efficiently than the others. The exact

mechanisms of Duffy negative erythrocyte invasion by *P. vivax* are still unclear and merit further investigation.

Amongst regions, a higher rate of Duffy positive infections had gametocytes compared to Duffy negatives ones. Gametocytes can infect mosquitoes and develop into sporozoites in the mosquito midgut and enter another host via a mosquito bite. The prevalence of gametocyte-positive infections is a proxy for transmissibility in malaria endemic courtiers. The detection of *P. vivax* gametocytes in Duffy negative infections in Amhara, Oromia, and SNNPR raises concern that these infections not only cause clinal symptoms but can also contribute to transmission, despite its lower prevalence than Duffy positive infected individuals. Given Duffy negative and Duffy positive individuals co-exist in Ethiopia, the extent of transmission remains uncertain. It is a possible that these cases being transform into gametocytes and spread from Duffy-negative to other people due to genetic similarities rather than only an infection [8], [49], [79]. Due to previously being exposed, the host may have acquired immunity against symptomatic blood-stage parasitemia; however, due to the early gametocyte development of P. vivax, long lasting sub-clinical illnesses may still contribute to continuous transmission [26], [80].

The proportion of parasite stage varies among each region. The majority of *P. vivax* samples in Amhara, Gambella, Sidama, and SNNPR have mixed parasite stages including gametocytes, whereas in Oromia and SNNPR trophozoite stage are prominent in most samples. Such difference could be due to the vector environmental preference, host availability, and environmental stability.

Our findings showed that PCR diagnosis by cytB was more sensitive than 18s rRNA gene in identifying *P. vivax* samples using microscopy as gold standard. Based on cytB qPCR, the number of threshold cycles (C_T) values were consistently lower when compared to 18s rRNA C_T values for the same patient samples. As C_T values is a relative measure of parasite quantity, the lower C_T values indicate higher level of parasite DNA or gene copy number that allow for detecting low-densities *P. vivax* infections, a biomarker superior to 18s rRNA. A more sensitive biomarker such as cytB will enable the identification of asymptomatic infections in communities and provide epidemiological information that is directly applicable to enhancing control measures [81], [82].

For all *P. vivax* confirmed infections, typical symptoms were fever as well as headache and fatigue. Other symptoms including muscle and joint pain, chills, sweating, and vomiting vary by individuals across the seven study regions. Interestingly, our analyses revealed that *P. vivax* cases were more likely occurred in men than women aged between 0-40 years old, and such demographic pattern could correlate to the vector mosquito feeding time and behavior (outdoor or indoor resting/biting), the form of occupation (outdoor or indoor), environment (rural or remote populations), and economic status (poverty) [83]. These factors are critical when identifying disease trends or at-risk populations.

4.2 Conclusion

The prevalence of Duffy-negative individuals among *P. vivax* malaria patients varies across Ethiopia. The proportion of infections that carry gametocytes is a proxy for human-to-mosquito

transmissibility and the documentation of *P. vivax* infections in Africa where the predominant population is Duffy-negative demonstrates the ability of *P. vivax* to replicate asexually in Duffy-negative hosts. This study confirms that Duffy-negativity does not completely protect against *P. vivax* infection and that these infections are frequently associated with low parasitemia, which may represent hidden reservoirs that can contribute to transmission. Understanding *P. vivax* transmission biology and gametocyte function via infectivity studies and in vitro assays especially in Duffy-negative populations would enhance the treatment and control strategies of vivax malaria in Africa. Further study is needed to quantify *Pvs*25 transcripts by qRT-PCR in order to measure gametocyte density in Duffy-negative infected samples and to expand sample size that will allow fair comparisons of gametocyte carriage between Duffy-positive and Duffy-negative infections. A deeper comprehension of the association between Duffy-negativity and the invasion processes of *P. vivax* would aid the development of *P. vivax*-specific eradication tactics, including substitute antimalarial immunizations other than a Duffy-binding protein-based vaccine.

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