JIMMA UNIVERSITY

SCHOOL OF GRADUATE STUDIES



EPIDEMIOLOGY OF MALARIA AND PERFORMANCE OF A NEW HIGHLY-SENSITIVE MALARIA DIAGNOSTIC TOOL IN JIMMA ZONE, ETHIOPIA

BY

ABDISSA BIRUKSEW HORDOFA

OCTOBER 2024

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EPIDEMIOLOGY OF MALARIA AND PERFORMANCE OF A NEW HIGHLY-SENSITIVE MALARIA DIAGNOSTIC TOOL IN JIMMA ZONE, ETHIOPIA

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October 2024

Jimma University, Ethiopia

DEDICATION

In loving memory of my hero, my late father:

To the unwavering giant who shaped my world, ***Buruksaa Hordofaa Roobii****: Though you left me for heaven in the midst of my PhD journey, your voice still echoes in my memory—a constant battle cry: '****Jabaadhu, Qeerransa eegee hin qabani****'—a warrior's wisdom urging me to face any challenge with unyielding spirit. Your unshakeable love and belief in my potential were the very wings that lifted me on this extraordinary journey. The journey wasn't paved with sunshine, Dad. Every day brought its challenges, '****no easy day '****at all. Yet, this PhD dissertation stands a testament to the steadfast foundation you built, brick by enduring brick, with every loving sacrifice. Your immense love remains a constant weight upon my shoulders, a comforting reminder of the path you tirelessly cleared for me. May your laughter echo eternally in the heavens. You are, and forever will be, my greatest inspiration.*

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Letter for Declaration (Dissertation Work)

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ABSTRACT

Background: Despite significant progress in malaria control efforts, the disease continues to pose a major public health challenge in Ethiopia due to its complex epidemiological landscape. A critical concern is the presence of a large proportion of the population, such as schoolchildren, who have asymptomatic malaria infections that go undiagnosed and untreated. This, coupled with a dearth of evidence on the performance of new, highly-sensitive histidine-rich protein-2 (HRP-2)-based malaria rapid diagnostic tests (hsRDTs), such as the NxTek™ Eliminate Malaria-Pf test, in detecting *Plasmodium falciparum* (*P. falciparum)* infections in asymptomatic schoolchildren, hinders accurate diagnosis and treatment. This contributes to the persistence of asymptomatic infections, which act as a silent reservoir, fueling ongoing transmission and thwarting the ambitious goal of malaria elimination.

Furthermore, a comprehensive examination of the spatiotemporal clustering of malaria incidence through modeling a decade of epidemiological data from the Jimma Zone is critical for informing resource allocation and targeted, locally effective intervention strategies. Taken together, this PhD project was aimed to investigate the epidemiology of malaria and evaluate the performance of a novel, highly-sensitive malaria diagnostic tool within the Jimma Zone of Southwest Oromia, Ethiopia.

Methods: To achieve this aim, a school-based cross-sectional study was conducted from September 2021 to January 2022, involving 994 healthy schoolchildren aged 6–15 years. A multi-stage sampling technique was employed to select schools and participants, with sample allocation done proportionally to each school and grade. Participants were randomly chosen from student records using a lottery method. Finger-pricked blood samples were collected for various diagnostic tests: microscopy, conventional rapid diagnostic test (cRDT) (SD Bioline Malaria Ag Pf/Pv), and the hsRDT. Additionally, dry blood spots (DBSs) were prepared on filter papers for quantitative real-time polymerase chain reaction (qPCR) assay. For the prevalence study, all diagnostic methods except hsRDT were utilized. Data on demographic and risk factors for asymptomatic malaria were collected through face-to-face interviews with the child's head of household by trained data collectors. The hsRDT was then compared to cRDT and microscopy. qPCR and microscopy were used as reference methods. Furthermore, to understand the broader epidemiological context, a decade of retrospective malaria data (2012-2021) from 15 districts in the Jimma Zone was analyzed. District-level annual parasite incidence (API) and standardized incidence ratio (SIR) were calculated, and malaria risk maps

were created. Spatial clustering (spatial autocorrelations) of malaria incidence was assessed using Moran's I and Monte Carlo statistics, while temporal patterns and future trends were with time series analysis using the Autoregressive Integrated Moving Average (ARIMA) model.

Results: the overall prevalence of asymptomatic malaria determined by qPCR was 5.03% (50/994). Of this, the prevalence rates using cRDT and microscope were 2.20% and 1.51% respectively. In the overall prevalence, *P. falciparum was* the dominant parasite species, accounting for 90%, followed by *P. vivax* (6%) and mixed infections (4%). Additionally, submicroscopic asymptomatic malaria infections constituted 70% (35/50) of the overall prevalence. Identified several risk factors that were significantly associated with asymptomatic malaria infections among schoolchildren, including the age of the household head, nighttime outdoor activities of household members, a family history of malaria, the absence of insecticide-treated nets (ITNs), and the presence of stagnant water around homes.

Moving to the next step that presents the diagnostic performance of the hsRDT. Hence, when qPCR was used as a reference, the sensitivity of hsRDT was higher (48.89%) than the microscopy (33.3%), and showed 100% specificity and a positive predictive value (PPV). Microscopy showed similar specificity and PPV as hsRDT. Using microscopy as a reference, the diagnostic performances of both hsRDT and cRDT were similar. Both RDTs also demonstrated identical diagnostic performances in both comparison methods. Finally, the prevalence of *P. falciparum* was 4.52%, 2.2%, 2.2%, and 1.51% by qPCR, cRDT, hsRDT, and microscopy, respectively.

In the end, the project examined the spatiotemporal distribution of malaria and forecasts trends for the next five years from 2022. To begin with, a total 1,718,471 individuals screened for malaria between 2012 and 2021. Of these, it was revealed that 359,655 confirmed cases, equating to 20.93% of those screened over the periods. The dominant parasite species was *P. falciparum,* responsible for 52.71% of these cases, while *P. vivax* accounted for 43.62%, and mixed infections constituted 3.67%. In terms of malaria incidence, a striking 96.13% reduction was observed. This dramatic decline is clearly reflected in the API, which dropped from 87.97 per 1,000 population at risk in 2012 to a mere 3.40 in 2021. Moreover, spatial autocorrelation analysis using Moran's I revealed random distributions of malaria incidence across the study districts, suggesting no significant spatial clustering of the disease in the Jimma Zone. Additionally, API and SIR maps proved invaluable in identifying both potential risk areas and cold spots. Despite these remarkable achievements, malaria remains a persistent threat in areas

like Chora Botor and Shebe Sonbo. The seasonal dynamics of the disease follow a distinct bimodal pattern, with peaks occurring from August to November (reaching a maximum in September) and again from March to June. Looking ahead, forecasting using the ARIMA (0, 1, 3) x (1, 1, 0) model for the years 2022-2026 predicts a stable case of malaria, characterized by seasonal fluctuations, yet without any significant upward or downward trends.

Conclusions: This study vividly paints the diagnostic challenges posed by conventional malaria diagnostic tools, which significantly underestimated the true prevalence of asymptomatic malaria within the study population. Remarkably, qPCR was proven adept at detecting even very levels of parasitaemia, thereby revealing a higher prevalence of submicroscopic malaria infections. These findings imply that schoolchildren with asymptomatic malaria infection are potential hotspots for malaria reservoirs that fuel ongoing transmission. Our finding suggests that relying solely on these conventional tools could hinder progress toward complete malaria elimination especially in regions with low malaria transmission where asymptomatic cases are prevalent. To enhance malaria control efforts, it is imperative to integrate schoolchildren and schools into intervention strategies. Equally crucial is the adoption of more advanced and sensitive diagnostic technologies such as qPCR, which are essential for effective malaria control and elimination. Targeted interventions for asymptomatic malaria-infected schoolchildren could provide significant support to the National Malaria Control Programme (NMCP), playing a vital role in the comprehensive control and eventual eradication of the disease. On the other hand, hsRDT has the same diagnostic performance as cRDT but improved diagnostic characteristics than microscopy for detection of the deadly parasite, *P. falciparum in* school children with asymptomatic malaria. Since it has demonstrated identical performance as the cRDT, it can be a useful tool for the national malaria elimination plan of Ethiopia. Moving on, the malaria incidence in Jimma Zone significantly decreased from 2012 to 2021, reaching notably lower risk levels by 2021. As the malaria incidence became lower, the spatial autocorrelation analysis showed random distribution of the disease in the zone. Nevertheless, malaria continues to pose a threat in regions such as Chora Botor and Shebe Sonbo, underscoring an ongoing risk. This highlights the urgent need for sustained surveillance and targeted interventions in historically high-risk areas to mitigate persistent threats effectively. The seasonal pattern of malaria underscores the need for targeted interventions during peak periods. Given that the 2022 to 2026 forecasted stability in malaria trends may hinges on the ongoing effectiveness of current control measures, it is crucial to use the model with caution, he authors advocate for unwavering vigilance and

the proactive adaptation of interventions in response to evolving trends. In this way, continued progress can be ensured and possible changes in malaria dynamics can be managed, thereby ensuring success in the fight against the disease.

Policy Implications of the Dissertation Findings

Our research highlights significant gaps in malaria control within Jimma Zone, Oromia. A major concern is the failure of conventional diagnostic tools to identify 56-70% of asymptomatic infections in schoolchildren, creating a hidden reservoir that perpetuates transmission and endangers the country's 2030 malaria elimination goal. Urgent integration of molecular diagnostic tools into routine surveillance, coupled with active community engagement, is crucial to addressing these risk factors effectively and closing the gaps in control efforts.

Second, strategic resource allocation must prioritize peak transmission seasons and historically vulnerable and malaria receptivity districts within Jimma Zone. This requires data-driven decision-making, leveraging real-time surveillance to identify emerging hotspots.

Finally, implementing a robust, adaptive surveillance system, incorporating real-time data like rapid diagnostic test results and climate information, is non-negotiable. This system must enable rapid response to evolving transmission patterns. To achieve malaria elimination, policymakers must act decisively, adopting these evidence-based strategies to safeguard the health of our children and communities.

For more information, contact the author Dr. Abdissa Biruksew: - abdissa.hordofa@gmail.com

LIST OF ABBREVIATIONS AND ACRONYM

API - annual parasite incidence

ARIMA - autoregressive integrated moving average

cRDT- Conventional Rapid Diagnostic Test

DBS - dried blood spots

GTS - Globa Technical Strategy

HHH – house hold head

HRP2/3 - histidine-rich protein-2/3

hsRDT – highly - sensitive Rapid Diagnostic Test

iRBCs - infected Red Blood Cells

ITNs - insecticidal treated nets

LAMP - loop-mediated isothermal amplification

LoD - limit of detestation

MSP-1 - Merozoite surface protein-1

NMCPs - National Malaria Control Programs

PfEMP-1- *P. falciparum* erythrocyte membrane protein-1

pLDH - Parasite lactate dehydrogenase

qPCR - quantitative Polymerase Chain Reaction

RBCs - Red Blood Cells

RDTs - Rapid Diagnostic Tests

uRDTs - ultra-sensitive RDTs

WHO - World Health Organization

COVID-19 - Coronavirus Disease 2019

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CHAPTER ONE: GENERAL INTRODUCTION

1.1 The global context of Malaria

Malaria is a disease caused by parasitic protozoans of the genius *Plasmodium* and has long history. Evidence suggest that the disease could have existed before the emergence of Homo sapiens, potentially affecting early hominins; thus, is an ancient disease witch may have evolved alongside the early human population and their ancestors (1, 2). Scientists proposed that it first appeared in Africa, likely in Ethiopian regions, and evolved along with human migration. It then spread from the Nile Valley to the Mediterranean, then to Asia and finally north across Europe (2, 3). By the early 19th century, the disease had become widespread throughout the world (4).

Based on that background, malaria most likely ruled humankind for more than half a million years, having a profound effect on human history (5, 6). For example, historical accounts such as the description of periodic fever attacks in the Hippocratic Corpus (5th - 4th centuries BCE) strongly suggest early cases of the disease (5, 7). As a matter of fact, it has casted a shadow over the rise and fall of empires over the centuries and shaped the course of wars and social unrest. The disease even claimed the lives of powerful figures, including kings, popes and military leaders at the height of their power (8).

Despite its profound influence on history and society, the true origins of malaria remained hidden for centuries (9). The term “malaria” is derived from the Italian words “mal’aria,” meaning “bad air or marsh fever (10),” and reflects the prevailing miasmatic theory. The theory attributed the disease to harmful fumes from swamps. However, the transition from this theory to the recognition of mosquitoes as the main vectors of malaria marked a significant paradigm shift and revolutionized approaches to prevention and control (11, 12). While the miasmatic theory prevailed for centuries and hindered progress in understanding the true cause of disease, a pivotal moment came in the late 19th century that led to the closing of this Pandora's Box(12).

That is, the *mosquito-malaria theory* was developed to answer the question of how malaria was transmitted. The theory stated that malaria was transmitted by mosquitoes, contrary to the centuries-old medical dogma of miasma (13). It then paved the way for discoveries and further advances in malaria research. In 1880, Charles Louis Alphonse Laveran discovered

Plasmodium parasites in human red blood cells (RBCs) as the true pathogens of malaria(14). In 1887, Sir Ronald Ross's groundbreaking discovery that mosquitoes were the vectors of the disease marked a clear departure from the miasmatic view and revolutionized the understanding of malaria transmission (15, 16). Based on this discovery, in 1900 Sir Patrick Manson reported his first experimentally proven mosquito infection through feeding on infected blood (14). Taken altogether, these breakthroughs marked an era of change in which the focus shifted from environmental to specific biological agents and their transmission cycle of malaria parasites (15, 17).

Building on these fundamental discoveries, it is important to recognize that malaria, a deadly vector-borne infectious disease is transmitted by female *Anopheles mosquitoes* while feeding on human blood (18, 19). This preventable and curable disease can cause a wide range of symptoms, from asymptomatic cases, mild fever and chills to severe and life-threatening complications. Infants, young children and pregnant women, travelers and people with HIV/AIDS are particularly at risk. If left untreated, malaria can cause severe illness and death within 24 hours (18, 19). Despite ongoing efforts to control its spread, malaria remains a significant barrier to socioeconomic development in endemic regions worldwide, with particularly severe impacts on the African continent (20).

While the health impacts of malaria are well documented, the economic burden is equally significant. A study by Haakenstad *et al.* (2016) highlights this issue, estimating annual global expenditure on malaria at a staggering US$4.3 million (21). In African countries in particular, it significantly hindered economic growth. Estimates suggest a decline in growth of 1.3% per year due to the disease (22). Further studies found that economic losses varied across the continent (23). Aside from the direct costs to households and health systems, malaria also wastes resources and discourages investment, reflecting its multiple economic impacts (24).

Aside from the economic consequences, malaria also significantly affects the education system and general health. As a leading cause of illness and death among children in sub-Saharan Africa, malaria impairs both preschool and schoolchildren's ability to concentrate and learn, while frequent school absence further hinders their education. The cognitive impairments associated with malaria can affect students' participation and academic performance, setting in motion a vicious cycle in which education, essential to escaping poverty, is undermined by the disease, ultimately affecting broader economic growth (25, 26). In addition, malaria during pregnancy contributes to child mortality in sub-Saharan Africa and has lasting effects on

survivors, including preterm birth, low birth weight, and impaired cognitive development in affected children (27). Furthermore, malaria is a risk factor for other diseases, as repeated infection with *P. falciparum* increases the likelihood of developing Burkitt's lymphoma by more than 50% in school-age children in endemic areas, especially with asymptomatic malaria complications (28, 29),

1.2 The *Plasmodium* parasites and their transmission

1.2.1 Biology and its life cycle

According to their biology, the causative agents of malaria are single-celled eukaryotic organisms, classified within the phylum *Apicomplexa* and belong to the genus *Plasmodium.* What is noteworthy is that these parasites can infect not only humans, but also a number of other vertebrates, including reptiles, birds and mammals. To date, over 200 *Plasmodium* species have been identified, each infecting a specific host range (30). Although they have different names, only five parasites are responsible for malaria in humans. Each species of these parasites is characterized by unique geographic distributions, clinical manifestations, drug resistance patterns, and epidemiological profiles. For example, *P. falciparum, P. vivax*, *P. malariae* and *P. ovale* are among the known species that cause serious diseases in different regions of the world. In contrast, *P. Knowlesi* is the newest addition to this list. This parasite, which naturally infects monkeys called M*acaques*, is increasingly recognized as a cause of zoonotic malaria in Southeast Asia (SEA) (31).

Plasmodium parasites have a complex life cycle (Fig. 1.1), which involves the complicated transition between two hosts, the female Anopheles mosquito, in which sexual reproduction occurs, and the human host, in which the parasites reproduce asexually, resulting in the symptoms of malaria (32, 33). First, infected female *Anopheles mosquitoes* inject *sporozoites* into human skin during a blood meal (34) that initially deposited at the injection site, these sporozoites exhibit remarkable motility and navigate the extracellular matrix through a process known as transmigration(35). From there they finally enter the bloodstream and make a quick journey to the sinusoids of the liver just a few minutes after the mosquito bite. Interestingly, not all sporozoites take this direct route into the bloodstream. About 50% remain trapped in the skin, where they can be engulfed and excreted by phagocytes. Furthermore, approximately 15% of inoculated parasites invade the lymphatic vessels, with the majority finding refuge in the lymph nodes, where they are often engulfed by dendritic cells (36).

Subsequently, about 35% of the sporozoites successfully infiltrate the blood vessel and rapidly migrate to the liver sinusoids. At this point, they express their circumsporozoite proteins (CSP) on their surface, which allows the parasites to adhere to highly sulfated heparan sulfate proteoglycans (HSPGs) presented by hepatocytes (37). Once the parasite is inside the hepatocytes, it begins to multiply. Within the first few hours of infection, the parasite undergoes a transformation from its elongated shape to a round shape called a trophozoite (38). Here they enter a phase of asexual replication known as exoerythrocytic schizogony. Approximately 20 hours after invasion, the parasite nucleus divides repeatedly and rapidly, resulting in a multinucleated schizont (39). It is important to note that *P. vivax* and *P. ovale* possess a unique property: a subpopulation of parasites differentiate into dormant stage known as the *hypnozoites* within the liver parenchyma. These hypnozoites can remain inactive for long periods of time - from weeks to years - before transforming into merozoites and causing relapses. After this initial liver replication, the mature schizonts eventually rupture, releasing thousands of new generations of merozoites into the bloodstream. Interestingly, however, the liver stage remains asymptomatic in all *Plasmodium* species (40).

Once merozoites are released into the blood, they immediately begin to attach to and subsequently infect erythrocytes, reticulocytes (*P. vivax*), or both (*P. falciparum*), depending on the *Plasmodium* species (41). Incredibly, these merozoites invade erythrocytes with a remarkable speed, requiring less than 30 seconds to minimize their exposure to the immune system. This rapid invasion initiates the erythrocyte schizogony cycle, in which the parasite matures through the ring, trophozoite, and schizont stages. As the schizonts mature, they rupture, releasing a new wave of merozoites that quickly invade new erythrocytes, continuing the cycle. Crucially, this erythrocytic schizogony causes the clinical symptoms of malaria, as the proliferation of the parasite leads to the destruction of the RBCs (42).

During the exponential phase of asexual blood stage development, a portion of the parasites begin sexual commitment, a small proportion of the parasites differentiate into sexual forms in the erythrocytes, so-called *gametocytes*. This process results in the formation of male microgametocytes and female macrogametocytes (43, 44).

When a female *Anopheles mosquito* feeds on an infected human, she ingests these gametocytes, triggering the sexual or sporogonic cycles in the mosquito's midgut. At this point, the microgametocytes undergo several rounds of division called exflagellation, producing eight flagellated microgametes from a single male gametocyte. These motile microgametes seek out

and fertilize the macrogametes, forming zygotes. After fertilization, the *zygotes* transform into motile *ookinetes* that traverse the midgut wall and develop into oocysts. Within the *oocyst*, the parasite undergoes further *sporogony* until the *oocyst* matures, ruptures, and releases thousands of *sporozoites*. These sporozoites then migrate to the mosquito's salivary glands, completing the parasite's life cycle and preparing the mosquito for transmission to a new human host during its next blood meal (45-47).

In addition to mosquito transmission, malaria can also be spread in other ways. For example, blood transfusions from an infected donor can transmit the disease. Unlike mosquito-borne malaria, transfusion-borne malaria skips the preerythrocytic cycle and doesn't involve hypnozoites. This means that there are no relapses, and the inoculation period is shorter (48). In addition, pregnant mothers can transmit the infection to their babies, resulting in congenital malaria (49). Moreover, rare cases, organ transplants or shared needles can also pose a risk of transmission (50).

Taken together, *Plasmodium* parasites rely on specific stages in their life cycle – gametocytes and sporozoites – for transmission between mammalian and mosquito hosts. However, significant bottlenecks occur at these stages as the parasite moves between both hosts - vulnerable points where survival and development become less likely. Consequently, these stages represent ideal targets for national malaria control programs (NMCPs), which focus on interrupting the parasite's life cycle and preventing transmission (51).

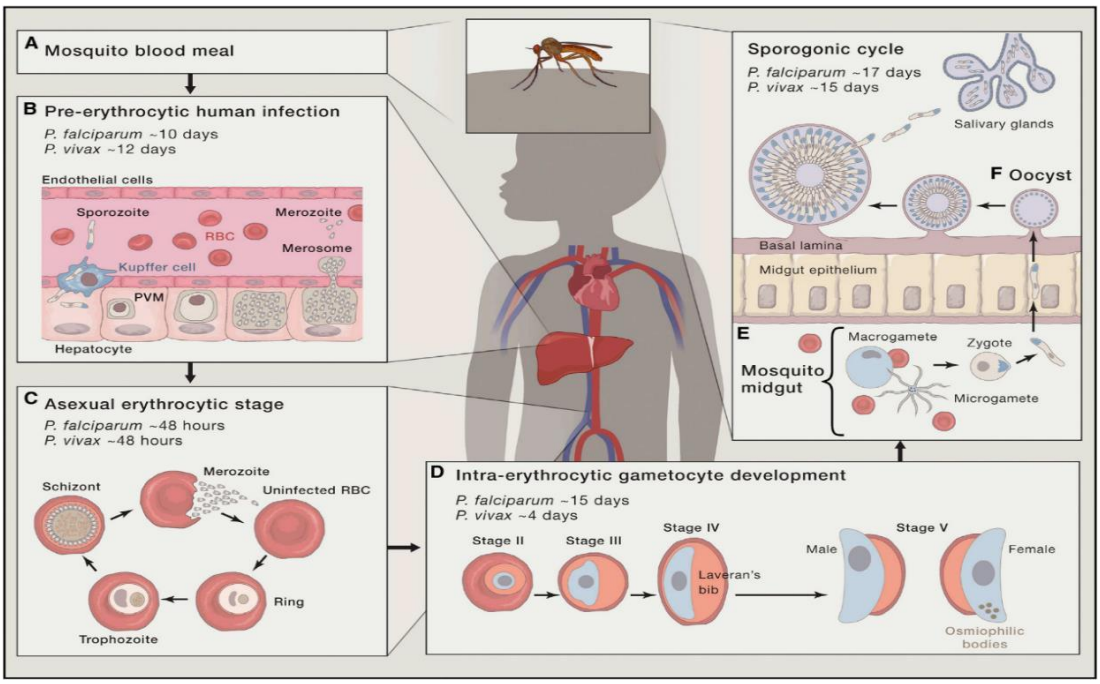


Figure 1. 1: Life cycle of *Plasmodium* parasites as adapted from Cowman *et al.***(**52**).**

Description of the figure: (A) Malaria infection is initiated with the injection of sporozoites (spzs) into the skin by blood feeding female *anopheline mosquito*. (B) The spzs enter the vasculature and are transported to the liver, where they exit the sinusoids through Kupffer or endothelial cells and enter a hepatocyte. Active invasion is preceded by cellular traversal until a suitable hepatocyte is found. They form a parassitoporous vacuole (PVM) and undergo schizogony until tens of thousands of daughters merozoites are released in packets of merosomes into the vasculature. (C) There they encounter RBCs and begin a chronic cycle of asexual schizogony in the bloodstream. (D) A proportion of asexually reproducing merozoites are reprogrammed to undergo gametocytogenesis. (E) Within a 15-day period, gametocytes sequester and develop within the bone marrow and, once mature, enter the peripheral circulation for ingestion by a mosquito where they emerge as extracellular male and female gametes in the midgut. (F) Mating occurs by fusion of micro- and macrogamete to form a zygote transforming over 24 hr. into a ookinete that migrates through the mosquito midgut epithelium and encysts to become an oocyst where asexual sporogonic replication occurs. Motile sporozoites are released into the hemocoel by oocyst rupture and pass into salivary glands where they can be injected into the next human host.

1.2.2 Pathogenesis and clinical presentations of malaria

The pathogenesis of malaria involves a complex series of events triggered by parasite infection and ultimately culminating in disease development (53). This complicated process clearly illustrates host-parasite interactions. Among *Plasmodium* species, the pathogenesis of *P. falciparum* is the most extensively studied due to its propensity to cause severe clinical disease. However, other species such as *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* can also cause life-threatening conditions (54).

During the liver stage, also known as intrahepatic or preerythrocytic schizogony, a single sporozoite can produce 10,000 to over 30,000 daughter merozoites (55). After this amplification, infected liver cells rupture and release thousands of merozoites into the bloodstream. These merozoites rapidly invade erythrocytes and initiate the blood stage of development. In contrast to *P. vivax* and *P. ovale*, which primarily target reticulocytes, *P. falciparum* exclusively infect all types of erythrocytes, regardless of their age. Once inside an erythrocyte, the merozoites go through a series of developmental stages, leading from ring forms to trophozoites and finally to mature schizonts. The duration of this maturation process varies depending on the parasite species and ranges from 24 hours for *P. knowlesi*, 48 hours for *P. vivax*, *P. ovale* and *P. falciparum* to 72 hours for *P. malariae* (35, 56).

Erythrocyte lysis releases not only the next generation of infectious merozoites but also various waste products, including fragments of erythrocyte membranes, hemozoin pigments, and toxic factors such as glycosylphosphatidylinositol (GPI). What is particularly noteworthy is that GPI stimulates immune cells such as macrophages and endothelial cells to trigger an inflammatory response. This response involves the secretion of cytokines, inflammatory mediators (e.g.,

tumor necrosis factor, interferon-gamma, interleukins), and free radicals (e.g., superoxide, nitric oxide). Several studies suggest that the GPI tail (MSP-1, MSP-2, and MSP-4), a common feature of several merozoite surface proteins, plays a critical role in the production of parasitic toxins (57, 58). Consequently, the systemic manifestations of malaria—including headache, fever, chills, nausea, vomiting, diarrhea, anorexia, fatigue, joint and muscle pain, and neurological symptoms—are attributed to this cytokine storm triggered by exposure to parasite products and erythrocyte debris (59) .

Similarly, intracellular parasites alter the erythrocyte membrane by obtaining energy from anaerobic glycolysis of glucose to lactic acid. This metabolic shift leads to clinical manifestations of hypoglycemia and lactic acidosis (60)]. As a result, the altered deformability of the erythrocyte membrane contributes to hemolysis and accelerated spleen clearance, which in turn worsens anemia. In addition, evidence shows that changes to uninfected RBCs—such as incorporation of *P. falciparum* GPI into the membrane—significantly increase the clearance of uninfected RBCs, thereby contributing to more severe anemia (61).

When newly formed daughter merozoites emerge from the schizont stage within infected erythrocytes, their emergence triggers a cascade of immune responses. The contents of the cell membrane and hemozoin crystals are immediately engulfed by circulating macrophages and serve as a critical trigger of the immune cascade. In addition, the release of free heme into the circulation acts as a potent stimulus for endothelial activation, potentially leading to endothelial cell damage in some patients (62-64). This cascade stimulates the release of proinflammatory cytokines, including tumor necrosis factor (TNF), which suppresses hematopoiesis and further exacerbates anemia. Consequently, severe hemolysis accompanied by high-density parasitemia leads to hepatosplenomegaly (65).

As the asexual cycle of merozoites progresses, they continue to invade new erythrocytes, with some of these differentiating into gametocytes. These immature gametocytes then leave the bloodstream within three to four days and develop in the bone marrow. In particular, studies have identified a specific niche in the extravascular space of the human bone marrow in which gametocytes form in erythroid progenitor cells. This location is advantageous because the bone marrow provides a nutrient-rich environment, facilitating proliferation and providing protection from the host immune response before the mature gametocytes reenter the circulation (Fig. 1.2) (43, 44).

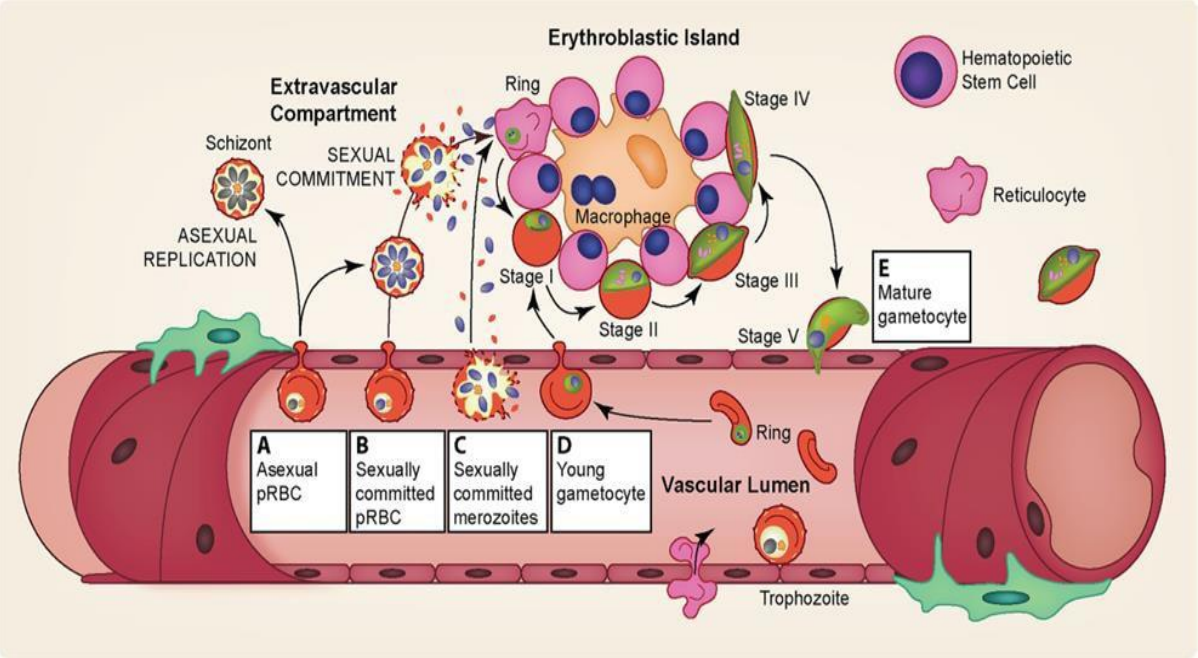


Figure 1. 2: Gametocyte sequestration and development in the bone marrow.

It shows several pathways may explain the enrichment of gametocytes in the extravascular compartment of the bone marrow and their subsequent release. Adapted from Nilson *et at.* (2015).

P. vivax exhibits a distinctive feature during the sporozoite stage, where sporozoites are categorized into ***tachysporozoites*** and ***bradysporozoites***. *Tachysporozoites* rapidly develop into exoerythrocytic schizonts and cause liver infection. In contrast, bradysporozoites transform into hypnozoites, a dormant form that can remain latent in the liver for extended periods of time, potentially causing relapses long after initial symptoms have resolved (66). However, the exact mechanism behind this dormant phase remained unknown. Conversely, *P. falciparum* and P. malariae do not include hypnozoite stages (66, 67). Instead, all of their sporozoites develop into schizonts, which subsequently release merozoites into the bloodstream, with no relapse infections with these parasites (68, 69). In addition, chronic infection with P. malariae can lead to the formation and deposition of immune complexes that can lead to kidney damage (70).

P. falciparum, the parasite responsible for the deadliest form of malaria, enhances its pathogenicity through virulence factors such as cytoadherence, sequestration, and rosette formation. Significantly, structural changes in iRBCs leading to increased stiffness and adhesion play a crucial role in the severity of falciparum malaria (58, 71, 72). As the parasite

matures in iRBCs, it forms sticky knobs on its surface, consisting primarily of parasite-produced proteins such as *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1), PfEMP-2, knob-associated histidine proteins (KAHRP) and ring-infected erythrocyte surface antigen (RESA), as well as host cell proteins such as spectrin and actin (73-75). These knobs facilitate cytoadherence, a critical process by which iRBCs adhere to capillary endothelium, driving the pathogenesis of *P. falciparum.*

In its attempt to evade immune detection, *P. falciparum* employs a sophisticated strategy in which its genome contains over 60-90 variable (var) genes. Importantly, only one of these genes is active at a time, which allows the parasite to continuously change its surface coat, the PfEMP-1 protein. This constant variation effectively protects the parasite from the host's immunity (76-78). In addition, the knobs of infected iRBCs bind to receptors of various cell types in blood vessels, including endothelial cells. This interaction is facilitated by receptors such as intercellular adhesion molecule-1 (ICAM-1), endothelial protein C receptor (EPCR), and chondroitin sulfate-A (CSA) in the placenta. As a result, this endothelial binding leads to the sequestration of iRBCs in small vessels, effectively clearing parasites from the peripheral circulation for prolonged periods (79-81).

Not only does this sequestration cause a partial blockage of blood flow, but it also damages the endothelial barrier, which in turn causes inflammation. (82). What is especially worrisome is that sequestration of iRBCs in the brain can result in cerebral malaria, a potentially fatal complication, and ultimately coma. (83). Furthermore, malaria can adversely affect the kidneys through multiple mechanisms, including the blockage of tiny vessels, immune-mediated damage, and disrupted blood flow, which ultimately results in fluid loss. (83).

Expanding upon this process, iRBs attach themselves to both endothelial cells and uninfected RBCs, resulting in the formation of **rosetting** clusters (Fig. 1.4). This rosette phenomenon is therefore results from the interaction between PfEMP-1 knobs on iRBCs and receptors such as complement receptor-1 (CR-1) on the surface of uninfected RBCs (84, 85).

Furthermore, the process of cytoadherence plays a crucial role in the sequestration of parasites in various organs such as the heart, lungs, brain, liver, kidneys, intestines, adipose tissue, subcutaneous tissue and placenta. This sequestration in deep tissues ensures an oxygen-poor environment that is particularly conducive to the maturation of the parasite. Furthermore, adhesion of iRBCs to endothelial cells facilitates evasion of spleen clearance and immune

surveillance, thereby promoting uncontrolled parasite proliferation and a significant increase in parasite numbers. As a result, only early-stage annular *P. falciparum* trophozoites are typically observed in the peripheral circulation, while more mature trophozoites and schizonts reside in the microvasculature and evade detection by routine blood smear testing. Should the process of cytoadherence, rosetting and sequestration of both iRBCs and un-iRBCs in vital organs continue uncontrolled, this can have serious consequences: obstruction of blood flow combined with reduced local oxygen levels impairs mitochondrial ATP production and triggers the release of proinflammatory cytokines. And finally, these factors contribute significantly to the progression of serious diseases (58, 86-91).

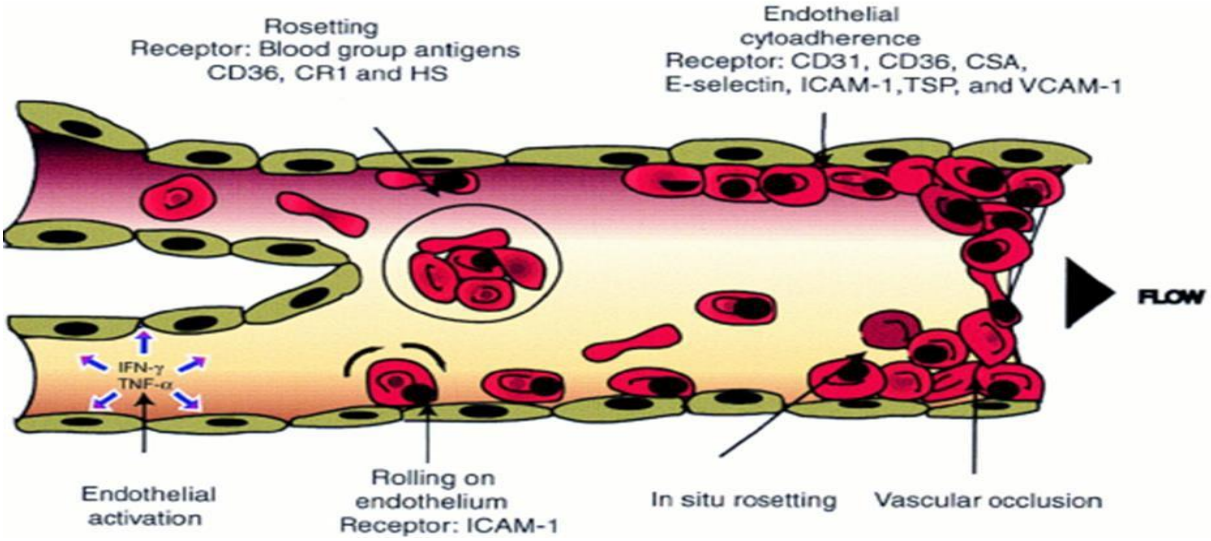


Figure 1. 3: *P. falciparum infection* triggers clumping of iRBCs in capillaries, blocking blood flow to vital organs.

When considering the crucial role of cytokines in severe malaria, it becomes clear that these molecules are central not only to the immune response, but also to the pathological process that determines the severity of the disease. Upon entering the host system, parasite antigens trigger the release of important cytokines such as IFN-γ and TNF-α. These cytokines, in turn, play an important role in modulating the endothelial surface and upregulating receptors that facilitate the binding of iRBCs. In particular, ICAM-1 is significantly involved in the initial binding of iRBCs to the endothelium, thus setting the stage for further interactions. After that, there are more robust adhesions, which are primarily brought on by CD36, which firmly attaches iRBCs to the vessel walls. This makes matters worse by enhancing the adherence of iRBCs to both iRBCs and the endothelium. This raises the risk of vascular blockage and intensifies the local inflammatory response, thereby generating a vicious cycle that exacerbates the disease (84, 92).

Moreover, procoagulant signaling pathways, upregulated selective adhesion molecules, and cytokine-induced vascular damage all play a role in potentially fatal side effects like organ failure, placental dysfunction, and cerebral malaria. The intricate relationship between pro- and anti-inflammatory responses determines how severe the disease is and emphasizes the potential of immunomodulation as a as a therapeutic strategy (57, 58, 93-95).

1.2.3 Classification of malaria

The consequences of infected with *Plasmodium* parasites vary in severity depending on the species and host-related factors, particularly the degree of host immunity, which is correlated with previous parasite exposure. Consequently, malaria manifests across three primary categories delineated by the severity of the disease: asymptomatic, uncomplicated, and complicated (96, 97).Sever malaria

In contrast to uncomplicated malaria, which can be caused by all *Plasmodium* species, severe malaria is caused primarily by *P. falciparum* (49, 98). However, recent insights have shed light on the potential for severe manifestations in patients infected with *P. vivax*, which sometimes results in fatal outcomes. (99-101). This life-threatening phenomenon arises from an increased parasite load, which triggers dysfunction of vital organs such as the central nervous system and complications such as coma and seizures (102), pulmonary compromise manifesting like pulmonary edema and respiratory distress (103), acute renal failure(104), severe anemia (105) (hemoglobin levels <5g/dL) with parasitaemia level around 10,000/uL, and metabolic acidosis (106). The pathogenesis of severe malaria is a complex process. Anemia, a hallmark of the disease, arises from destruction of iRBCs during merozoite release, which is exacerbated by bone marrow suppression or dyserythropoiesis. It is noteworthy that a hemoglobin level of ≤ 3 g/dL is strongly correlated with an increased risk of mortality (107), highlighting the critical importance of prompt detection and intervention in the treatment of severe malaria.

The simultaneous rupture of mature schizonts during malaria infection triggers a cascade of physiological responses, including a sharp increase in body temperature that marks the onset of the characteristic malaria attack. This event is accompanied by pronounced symptoms such as severe chills, chills and elevated fever (sometimes over 40 °C). The synchronized release of merozoites and pyrogenic substances from lysed RBCs is thought to be the underlying mechanism that triggers these seizures. In uncomplicated *falciparum* malaria, the mortality rate

is less than 0.1% with timely and appropriate treatment. However, the prognosis changes significantly once vital organ dysfunction occurs or the proportion of iRBCs exceeds 2%, which corresponds to a total parasite load (biomass) of more than 10^{12} parasites in an adult host. In such cases, the risk of death increases significantly, with the outcome highly dependent on the host's immune response (108).

1.2.3.1 Uncomplicated malaria

Unlike asymptomatic malaria, uncomplicated malaria is characterized by periodic bouts of nonspecific symptoms such as fever, moderate to severe chills, profuse sweating, headache, nausea, vomiting, diarrhea, and anemia, in the absence of clinical or laboratory evidence of severe organ dysfunction(98). This spectrum illustrates the dynamic interaction between parasite and host, in which a partially effective immune response controls parasite replication and thus prevents severe disease (84).

1.2.3.2 Asymptomatic Malaria

Asymptomatic malaria (which we have attempted to explain in detail in the subsequent sections), also known as "the silent threat to malaria control and elimination," refers to people who are infected with malaria but do not exhibit any obvious symptoms of disease, such as fever or chills, despite having the parasite in their blood. Nonetheless, some asymptomatic infections have the potential to develop into symptomatic cases (109-112).

1.3 The epidemiology of malaria

Malaria remains a significant threat to public health, with millions of cases reported globally each year. The entomological inoculation rate (EIR) measures the number of infectious female Anopheles mosquito bites per person per year. An EIR of <10/year indicates low transmission, 10 to 49/year indicates intermediate transmission, and >50/year indicates high transmission areas, regardless of seasonal or geographic variations. Malaria is typically more prevalent in areas with higher EIR values (113). In this context, disease transmission therefore depends on three key factors related to female Anopheles mosquitoes: density, biting habits and longevity. The more abundant these mosquitoes are, the greater the risk of malaria transmission (114).

1.3.1 The global burden of Malaria

Globally, malaria transmission persists across much of the tropics, affecting 85 countries, thereby remaining a significant global public health challenge. Despite efforts, progress in reducing malaria cases and deaths has been slower than anticipated (49, 115). According to the latest World health Organization (WHO) world malaria report report (49), there were 249 million malaria cases in 2022, marking a concerning increase of 5 million cases compared to 2021. Notably, this rise continues a worrying trend, with a 5 million increase from 2021 and 18 million from the baseline year of the GTS for malaria 2016-2030, established in 2015, which reported 231 million malaria cases (116). In 2022, Pakistan (+2.1 million, +48%), Ethiopia (+1.3 million, +20%), Nigeria (+1.3 million, +10%), Uganda (+597,000, +12%), and Papua New Guinea (+423,000, +17%) were identified as the leading contributors to the increase in global malaria case. Additionally, there has been a notable decline in malaria incidence worldwide, falling from 81.0 per 1000 population at risk in 2000 to 56.8 in 2019. Despite a small increase of 3% in 2020, possibly due to disruptions caused by the COVID-19 pandemic, there has been a notable decline in malaria incidence rates. Rates have largely stabilized over the past three years and are in the year increased slightly to 58.4 per 1000 in 2022. Between 2000 and 2019, malaria mortality declined steadily from 864,000 to 576,000. However, in 2020, there was a notable increase from 55,000 malaria deaths to an estimated 631,000, due in part to disruptions in access to malaria prevention and case management due to the COVID-19 pandemic. There was then a slight decline to 610,000 and 608,000 deaths in 2021 and 2022, respectively. The malaria mortality rate halved between 2000 and 2019, from 28.8 per 100,000 at-risk population to 14.1. In 2020, the mortality rate rose to 15.2 per 100,000 residents at risk, before falling slightly to 14.5 in 2021 and 14.3 in 2022 (49).

1.3.2 Geographical distribution of malaria

The 2023 world malaria report sheds light on the persistent challenge of malaria on a global scale, revealing stark regional differences in burden and impact. Across various regions, the picture painted is one of inequality and urgency in combating this disease. In the Americas, where *P. vivax* is the predominate parasite, the burden of malaria appears relatively light, with documented cases representing a mere 0.22% of global figures and a comparatively low mortality rate of 343 deaths. However, as we move towards SEA, the situation becomes more concerning. Here, while malaria cases constitute 2% globally, the proportion of deaths rises to 1.3%, indicating a higher severity of the disease. The Eastern Mediterranean region, though

not the most severely affected, still faces significant challenges in malaria control. It contributes 3.33% of global cases and 2.6% of deaths, emphasizing the ongoing struggle in this area. However, the most alarming statistics come from the WHO African Region, which stands as the epicenter of the global malaria crisis. Here, an overwhelming 94% of reported cases and a heartbreaking 95.4% of global malaria deaths reported. This glaring disproportion underscores the urgent need for intensified control efforts and continued research and development of innovative prevention and treatment strategies, particularly focused on Africa, where the majority of the burden is due to *P. falciparum* (49).

1.3.3 The burden of asymptomatic malaria and diagnostic challenges

Understanding the importance of asymptomatic malaria carriers is vital for devising effective malaria control strategies, particularly in regions with low transmission rates (117). Although symptomatic cases and malaria-related deaths draw considerable attention, they account for only a small portion of the total infected population, much like the visible tip of the iceberg. This analogy illustrates that symptomatic malaria cases alone do not capture the full extent of the malaria burden (118). Furthermore, asymptomatic malaria, who often remain undiagnosed and untreated due to their lack of symptoms, form a significant and frequently overlooked reservoir that perpetuates the transmission of *Plasmodium* parasites to mosquitoes. In addition, recent advancements in diagnostic techniques have unveiled an expectedly widespread prevalence of both microscopic and submicroscopic asymptomatic malaria infections, offering critical insights into their role in sustaining transmission. Notably, Churcher *et al.* (2015) provided strong evidence that both asymptomatic and submicroscopic malaria infections significantly contribute to the persistence of malaria (119).

A groundbreaking longitudinal study by Andolina *et al.* (2021), vividly paints this phenomenon. They followed 531 individuals from 80 households between October 2017 and October 2019. The study found that at baseline, *P. falciparum was* detected in 5.3% of the participants by microscopy and 12.1% by quantitative Polymerase Chain Reaction (qPCR), with prevalence declining overtime. In 538 mosquito feeding experiments involving 107 individuals, correlates with gametocyte densities. The study revealed that the human infectious reservoirs was predominantly composed of microscopic asymptomatic infections (83.8%), with school-aged children (5-15 years of age) contributed the most to infections constituted to transmission (58.7%). Notably, four children were responsible for 62.2% of infected

mosquitoes (120), underscoring the disproportionate impact that certain individuals have on transmission dynamics.

However, in contrast to the earlier evidence that highlighted the role of cytoadherence in evading the immune detection (121), Andrade *et al.* (2021) proposed a different mechanism during the dry season. Rather than relying on sequestration, which is typically associated with acute malaria, the parasites during the dry season were found to circulate longer within the blood without adhering to the vascular endothelium. This low-density circulating *P. falciparum was* persisting in asymptomatic individuals without threatening host survival and with minimal impact on the immune response. These parasites were found to be transcriptionally different from those during the transmission season and circulate longer in the blood. This lack of cytoadherence increased splenic clearance, which keeps parasite at low density, below both clinical and immunological detection. Andrade and colleagues proposed that this regulation of virulence, characterized by decreased endothelial binding capacity, enables *P. falciparum* to persist asymptomatically for several months, thereby bridging the gap between transmission seasons in area of seasonal malaria transmission (122).

We get a completely different impression from recent scientific findings that have uncovered a hidden malaria life cycle in the spleen. These discoveries significantly expand our understanding of the biology of the parasites and open up new opportunities for the development of targeted treatment and diagnostic tools. In particular, persistent infection of the spleen has significant clinical and public health implications. In particular, these recent findings have revealed a previously unknown fourth life cycle of *Plasmodium* in the human spleen (123, 124). These findings contradict the conventional wisdom that malaria parasites mainly spread through the bloodstream. For many years, scientists believed that the bloodstream served as the primary location for parasite replication. Studies on spleens from people undergoing spleen removal following car accidents in Papua, Indonesia, however, paint a different picture. Despite not having any symptoms of malaria before the incident, 95% of these patients had a high biomass of live parasites in their spleens, which redefined the malaria life cycle.

Furthermore, the hidden reservoir suggests that chronic malaria may occur predominantly in the spleen. Interestingly, the two main *Plasmodium* species, *P. falciparum* and *P. vivax*, have been found to accumulate in the spleen. However, *P. vivax* showed a remarkable preference for this "safe haven," with over 98% of the parasite population living there, compared to 93% for

P. falciparum. This preference may be attributed to the role of the spleen in filtering reticulocytes, which are particularly susceptible to *P. vivax* infection. Consequently, the discovery revises our understanding of the spleen's role in malaria, moving from viewing it as a parasite adversary to a surprisingly suitable environment for long-term survival and proliferation.

Furthermore, Toti *et al*. (2020) introduced a new concept called "***spleen pitting***" in their review published in Cell Press (125). In this process, the spleen repairs iRBCs by mechanically removing dead ring-stage parasites, allowing the once iRBCs to reenter the circulation. Persistent splenic infection has serious implications for clinical practice, management, public health strategies, and malaria control efforts. Moreover, Joice *et al.* (2014) showed that gametocytes [of *P. falciparum*] are concentrated not only in the bloodstream but also in other tissues such as bone marrow, posing a potential challenge for interrupting transmission (126). Building on this, Nelson *et al*. (2015) further elucidated the extravascular life cycle of malaria parasites in other tissues such as in bone marrow, shedding light on their to evade the immune responses and thrive in nutrient-rich environments (44). Therefore, in malaria-endemic regions, repeated infections lead to partial immunity, which establishes asymptomatic reservoirs of the parasites. These symptomatic carriers are reportedly more infectious than symptomatic patients (110, 127). In support of this, a striking report from Uganda shows how asymptomatic carriers affect malaria transmission. That is, schoolchildren with asymptomatic malaria reservoirs were found to account for 50.4% of malaria transmission to mosquitos (128). Yet, Okell *et al*. (2012) also unveiled that asymptomatic malaria carriers could contribute potentially to accounting for 20–50% of human-to-mosquito transmissions (129). Furthermore, an experimental study conducted in a low malaria transmission setting in Ethiopia found that symptomatic, microscopically detected asymptomatic and submicroscopic malaria carries contributed 8.0%, 76.2% and 15.8%, respectively, to human-to-mosquito transmission (130).

Interestingly, comprehensive systematic review and meta-analysis by Whittaker *and* colleagues shed light on the global burden of asymptomatic submicroscopic *P. falciparum malaria*. Their findings reveal that in in regions with lower malaria transmission, the proportion of such infections is significantly higher, ranging between 60 to 70%. This contrasts sharply with areas of high transmission, where the proportion of submicroscopic malaria infections is significantly lower, between 10% and 20%. Nevertheless, age related burdens across both transmission settings, a compelling trend emerges: submicroscopic malaria infections are more

prevalent in children than compared to adults. In high-transmission areas, where Polymerase Chain Reaction (PCR) prevalence is around 70%, the prevalence of such infections in young children is projected to be 1.42 times higher than adults. Conversely, in low-endemic areas with 10% overall PCR prevalence, the ratio of prevalence in children compared to adults rise even more sharply, reaching 1.92 times (131).

Therefore, diagnostic challenges with low-density infections are one of the roadblocks to the move to malaria elimination efforts. In healthcare systems, conventional malaria diagnostic tools (microscopy and routine malaria rapid diagnostic tests, RDTs) are typically employed to screen symptomatic and self-presented patients. However, these tools are inherently limited in their ability to detect low-density parasitemias, which contributes to the continues spread of the disease, as discussed earlier. For instance, the limit of detestation (LoD) for microscopy has been suggested to be up 100 parasites/µL by an expert microscopist, resulting in the average miss of 20–50% of cases, and usually misses, in average 20 - 50 % of the cases (129). Similarly, routine malaria RDTs have a detection limit of 100 – 200 parasites/µL, meaning parasite densities below these thresholds can be easily missed (132, 133).

A handful of studies have showed the significant presence of asymptomatic malaria infections within specific demographics. Yimam *et al.* (2021) conducted a comprehensive meta-analysis on *pregnant* women in sub-Saharan Africa, revealing a concerning prevalence of (26.1%) for all malaria infections, with *P. falciparum* being the dominant species at 22.1% (134), highlights a substantial hidden reservoir of malaria parasites capable of onward transmission. This underscores the influence of local transmission intensity on asymptomatic carriage (135). Moving to West Africa, with its characteristically low transmission seasons, a community-based study documented a 12.7% prevalence (136). Interestingly, the study also associated asymptomatic malaria with age where it was higher in school-age children (5–14-year-olds) and in dry seasons. *P. falciparum remained* the dominant parasite (99.2%).

In Ethiopia, few studies have explored the burden of asymptomatic malaria in different populations, highlighting variations across demographics. A systematic review by Tegegne *et al.* (2021) analyzing seven studies found a pooled prevalence of asymptomatic malaria in children of 6.67% (137). At the same period, Kendie *et al.* (2021) also investigated eight published studies on malaria infections among adults in Ethiopia. The authors showed that an overall prevalence of 13.61%, of which significant proportions were asymptomatic carriers, 11.99% (138).

A wealth of published evidence on asymptomatic malaria further emphasized these diagnostic shortcomings. Studies revealed that these conventional methods fall short in capturing the true extent of this hidden reservoirs (139, 140). For example, Fransica *et al.* (2015) found that over 70% of asymptomatic carries evaded detection by these standard tests (141). Bousema *et al.* (2014) also highlighted the importance of molecular methods over these conventional tools , particularly for detecting asymptomatic infections linked to submicroscopic densities (140). These limitations, coupled the prevalence of asymptomatic malaria —particularly in schoolchildren—pose significant challenges to the elimination efforts. As Whittaker *et al.* (2021) highlighted, these infections are not only difficult to detect but also have far-reaching implications. In particular asymptomatic infections in schoolchildren hinder elimination efforts and are associated with long-term health consequences, such as school absenteeism and cognitive impairments (142).

Diagnostic challenges in malaria extend beyond the LoD of conventional diagnostic tools. Biological threat due to parasite factors such as *P. falciparum* parasites associated with histidine-rich protein-2/3 (HRP2/3) gene deletions, which result in false-negative result (49). A recent report from Ethiopia revealed that widely used HRP2/3-based RDTs failed to detect approximately 9.7% of cases due to these deletions in the parasites (143). Additionally, Fola *et al.* (2023) reported that about 8.2% of parasites from Ethiopia carried double both artemisinin and diagnostic-resistance genes (pfhrp2/3 deletions) (144). Similar findings have been reported in Peru (145)**,** Ethiopia (146), Eritrea (147, 148), Kenya (149), Zambia (150), Uganda (151), and even in Germany (152) from a patient returning from Ethiopia.

1.3.4 The global malaria eradication efforts

Throughout the annals of history, numerous efforts have been made to eradicate or control malaria. A pivotal moment in this ongoing battle occurred in 1955 with the launch of the first Global Malaria Eradication Program (GMEP). This initiative was fueled by the discovery of two game-changing chemicals in 1930s: dichlorodiphenyltrichloroethane (DDT), a potent insecticide discovered in 1939, and chloroquine, an effective antimalarial drug unearthed in 1934 (153). The GMEP embodies the triumphs and tribulations of public health and is a testament to both the successes and challenges of disease eradication (154).

On one hand, the program was a resounding success, achieving the monumental achievement of eradicating malaria from 35 countries. However, its ultimate failure was attributed to several

technical, operational and financial challenges slowed and ultimately stalled progress. On the other hand, the GMEP also faced its share of setbacks. Despite the initial progress, the program ultimately did not achieve its global eradication goal. This was largely due to a series of technical, operational, and financial challenges that emerged along the way. These obstacles not only slowed the momentum but eventually brought the progress to a standstill. These challenges underscore the complexity of global health initiatives and serve as a reminder that success in such endeavors requires not just initial momentum, but also sustainable strategies and resource. These included technical challenges, the emergence of insecticide-resistant and drug-resistant of both the parasite and its vectors, a lack of sustained political and financial support, and an inadequate understanding of the parasite and vector biology, ecology, and climatology specific to a country.

Additionally, variations in the distribution and incidence of parasite and vector species across different countries also contributed to the program's downfall. This complex interplay of factors underscores the multifaceted challenges faced by such a global health initiative (154). Because of these factors, eradicating malaria across large areas therefore, sparked debate and uncertainties surrounding the campaign's long-term impact (154, 155).

Next, proponents full of optimism saw a chance to finally completely eradicate malaria based on local eradication in places like ***Sardinia***, Italy, reinforcing their belief that global eradication was possible, they believed a "one-size-fits-all" approach would work. Additionally, eradication promised significant long-term cost savings compared to ongoing control programs. Finally, concerns about the sustainability of existing control methods like DDT spraying, which drained resources and faced growing resistance, made eradication seem like a more permanent solution (154-156).

On the other hand, critics voiced significant concerns. They argued that the massive difficulty and cost of achieving global eradication were being underestimated. Malaria's complex life cycle and the program's ambitious scale raised doubts about securing and maintaining the necessary resources. Additionally, the rapid development of insecticide resistance threatened to render the program ineffective in the long run. Critics advocated for a more cautious approach that considered these challenges (154-156).

The truth is that the challenges of eradicating malaria and the potential uncalculated consequences of a global campaign pose high risks, and critics had foreseen the various

problems the campaign would ultimately suffer from. When the GMEP was launched, no clear implementation strategy had been developed and no roadmap had been outlined. Africa, the continent with the highest disease burden, was excluded from the program at the beginning. Additionally, an excessive dependence was observed on a solitary intervention, specifically the application of DDT, despite uncertainties regarding its ability to accomplish campaign objectives across all environments, especially considering the potential threat of insecticide resistance. Even after an eradication strategy had been developed, potential obstacles were downplayed, giving the impression that eradication could be accomplished within a limited time-frame and budget (154-156).

The mid-20th century therefore witnessed a dramatic change in the worldwide approaches to controlling malaria. As evidenced by numerous reports from 1955 to the early 1960s (154, 157), chloroquine and insecticide resistance emerged, shattering hopes of eradication. This worrying trend forced a costly global switch from eradication to control (155, 156, 158, 159).

Over the past century, malaria was a widespread threat, casting a long shadow over much of the world (Fig. 1. 4A). However, relentless scientific progress has led to remarkable global efforts to control malaria cases. This inspiring story of triumph is beautifully illustrated by two starkly contrasting maps (Fig. 1.4). However, relentless scientific progress has led to remarkable global efforts to control malaria cases. This inspiring story of triumph is beautifully illustrated by two starkly contrasting maps (Fig.1.4). The map on the left (Fig. 1. 4A) shows a world a century ago in which malaria transmission covered vast areas, with only Greenland and Mongolia remaining beyond its reach (160). The map on the right (Fig. 1.4 B) paints a very different picture and shows the dramatic reduction in malaria transmission areas by 2023 (49).

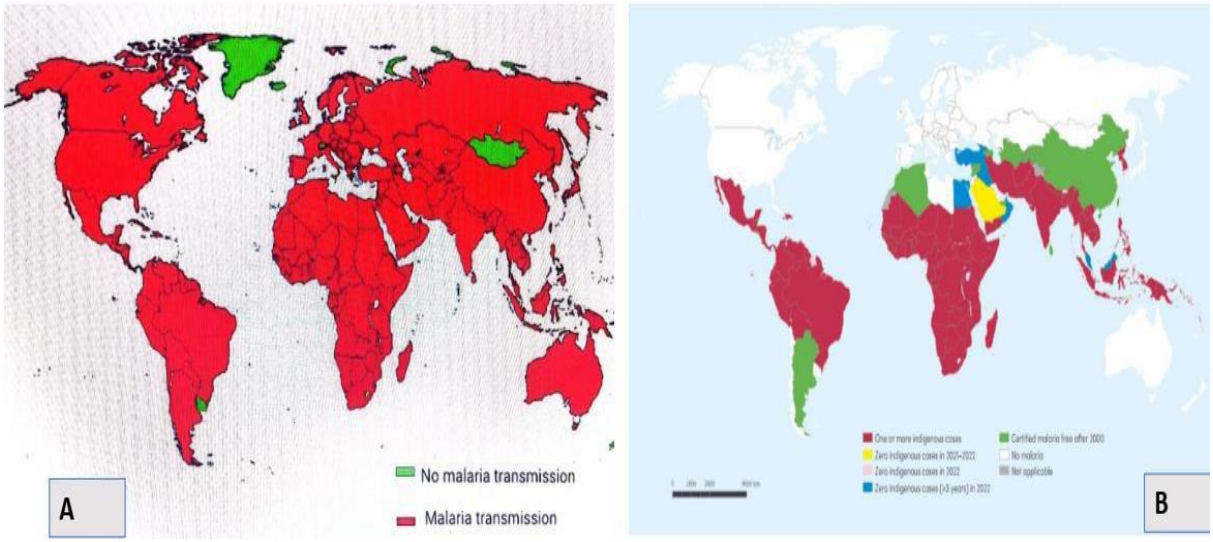


Figure 1. 4: Global malaria distribution in 1900 vs. 2023.

More recently, in its collective effort to eliminate malaria, WHO shared a bold vision: a world free of the disease. The establishment of the Global Technical Strategy (GTS) for Malaria 2016-2030 serves as a roadmap and sets ambitious but global targets for 2030. These goals include a 90 percent reduction in both malaria incidence and mortality rates compared to 2015. Furthermore, the strategy aims to eliminate malaria transmission in at least 35 countries and prevent its re-establishment in malaria-free regions. Recognizing the diverse needs of different regions, the GTS allows countries to set their own national targets that complement the global strategy (161) as country-led and country-driven malaria elimination programmes (20).

Briefly, to achieve these goals, the GTS adopted three pillars (161): First, to ensure equitable access to malaria prevention, diagnosis, and treatment as part of universal health coverage (UHC), which means that all individuals and communities receive the health services they need without suffering financial hardship (161, 162).This can significantly reduce morbidity and mortality, as evidenced by the WHO-recommended package of interventions such as vector control, chemoprevention, diagnostic testing, and treatment.

Second, accelerating efforts towards elimination and achieving malaria-free status. Accordingly, countries are intensifying efforts to interrupt transmission of new infections in defined geographical areas, particularly in settings with low transmission. To achieve this objective, alongside continued efforts in prevention, diagnosis, and treatment as part of UHC, targeted interventions against both parasites and vectors in transmission foci are crucial. Active case detection and case investigations should guide these efforts as part of a robust malaria surveillance and response programme.

Third, to transform malaria surveillance into a key intervention. Effective malaria surveillance is central to program planning, implementation, and accelerating progress. Therefore, countries with endemic malaria and those at risk of re-establishment need a robust health management information system. This system should assist national malaria programs in several ways: directing resources to the most affected populations, identifying gaps in program coverage, detecting outbreaks, and assessing the impact of interventions. Ultimately, this will lead national strategic planning and delivery of malaria control effort (161). In addition to the above strategic framework, the GTS emphasized the need to harness innovation and research and strengthen the enabling environment.

1.3.5 Malaria elimination efforts in Africa

More than half a century after the launch of the GMEP in the 1950s, a new optimism emerged at the turn of the millennium. Given the success stories of countries defeating malaria, a wave of enthusiasm for total elimination broke out around the world (163). In 2015, the WHO set an ambitious goal: a 90% reduction in malaria cases and deaths, along with complete elimination from 35 countries by 2030 (159). African nations, embracing this challenge, ramped up existing control efforts. Their dedication has paid off. Success stories like Cabo Verde achieving zero malaria cases in 2024 and Algeria becoming WHO-certified malaria-free in 2019 illuminate a brighter path forward. Inspired by this progress, several African countries, including Ethiopia, are setting their sights on eliminating malaria by 2030 (164, 165).

1.3.6 The global malaria prevention and control strategies

Fighting malaria requires a multifaceted approach that combines established methods with innovative solutions, supported by global collaboration between governments, NGOs and international organizations. Continued investment in research, health infrastructure and community engagement is critical, as highlighted in WHO's comprehensive strategies for malaria prevention and control, set out in the following sections (166).

1.3.6.1 Vector Control methods

Insecticide-Treated Nets (ITNs) and Indoor Residual Spraying (IRS): The two most important methods for malaria vector control methods are indoor residual spraying (IRS) and insecticide-treated nets (ITNs). Whereas IRS applies insecticides to interior walls to eradicate mosquitoes,

ITNs kill or repel mosquitoes to form a protective barrier. When combined, these methods have been successfully lowering the risk of malaria transmission (166).

1.3.6.2 Malaria diagnostic methods

Malaria diagnosis involves identifying which pathogens are causing an illness, such as a fever. This involves the used both clinical diagnosis and diagnostic tests (167). Relying on clinical signs and symptoms alone to diagnose malaria is unreliable due to its nonspecific nature, leading to overdiagnosis of malaria and inappropriate treatment of non-malarial diseases with antimalarial drugs (168, 169). As a result, ensuring accurate and prompt diagnosis is critical in managing timely treatments and reducing risks of malaria-associated morbidity and mortality (170). Besides that, high diagnostic specificity will reduce the unnecessary use of antimalarial medications and enhance the diagnosis of other febrile illnesses in all malaria transmission settings (171).

Diagnostic tests are therefore parasitological diagnoses in which malaria parasites are directly detected or Plasmodium-specific antigens or genes are detected in the blood of an infected person (170-172). Microscopy, malaria RDTs, molecular tools such as loop-mediated isothermal amplification (LAMP), and polymerase chain reactions (PCRs) are used to definitively identify *Plasmodium* species. As a result, the choice of a diagnostic method is determined by factors such as existing infrastructure, professional expertise, patient influx, and the prevalence of specific *Plasmodium* species in the region (173). In malaria-endemic areas, malaria is suspected when individuals have a fever or a body temperature $\geq 37.5°C$ and with no obvious alternative causes. In addition, in regions with stable malaria transmission or during the peak transmission season, malaria is also suspected in children with a hemoglobin level below 8 g/dL (171).

1.3.6.2.1 Light Microscopy

Microscopy is the cornerstone of malaria diagnosis and has been considered the gold standard for over a century (174). This technique detects malaria parasites directly in thick and thin blood smears prepared from blood samples. It plays a crucial role not only in detecting parasites, but also in identifying the specific *Plasmodium* species and their developmental stages in RBCs Additionally, it allows quantification of parasites by manually observing the percentage of iRBCs, allowing healthcare providers to tailor treatment strategies(175).

The definitive diagnosis of malaria depends on the identification of the developmental stages of the parasite in stained blood smears(176). Among the available staining techniques, Giemsa staining with a specific pH (7.2) is preferred for optimal parasite visualization(177, 178). For a thorough examination, two kinds of blood smears are essential: thick and thin(179):

Thick smears: Thick smears, containing a significantly larger blood volume (around 35-40 µL, about 20-30 times more than thin smears), offer a 20-40 times greater chance of detecting parasites, making them crucial for initial diagnosis (180). In contrast to thin smears, thick smears are characterized by uneven thickness and require thorough drying before staining, without any fixation step being provided. During the staining process, the RBCs lyse, allowing for the detection of parasites that are now located outside the cells. The key advantage of thick smears lies in their ability to concentrate parasites, an increased layering of multiple erythrocytes. This significantly enhances the sensitivity of the diagnostic test. Here, parasite density can be estimated by counting both parasites and white blood cells (WBCs), followed by calculating the number of parasites per unit volume based on the total leukocyte count (179).

Following the identification of *Plasmodium* species' asexual stages, parasite density (defined by WHO as "number of asexual parasites per unit volume of blood or per number of RBCs (167) is a useful indicator of infection severity and treatment response. Unless the protocol requires otherwise, gametocytes are not counted, but their presence is always reported along with all identified parasite species. Most importantly, thick blood films are the recommended method for parasite count due to reasons indicated above. In addition, thin film counts are used to identify and report *Plasmodium* species, as well as to study RBC abnormalities (176).

Interpreting blood smears, especially thick films, requires experience due to the presence of artifacts that can resemble parasites. Examination of a minimum of 100-200 fields within the smear is mandatory before a thick smear can be ruled negative for malaria parasites.

It's important to note that in areas with high malaria transmission, individuals may harbor parasite densities as high as 10,000 parasites/µL of blood without experiencing any symptoms. Consequently, in such locales, the identification of low-density parasitemia (defined by the WHO as "presence of asexual forms of *Plasmodium* parasites in the blood at parasite density below 100 parasites/µL microscopy (167)" may exhibits sensitivity but lacks specificity in pinpointing malaria as the underlying cause of illness. Given the prevalent occurrence of

asymptomatic parasitemia, low-density parasitemia frequently emerges as an incidental finding in other fever-inducing conditions (176).

Accordingly, the following formula is applied for the estimation of parasite density from thick blood films (181).

$$Number\ of\ parasites/uL = \frac{No.of\ \ parasites\ counted\ x\ 8000\ WBCs/\mu L}{No.of\ WBCs\ counted}$$

The peripheral blood smear offers a wealth of information about the infection, including parasite species, stage, and density. However, the effectiveness of this test depends on several factors: equipment and reagent quality, smear preparation, technician skill, parasite density in the bloodstream, and analysis time (182). Notably, parasite density influences disease severity and prognosis. In falciparum malaria, a density exceeding 5% indicates severe and the presence of late-stage asexual parasite in RBCs suggests a severe course and poor prognosis (175).
Thin smears: These smears undergo a thorough preparation process involving air-drying, followed by fixation with pure methanol and then staining. Examining the smear under high magnification (1000x) with oil immersion is essential, focusing particularly on RBCs at the smear's edge (176).

When a thick blood film is unavailable or damaged, the WHO recommends quantifying parasite density on a thin film, adhering to established protocols. Following this protocol, a systematic examination of approximately 20 fields on the thin blood smear is recommended. Each field typically contains approximately 250 RBCs, resulting in an estimated total of 5,000 RBCs examined (20 fields x 250 RBCs/field). Ideally, the parasite density calculation should utilize the patient's actual red blood cell count for the most accurate results. However, if this information is unavailable, a standard assumption of 5,000,000 RBCs/µL of blood can be employed. The following formula facilitates this calculation, with the final result rounded to the nearest whole number (181).

$$Parasites/\mu L = \frac{Number\ of\ iRBCs * 5000000}{Number\ of\ RBCs\ counted}$$

The relationship between parasite density and prognosis is intricate and variable. Generally, patients with over 10^5 parasites/µL face an elevated risk of mortality. However, nonimmune individuals may experience fatal outcomes even at lower parasitemia levels, while partially

immunized persons can tolerate considerably higher parasitemia levels with only mild symptoms.

While indispensable, microscopy has limitations. It struggles to detect parasite levels below its threshold (around 50 parasites/μL), potentially leading to missed infections or unnecessary treatments (183) Additionally, it's time-consuming and laborious, lacks the ability to detect sequestered parasites like *P. falciparum* , and relies on experienced technicians for accurate interpretation (184). These limitations highlight the need for improved diagnostic tools, particularly RDTs that don't require extensive training or sophisticated equipment.

1.3.6.2.2 The malaria RDTs

While light microscopic peripheral blood smear examination has long been the "gold standard" for diagnosing malaria because it provides the most comprehensive information in a single test format, immunochromatographic tests for the detection of malaria parasite antigens have been developed in the last decade. This opens up new and exciting possibilities for malaria diagnosis (125). Malaria RDTs are immunochromatographic lateral flow tests for the detection of *Plasmodium*-specific antigens in finger-pricked or whole blood samples. Currently, these tests target various markers, including *P. falciparum* HRP2, pan-malarial *Plasmodium* aldolase (PMA) and parasite-specific lactate dehydrogenase(pLDH) *(*185*)*.

HRP2, a water-soluble protein produced in both the asexual and sexual stages of *P. falciparum* , exhibits distinct expression patterns throughout the parasite's life cycle(186). Notably, its concentration increases sharply in the early ring stage, followed by a sustained increase in the trophozoite and schizont stages. This protein is expressed on the erythrocyte membrane and is characterized by a remarkable persistence in the bloodstream, lasting at least 28 days after the start of antimalarial treatment and possibly up to 36 days after parasite clearance. These unique properties have driven the development of numerous RDTs that target HRP2 for effective detection of *falciparum* malaria (187, 188).

While HRP2 is a useful marker for falciparum malaria, additional enzymes produced by blood-stage parasites have shown broader importance in malaria diagnosis. These include *Plasmodium aldolase*, an important enzyme in the parasite's metabolic pathway that is abundantly expressed by all *Plasmodium* species and is called PMA. To take advantage of this, scientists have developed highly specific monoclonal antibodies that recognize aldolase in

various *Plasmodium* species. These antibodies integrate into advanced immunochromatographic "P.f/P.v" assays that enable simultaneous detection of PMA alongside PfHRP2, facilitating comprehensive malaria diagnosis (189).

pLDH is another important diagnostic target because it is a soluble enzyme found in both the asexual and sexual stages of living parasites and is found in and released from infected red blood cells(190). Interestingly, each of the four human malaria species has different pLDH isoforms (190), highlighting the diverse approaches to accurate malaria detection. The combination of diagnostic tools targeting HRP2, *Plasmodium* aldolase and pLDH forms a comprehensive and adaptable strategy to combat malaria. In contrast to microscopic examinations, RDTs offer the advantage of being easy to use without the need for laboratory infrastructure, electricity or specialist equipment. Minimal training is required to perform these tests, making them essential for diagnosing malaria in resource-limited settings (191, 192).

Since 2010, WHO has recommended prompt parasitological confirmation of all suspected malaria cases by microscopy or RDTs before starting treatment. This recommendation emphasizes the critical role of accurate diagnosis in ensuring appropriate treatment of malaria cases and in curbing the unnecessary use of antimalarial drugs for non-malarial diseases(193). According to the latest WHO report, manufacturers sold 3.9 billion RDTs worldwide between 2010 and 2022, with a significant share (82%) of these sales occurring in sub-Saharan Africa. Among these, the National Malaria Programs (NMPs) distributed 2.9 billion RDTs, about 90% of which were deployed in sub-Saharan Africa(49). In particular, the report highlights that around 415.5 million RDTs based on HRP2 were sold in 2022, indicating the widespread adoption of this particular type of test. As a result, Ethiopia received 7.6 million RDTs in 2022.

1.3.6.2.3 Nucleic Acid Amplification methods (NAA)

Molecular diagnostics using NAA-based m to detect the presence of malaria parasites(167). These instruments are limited to reference laboratories and are intended primarily for research and epidemiological purposes. They currently have no role in the treatment of clinical malaria(171). NAATs, especially PCR, are the gold standard for efficacy studies of antimalarial drugs, vaccines, and diagnostic evaluations due to their high sensitivity and specificity (194-196).

The LoD for PCR has been estimated to range from 0.02 to 1 parasite/µL (197). It should be noted however that Nested PCR is the most sensitive nucleic acid amplification technology with the sensitivity to detection is 0.4 parasites/µL (198, 199). *Plasmodium* parasite DNA can be amplified after extraction from small volumes (typically 50 to 200 µL) of whole blood collected on filter paper as dried blood spots (DBS). This method offers a convenient and stable way to store blood samples for later analysis (200, 201). Scientific literatures on show that PCR assays commonly target genus-specific and species-specific sequences. These targets typically include the 18S small-subunit ribosomal RNA (a highly conserved gene in *eukaryotes*), and other markers like the CSP (essential for parasite invasion of the host's liver cells), and the cytochrome-b gene (a mitochondrial gene with high variation between *Plasmodium* species) (189, 202-205).

according to the most recent evidence on the dynamics of malaria transmission, low-density malaria infections, which are detectable by PCR but fall below the detection threshold of microscopy or RDTs, may contribute to transmission (118, 206-209). As some malaria-endemic areas approach elimination, accurate detection of low-density malaria infection becomes increasingly important (210), with surveillance and screening playing larger roles in program management. However, the availability of infrastructures and training required to operate PCR limit its usefulness for these applications (210-212).

A handful of evidence also shows that LAMP represents a paradigm shift in malaria diagnosis for resource-limited settings (213-216). Unlike PCR, LAMP offers a field-deployable solution for detecting *Plasmodium* parasite DNA. This eliminates the need for expensive thermocyclers, as LAMP operates at a constant temperature. Furthermore, LAMP boasts rapid amplification, generating 10^9 to 10^{10} target copies within 15-60 minutes. The resulting turbidity change allows for visual detection by turbidimetry, bypassing complex post-amplification procedures. However, it's crucial to acknowledge that LAMP assays targeting various *Plasmodium* species and processing methods exhibit variability in sensitivity and specificity (217-222).

Overall, the WHO recommendations for selecting a malaria diagnostic test depend on the specific situation. Here is a breakdown of these recommendations. In uncomplicated malaria with parasite density assessment, both RDTs and microscopy are recommended. However, in severe malaria, microscopy is critical for a definitive diagnosis at the time of admission, as RDTs alone may be insufficient(171).

Microscopy, which provides detailed information about parasites, is the the method of choice for patient follow-up after admission, whereas RDTs are not. Furthermore, microscopy is very useful for diagnosing treatment failure. It enables the detection of persistent parasites following confirmed malaria, indicating inadequate treatment efficacy. Similarly, in cases where treatment is incomplete or the diagnosis is initially ambiguous, a combination RDT and microscopy approach is recommended for a thorough assessment. Resource constraints also influence test selection.

Conversely, in resource-limited settings like health posts with community health extension Workers (HEWs), RDTs become the primary diagnostic tool due to their simplicity and minimal technical expertise required. Finally, factors like workload and technical expertise come into play. RDTs offer a faster turnaround time and user-friendliness, making them ideal for high patient loads and settings with limited training and supervision. While microscopy provides a more in-depth analysis, it demands a longer processing time and a higher level of technical skill. At the same time, while malaria RDTs offer a rapid and convenient diagnostic tool, their utility is constrained by inherent limitations. These include the inability to quantify parasitemia, distinguish between the sexual and asexual life stages of *Plasmodium* species, and inability to effectively monitor treatment outcomes due to persistent positivity lasting up to 36 days after treatment (223).

In line with this WHO recommendations, Ethiopia's National Malaria Elimination Strategic Plan prioritizes the nationwide implementation of early diagnosis and prompt treatment in all health sectors, regardless of local malaria transmission intensity. Parasitological diagnosis is being the basis for treatment decisions, with RDTs used in health posts for rapid detection and microscopy used in health centers and hospitals for confirmation and possible species differentiation to guide targeted treatment plans. This ensures universal access to confirmatory testing and treatment, with management following established national guidelines (164). However, the updated version (August 2023) of the National Strategic Plan for Malaria Elimination suggests that areas with high levels of HRP2/3 gene deletions should be replaced with non-HRP2 based malaria RDTs to ensure effective diagnosis (224).

1.3.6.3 Antimalarial Medications

1.3.6.3.1 Treatment of uncomplicated malaria

The WHO (171, 225) recommends artemisinin-based combination therapy (ACT) as the primary treatment for uncomplicated *P. falciparum* malaria in endemic regions, with ACT

also recommended for *P. knowlesi* infections and either chloroquine or ACT for other malarias. Despite chloroquine resistance in *P. vivax* in certain regions, it remains effective in many areas, along with *P. ovale* and *P. malariae* infections. Artemisinin resistance in *P. falciparum has* emerged in SEA, leading to piperaquine and mefloquine resistance. ACT failures are noted in SEA, with significant artemisinin resistance in the Greater Mekong subregion (226) and emerging resistance in East Africa, particularly recent studies showed resistance emerged in Ethiopia (144, 227) The prevalence of counterfeit antimalarial drugs is a concern in Asia and Africa, potentially contributing to treatment failures (228).

Therefore, ACT is an effective treatment regimen that combines a number of medications, including artemether - lumefantrine, artesunate - amodiaquine, artesunate - mefloquine, dihydroartemisinin - piperaquine, and artesunate – sulfadoxine - pyrimethamine. These drug combinations effectively target and eliminate the *Plasmodium* parasites that cause the disease in children and adults. However, there are some exceptions. Because of potential drug interactions or safety concerns, pregnant women—particularly those in the first trimester—and those co-infected with HIV may require alternative treatment options (171, 225). While ACT effectively treats acute malaria infection, in some cases primaquine may be added to further reduce the spread of the disease, particularly in areas with low malaria transmission (171, 225).

Furthermore, WHO recommends short-course primaquine treatment as a radical cure (0.5 mg/kg/day for 7 days) to prevent relapse of *P. vivax* or *P. ovale* malaria in children and adults, with the exception of pregnant women, infants under 6 months, breastfeeding mothers of young infants without confirmed G6PD-normal status, and people with G6PD deficiency (225). However, when G6PD status is unknown and testing is not available, the decision to prescribe primaquine must be based on a risk-benefit analysis (171).

For the blood stage infection, in areas where chloroquine-susceptible infections exist, adults and children with uncomplicated non-falciparum malaria should be treated with either an ACT or chloroquine. In contrast, in areas with chloroquine-resistant *P. vivax* infection, ACTs containing piperaquine, mefloquine, or lumefantrine are recommended for children and adults, though artesunate + amodiaquine may also be effective in some cases. ACTs are the most effective and treatment of choice for mixed infections caused by all malaria species. In such areas, adults and children are recommended to be treated with ACT for uncomplicated non-falciparum malaria (171).

1.3.6.3.2 Treatment of severe malaria

Untreated severe malaria, particularly the cerebral one, can be almost completely fatal. However, with prompt and effective antimalarial treatment and supportive care, the survival rate rises dramatically to 80-90%. This can be significantly improved by treating adults and children with severe malaria (including infants, pregnant women in all trimesters, and lactating women) with intravenous or intramuscular artesunate for at least 24 hours or until they can tolerate oral medication. After a patient has received at least 24 hours of parenteral therapy and can tolerate oral therapy, complete treatment with 3 days of ACT (171).

1.3.6.3.3 Intermittent preventive treatment in school-aged children (IPTsc)

Intermittent preventive treatment is the administration of a full course of treatment with an antimalarial drug at regular intervals to treat and prevent malaria infections in schoolchildren who are old enough to attend school. Thus, school-age children in malaria-endemic areas with moderate to high perennial (year-round) or seasonal transmission can be given a full course of antimalarial treatment at specified intervals as chemoprevention to minimize disease burden(225). Effective drugs for IPTsc include sulfadoxine-pyrimethamine (SP) + aminoquinoline such as amodiaquine (AQ) or piperaquine, SP + AS and ACT (AS-AQ or dihydroartemisinin (DHAP)).

1.3.6.3.4 Perennial malaria chemoprevention (PMC)

PMC is the administration of a full course of an antimalarial drug at predetermined intervals, regardless of whether the child is infected with malaria. This aims to protect young children by establishing preventive antimalarial drug concentrations in the blood that clear existing infections and prevent new ones during the age of greatest risk of severe malaria in moderate to high perennial malaria transmission settings (229). Originally referred to as intermittent preventive treatment in infants (IPTi) (230), subsequent evidence highlighted its efficacy in children aged 12 to 24 months. Hence, the name change to 'PMC' reflects a broader scope of application that extends beyond infancy and aligns with the malaria transmission contexts where the intervention is applicable. In this context, SP is widely used for chemoprevention in Africa (231).

1.3.6.3.5 Seasonal malaria chemoprevention (SMC)

In certain endemic regions, malaria exhibits a highly seasonal pattern, with the majority of cases occurring during rainy seasons. SMC is tailored to protect children by clearing existing

infections and preventing new ones during the high-risk period. This is accomplished by administering antimalarial medications on a monthly basis, typically sulfadoxine-pyrimethamine plus amodiaquine (SP+AQ) throughout the rainy season. Eligibility for SMC is determined by the seasonality of malaria transmission and the age groups at risk of severe malaria (225).

1.3.6.3.6 Intermittent preventive treatment of malaria in pregnancy (IPTp)

IPTp involves administering antimalarial medication at set intervals, irrespective of the pregnant woman's malaria infection status. Malaria infection during pregnancy poses significant risks to both to the mother and her fetus and the newborn. In this regard, SP is widely used for malaria chemoprevention during pregnancy and remains effective in improving key pregnancy outcomes (231).

Overall, there is no clear guidance explicitly in the WHO malaria guidelines regarding the treatment of asymptomatic malaria.

cases. However, in countries with very low malaria transmission, cases tend to concentrate in people with shared risk factors. Hence, the WHO recommends targeted strategies where interventions are applied to this subset of the population, which is thought to include the reservoir of infection (including asymptomatic malaria cases). This aims to reduce overall transmission. People who exercise at night and outdoors, or sleep outside, are also at higher risk of developing the disease. Targeted strategies to reach these populations could include targeted drug delivery and targeted testing and treatment (232).

1.3.7 Malaria situation in Ethiopia

1.3.7.1 Malaria epidemiology

Since the first launch of the GMEP (233), Ethiopia has made significant contribution in the fight against malaria, distinguishing itself as one of the few African countries that have successfully maintained their efforts toward nationwide elimination of the disease (234). This success is due to the focus on capacity building of national malaria programs since 1993. As of 2004, access to diagnosis and treatment has improved through the deployment of large numbers of health extension workers in each district. Additionally, the introduction of effective treatment options such as ACT and RDTs was crucial. This, along with effective vector control

tools such as indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs), has led to a significant decrease in malaria cases and deaths (224).

Despite remarkable progress over the past decade, recent trends indicate a worrying shift, the country has experienced a worrying rise in malaria cases. According to recent data, malaria incidence has increased by 32%, equivalent to 60.9 cases/1,000 population at risk in 2022, totaling a staggering 1.3 million cases. While the specter of malaria-related deaths looms, the current toll stands relatively low at 180 (49).

In Ethiopia , *P. falciparum* is the dominant parasite species distributed throughout the country, accounting for 71.3% of cases, followed by *P. vivax*, accounting for 21.9% (224). Although over 27 % of Ethiopians live in areas with high malaria risk (49, 165), factors influencing transmission vary greatly across the country. Geography, climate, hydrological processes, human settlements, and even socioeconomic activities all play a role (235). Traditionally, areas above 2000 meters above sea level (masl) were considered malaria-free. However, recent studies have detected cases at higher altitudes, likely due to rising temperatures causing a shift in transmission patterns. Furthermore, Ethiopia experiences distinct seasonal variations in malaria intensity, with higher risks at lower altitudes where mosquito breeding flourishes after rains (236, 237). Antimalarial interventions are currently focused on areas below 2000 meters above sea level, as these locations have a well-documented risk of malaria transmission.

The detection of infections at higher elevations however suggests a need to adapt these interventions as ecological changes, like deforestation linked to agriculture, and unusual weather patterns may be creating new areas suitable for occasional malaria transmission (237, 238).

Therefore, the rhythm of transmission peaks with the end of the main rainy season (June to August), peaking from September to December. During this window, stagnant waters offer ideal breeding grounds for mosquito, propelling transmission rates skyward (239). A secondary, albeit minor, peak may occur from March to June, coinciding with a shorter rainy spell (224, 240). These cyclical outbreaks, both minor and major, are intricately woven into Ethiopia's seasonal narrative, influencing its epidemiological tapestry (241).

The major malaria vector in Ethiopia is *An. arabiensis* with a wide geographical distribution. Other mosquito species with limited distribution include *An. funestus complex*, *An. pharoensis*, and *An. nili (*224, 239*)*. In most parts of the country, the population of *An. arabiensis* increases

following the major rainy season. However, in some areas of southwest Ethiopia, it occurs throughout the year, sustaining year-round (perennial) malaria transmission. On the other hand, a study confirmed that *An. stephensi* is becoming widely distributed and established in eastern Ethiopia (242), and Hawaria *et al.*(2023) also recently revealing its distribution in the Sidama region (243).

1.3.7.2 Malaria control and elimination efforts in Ethiopia

Ethiopia has set a bold vision to be malaria-free by 2030 (165). The recently revised NMSP for 2024/25-2026/27 reflects this ambition, with three-pronged objectives to achieve significant progress by 2027. The first two goals focus on reducing the overall malaria burden: a 60% decrease in cases and a 50% reduction in deaths compared to the 2022/23 baseline. The third objective is to achieve zero indigenous malaria cases in 100% of the kebeles within the elimination-targeted districts that report <1 API (224).

Furthermore, it emphasizes preventing the disease's re-introduction in these successfully cleared areas. To achieve these goals, the NMSP implements a comprehensive approach that focuses on empowering community mobilization, ensuring early diagnosis and prompt treatment, strengthening mosquito control, and improving malaria surveillance systems; improving supply chains for quality antimalarial commodities; ensuring human rights and gender equality in accessing malaria services; program management; private sector and stakeholder engagement; and finally, research, monitoring, and evaluation (164, 224).

Ethiopia's diverse ecological landscapes create a wide range of malaria transmission intensity, reflected by the Annual Parasite Incidence (API) varying significantly across the country. Recognizing this heterogeneity, the recent national malaria elimination strategic plan (224) adopted a stratified approach. The plan categorizes the country into five malaria risk zones (Fig. 1.5): malaria-free (API = 0), very low (API > 0-5), low (API > 5-10), moderate (API > 10-50), and high (API > 50).

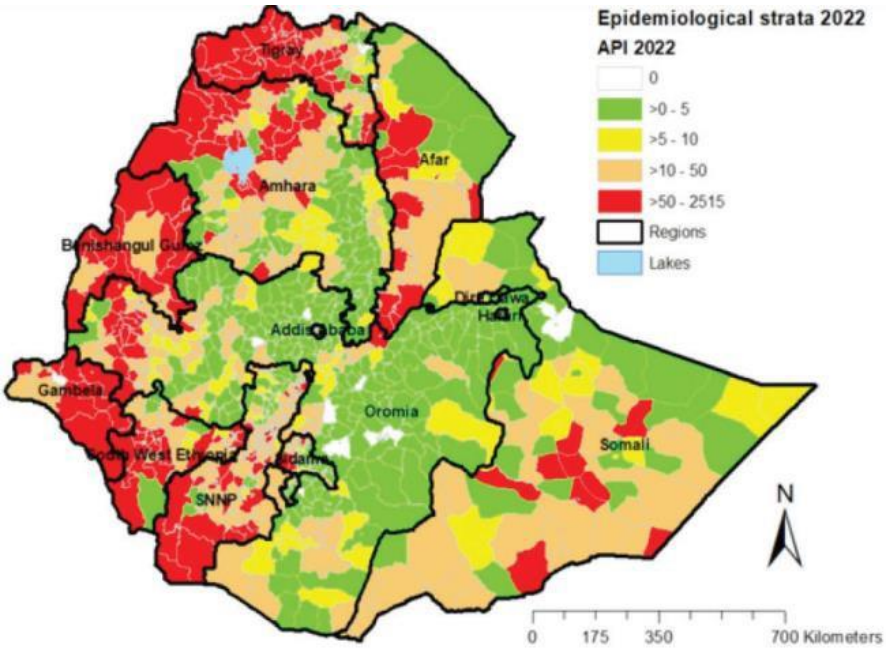


Figure 1. 5: Stratification of malaria risk Map of Ethiopia. Source NMSP (2024/25-2026/27) (224).

In its strategic framework, Ethiopia has emphasized a multi-faceted approach to malaria elimination, incorporating various interventions to combat the disease effectively. Vector control remains a cornerstone of this strategy, with a focus on new generation insecticidal treated nets (ITNs) to address pyrethroid resistance. Indoor spraying (IRS) and larval source management further target adult mosquitoes and breeding grounds, respectively.

Parasite control measures include chemoprophylaxis for high-risk individuals, RDTs or microscopy, and molecular tools for asymptomatic infections. The framework also prioritizes the intensification of malaria elimination through LLINs for all at-risk populations, IRS, larval source management, chemoprophylaxis, accurate diagnosis using RDTs, microscopy, or PCR, ACT treatment, and primaquine for both gametocytocidal and radical treatment of *P. falciparum and P. vivax,* respectively. On top of that, a robust surveillance database and operational research are set to be essential for enhancing elimination efforts, while a well-functioning supply chain ensures the availability of necessary tools. Thus, this comprehensive strategy positions Ethiopia for significant progress in malaria elimination (164, 224).

1.4 Statement of the problems

1.4.1 Asymptomatic malaria infection as the biological threats to malaria elimination

Asymptomatic malaria infections pose a significant challenge to global malaria control and elimination efforts (109, 244). For instance, *P. falciparum* in particular, individuals without symptoms can still transmit the parasite under favorable conditions, making them a hidden reservoir of infection (120). Consequently, in endemic areas, a high proportion of cases go undiagnosed and untreated due to the absence of clinical symptoms. This leads to a significant underestimation of the true prevalence of malaria in populations, resulting in less effective control strategies (244).

Traditionally, malaria control efforts have focused mainly on symptomatic infections, leaving asymptomatic malaria largely unaddressed. Although most research and national malaria control programs (NMCPs) prioritize febrile patients, asymptomatic carriers remain a critical missing piece in the puzzle. Studies, such as Okell *et al.* (2012), emphasize the importance of these hidden infections. Asymptomatic carriers can unknowingly harbor malaria parasites and continue to transmit the disease. Furthermore, estimates suggest that such individuals may account for 20-50% of infections transmitted to mosquitoes, particularly in low-transmission settings (129).

In areas with seasonal transmission, *P. falciparum* infections can persist without symptoms during the low transmission period (245). Moreover, research indicates that these infections may last over 18 months in individuals with some degree of immunity (246). The presence of this asymptomatic reservoir, as highlighted by Smith *et al.* (2007), presents a substantial challenge. Their findings show that even a small number of asymptomatic but infectious individuals can sustain transmission if competent mosquito vectors are present (247).

The overwhelming majority of asymptomatic malaria carriers remain unidentified and untreated due to factors such as healthcare-seeking behavior and the absence of acute symptoms (133). Furthermore, as indicated in the introductory section, conventional diagnostic tools are limited to detecting infections with higher parasite densities (129, 140). Fortunately, advances in diagnostic technology, including PCR, LAMP, and highly sensitive hsRDTs, offer the potential to revolutionize malaria detection by identifying low-density infections (248).

Studies have explored the diagnostic methods and their respective detection thresholds. Lucy *et al.* (2012, 2009) conducted a comprehensive meta-analysis, revealing that microscopy misses roughly more than 50% of *P. falciparum* infections in endemic regions compared to PCR (129, 206). Moreover, the probability of detecting malaria parasites is influenced by parasite density and blood volume examined (249). For example, LAMP typically examines a blood volume of 50 µL (219), whereas high-power field microscopy (1000x) examines only 0.1–0.25 µL (250, 251), In most PCR protocols, DNA is extracted from 5 to 100 µL (252-255), while RDTs use about 5 µL of blood for diagnosis (256).

Microscopy and RDTs, while being the mainstay of malaria diagnosis, have practical limits in terms of detection, typically missing infections below 10-100 parasites/µL of blood (174). Most RDTs detect 100-200 parasites/µL (257), meaning that lower parasite densities can escape diagnosis (202, 258). In contrast, more sensitive tools like PCR and LAMP can detect low-density infections, with PCR identifying as few as 0.01-0.2 parasites/µL (202, 259) nd LAMP detecting down to 1 parasite/µL (257). This disparity has major implications in low transmission settings, where microscopy and RDTs may miss low-density but transmissible infections (260).

While studies have focused on the prevalence of asymptomatic malaria, the associated factors remain less explored. Most research concentrates on specific population groups. For instance, a cross-sectional survey in Malawi found that school-age children are at higher risk of asymptomatic infections (261). In Rwanda, a study demonstrated a 22.4% prevalence among children in 12 elementary schools, identifying low altitude, older age, and previous antimalarial treatment as risk factors (262). However, in the Central African Republic, a study of asymptomatic children reported infection rates of 32.4% and 40.6%, but its reliance on RDTs might have underestimated prevalence (263).

n Ethiopia, Worku *et al.* (2014) found a 6.8% prevalence of asymptomatic malaria among school children, linking factors such as school grade, age, bed net use, and frequent malaria exposure to increased risk. However, diagnostic tool limitations were noted (264). Similarly, Minwuyelet *et al.* (2020) found a 2.8% prevalence among children aged 5-14, with factors like age group, bed net usage, and previous malaria history being significant (265). Socioeconomic factors such as gender, age, occupation, healthcare access, mosquito bite prevention, and housing conditions have also been linked to malaria prevalence (266).

A study in Kenya by Sultana *et al.* (2017) found higher malaria prevalence in rural children, with anemia, rural residence, lack of bed nets, and low household educational levels being significant risk factors (267). Similarly, Mohan et al. (2021) in India identified risk factors such as living in rural areas, low adult education levels, and working in agriculture (268). Along the China-Myanmar border, male gender, being under 15 years old, poor housing conditions, and distance from healthcare clinics were significant risk factors (269).

Hence, to achieve malaria elimination, addressing the knowledge gap on asymptomatic infections, particularly among schoolchildren (aged 6 to 15 years) is critical. This group is often overlooked despite their vulnerability and the socio-economic impact of malaria. Prioritizing targeted interventions for this population is essential. The UN's 2022 Revision of World Population Prospects highlight that school-age children represent over 27% of the population in sub-Saharan Africa, with nearly 45% of Ethiopia's population being under age 15 years (270). These children, particularly in rural settings, are more exposed to mosquito bites and less able to access preventive resources (224). As a result, they are more likely to be infectious to mosquitoes, exacerbating community transmission. Additionally, the consequences of repeated malaria infections can be particularly severe for children, leading to increased morbidity and mortality rates, as well as long-term developmental issues (26, 122, 142, 271, 272). Hence, lack of school-based interventions thus creates a significant vulnerability, despite evidence that such interventions can disrupt transmission and improve child health simultaneously (142, 273-276).

Favorable risk factors (Fig. 1.6) such as environmental conditions, proximity to water bodies, and low ITN usage, have also been linked to malaria-related childhood mortality (277) around Gilgel Gibe Field Research Center, Southwest Ethiopia. Graves *et al.* (2009) also demonstrated that factors such as family wealth, the number of LLINs per household, and the altitude above 2000 meters were protective against malaria, though peak monthly rainfall increased the risk of infection for people of all ages (278). In addition, living within 450 meters of a vector-breeding site significantly increased malaria incidence in both adults and children in peri-urban areas of the Ethiopian highlands (279). In the past decade, significant intervention measures have reduced global malaria incidence by 37%, with malaria-specific mortality declining by 60% (280, 281). Despite these gains, malaria remains a major public health challenge in Ethiopia (280, 281). Despite these gains, malaria remains a major public health challenge in Ethiopia (282).

In conclusion, malaria elimination requires a holistic approach. However, many national interventions, including Ethiopia, fail to address key populations that contribute to transmission, especially asymptomatic malaria-infected schoolchildren. This oversight is critical because schoolchildren are often silent carriers of the disease. Furthermore, schools provide a unique, cost-effective platform for malaria interventions, ensuring high participation, easy identification, and broad reach. Therefore, integrating schools into malaria control frameworks can enhance planning, monitoring, and evaluation, particularly in resource-limited settings like sub-Saharan Africa including Ethiopia (275, 283, 284). Moreover, numerous scholarly works have highlighted the critical role schools and schoolchildren play in malaria elimination efforts. In addition to being ideal sites for direct intervention, schools also serve as strategic hubs for assessing and refining malaria control measures. Consequently, by incorporating this model into national strategies, malaria programs can increase effectiveness, enhance sustainability, and accelerate progress toward elimination (25, 26, 142, 271, 272).

1.4.2 Malaria diagnostic challenges in asymptomatic malaria infections

The success of malaria elimination programs hinges on the routine use of high-quality, highly sensitive diagnostic tools(285). However, conventional tools like rapid diagnostic tests (cRDTs) are increasingly undermined by a rise in false-negative results. A primary concern is the inherent limitation of these tools in detecting low-density parasitemia, particularly in asymptomatic individuals, which allows infections to go undetected (286, 287).

In response to these challenges, innovative diagnostic solutions have come up (emerged). For example, the cRDT, SD Bioline Malaria Ag P.f (HRP2/pLDH) test improves detection capabilities by incorporating dual-antigen detection—combining HRP2 with lactate dehydrogenase (pLDH). This advancement enhances the identification of P. falciparum infections, including those with HRP2 gene deletions. However, such cRDTs are reported to fall short in detecting low-circulation parasites due to their lower LoDs (288).

As a result, diagnostic challenges in asymptomatic malaria infections are significant roadblocks to malaria elimination efforts. Conventional malaria diagnostic tools, such as microscopy and cRDTs, are typically employed in healthcare systems to screen symptomatic and self-presented patients. However, these diagnostic tools are inherently limited in detecting low-density parasitemias, which contributes to the ongoing spread of the disease, especially in asymptomatic infections. For example, as discussed in earlier sections, the limit of detection (LoD) for microscopy is generally around 100 parasites/µL when performed by an expert

microscopist. This limitation leads to an average miss rate of 20–50% of cases (129). Similarly, cRDTs have a detection limit of 100–200 parasites/µL, meaning that parasite densities below these thresholds can easily go undetected (132, 133).

While this advancement addresses certain limitations, challenges remain—particularly in detecting asymptomatic malaria infections, which often involve very low parasite densities. These densities frequently fall below the LoD of current RDTs, including the improved SD Bioline test. This underscores the need for even more sensitive diagnostic tools to support malaria elimination efforts. Consequently, highly sensitive RDTs (hsRDTs) are critical for accurately detecting low-density infections, especially in asymptomatic individuals who can unknowingly sustain malaria transmission. In this regard, a promising development in the field is the NxTek™ Eliminate Malaria Ag Pf [Abbott Diagnostics Inc., Republic of Korea (05FK140)], an hsRDT designed to detect HRP2-based P. falciparum in low-density infections (288). Prequalified by the WHO (289), this test has demonstrated its efficacy in detecting clinical malaria infections (parasitemia > 200 parasites/µL). Hence, the hsRDT is specifically designed to detect low-density parasite levels, a characteristic of asymptomatic infections during low transmission periods. Early studies and company reports suggest that hsRDTs offer significant improvements in detecting low parasitemia, regardless of the presence of symptoms (290-294).

Field studies have underscored the potential future reliance on such advanced diagnostic tools for malaria. For instance, Slater et al. (2021) demonstrated that hsRDTs exhibited significantly higher sensitivity (56.1%) compared to conventional RDTs (44.3%) in detecting asymptomatic malaria in pregnant women in Uganda, using PCR as the reference standard (248). Similarly, Landier *et al.* (2018) found that ultra-sensitive RDTs (uRDTs) in Eastern Myanmar significantly increased detection rates compared to conventional RDTs (51.4% vs. 25.2%) (288). A meta-analysis of cross-sectional surveys further indicated that hsRDTs have a sensitivity of 56.1%, an improvement over the 44.3% sensitivity of routine RDTs used on the same samples (295). Additionally, a study by Turnbull et al. (2022) in Kenya highlighted the potential of hsRDTs to detect low-density P. falciparum infections in asymptomatic schoolchildren (296).

1.4.3 Spatiotemporal distribution of malaria and modeling its epidemiolocal data

Malaria is still transmitted in many tropical regions, affecting 85 countries, including Ethiopia, and posing a persistent global health challenge. Despite ongoing efforts, the reduction in malaria cases and death has been slower than expected (49, 115). As indicated elsewhere in the previous section, the WHO has set new goals in the GTS to reduce global malaria incidence and mortality rates by 90% and eliminate malaria in 35 countries by 2030 (161).

In accordance with its malaria epidemiology, Ethiopia also launched its malaria elimination program in 2017 to achieve elimination within the same timeframe, as outlined in its series of updating documents (297-299). Ethiopia's topography is hence highly varied, with elevations reaching up to 4,550 meters above sea level at Ras Dashen and dropping to as low as 110 meters above sea level in the Afar Depression. Furthermore, the Great East African Rift Valley divides the Ethiopian highlands into two distinct regions: the Northwestern and Southeastern highlands (299). As a result of these geographical features, combined with ecological and climatic variations, the country exhibits a diverse eco-epidemiology. This diversity supports heterogeneous transmissions of infectious diseases, including malaria(300, 301), Consequently, such variability reduces the effectiveness of the disease control and elimination strategies (247).

In these scenarios, various studies employed spatiotemporal analysis tools to examine the distribution and dynamics of malaria in many endemic countries (302-305). Hence, identifying spatial clusters of malaria incidence and understanding its temporal dependence of cases are suggested to be crucial for guiding targeted interventions and accelerating malaria elimination efforts (247, 300, 302, 306). In similar fashion in Ethiopia, where malaria transmission is often seasonal and unstable, analyzing the spatiotemporal distribution and tracking disease trends is crucial for effective malaria control planning and implementation (307-309).

Hence, despite substantial efforts to control malaria, the disease remains a persistent challenge (49), particularly in regions where its spatiotemporal distribution is poorly understood(309). Over recent years, malaria trends have shown fluctuating patterns, with periods of decline followed by unexpected resurgences. These fluctuations are influenced by various factors, including changes in climate, population movements, and local intervention strategies. The lack of a comprehensive understanding of these trends hampers the effectiveness of targeted interventions and impedes progress towards malaria elimination (310-312). Addressing these issues requires a detailed analysis of both spatial and temporal dimensions and future trajectory of malaria in the Jimma zone.

1.4.4 The knowledge gaps

School-age children in sub-Saharan Africa (SSA), comprising 27% of the population, and in Ethiopia (~45%), have been largely overlooked in malaria interventions, particularly in the Gomma district, Jimma zone, Oromia of Ethiopia. Repeated infections in this group can lead to asymptomatic infection and could play a significant role in sustaining the transmission of the disease, yet the prevalence and risk factors for such infections remain poorly understood. This represents a critical research gap, emphasizing the urgent need for studies to address these issues and inform more effective, targeted malaria interventions in the district.

The hsRDT, a newly developed HRP-2-based malaria diagnostic tool donated by Abbott Diagnostics Inc. for research purposes, is specifically designed to detect *P. falciparum* infections. The company claims it is effective at detecting low-density parasitemia. Despite being pre-qualified by the WHO, the hsRDT has not yet been integrated into routine malaria diagnostics. Hence, there is a significant lack of data regarding its effectiveness in detecting P. falciparum infections in asymptomatic schoolchildren, particularly in the Gomma District of Jimma Zone, Ethiopia, where this population remains largely underexplored. This creates a critical gap in malaria control, hindering effective interventions for these overlooked infections and a population that has not received adequate attention in malaria control program.

While extensive studies conducted on various aspects of malaria infection in the Jimma zone, the spatiotemporal dynamics of malaria and modeling a decade of its epidemiological data at the zone level remain largely unexplored. Addressing this pressing issue need for a comprehensive investigation into the complexities of malaria epidemiology in this zone. By addressing these critical knowledge gaps, can help enhance our understanding of malaria dynamics in the Jimma Zone and inform evidence-based interventions.

1.5 Conceptual framework

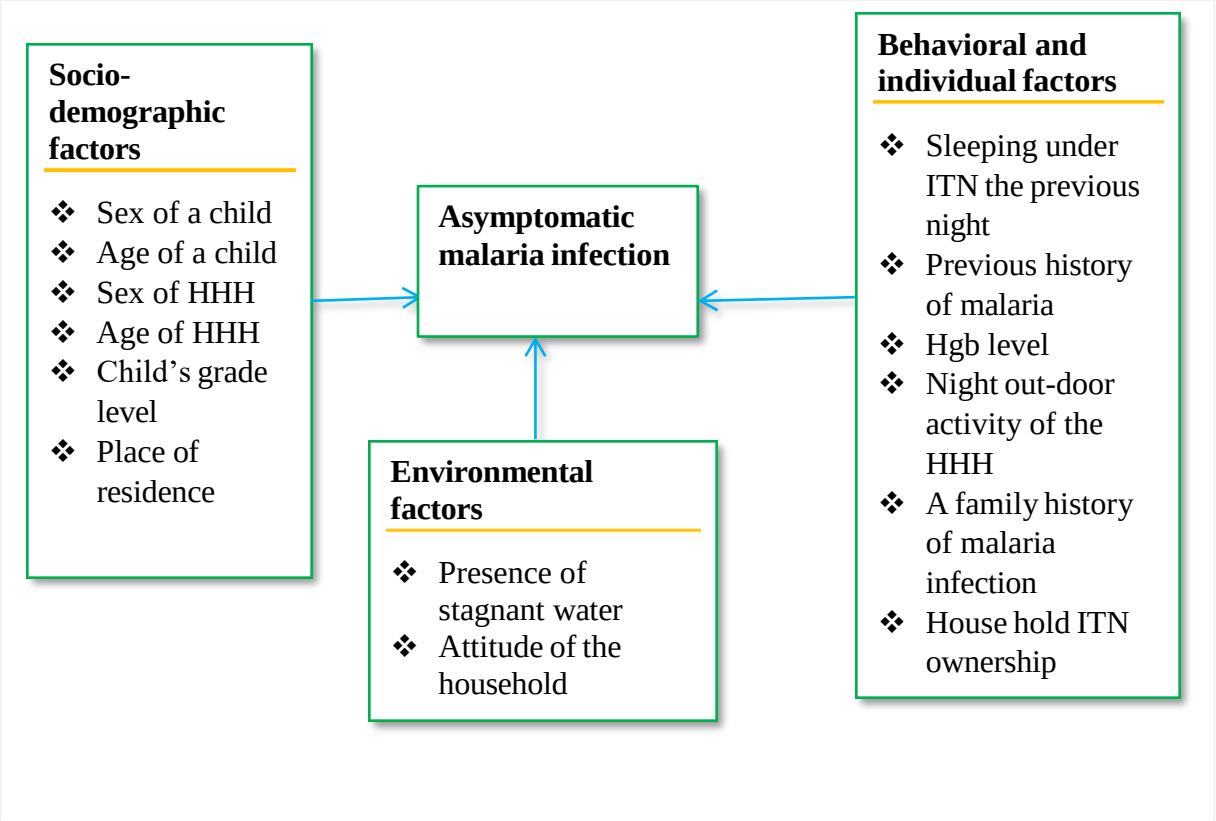


Figure 1. 6: Conceptual framework

1.6 Significance of the studies

Despite substantial control efforts, malaria remains a significant public health challenge in Ethiopia, particularly in the Jimma Zone. The results of this PhD project present a comprehensive analysis of the disease's epidemiology in the region, offering crucial insights that can inform tailored malaria control and elimination strategies aligned with local transmission patterns.

The results of the first objective revealed that healthy schoolchildren were harboring hidden malaria infections in the Gomma district, emphasizing the critical need for expanded screening programs that include schoolchildren at their respective schools. In addition, the study identified risk factors associated with these infections, paving the way for targeted interventions and surveillance in schools. These measures are essential to safeguard the health of this vulnerable population and to guide the NMCP in breaking transmission chains and advancing the vision of a malaria-free Ethiopia.

The second objective focused on the evaluation of the next generation, HRP2-based highly sensitive malaria RDT (hsRDT) represents a critical advancement in malaria diagnostics with significant public health implications. This study demonstrates that the hsRDT not only matches routine RDTs but also outperforms microscopy in sensitivity, crucial for detecting asymptomatic infections in schoolchildren—often overlooked by microscopy. Enhanced detection can lead to quicker treatment initiation and more effective control strategies, ultimately transforming malaria management. These findings can drive policy changes advocating for the widespread implementation of hsRDT, strengthening malaria surveillance and control efforts, and moving closer to a malaria-free future.

Proceeding sequentially, the results of our 3rd objective provided evidence of where and when malaria is most prevalent, are critical for strategic resource allocation and intervention planning. We not only elucidated historical transmission trends but also developed a robust predictive framework that is essential for formulating effective public health interventions. Our findings delineated areas of persistent transmission and seasonal fluctuations, enabling malaria control programs to implement timely, targeted preventive measures. This crucial information enhances the optimization of resource allocation, thereby strengthening overall strategies for malaria prevention and control. By clarifying these transmission dynamics, this study serves as an invaluable tool for both policymakers, ensuring that interventions are both efficient and impactful during peak transmission periods. Ultimately, these insights make a significant contribution not only to the broader global effort but also to national and local initiatives aimed at controlling and eliminating malaria, thereby safeguarding public health and fostering community resilience.

CHAPTER TWO: OBJECTIVES OF THE STUDY

2.1 General objective

The main objective of this PhD project was to investigate the epidemiology of malaria and assess the performance of the NxTek™ Eliminate Malaria-Pf Test (hsRDT) in the Jimma Zone of Oromia, Ethiopia, from September 2021 to January 2022.

2.2 Specific objectives

1. To assess the prevalence of asymptomatic malaria infection and its associated factors in apparently healthy schoolchildren within the Gomma District of Jimma zone, southwestern Oromia of Ethiopia
2. To evaluate the performance of the NxTek™ Eliminate Malaria-Pf Test for the detection of *P. falciparum* in school children with asymptomatic malaria infections in the Gomma District of Jimma zone
3. To analysis and modeling of the spatiotemporal patterns of a decade of epidemiological malaria data (2012 -2021) in Jimma Zone, Southwest Oromia, Ethiopia

CHAPTER THREE: GENERAL METHODS

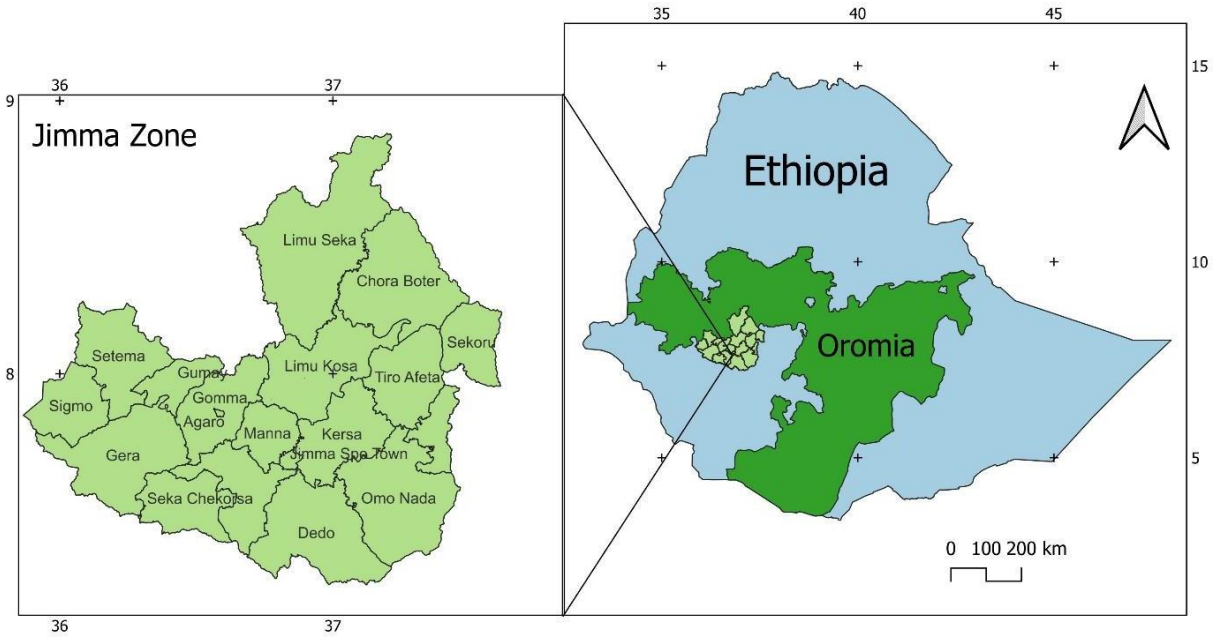
3.1 Description of the study setting

This section is adapted from Biruksew *et al*. (2024) – manuscript under review.

This PhD study was conducted in the Jimma Zone (Fig. 3.1) the largest zone situated in the southwestern Oromia regional state in Ethiopia. The zone is located at 7°13' to 8°56' north latitude and 35°49' to 38°38' east longitude, bordered by the East Wollega Zone (north), East and Southwest Shawa Zones (northeast), Southern Nations and Peoples' Region (south and southeast), and the Illubabor Zone (west). Administratively, it comprises 20 rural districts and two urban centers: Jimma town (capital) and Agaro town **(**313**).** As of July 2023, recent estimates show that the Jimma Zone has a population of 3,638,103, with nearly equal male and female distribution (314). The zone boasts a comprehensive healthcare system, with the public sector offering eight government hospitals (5 primary, 3 general), 122 health centers, and 513 health posts supported by about 1461 health extension workers. Additionally, the private sector contributes with 3 hospitals and 156 small and medium-sized clinics (315).

Beyond its administrative characteristics, the Jimma Zone is known for its rich natural resources and distinct climate. Renowned for its coffee production within the Oromia region, the zone also receives abundant rainfall, making it one of the wettest areas in the region. Annual precipitation reaches up to 1,500 mm (60 inches), with over 100 mm (4 inches) falling monthly between April and September. Jimma also experiences year-round warmth, with temperatures ranging from 9°C to 28°C. It rarely dips below 6°C or exceeds 31°C. The warmest period, lasting from December to April, sees average daily highs surpassing 27°C (316).

Communicable diseases, including malaria, account for approximately 60-80% of health concerns in the Jimma Zone. Malaria is endemic, exhibiting seasonal variations linked to Jimma's climatic factors. These factors are believed to contribute to the disease's persistence in the zone. Local health facilities are currently reporting characteristics of local malaria outbreaks within districts (317).



3. 1: Map of the Jimma Zone in the southwestern Oromia Regional State, Ethiopia. Map created with QGIS 3.36.2-Maidenhead (QGIS code revision 6d250527).

3.2 A brief description the study design

This dissertation delves into the complexities of malaria epidemiology in the Jimma Zone, Ethiopia. The research employs a mixed-methods approach, strategically combining two distinct study designs to gain a comprehensive understanding of this disease (details further explored in Table 2. 1). The first two objectives of this dissertation focus on understanding the current state of asymptomatic malaria and evaluating a new diagnostic test among schoolchildren in Gomma District. To achieve this, a cross-sectional design with multistage sampling was employed. This specific research design involves collecting data from a representative sample of healthy schoolchildren at a single point in time. Accordingly, the sampling process were detailed strategically as follows:

Briefly, the study employed a single population proportion formula, considering a previous estimate of 6.8% asymptomatic malaria prevalence in schoolchildren (264). To obtain a representative sample, a multistage sampling approach was used. Gomma District was randomly chosen as the primary sampling unit from the 21 districts in the Jimma Zone. Within Gomma District, a list of 70 schools served as the sampling frame for secondary sampling units (SSUs). Twenty-one schools (30%) were then selected using a lottery method.

The target sample size of 1002 schoolchildren were then proportionally distributed across the selected schools, grades, and sections within grades. Finally, individual student was selected

as the final sampling unit through lottery method from student rosters within their respective schools/grades. This process ensured balanced representation by stratifying participants based on age (6-15 years) and grade level (1-4 and 5-8), following methods described by Brooker *et al.*(275).

By collecting data from 1002 schoolchildren across 21 primary schools in Gomma District, we obtained a robust sample for analysis. This comprehensive data collection involved two key components:

First blood Samples: collected as blood smear for malaria diagnosis by microscopy, RDTs (5µl for each) and on Watmann filter paper DBS for molecular analysis. Second one was risk factor information: questionnaire was distributed to each schoolchild's house head and information was gathered on potential factors that might increase the risk of schoolchildren having asymptomatic malaria. In this case the roles of health extension workers were paramount.

This objective focuses on asymptomatic malaria infection, a condition where these children harbor malaria infections without experiencing the typical symptoms like fever. In this regard, the study aimed to uncover two key aspects of this hidden reservoir of malaria:

Prevalence: How common is asymptomatic malaria among apparently healthy schoolchildren in Gomma District? This helped quantify the number of children who might be infected without showing any outward signs.

Risk Factors: What factors increase the risk of schoolchildren contracting asymptomatic malaria? Identifying these factors was crucial, as it can inform strategies to prevent this hidden form of the disease. Finally, logistic regressions models (both bivariate and multivariate) were used to assess factors associated with asymptomatic malaria infection in apparently healthy schoolchildren. Variables with a p-value of < 0.25 in the bivariate model were included in the final model. Variables with a p-value < 0.05 from the multivariate model were considered statistically significant association with the outcomes. An adjusted odds ratio with 95% confidence interval (CI) were used as the measure of the association.

Objective 2**:** Performance evaluation of a novel hsRDT:

Building upon the same group of schoolchildren from Objective one, this objective focused on how performed a new an HRP2-based malaria hsRDT. This information is critical for determining the reliability of the hsRDT in diagnosing P. falciparum infections within this population. In general, metric for the performance evaluation of this RDT were sensitivity,

specificity, positive predictive value (PPV), and negative predictive value (NPV) of the new malaria hsRDT were determined using microscopy and PCR as references methods (318).

A crucial aspect of any diagnostic test is its sensitivity. By definition, it is the probability that a test result will be positive given the disease is present (true positive rate). Simply to put, it measures how well the test can correctly identify people with the disease. Here is the formula to calculate sensitivity (318): $\boldsymbol{Sensitivity} = \frac{True\ Positives}{(True\ Positives + false\ Negatives}$

Alongside sensitivity, another crucial aspect of a diagnostic test is its specificity. It refers to the proportion of true negative results among individuals who do not actually have the disease (true negative rate). In simple terms, it measures how well the test correctly identifies those who are free of the disease. Here is the formula for calculating specificity:

$\boldsymbol{Specificity} = \frac{True\ Negatives}{(True\ Negatives + false\ positives)}$, On the other hand, PPVs is the probability that the disease is present when the test is positive: $\boldsymbol{PPV} = \frac{True\ positive}{(True\ Positives + False\ Positives)}$

And NPV is probability that the disease is not present when the test is negative:

$$\boldsymbol{NPV} = \frac{True\ negative}{(True\ Negative + False\ negative)}$$

Test Accuracy is overall probability that an individual is correctly classified:

$$\boldsymbol{Accuracy} = Sensitivity \times Prevalence + Specificity \times (1 - Prevalence)$$

Objective three addressed the broader aspects of malaria epidemiology through comprehensive historical malaria data analysis. This study focused on two key areas: trend analysis that (included total malaria cases and proportions (%), *Plasmodium* species distribution and sociodemographic characteristics of malaria patients, and API of each district over time), and two modeling approaches: spatial autocorrelation analysis using Moran's I and time series modeling of future malaria trends (2022-2026) using ARIMA.

Table 2. 1: Summary of the study population, study design, sample size and (diagnostic) methods used in each objective

Objective	Study design	Study population	Study duration	Sampling method	Sample size	Malaria diagnostic tools / methods used	Publications status
To assess the prevalence of asymptomatic malaria infection and its associated factors in apparently healthy schoolchildren	Cross-sectional	Schoolchildren	September 2021 to January 2022	Multistage	1002	Microscopy, Malaria RDT (SD Bioline Pf/Pv (pLDH), and qPCR	Published (319)
To evaluate the performance of Nxtek™ Eliminate Malaria P.f Test for the detection of *P. falciparum asymptomatic* malaria infections in Schoolchildren	Cross-sectional	Schoolchildren	September 2021 to January 2022	Multistage	1002	Microscopy, Malaria RDT (SD Bioline Pf/Pv (pLDH), hsRDT and qPCR	Published (320)
To analysis and modeling of the spatiotemporal patterns of a decade of epidemiological malaria data (2012-2021) in Jimma Zone, Southwest Oromia, Ethiopia	Retrospective	Data 2012 to 2021 obtained from Jimma Zone Health Office	NA	Ten years (2012-2021) retrospective malaria data	NA	- Trend analysis (Temporal and Spatial) - Modeling: o Moran's, I modeling of the Spatial autocorrelation of malaria incidence across the districts in Jimma zone. o Forecasting Using ARIMA Modeling for the Next Five Years (2022 to 2026) in Time Series Analysis	Under review (Scientific Reports)

3.3 Ethical considerations

To ensure the well-being of all participants, this PhD research underwent a rigorous ethical approval process. The Institutional Review Board (IRB) at Jimma University's Institute of Health meticulously examined the study (Ref: IHRPED/550/2). Following approval, permission letters were submitted to and endorsed by the Jimma Zone Health and Education Offices. These offices then forwarded official letters to the Gomma District Health and Education Offices, who ultimately notified individual primary school directors and health extension workers. Furthermore, the research team obtained final approval from each participating school child's head of household. Written informed consent was acquired from parents or guardians of the children involved in the study. Additionally, children under 15 years old provided their assent.

Prioritizing a child's health: Children diagnosed with malaria received confidential details (name, age, sex, grade, parasite species/stage, and level of anemia) communicated to the school directors and nearby healthcare facility for prompt treatment.

CHAPTER FOUR: SCHOOLCHILDREN WITH ASYMPTOMATIC MALARIA ARE POTENTIAL HOTSPOT FOR MALARIA RESERVOIR IN ETHIOPIA: IMPLICATIONS FOR MALARIA CONTROL AND ELIMINATION EFFORTS

(Adapted from Biruksew *et al.* (2023))

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Schoolchildren with asymptomatic malaria are potential hotspot for malaria reservoir in Ethiopia: implications for malaria control and elimination efforts

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Abstract

Background

Schoolchildren with asymptomatic malaria infections often go undiagnosed and untreated, serving as reservoirs for infection that hamper malaria control and elimination efforts. In this context, little is known about the magnitude of asymptomatic malaria infections in apparently healthy schoolchildren in Ethiopia. This study was aimed at determining the prevalence of asymptomatic malaria infection and its associated factors in apparently healthy schoolchildren in Ethiopia.

Methods

From September 2021 to January 2022, a school-based cross-sectional study was conducted on 994 apparently healthy schoolchildren (aged 6–15 years) selected from 21 primary schools in the Gomma district, of Jimma zone, southwestern Oromia, Ethiopia. A multi-stage sampling technique was used to select schools and participants. After allocating the total sample proportionally to each school and then to each grade, participants were selected using the lottery method from a list of

student records (rosters). Finger-pricked blood samples were collected for microscopy blood film preparation and malaria rapid diagnostic test (RDT) (SD Bioline Malaria Ag Pf/Pv). Moreover, dry blood spots (DBSs) were prepared onto filter papers for quantitative real time polymerase chain reaction (qPCR) analysis.

Results

As determined by RDT and microscopy, the prevalence of asymptomatic malaria was 2.20% and 1.51%, respectively. Using qPCR, the overall prevalence was 5.03% (50/994). Of this, *Plasmodium* falciparum, *Plasmodium* vivax and mixed infections accounted for 90%, 6% and 4%, respectively. Submicroscopic asymptomatic malaria infection was also accounted for 70% (35/50) of the overall prevalence. Household head age, nighttime outdoor activities of household heads, family history of malaria, absence of insecticide-treated nets (ITN), and presence of stagnant water around the houses are all significantly associated with asymptomatic malaria infections among schoolchildren.

Conclusions: This study found that both RDT and microscopy underestimated the prevalence of asymptomatic malaria in schoolchildren. However, qPCR was able to detect even low levels of parasitaemia and revealed a higher prevalence of asymptomatic submicroscopic malaria infections. The findings imply that schoolchildren with asymptomatic malaria infection are potential hotspot for malaria reservoir that fuels ongoing transmission. Therefore, it is imperative to include schoolchildren and schools in malaria intervention package and equally important is the adoption of more advanced and sensitive diagnostic tools, which would be crucial for successful malaria control and elimination efforts. Targeted interventions for asymptomatic malaria-infected schoolchildren can provide invaluable support to the National Malaria Control Programme in controlling and eventually eliminating the disease.

Keywords Malaria prevalence, Asymptomatic malaria, Schoolchildren, Real-time PCR, Gomma district.

4.1 Background

Malaria, an ancient scourge that has plagued humanity for half a million years (321), holds a dark legacy as it is estimated to have claimed the lives of half of all people who have ever lived (322). The causative agents of this disease are five different species of *Plasmodium* parasites (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*), which are transmitted into the human body through the bite of female *Anopheles* mosquitoes, which seek a blood meal to sustain themselves (20).

Malaria can present in various forms, from severe and complicated cases to mild and uncomplicated instances to asymptomatic malaria (110). Therefore, asymptomatic malaria, often referred to as "silent threat" (323), is a significant portion of the population where individuals are infected with the malaria parasites but do not exhibit any visible symptoms of the disease, and represents a reservoir for sustained transmission (109). This makes them invisible to healthcare systems and serves as a source of persistent transmission (120, 324).

In this regard, the demography in sub-Saharan Africa is bottom-wide, with more than 27% of the population being young or of school age (5-15 years) (270), and nearly 40% of Ethiopians are under the age of 15 (325). Hence, school-age children, the proportion of the population serving as malaria reservoirs, are more attractive and available to mosquito bites, contributing to the overall infectious reservoir for malaria (326), as they have received little attention and have benefited only marginally from the universal malaria intervention(142, 274, 327). In this age group, repeated infections lead to the development of partial immunity against future complications, which aids in the establishment of asymptomatic reservoirs (127), and are reportedly more infectious than symptomatic patients (110).

In Uganda, for instance, a study conducted in areas with rigorous mosquito control measures and high *P. falciparum* transmission has revealed a startling finding: school-age children carrying asymptomatic malaria reservoirs were responsible for a staggering 50.4% of malaria transmission to mosquitoes (128). In the same vein, Okell *et al.* (129) estimated that asymptomatic submicroscopic carriers (the proportion of malaria cases missed by microscopy) account for 20-50% of human-to-mosquito transmission. Furthermore, an experimental study conducted in low-

transmission areas of Ethiopia found that both microscopic and submicroscopic asymptomatic malaria-infected individuals contributed 76.2% and 15.8%, respectively, of human-to-mosquito transmissions (130). The infections are particularly prevalent in stable malaria transmission regions of sub-Saharan Africa, possibly due to naturally acquired immunity that develops over time from repeated infections (264, 328-330).

Since 2004, Ethiopia has significantly reduced burden of malaria through deploying numerous health extension workers for extensive community interventions and putting control measures into place (331). As a result, it has succeeded in reducing malaria incidence by 40% by 2021, as set forth in the GTS. In 2021, the country estimated to account for 1.7% of cases and 1.5% of deaths of malaria in 2021, making it a serious public health concern. Roughly, 75% of the landmass is malarious, and about 27.20% of the population lives in high-risk areas. Besides this, transmission in the country is highly seasonal and unstable (115, 332, 333). As part of its country-owned, country-driven malaria elimination program, Ethiopia has adopted a National Malaria Elimination Roadmap, which was recently revised as the National Malaria Strategic Plan 2021-2025 (333, 334) with the vision of a malaria-free Ethiopia by 2030. To this end, conventional malaria diagnostic tools (microscopy and malaria RDT), vector control, case treatment, and single-dose primaquine as a transmission blocker are used in the framework of universal health coverage (334). National malaria control programmes (NMPs) and the global research community have largely been focused on symptomatic (clinical) malaria, however, it appears to have neglected the contribution of asymptomatic malaria-infected individuals to the global disease burden, undermining the true prevalence of the disease (335).

In healthcare systems, conventional malaria diagnostic tools are usually used for screening of symptomatic and self-presented patients, but they are inherently incapable of detecting such low-density parasitaemias, allowing the disease to spread. For instance, the limit of detection (LoD) of malaria microscopy up to 100 parasites/µL by expert microscopist, missing on average between 20-50% of the cases (129) and that of the most malaria RDTs is 100-200 parasites µL) (132, 336). Such chronic infections in schoolchildren not only pose a challenge to the elimination efforts, but they are also associated with long-term health consequences, such as school-absenteeism and cognitive impairment (142). Addressing the burden in asymptomatic schoolchildren through targeted interventions offers two broad benefits. First, it can help achieve Sustainable

Development Goals 3 and 4: promoting healthy living and well-being for people of all ages and quality education for all (271, 337). Second, prioritizing malaria control in this large demographic can be used as a strategy to reduce transmission, since this segment of the population is vulnerable to malaria and a significant source of ongoing infection (338). Generating evidence of the magnitude of asymptomatic malaria reservoirs in schoolchildren is therefore critical to informing the national malaria control programme and justifying the inclusion of schools in malaria control packages. However, little is known about asymptomatic malaria infection and its associated factors in apparently healthy schoolchildren in the Gomma District of the Jimma Zone in southwestern Oromia, Ethiopia.

4.2 Methods

4.2.1 Details of study setting and design

In the earlier work (320), a thorough description of the study was provided. Additionally, a concise explanation of the background with some additional information pertinent to the current work is presented here. According to the Ethiopian Statistical Service, southwestern district office (339), there are a total of 300,266 population dwelling the Gomma district, of which 152,402 are males and 147,864 are females, with 20.24% semiarid urban areas and the majority are rural residents. The district is composed of 42 lower-level administrative villages (known as *Ganda* in Afaan Oromoo). The district is well-known for its extensive coffee growing including moist Afromontane forests with various tree species as its climax vegetation. Gomma district is therefore receives rain all year-round and is endemic to malaria (unpublished, Jimma Zone Health and agriculture Departments, unpublished data]. Furthermore, according to data obtained from the Ethiopian Meteorology Institute, Southwestern Oromia Meteorology Research Center [unpublished data, 2021], Gomma district received an annual temperature range from 25 to 28°C, creating an ideal environment for mosquito breeding. As a seasonal disease, malaria has a peak immediately following the main rainy season and then gradually declines over the following months. There are only two *Plasmodium parasite* species endemic to Jimma zone in general: *P. falciparum and P. vivax,* with *P. falciparum is* the leading cause of malaria. Malaria prevention and control are active in the district, except that ITN distribution has been halted for four years (2018-2022) due to a decrease in malaria incidence in the area. As a result, some households were still using old ones. Based on the country's malaria stratification [7] system, Jimma zone, including the Gomma

district, is classified as having low malaria transmission depending on its Annual Parasite Incidence (API).

4.2.2 Sample size and sampling technique

As described in the previously published work (320), the sample size (n) for this study was determined using the single population proportion formula,

$$n = \frac{z\left(\frac{\alpha}{2}\right)^2 p(1-p)}{d^2}$$

which took into account z = 1.96 at the desired confidence interval (CI) of 95% along with a 2% margin of error (d), and the expected prevalence rate(p) of asymptomatic malaria infection among apparently healthy schoolchildren in Gomma district was 6.8% (264). Using these parameters, the sample size was calculated to be 608. However, to account for potential non-response, a 10% non-response rate was added, resulting in a total sample size of 668. To account for possible variability in the multi-stage sampling technique, a design effect of 1.5 was applied. This adjustment increased the final sample size to 1002 schoolchildren. This study utilized a multi-stage sampling technique. The Gomma district was randomly selected as the primary sampling unit from a list of 21 districts in Jimma zone. From a list of 70 schools, 21 secondary sampling units were selected using lottery method. The sample size was then allocated proportionally to each primary schools, grades, and sections (within grade). Each school child was selected as the final sampling unit using the lottery method from the sampling frames (student rosters), stratified by age (6–15 years) and grade level (1–4 and 5–8) according to methods described by Brooker *et al* (275) and previous works (264, 340).

4.2.3 Inclusion and exclusion criteria

Schoolchildren aged 6 to 15 years, whose family/guardian had consented to the study, who were also not taking anti-malarial medication and had an auxiliary body temperature of < 37.5°C were included. Schoolchildren who were taking anti-malarial medication within one month of enrollment, had a fever at the time of data collection, or were over 15 years old were excluded from the study.

4.2.4 Demographic data collection

Using a structured questionnaire, both demographic and malaria risk factor data were collected through face-to-face interviews with a child's head of household while observational data were recoded. The data were collected by trained data collectors.

4.2.5 Blood film examinations

Finger-pricked blood was collected from each student, and both thick and thin blood smears were prepared on a frosted-ended microscopic slide. After air-drying, the smears were stained for 10 minutes with 10% buffer-diluted Giemsa and examined under the microscope with a 100X oil immersion objective. Following the standard protocol (341) and also described the previous work (320), skilled malaria microscopists at Jimma University detected, identified *Plasmodium* species, and determined asexual parasitaemia after reading the slides. Any discrepancies were confirmed by a third experienced microscopist who was unaware of the first two microscopists' readings. In addition, the HemoCue® Hb 301 System (with curves product date: 2020.10.29 and expiry date: 2022.10.29; lot number: 2101570; HemoCue AB Kuvettgattan 1, SE-262 71, REF 111801, Ängelholm, Sweden) was used to measure the haemoglobin (Hb) concentration (in g/dL) from finger-pricked blood samples blood samples (342). In accordance with WHO recommendations, anemia (after Hb adjusted for altitude) is then categorized as having an Hb concentration of less than 11.5 g/dL in children aged 5 to 11 years and less than 12.0 g/dL in those aged 12 to 15 years (343).

4.2.6 Malaria Rapid Diagnostic Test (RDT)

In this study, a malaria RDT, the SD Bioline Malaria Ag (Pf-HRP2 and Pv-pLDH) was used in the field to screen children for possible malaria infections. It is a three-banded device containing a *P. falciparum HRP*2, a *P. vivax* pLDH, and a control band, manufactured by Standard Diagnostics Inc. (now Abbot Diagnostic Korea Inc.) with LOT number: 05DDF011A, and product code 05fk80, Korea. The kit was developed in order to detect histidine-rich protein-2 (pfHRP2) of *P. falciparum and* lactate dehydrogenase (pLDH) of *P. vivax*. As previously stated (320), after

labelling the device with the students' ID, 5µL of blood samples were collected for onsite testing, and the kit provided results in 15 minutes (per the manufacturer's instructions). It is commonly utilized in the country's rural healthcare facilities (heath post and health centers) to rule out malaria in febrile individuals.

4.2.7 Molecular assays

The same procedures described in the earlier publication of a section of this work was applied in this section as well (320). Using a round hole puncher, approximately 3-5 mm diameter pieces of DBS were cut into a labeled 1.5 ml Eppendorf tube. The previously described Chelex-100 -resin-saponin method was then used to extract parasite DNA from DBS. The purified DNA was transferred to Nunc tubes with a volume of 0.5 mL and stored at -20 °C until it was ready for use in the PCR assay. The PCR assay was then conducted using QuantStudio™ 3 Real-Time Multiplex PCR. This molecular tool was used to amplify the 18S rRNA genes, allowing for the detection of both *P. falciparum and P. vivax*. The amplification process involved the use of a pair of forward and reverse primer sequences and probes that were specifically designed for this purpose.
For *P. falciparum* specific primers (5'-3') (344): F-F (forward):
TATTGCTTTTGAGAGGTTTTGTTACTTTG, and
F-R (Revers): ACCTCTGACATCTGAATACGAATGC. And probe used was: Pf-fam (MGB):
ACGGGTAGTCATGATTGAGTT. For *P. vivax* specific primers used was:

Pv-1 (forward): CGCTTCTAGCTTAATCCACATAACTG, and Pv-2 (Reverse):
AATTTACTCAAAGTAACAAGGACTTCCAAG. The probe used was: Pv-probe (VIC-MGB):
CGCATTTTGCTATTATGT (345).

To ensure the utmost precision in the amplification process, we included positive controls consisting of DNA from *P. falciparum and P. vivax* isolates (Pf and Pv MR4(BEI) whereas molecular grade water was used as a negative control. For the qPCR master mix, the PerfeCTa® qPCR ToughMix® (Low ROX™, Quanta Bio vwr, catalog number: 97065–968) was utilized to guarantee optimal performance. The PCR amplification was conducted in a total reaction volume of 12 µL, comprising of 6 µL of the PerfeCTa master mix. To target *P. falciparum and P. vivax,* 0.5 µL (× 2) each of the Pf-Fam and Pv-vic probes, along with 0.4 µL (× 4) each of the forward and reverse primers were used. Furthermore, 2 µL of extracted DNA and 1.4 µL of molecular

grade water were added. Under the specified PCR cycling conditions, which involved 45 cycles, the amplification process commenced with an initial denaturation at 50ºC for 2 min. Subsequently, 45 cycles of amplification were performed at 95ºC for 2 min, followed by 2 s at 95ºC and 30 s at 60ºC. The entire run-time for this process lasted approximately 60 min.

4.2.8 Data management and statistical analysis

Data were coded, entered, and cleaned using EpiData version 3.1 and exported to SPSS version 26.0 for analysis. Descriptive and inferential statistics were done as frequencies and percentages to determined socio-demographic characteristics and prevalence of malaria among asymptomatic schoolchildren. A chi-square test was used to determine whether there was a statistically significant association between schoolchildren characteristics and asexual parasite density as measured by microscopy. Finally, both bivariate and multivariate logistic regressions model were used to assess factors associated with asymptomatic malaria infection in apparently healthy schoolchildren. Variables with a p-value of < 0.25 in the bivariate model were included in the final model. Variables with a p-value < 0.05 from the multivariate model were considered statistically significant association with the outcomes. An adjusted odds ratio with 95% confidence interval (CI) was used as the association measure. The goodness of fit of the model was assessed using the Hosmer–Lemeshow test.

4.2.9 Ethical consideration

This project received ethical approval from the Institutional Review Board (IRB) (Ref: IHRPED/550/2) of the Institute of Health at Jimma University. The study was also approved by the Jimma zone Health and Education offices, and official letters were submitted to the Gomma district Health and Education offices, then to each school director. Furthermore, final permission was obtained from each schoolchild's household head.

4.3 Results

4.3.1 Characteristics of the study participants

Beforehand, 1002 healthy schoolchildren were enrolled in the study; however, eight were excluded based on exclusion criteria, which gave a response rate of 99.20%. Thus, a total of 994 healthy children with normal auxiliary temperature (<37.5°C) were included for this analysis. Of the study participants, 52.40% (521/994) were males and 47.60% (473/994) were females. The mean age of the study participants was 11.54 years (±2.5 SD). Over half (56.40%) of the children were in their 1st cycle primary education (grade 1-4) while 43.60% were in their 2nd cycle primary education (grades 5 – 8). The majority of schoolchildren (77%) were from rural areas and 23% from semi-urban areas. The locations of study sites lie at an altitude between 1428 and 1749 meters above sea level (masl) with a mean altitude of 1623 masl (Table 4. 1).

Table 4. 1: Socio-demographic characteristics of the study participants in Gomma District, Jimma Zone, Southwestern Oromia, Ethiopia, 2022.

Socio-demographic characteristics		
Characteristics of participants	Category	n (%)
Student's Sex	Male	521 (52.40)
	Female	473 (47.60)
Student's age group	6 -11	463 (46.6)
	12-15	531 (53.40)
Student's grade level	1-4	561 (56.40)
	5-8	433 (43.60)
Sex of the household head (HHH)	Male	915 (92.10)
	Female	79 (7.90)
Age of the HHH	<35	72 (7.20)
	35-44	295 (29.70)
	45-54	374 (37.60)
	≥55	253 (25.50)
Marital status of the HHH	Married	911 (91.60)
	Widowed	49 (4.90)
	Divorced	30 (3.00)
	Single	4 (0.40)
Educational status of the HHH	Cannot read and write	326 (32.80)
	1-8	544 (54.70)
	9-12	49 (4.90)
	Tertiary level	75 (7.50)
Occupation of the HHH	Farmer	794 (79.90)
	Gov't employee	94 (9.50)
	Merchant	76 (7.60)

	Daily labor	30 (3.00)
Household Size	< 5	442 (44.50)
	≥ 5	552 (55.50)
Residency of the household	Rural	765 (77)
	Semi-urban	229 (23)

n = number

4.3.2 Prevalence of asymptomatic malaria reservoirs

Of the 994 healthy school children from 21 primary schools included in the study (Additional file 1: Tables A and B), using RDT, the prevalence of asymptomatic malaria was 2.20% (95% CI: 1.40, 3.30) whereas using microscopy, it was 1.51% (95% CI: 0.80, 2.50), However, only *P. falciparum mono* infections were detected with both conventional malaria diagnostic tools. The overall prevalence determined by qPCR (Table 4. 4) was 5.03% (95% CI: 3.80, 6.60). Of these, 6.33% (33/521) were males and 3.60% (17/473) were females. The majority [80% (40/50)] of children were from rural while 20% (10/50) were from semi-urban areas. There was no difference in the proportion of children infected in either age group (6–11 years or 12–15 years), with each group accounting for 50% (25/50). The prevalence in grades 1–4 was 54% (27/50), while it was 46% (23/50) in grades 5–8. *P. falciparum*, *P. vivax*, and mixed infections accounted for 90% (45/50), 6% (3/50), and 4% (2/50) of all infections, respectively. Of *P. falciparum infected* schoolchildren, 64.44% (29/45) was males whereas 35.56% (16/45) was females (Fig. 4. 2).

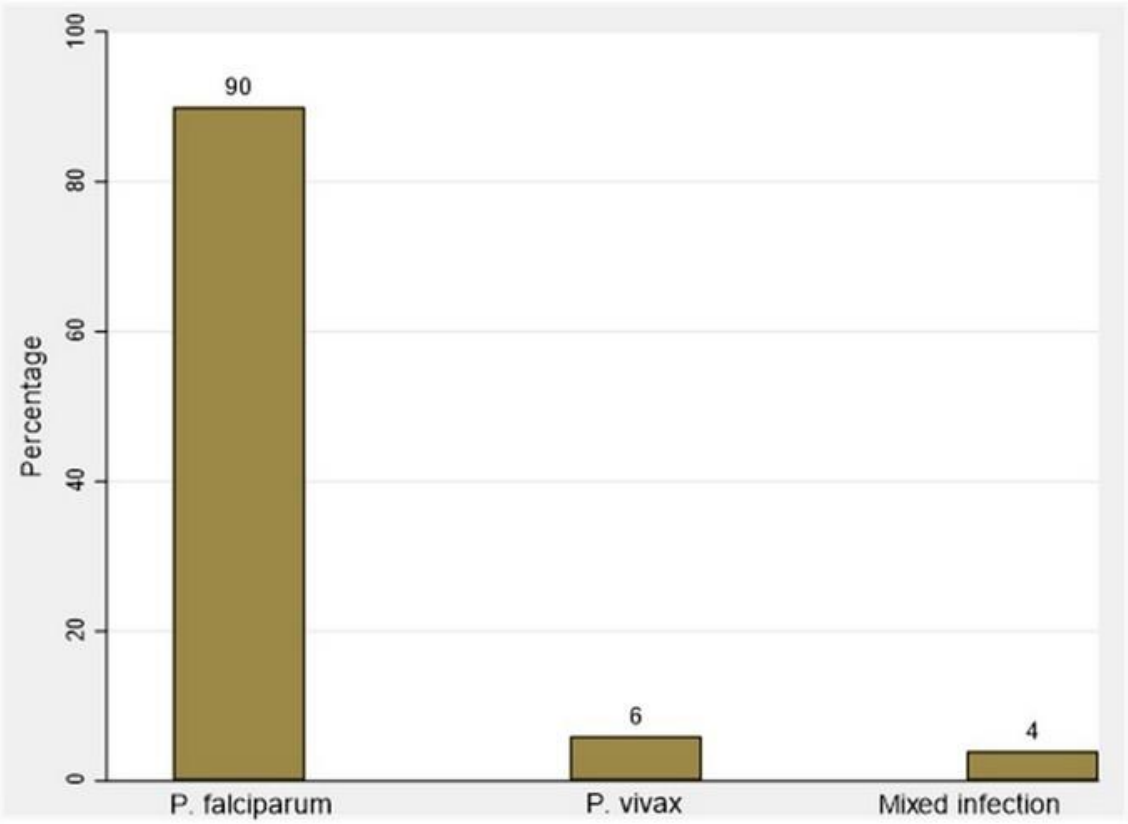


Figure 4. 1: Prevalence of *Plasmodium* species as determined by qPCR in asymptomatic malaria infected school children in Gomma zone, Oromia, Ethiopia, 2022.

Prevalence of *Plasmodium* species as determined by qPCR in asymptomatic malaria infected school children in Gomma zone, Oromia, Ethiopia

Furthermore, Hb concentration was measured to determined level of anaemia, which ranged between 10.5 and 14.80 g/dl, with a mean of 12.62 g/dl (± 0.82 SD). As a result, children with moderate anaemia (< 11.5 g/dl) accounted for 5.84% (n = 58), of whom 32.76% (n = 19) were infected with malaria. Of these malaria-infected, mildly anaemic children, 78.95% (n = 15) were infected with *P. falciparum*, 15.80% (n = 3) with *P. vivax*, and a mixed infection. On the other hand, the proportion of children with normal Hb levels (> 11.5 g/dl) was 94.16% (n = 936), of which 31 (3.31%) were asymptomatic malaria carriers. The majority of these infections (n = 30) were caused by *P. falciparum (*n = 30), with one mixed infection.

P. falciparum asexual parasite densities were determined in all microscopically positive children, and ranged from 64 to 8080 parasites/uL (Table 4. 2). Furthermore, it was observed that more than half of these children were gametocyte carriers, infective stage of *Plasmodium* species to female *Anopheles* mosquito. As demonstrated in Table 4. 2, there was no statistically significant association between parasite density and schoolchildren.

Table 4. 2: Distribution of asexual *P. falciparum density* by sex, age group, grade level, place of residence and altitude in asymptomatic malaria infected schoolchildren in Gomma district, Jimma Zone, 2022.

Characteristics		**Parasite density /µL**					
		< 200	201 - 500	501- 2000	2001 - 5000	5001 - 9000	p-value
Sex	Female	2	1	0	0	0	0.504
	Male	3	2	3	3	1	
Age (in years)	6-11	2	1	2	1	1	0 .710
	12 –15	3	2	1	2	0	
Grade level	1 – 4	2	1	2	2	1	0 .710
	5 – 8	3	2	1	1	0	
Residence	Semi-urban	2	0	0	0	0	0 .329
	Rural	3	3	3	3	1	
Altitude (masl)	<1600	2	1	3	1	0	0.306
	≥1600	3	2	0	2	1	

Statistically significant when the p-value of the chi-square (X^2) test is < 0.05; masl = meters above sea level

4.3.3 Proportions of malaria cases missed by microscopy and malaria RDT

In this study, the proportion of cases missed by microscopy is translated to asymptomatic submicroscopic malaria infection, which is obtained by subtracting the microscopic prevalence from the qPCR prevalence (50–15 = 35). This means that 35 children went undiagnosed and left untreated. (Table 4. 3). As a result, the prevalence of submicroscopic asymptomatic malaria was 70% (35/50), with *P. falciparum made* up 85.70% (30/35), followed by *P. vivax* [8.60% (3/35)] and mixed infections [5.70% (2/35)]. Although the RDT detected seven more cases than microscopy, it also missed 56% (28/50) of infection reservoirs detected by qPCR. Generally, both

the conventional malaria diagnostic tools missed a high number of infection reservoirs, which might jeopardize malaria elimination efforts.

Table 4. 3: Proportions of asymptomatic malaria infections missed by conventional malaria diagnostic tools in the total qPCR prevalence in asymptomatic malaria infected schoolchildren in Gomma district.

		qPCR			% cases missed
		+ve	-ve	Total	
Microscopy	+ve	15	0	15	$\frac{35}{50} * 100 = 70\%$
	-ve	**35**	944	979	
	Total	50	944	994	
RDT	+ve	22	0	22	$\frac{28}{50} * 100 = 56\%$
	-ve	**28**	944	972	
	Total	50	944	994	

+ve: positive; -ve: negative.

4.3.4 Factors associated with asymptomatic malaria infection in schoolchildren

Bivariate and multivariate logistic regressions were employed to identify factors associated with asymptomatic malaria among schoolchildren. As the summary of results can be seen in Table 4.4, the analysis identified six variables that are significantly (P<0.05) associated with an increased risk of malaria infection in asymptomatic schoolchildren: anemic schoolchildren, household head's (HHH) age, HHH nighttime outdoor activities, family history of malaria, household ITN ownership, and presence of stagnant water near the household.

Accordingly, apparently healthy anemic schoolchildren were 12 times more likely to be asymptomatic malaria carriers than non-anaemic children [AOR = 12.05 (95% CI: 5.329, 27.274)]. Also, children whose HHHs were 55 years or older were five times more likely to be infected with malaria than children whose HHHs were younger than 35 years [AOR = 4.98 (95% CI: 1.218, 20.645)]. In the same vein, children whose HHHs engaged in nighttime outdoor activities had a 3.27 [AOR = 3.27 (95% CI: 1.602, 6.687)] times greater risk of developing asymptomatic malaria compared to whose heads of households did not engage in such activities. What stands out in the table 4 is that children with a family history of malaria were 98% less likely to be infected with malaria infection than children without a family history of malaria [AOR= 0.021 (95% CI: 0.003, 0.159)]. The study found that there were 3.6 times higher likelihood of malaria infection in children living with a family who did not have ITNs compared to children with a family who owned ITN

[AOR = 3.624 (95% CI: 1.676, 7.838)]. Notably, children who lived in homes near stagnant water were 4.65 times highly likely [AOR = 4.986 (95% CI: 2.042, 12.178)] contracting malaria than children who lived in homes without nearby stagnant water.

Table 4. 4: Bivariate and multivariate analysis of factors associated with asymptomatic malaria in schoolchildren in Gomma district, Jimma Zone, Oromia, Ethiopia.

Individual child factors	**Categories**	**All**	**n (%)**		**COR (95% CI)**	**p-value**	**AOR (95% CI)**	**p- value**
			+ve	-ve				
Sex	Female	473	17 (3.60)	456 (96.40)	1		1	
	Male	521	33 (6.33)	488 (93.66)	1.814 (0.99, 3.30)	0.051		
Age (in years)	6 - 11	463	25 (5.40)	438 (94.6)	1.16 (0.65, 2.04)	0.621		
	12 – 15	531	25 (4.70)	506 (95.30)	1			
Grade level	1-4	561	27 (4.81)	534 (95.19)	0.90 (0.51, 1.59)	0.72		
	5- 8	433	23 (5.30)	410 (94.70)	1			
Slept inside ITN the previous	No	74	10 (13.50)	64 (86.50)	0.589 (0.212, 1.64)	0.31		
	Yes	83	7 (8.43)	76 (91.57)	1			
Previous history of malaria	No	942	42 (4.58)	900 (95.42)	1			
	Yes	52	8 (15.38)	44 (84.62)	3.89 (1.72, 8.79)	0.001		
Hb level(g/dl)	< 11.5	58	19 (32.76)	39 (67.24)	14.22 (7.38, 27.37)	<0.0001	12.05 (5.329, 27.274)	***<0.0001****
	≥11.5	936	31 (3.31)	905 (96.69)	1		1	
Household factors								
Sex of the household head (HHH)	Female	79	1 (1.27)	78 (98.73)	1			
	Male	915	49 (5.35)	866 (94.65)	4.41 (0.60, 32.40)	0.14		
Age of the HHH	<35	72	3 (4.17)	69 (95.83)	1			
	35-44	295	11 (3.73)	284 96.27))	0.89 (0.24, 3.28)	0.86		
	45-54	374	13 (3.50)	361 (96.50)	0.83 (0.23, 2.98)	0.77		

	≥55	253	23 (9.10)	230 (90.90)	2.30 (0.67, 7.89)	0.18	4.98 (1.218, 20.645)	***0.027****
Educational status of HHH	Cannot read and write	326	16 (4.90)	31 (0 95.10)	0.723 (0.256, 2.038)	0.54		
	1-8	544	25 (4.60)	519 (96.40)	0.674 (0.250,01.819)	0.44		
	9-12	49	4 (8.20)	45 (91.80)	1.244 (01.819, 1.819	0.75		
	Tertiary	75	5 (6.70)	70 (93.80)	1			
Night-time outdoor activities of HHH	No	643	21 (3.27)	622 (96.73)	1		1	
	Yes	351	29 (8.26)	332 (91.74)	2.67(1.50, 4.75)	0.001	3.27 (1.602, 6.687)	***0.001***
Family history of malaria infection	No	742	49 (6.60)	693 (93.40)	1		1	
	Yes	252	1 (0.40)	252 (99.4)	0.056 (.008, 0.410)	0.005	0.02 (0.003, 0.159)	< ***0.0001****
Household ITN ownership	No	837	33 (3.94)	804 (96.06)	2.958 (1.604, 5.456)	0.001	3.62 (1.676, 7.838)	***0.001****
	Yes	157	17 (10.83)	140 (89.17)	1			
Environmental factors								
Presence of stagnant water around home	No	446	9 (2.00)	437 (98.00)	1		1	
	Yes	548	41 (7.50)	507 (92.50)	3.93(1.89, 8.17)	0.0001	4.98 (2.042, 12.178)	< ***0.0001****
Place of residency	Rural	765	40 (5.23)	725 (94.77)	0.83 (0.41, 1.68)	0.60		
	Semi-urban	229	10 (4.37)	219 (95.63)	1		1	
Altitude of the house (masl)	< 1600	312	20 (6.41)	292 (93.59)	1.489 (0.831, 1.266)	0.18		
	≥ 1600	682	30 (4.40)	652 (95.60)	1			

Key: - COR: Crude Odds ratio; AOR: Adjusted odds ratio, *significant at $p < 0.05$, ** significant at $p < 0.01$, *** Signiant at $p < 0.001$

4.4 Discussion

Asymptomatic malaria infections pose a hidden threat to malaria elimination efforts. This is because large segments of the population, asymptomatic schoolchildren with malaria, are sources of persistent infection and contribute to continued transmission (120, 142, 273, 275). To ensure the success of the malaria elimination program, it is critical to address this segment of the population, which did not receive special attention in the malaria control and elimination strategy (120, 142) and the burden of infections in this age group remains unaddressed across endemic countries (346). As a result, the current study investigated the prevalence of asymptomatic malaria infection and its associated factors in healthy schoolchildren in the Gomma district of Jimma zone, Southwestern Oromia, Ethiopia.

In this study, the overall prevalence of asymptomatic malaria infection was 5.03%, as determined by qPCR. The majority of infections were caused by *P. falciparum (*90%) followed by *P. vivax* (6%), and mixed infections (4%). The prevalence of *falciparum* malaria infection was 2.20% and 1.51% using RDT and microscopy, respectively. This is consistent with other reports (115, 331), according to which *P. falciparum* infections account for the vast majority of infections in sub-Saharan countries. Importantly, 70% of the schoolchildren in this study were asymptomatic submicroscopic malaria reservoirs. The majority of infections (85.70%) were caused by *P. falciparum* , followed by *P. vivax* (8.60%) and mixed infections (5.70%), highlighting that microscopic asymptomatic infection is only the tip of the iceberg, consistent with previous findings in Ethiopia (118). The result of this study is in line with those of Okell *et al.* (129), that looked at data from a number of different studies that had been conducted in areas with low malaria transmission. They also found that in areas with a microscopy prevalence of < 10%, the prevalence of submicroscopic infections could range from 70 to 80%. As previously described (129), the approach to calculating the asymptomatic submicroscopic malaria prevalence was as follows:

$$Submicroscopic\ prevalence\ =\ qPCR\ prevalence - Microscopic\ prevalence.$$

Whitttaker *et al.* (324) also conducted systematic review and meta-analysis showed that asymptomatic submicroscopic infections are more common in low malaria transmission areas. In this study, the RDT also missed a significant proportion (56%) of infections detected by qPCR.

In broad sense, the current study showed that males had a higher prevalence of asymptomatic malaria than females, which is consistent with previous research from Ghana (347). Despite what might be expected, infections are typically similar regardless of age, grade level, place of residence or altitude of the household. A large body of evidence (120, 129, 140, 272, 348) suggests that such hidden infection are important sources of sustained transmission, accounting for 20-50% of all human-mosquito transmissions (129, 140, 206, 349) and *P. falciparum* infection demonstrates that it can remain in the circulation for months, up to 200 days (350). The findings in this study imply that infections were actively and covertly spread among the study population, going undiagnosed as a result absence of health seeking behavior at healthcare systems.

A handful of scientific studies explores the potential causes of these infections: From the immunological viewpoint, older children develop asymptomatic states as a result of intermittent, non-sterilizing immunity following repeated exposure, leading to low-density parasitaemias (351). Because of this, once infection is established, both anti-parasite and anti-disease immune reactions are triggered, and the parasite reacts in accordance with its priority. In low-transmission areas with few mosquito bites, the parasite opts to be low-parasitaemic and increase transmission at the cost of decreased pathogenicity in order to thwart an anti-parasitic immune response. However, because mosquito bites are common in high-transmission areas, the parasite favors overriding host immunity over transmission (122, 352, 353).

Another state of knowledge (354, 355) deals with the virulence trade-off hypothesis. According to this theory, parasites are typically less virulent in low-transmission settings to avoid competition from more virulent strains. They consequently persist at low densities and go undiagnosed, making them a transmission candidate. Therefore, less virulent strains would prevail over more virulent ones, with the latter being cleared after diagnosis thanks to anti-malarial treatment and a stronger immune response. The most recent discovery also revealed the fourth *Plasmodium* parasite life cycle, particularly *P. vivax*, in the human spleen (123, 124). The scientists conducted an empirical study on splenectomized Indonesian patients who also had chronic, asymptomatic malaria infections. The PCR results revealed that 98.7% of *P. vivax* and 93.1% of *P. falciparum hidden* biomasses were found in the human spleen rather than in the circulation. It is therefore safe to conclude that malaria parasites escape detection by conventional methods by circumventing circulation in chronically asymptomatic carriers and evolving a novel survival strategy.

Additionally, Andrade *et al.* (122) showed that the longer *P. falciparum* circulating time is linked to increased splenic clearance in asymptomatic carriers during the dry season, and maintains parasitaemia below clinical and immunological thresholds. A similar study in Uganda reported that in areas where malaria is effectively controlled, asymptomatic school-age children were sources of persistent infections (120).

The microscopic result of this study is lower than reports from northern Ethiopia (130, 264, 356), Uganda (128), Democratic Republic of Congo, DRC (357), Cameroon (358), Ghana (359, 360), Tanzania (361), India (362). The variations could be attributed to different malaria transmission settings, study periods, and host immunity. In addition, since 2015, Ethiopia made significant strides in drastically reducing the incidence of malaria through the comprehensive implementation of control strategies and the implementation of the elimination programme. In contrast, this result is higher compared to previous study conducted in Ethiopia (118, 363). In this study, the RDT-based result is comparable to a recent finding by Zerdo *et al* (348) in asymptomatic schoolchildren in southern Ethiopia and lower than that of in the DRC (357), Kenya (364), Ghana (359) Similarly, the PCR result in this study is lower than the study reported in Ethiopia (118, 363), Uganda (120, 365) Ghana (272, 366), DRC (357), Cameron (358), Tanzania (361) and India (362). However, it is higher compared to a study conducted by Tadesse *et al.*(367) on school-age children in Ethiopia. These differences appear to be due to differences in host immunities, parasite densities, and differences in studies conducted in different malaria transmission settings.

Furthermore, the present study identified several risk factors for asymptomatic malaria infection in schoolchildren, which may help malaria control programmes focus on targeted interventions. Proceeding sequentially, anemic schoolchildren were at a significantly higher risk of having asymptomatic malaria infection, which is consistent with reports from previous studies (267, 347, 368). A possible explanation for this might be that an increased risk of anemia can lead to a transition from asymptomatic to severe malaria. Anaemia weakens the immune system, makes it harder for the body to fight off malaria parasites, and leaves the children more susceptible to serious infections. Similarly, children whose household heads were older than 55 years had a higher risk of developing asymptomatic malaria. As a result of the lack of access to diagnosis and treatment, malaria in children is influenced by the ages of the household heads (369). Additionally, adults are more likely to be exposed mosquito bite (370) as older people tend to spend more

evening hours active, unprotected by ITNs and occasionally engaged in nighttime outdoor activities, increasing the likelihood of exposing children to malaria-transmitting mosquito bites (371, 372).

One striking result of this study is that children who had a family history of malaria were 98% less likely to be infected with malaria infection than those with no history. This is mainly due to the fact that children who have a family history of malaria are more likely to have been exposed to the disease in the past, this exposure helps them build up their acquired immunity and makes them less likely to contract the disease (373). In addition, once experiencing the disease, families would likely to take preventive measures to protect their children. It was noticed that schoolchildren living in households without ITN were more likely to contract malaria than those living in households with ITN, consistent with a previous study (347, 356). The use of ITN is one of the effective ways to fight against malaria; however, studies show that this age group uses bed nets less frequently than the rest population (273) and benefits the least from universal malaria interventions (142). It should be noted that according to the Gomma district Malaria Control department (unpublished data), the distribution of ITNs had been suspended prior to the start of this study (2018-2021) and the study presents here only 157 households using an older ITN. It's interesting to note that children whose families had not used IRS in the previous 3-12 months had a higher risk of having asymptomatic malaria than those whose families had used the IRS in that time. Likewise, school children who lived in a house where there was standing water nearby were at odds of the infection than those who did not live in such houses. This is supported by a study conducted in northwest Ethiopia (374, 375). It could be due to the fact that such water bodies provide ideal mosquito breeding grounds, increasing the risk of children being bitten by mosquitoes carrying the malaria parasite (376).

However, this finding contradicts with a systematic review and meta-analysis of asymptomatic malaria in Ethiopia, which found no statistically significant association between stagnant water and asymptomatic malaria infection (377). In contrast to earlier reports (347, 378, 379), this study found no statistically significant association between schoolchildren's sex and asymptomatic malaria infection. However, it seems that male children had more infections than female children. This disparity in malaria infections could be attributed to gender roles related to labor division and hormonal influence(380). In experimental studies, male mice developed testosterone-linked

susceptibility to and estrogen-linked resistance to malaria after infecting malaria-free mice (381, 382), as well as immunological differences (383), genetic factors, behavioral factors, and sleeping patterns (372). This study, however, has certain limitations that should be taken into consideration. While this study provides valuable insights into the association between predictor variables and asymptomatic malaria infection schoolchildren, it is important to note that it is a cross-sectional study. This means that the data on anaemia and malaria were collected simultaneously, making it impossible to ascertain a cause-effect relationship between the two conditions. Therefore, readers should interpret these findings with caution.

4.5 Conclusion

The current study revealed that apparently healthy schoolchildren were potential hotspot malaria infection reservoirs. The study also showed that infection was more common in male children than females and that both RDT and microscopy missed a significant portion of infection which tested positive with qPCR. The study also uncovers a multitude of factors associated with increased risks of apparently healthy schoolchildren to asymptomatic malaria. Hence, it was revealed that anaemia in children, the age of household heads, their nighttime outdoor activities, and the presence of stagnant water around housed all play pivotal roles in this phenomenon. Moreover, the study highlights the significance of a family history of malaria and the absence of ITNs as crucial contributors to this alarming issue. By shedding light on these interconnected factors, this study provides invaluable insights into the intricate web of connections associated with the increased risks of asymptomatic malaria in schoolchildren.

Inclusion of schoolchildren and schools in the malaria intervention package would be the most effective strategy for the success of malaria control and elimination programme. Given that asymptomatic malaria infected schoolchildren are the source of persistent infections, targeted intervention in conjunction with identified risk factors provides an additional opportunity to strengthen the National Malaria Control Programme. Hence, to ensure the elimination goal, such new interventions are strongly recommended to support and improve existing measures and it has the potential to provide both immediate and long-term benefits to schoolchildren. The authors also strongly recommend using molecular tools to screen for low-density parasitaemias in the malaria elimination programme.

CHAPTER FIVE: DIAGNOSTIC PERFORMANCE OF NxTek™ ELIMINATE MALARIA-PF TEST FOR THE DETECTION OF PLASMODIUM FALCIPARUM IN SCHOOL CHILDREN WITH ASYMPTOMATIC MALARIA

(Adapted from Biruksew *et al.* (2023))

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Diagnostic performance of NxTek™ Eliminate Malaria-Pf test for the detection of *Plasmodium falciparum* in school children with asymptomatic malaria

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Abstract

Background: One of the major roadblocks to the falciparum malaria elimination programme is the presence of a portion of the population, such as school children, with asymptomatic malaria infection. Targeting such reservoirs of infections is critical to interrupting transmission and enhancing elimination efforts. The NxTek™ Eliminate Malaria Pf test is a highly sensitive rapid diagnostic test (hsRDT) for the detection of HRP-2. However, knowledge gaps exist in Ethiopia on the diagnostic performance of hsRDT for the detection of *Plasmodium* falciparum in school children with asymptomatic malaria.

Methods: A school-based cross-sectional study was conducted from September 2021 to January 2022 on 994 healthy school children (aged 6–15 years). Finger-pricked whole blood samples were collected for microscopy, hsRDT, conventional RDT (cRDT or SD Bioline Malaria Ag Pf/P.v),

and QuantStudio™ 3 Real—Time PCR system (qPCR). The hsRDT was compared to cRDT and microscopy. qPCR and microscopy were used as reference methods.

Results: The prevalence of *P. falciparum* was 1.51%, 2.2%. 2.2% and 4.52%, by microscopy, hsRDT, cRDT and qPCR, respectively. Using qPCR as reference, the sensitivity of hsRDT was higher (48.89%) than the microscopy (33.3%), and showed 100% specificity and a positive predictive value (PPV). Microscopy showed similar specificity and PPV as hsRDT. Using microscopy as a reference, the diagnostic performances of both hsRDT and cRDT were similar. Both RDTs demonstrated identical diagnostic performances in both comparison methods.

Conclusions: hsRDT has the same diagnostic performance as cRDT but improved diagnostic characteristics than microscopy for detection of *P. falciparum in* school children with asymptomatic malaria. It can be a useful tool for the national malaria elimination plan of Ethiopia.

Keywords: *NxTek™ Eliminate Ag Pf, hsRDT, qPCR, Asymptomatic falciparum malaria, School children, Ethiopia*

5.1 Background

Malaria continues to be a major public health problem putting nearly half of the world's population at risk despite years of significant rollback efforts (384, 385). A total of 241 million cases were reported from 85 endemic countries in 2020, the number of cases rose to further 14 million with mortality rose from 534,000 to 602,000 compared to 2019, with the most dominant species of these cases being attributed to *P. falciparum* (384).

In Ethiopia, malaria is a major public health problem with approximately 60% of the population is at risk (386). The peak transmission occurs from September to December after the main rainy season (June–August), while the minor one is from March to June every year after a rain shower, and is typically endemic below an altitude of 2000 m above sea level. As a result, it is often characterized by localized and broader epidemic cycles (165, 387, 388).

Over the past decade, Ethiopia has committed to strengthened malaria control efforts and significant progress has been made in reducing malaria-related morbidity and mortality. In line with the Global Technical Strategy on Malaria 2016–2030 (GTS), the country adopted a National Malaria Elimination Roadmap (later launched as National Malaria Strategic Plan 2021–2025) in 2016, envisaging a malaria-free Ethiopia by 2030 (164, 165). To achieve the vision, the country's key elimination strategies are to use conventional malaria diagnostic tools such as microscopy and malaria rapid diagnostic tests (RDTs), vector control, case treatment with artemisinin-based combination therapy (ACT), and use of a gametocytocidal agent, such as a single dose of primaquine (165). However, a significant portion of the population is infected with *P. falciparum* malaria parasite, but is asymptomatic and is, therefore, invisible to the healthcare system and serves as a source of persistent transmission (131). Among this segment of the population are a large number of asymptomatic school-age children, who have not received much attention and have benefited the least from universal malaria intervention (142, 274, 327). It is one of the major challenges facing the national malaria eradication programme. The identification and targeting of such transmission reservoirs have major implications for the program. Currently being used successfully for clinical malaria diagnosis and immediate treatment, but are inherently unable to detect such a large population, which is suboptimal, allowing for sustained transmission of the disease to mosquitoes. For example, the limit of detection (LOD) for microscopy is 50–100

parasites/µL blood, reportedly missing 20–50% of infected individuals (129), while most conventional cRDTs fail to detect malaria when parasitaemia falls below the LOD 100–200 parasites/µL in asymptomatic carriers (336), leading to misdiagnosis and clinical decisions.

On the other hand, among the highly sensitive molecular tools for detecting low-density parasitaemias include the loop-mediated isothermal amplification method (LAMP) (213), conventional polymerase chain reaction (PCR), nested PCR and multiplex real-time PCR (215). They are also important tools for detecting *P. falciparum* in areas where HRP2/3-based malaria RDTs are reported to be circumvented (387, 389, 390). Unfortunately, these tools are expensive, require highly skilled personnel and a continuous power supply (391)], and are only operated in research settings (215).

Therefore, there is an urgent need for a malaria RDT that is highly-sensitive to detect low-density parasitaemias, quality-assured, and easy to use in remote settings with limited resources (335, 392). Recent scientific recommendations also underlined the need to use such RDTs to identify asymptomatic infection reservoirs to achieve the goal of malaria elimination (393). In this regard, the NxTek™ Eliminate Malaria Ag Pf [formerly Alere ultra-sensitive Malaria Ag Pf RDT/Abbott Diagnostics Inc., Republic of Korea (05FK140)], is an hsRDT developed to detect *P. falciparum* HRP2 (394) in low-density infections. It has been prequalified by the World Health Organization (WHO), confirming its performance in detecting clinical malaria infections (parasitaemia > 200 parasites/µL) and the evaluation of this kit in population with asymptomatic malaria has not been validated (395). A systematic review was conducted on its field performance revealing the hsRDT had higher sensitivity than cRTDs (396). Few studies conducted field evaluation of the kit in Uganda and Myanmar in detecting HRP2 among malaria infected individuals including asymptomatic population in varying geographical settings showed superior sensitivity of the hsRDT over conventional malaria diagnostic tools (264, 293, 396).

In the Gomma District of the Jimma Zone in Ethiopia, microscopy and SD Bioline Malaria Ag Pf (HRP2/ Pv pLDH) are the routinely used malaria diagnostic tools. In Ethiopia, hsRDT has never been used as malaria diagnostic tool and data on the extent of asymptomatic malaria in school children is scarce. So far, no work has been published on the diagnostic performance of the hsRDT in detecting *P. falciparum in* school children with asymptomatic malaria in Ethiopia.

This study aimed to evaluate the diagnostic performance of the NxTek™ Elimination Malaria Pf Ag in detecting *P. falciparum malaria* in asymptomatic school children in Gomma District, Jimma Zone, Ethiopia.

5.2 Methods

5.2.1 Study setting and design

A school-based cross-sectional study was conducted as part of the main project entitled "Asymptomatic malaria and its determinants in school children in Gomma district, Jimma zone, Oromia, Ethiopia". The study site was 395 km southwest of Addis Ababa, Ethiopia and about 45 km west of Jimma town, the capital of the Jimma Zone (Fig. 5. 1). It is also located at 7 49 59.99 north latitude and 36 39 59.99 east longitude and at an altitude of 1,380–1,680 m above sea level. The district consists of 42 administrative villages (known as Ganda, the lowest administrative level). Of the 80 schools in the district, five are high schools (9–12 grade) and 75 are primary schools (1–8 grade). There are 49 health facilities in the district. There are also 86 active health workers in the district [Jimma Zone Health Department, unpublished data]. The district receives rain all year-round and is endemic to malaria, primarily caused by *P. falciparum (*unpublished, Jimma Zone Health Department unpublished data). Malaria is high immediately after the main rainy season and then gradually declines thereafter. Only two *Plasmodium* parasite species are prevalent in the area, with the dominant species *P. falciparum followed* by *P. vivax*. Malaria prevention and control in the district is active. According to malaria stratification of the country (164), Gomma is classified as an area of low malaria transmission based on the Annual Parasite Incidence (API), i.e., API > 5 & < 10. Depending on the parasite species, age and situation of the patient, primaquine is administered as a radical cure and transmission blocking, respectively.

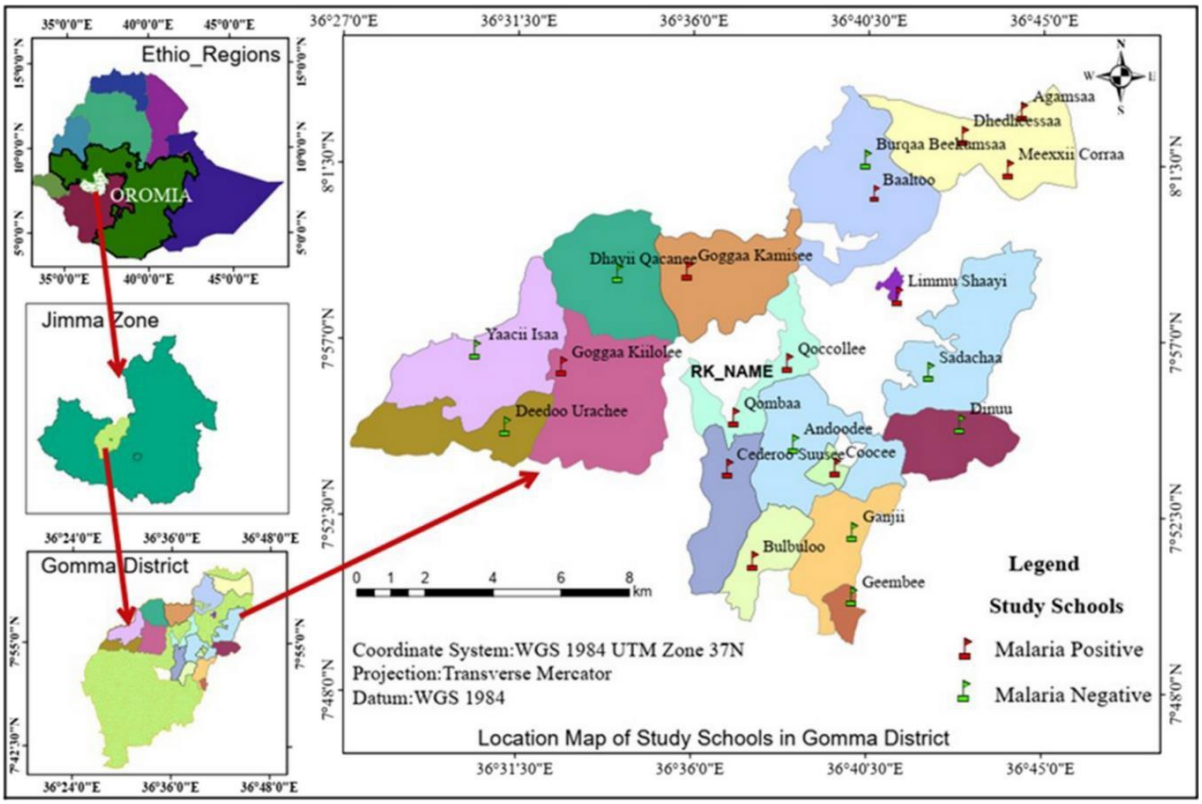


Figure 5. 1: Map of study area and schools in Gomma district, Jimma Zone.

5.2.2 Sample size and sampling technique

The sample size was calculated based on single population proportion formula:

$$n = \frac{(z_{\frac{\alpha}{2}})^2 \, p(1-p)}{d^2}$$

where n = the sample size, z = 1.96 at 95% Confidence Interval (CI), d is the margin of error at 2% (to maximize sample size since asymptomatic malaria is a rare infection), p is the expected prevalence rate for falciparum malaria among asymptomatic school children in the district was 6.8% (264). Therefore, the calculated sample size (n) became 608. A non-response rate of 10% was added, resulting in 668. Finally, a design effect of 1.5 was applied to estimate the total sample of 1002 school children included in this study. In this study, multi-stage sampling technique was used. That is, Gomma district was randomly selected as the primary sampling unit from the list of 21 districts in Jimma zone. Of the 75 primary schools (1st through 8th grade), five were not accessible, so 21 were also selected as secondary sampling units from the list of 70 schools. The

sample size was then distributed proportionally to the individual primary schools and then to the individual grades and sections. Finally, each school child was selected as the final sampling unit using the lottery method from lists of student records (rosters) and then stratified by age (6–15 years) and grade level (1–4 and 5–8) according to methods described by Brooker et al. (275) and previous works (264, 340).

5.2.3 Inclusion and exclusion criteria

A school child aged 6–15 years whose family/guardian consented for and was not taking anti-malarial medication and had an auxiliary body temperature of < 37.5 °C included in the study. School children who were on anti-malarial medication within 1 month of recruitment, had a fever at the time of data collection, and were older than 15 years were excluded from the study.

5.2.4 Demographic data and blood sample collection

Following collecting demographic data (age sex, grade level and place of residency), finger-pricked whole blood samples were obtained from each study participant at each study school. After labeling with each participant's identification code (ID), 5 µL of blood was used for field detection of *P. falciparum* with hsRDT and cRDT and testing was performed according to the manufacturer's instructions (292). For microscopic examination, a labelled slide with frosted ends was used for both thick and thin blood smear preparation, air-dried and alcohol-fixed, and transported to the Medical Parasitology Laboratory at Jimma University, Institute of Health. Four drops of blood were also collected on Watmann filter paper-2 as dried blood spots (DBS), and stored at −20 °C in individual sterile plastic bags for PCR analysis.

5.2.5 Microscopic examination

Blood smears were stained with 10% buffer diluted Giemsa for 10 min and examined through a 1000X oil immersion objective. Species identification and parasite density were determined after slide reading by experienced laboratory technologists (who have experience in malaria microscopy as senior technicians at Jimma University), and each negative slide was declared after 100 fields were thoroughly examined (397). Asexual blood stage parasite counts (density) were determined from a thick blood smear using the standard WHO formula (341) as follows:

$$Parasites\ /\ \mu L\ of\ \ blood = \frac{No\ of\ asexual\ parasite\ couted * 8000\ White\ Blood\ Cells/\mu L}{No\ of\ White\ Blood\ Cells\ counted}$$

That is, the parasite count was performed against 500 white blood cell (WBC) counts, assuming a mean WBC count of 8,000/µL. Quality control was carried out at every step of the process. Each blood smear was read independently by two experienced microscopists, each blinded to the other reader's results. Discordant results were resolved by a third expert microscopist who was blinded to the readings of the first two microscopists. And all the data collectors took training to ensure quality of the data as the study flowchart was followed (figure 5.2).

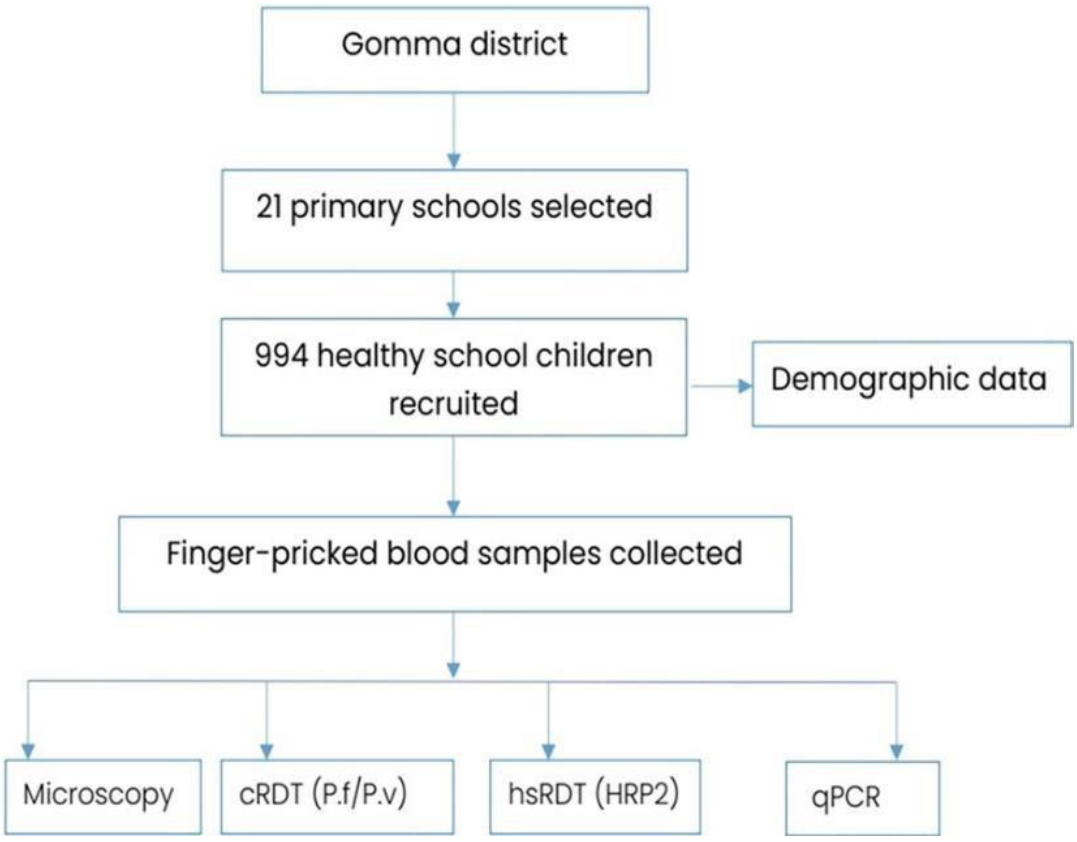


Figure 5. 2: Study flowchart of diagnostic performance of hsRDT in school children with asymptomatic malaria in Jimma zine, Southern Oromia, Ethiopia

5.2.6 NxTek™ Eliminate Malaria Pf Ag test (hsRDT)

NxTek Eliminate Malaria Pf is an hsRDT (Product Code 05FK140, Lot No. 05LDF009F, Expiry Date 22 Apr 01, manufactured by Abbott Diagnostics Korea Inc.) (395)], a two-band test and a qualitative and differential test for the detection of *P. falciparum* HRP2 in human whole blood.

5.2.7 The conventional RDT (cRDT)

SD Bioline Malaria Ag (Pf- HRP2 and Pv-pLDH) is a triple band (one for *P. falciparum HRP*2, one for *P. vivax* pLDH and a control band), manufactured by Standard Diagnostics Inc. (now Abbot Diagnostic Korea Inc.), LOT number: 05DDF011A and product code 05fk80, Korea), developed for routine diagnosis of both *P. falciparum HRP*2 and pLDH of *P. vivax*, was used as a comparator. The test provides results within 15 min and is routinely used in remote healthcare facilities in Jimma zone, Ethiopia.

5.2.8 The molecular assay

Approximately 3–5 mm diameter pieces of DBS were cut with a round hole puncher into a labeled 1.5 ml Eppendorf tube. Parasite DNA was then extracted from DBS using the Chelex100 resin saponin method as previously described **(**398**)** and the purified DNA was then transferred to Nunc tubes (0.5 mL) and stored at −20 °C until used for the PCR assay**.** For this PCR assay, the TaqMan probe for species-specific detection of *Plasmodium* parasite was used. QuantStudio™ 3 Real-Time Multiplex PCR system (Thermo Fisher Scientific) was operated to amplify the 18S rRNA genes for detection of *P. falciparum and P. vivax* using a pair of forward and reverse primer sequences and probes. For *P. falciparum* specific primers (5'-3') (344):

F-F (forward): TATTGCTTTTGAGAGGTTTTGTTACTTTG and.
F-R (Revers): ACCTCTGACATCTGAATACGAATGC. And probe used was: Pf-fam (MGB): ACGGGTAGTCATGATTGAGTT. For *P. vivax* specific primers used (344) was:
Pv-1 (forward): CGCTTCTAGCTTAATCCACATAACTG, and Pv-2 (Reverse): AATTTACTCAAAGTAACAAGGACTTCCAAG. And the probe used was: Pv-probe (VIC-MGB): CGCATTTTGCTATTATGT.

For quality control, DNA from *P. falciparum and P. vivax* isolates (Pf and Pv MR4(BEI)) were used as positive controls, while molecular grade water was used as a negative control. In brief, PerfeCTa® qPCR ToughMix® (Low ROX™, Quanta Bio vwr, catalog number: 97065–968) was used as qPCR master mix. Briefly, PCR amplification was performed in a total reaction volume of 12 µL which contained 6 µL of PerfeCTa master mix, 0.5 µL (× 2) each of probe for *P. falciparum (*Pf-Fam) and *P. vivax* (Pv-vic), 0.4 µL (× 4) each of the forward and reverse primers, 2 µL extracted DNA and 1.4 molecular grade water under the PCR cycling conditions for 45 cycles. The initial denaturation was 50 ºC for 2 min, followed by 45 cycles of amplification at 95 ºC for 2 min, 95 ºC for 2 s and 60 ºC for 30 s with the total run-time of 60 min.

5.2.9 Data analysis

Data were entered using Epi-data version 3.1, exported to Statistical Package for Social Sciences (SPSS) version 26, and analyzed. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy were first determined against microscopy. This because of microscopy is the gold standard for malaria diagnosis. Then, against the qPCR due to its higher sensitivity to detect low-density parasitaemia. In all parameters, 95% CI was taken. Kappa values were used to assess the agreement between malaria diagnostic methods. The Chi-Square test was used to assess the association between characteristics of children and the positivity of each malaria diagnostic tool. A p-value of 0.05 was considered statistically significant.

5.2.10 Ethics statement

Ethical approval was obtained from the Jimma University Institute of Health Research and Ethical Review Committee/Internal Review Board (IRB) (Ref: IHRPED/550/2). In addition, letters of permission were obtained from the Gomma District Health and Education Departments and presented for selected primary schools and local health departments. Information on the purpose, potential risks and benefits of the study was well addressed to each school child's family/guardian and presented in their own language. Fingerstick blood samples were taken after obtaining written informed consent from the parents or legal guardians of each school child. All malaria-positive children were treated at a nearby health facilities in accordance with the Federal Ministry of Health's malaria treatment guidelines after sending cover letters stating their name, age, gender,

school and grade level. Confidentiality of each study participant's data was maintained throughout this project.

5.3 Results

5.3.1 Characteristics of the study participants

A total of 1002 healthy school children included in the study and eight were excluded after field works based on exclusion criteria (for anti-malarial drug treatment), resulting in a total of 994 participants with normal axillary body temperature (< 37.5 °C) completing the study with the response rate is 99.2%. Of 994 school children who completed the study, as presented in Table 5. 1, 52.4% (521/994) of them were males and 47.6% (473/994) were females with a mean age of 11.54 (± 2.5 std). The majority of students (77%) were from rural areas, while 23% were from semi-urban areas.

Using qPCR as a reference method (Table 5. 3). The sensitivity of the hsRDT was 48.89% (95% CI 33.70, 64.23) and that of the microscopy was 33% (95% CI 33.33 (20; 48.95)]. The hsRDT demonstrated similar PPV 100% (95% CI 85.00, 100) and specificity [100% (95% CI 99.61, 100)] with microscopy [specificity 100% (95% CI (99.61, 100) and PPV of 100% (95% CI 79.60, 100)]. In contrast, it achieved a better NPV [70.51% (95% CI 64.25; 76.09) than the microscopy [64.71% (95% CI 59.86; 69.27)]. The RDT showed good level of agreement with results of qPCR at kappa value 64% (95% CI 51.25, 77.99). The microscopy on the other hand showed moderate level of agreement with qPCR results [kappa = 49% (95% CI 33, 64.3). The overall probability that a school child with asymptomatic malaria was correctly classified (test accuracy) by the new kit was 74.4% (95% CI 74.26, 79.58) than microscopy [66.7% (95% CI 67.04, 72.84).

Table 5. 1: Malaria diagnostic tools and characteristics of the participants

			Malaria diagnostic tools							
			Microscopy		**hsRDT (pfHRP2)**		**cRDT (PfHRP2)**		**qPCR**	
		Total No.	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Characteristics		994	15	979	22	972	22	972	45	949
Sex	Male	521 (52.40)	12 (80.00)	509 (52.00)	18 (82.00)	503 (51.70)	18 (82.00)	503 (51.70)	29 (64.40)	492 (51.84)
	Female	473 (47.60)	3 (20)	470 (48.00)	4 (18.00)	469 (48.30)	4 (18.00)	469 (48.30)	16 (35.60)	457 (48.16)
P-value			0.030*		0.005*		0.005*		0.61	
Age	5 - 11	463 (46.60)	7 (46.70)	456 (46.58)	12 (54.50)	451 (46.40)	12 (54.50)	451 (46.40)	22 (48.90)	441 (46.47)
	12 - 15	531 (53.40)	8 (53.33)	523 (53.42)	10 (45.50)	521 (53.60)	10 (45.50)	521 (53.60)	23 (51.10)	508 (53.53)
P-value			0.99		0.44		0.44		0.7507	
Grade level	1-4	561 (56.40)	8 (53.33)	553 (56.50)	13 (59.10)	548 (56.40)	13 (59.10)	548 (56.40)	24 (53.30)	537 (56.59)
	5 - 8	433 (43.60)	7 (46.67)	426 (43.50)	9 (41.90)	424 (43.60)	9 (41.90)	424 (43.60)	21 (46.70)	412(43.41)
P- value			0.8070		0.79		0.79		0.66	
Residence	Rural	765 (77.00)	13(86.70)	752 (76.80)	17 (77.27)	748 (77.00)	17 (77.27)	748 (77.00)	35 (77.80)	730 (77.00)
	Semi-urban	229 (23.00)	2 (13.30)	227 (23.20)	5 (22.73)	224 (23.00)	5 (22.73)	224 (23.00)	10 (22.20)	219 (23.00)
P- value			0.36		0.97		0.97		0.89	

Legend: +ve: Positive, -ve: negative, qPCR: quantitative Polymerase Chain Reaction, X^2**- test:** Chi-squared test, Total No.: Total number; * Statistically significant if the P value for a chi-square test is less than 0.05.

5.3.2 The diagnostic performances of malaria RDTs

Using microscopy as a reference method, the diagnostic performances of the hsRDT were identical with the cRDT (Table 5. 2). Accordingly, the sensitivity of the RDTs was 100% (95% CI 78.20, 100) and their specificity was 99.28% (95% CI 98.53, 99.71). It also demonstrated a PPV of 68.20 (50.62, 81.77) and an NPV of 100% (95% CI (99.60, 100). The RDTs also showed a strong agreement with results of microscopy at kappa value 81% (95% CI 67.00, 94.69). Thus, the overall probability of the RDTs to correctly classifying a school child with asymptomatic malaria (accuracy) was 99.30% (95% CI 98.55, 99.72).

Table 5. 2: Diagnostic performance of malaria RDTs using microscopy as a reference

		Microscopy								
Malaria RDTs		+ve	-ve	Total	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Kapa (95% CI)	Accuracy (95% CI)
hsRDT	+ve	15	7	22	100 (78.20, 100)	99.28 (98.53, 99.71)	68.20 (50.62, 81.77)	100 (99.60, 100)	81 (67.00, 94.69)	99.30 (98.55, 99.72)
	-ve	0	972	972						
Total		15	979	994						
cRDT	+ve	15	7	22	100 (78.20, 100)	99.28 (98.53, 99.71)	68.20 (50.62, 81.77)	100 (99.60 100)	81 (67.00, 94.69)	99.30 (98.55, 99.72)
	-ve	0	972	972						
Total		15	979	994						

+ ve positive, -ve negative, PPV: Positive predictive value, NPV: Negative predictive value

Using qPCR as a reference method (Table 5. 3). The sensitivity of the hsRDT was 48.89% (95% CI 33.70, 64.23) and that of the microscopy was 33% (95% CI 33.33 (20; 48.95)]. The hsRDT demonstrated similar PPV 100% (95% CI 85.00, 100) and specificity [100% (95% CI 99.61, 100)] with microscopy [specificity 100% (95% CI (99.61, 100) and PPV of 100% (95% CI 79.60, 100)]. In contrast, it achieved a better NPV [70.51% (95% CI 64.25; 76.09) than the microscopy [64.71% (95% CI 59.86; 69.27)]. The RDT showed good level of agreement with results of qPCR at kappa value 64% (95% CI 51.25, 77.99). The microscopy on the other hand showed moderate level of agreement with qPCR results [kappa = 49% (95% CI 33, 64.3). The overall probability that a school child with asymptomatic malaria was correctly classified (test accuracy) by the new kit was 74.4% (95% CI 74.26, 79.58) than microscopy [66.7% (95% CI 67.04, 72.84).

Table 5. 3: Diagnostic performance of malaria RDTs using qPCR as a reference method

Malaria diagnostic tools		qPCR			Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Kapa (95% CI)	Accuracy (95% CI)
		+ve	-ve	Total						
hsRDT	+ve	22	0	22	48.89	100	100	70.51	64	74.4
	-ve	23	949	972	(33.70, 64.23)	(99.61, 100)	(85.00, 100)	(64.25, 76.09)	(51.00, 78.00)	(74.26, 79.58)
Total		45	949	994						
cRDT	+ve	22	0	22	48.89	100	100	70.51	64	74.4
	-ve	23	949	972	(33.70, 64.23)	(99.61, 100)	(85.00, 100)	(64.25,76.09)	(51.00, 78.00)	(74.26, 79.58)
Total		45	949	994						
Microscopy	+ve	15	0	15	33.33	100	100	64.71	49 (33.00, 64.30)	66.7
	-ve	30	949	979	(20, 48)	(99.61, 100)	(79.60,100)	(59.86,69.27)		(67.04,72.84)
Total		45	949	994						

Legend: +ve – positive; -ve – negative; PPV: positive predictive value; NPV: negative predictive value

5.3.3 Prevalence of asymptomatic *P. falciparum* malaria in schoolchildren using qPCR, RDTs and microscopy

Detection rates of asymptomatic *falciparum* malaria varied depending on the method used (Table 5. 1). The overall prevalence of the infection was 4.52% (45/994) as determined by qPCR, of which 2.2% was detected by hsRDT/cRDT and 1.51% by microscopy. Across all malaria diagnostic tools, high infections were observed in males, grades 1–4, and children living in rural areas. There was statistically significant association between malaria positivity by conventional diagnostic tools (microscopy and RDTs) and sex of children ($p < 0.0.05$). Despite both RDTs had matching results, few cRDTs showed more fainter positive band lines than hsRDT (Fig5. 3). On the other hand, the cRDT did not detect *P. vivax* in the current study population.

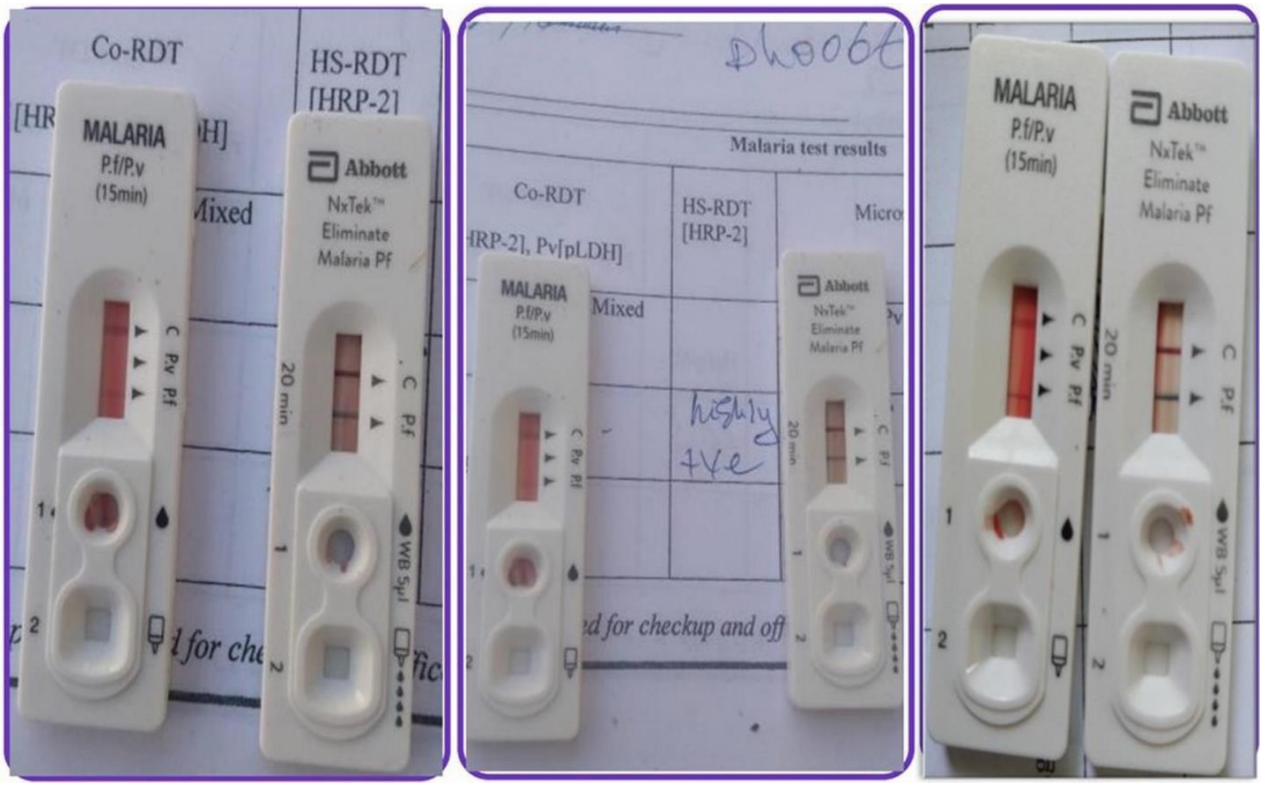


Figure 5. 3: Plate 1 showing the differences in band line positivity of hsRDT and cRDT for detection of *P. falciparum HRP*2 from whole blood for detection of asymptomatic malaria of different study participants.

The Venn diagram (Fig. 5. 4) shows a summary of the overall positive intersections of the malaria diagnostic methods used in the current study. Of the 22 cases identified by hsRDT, microscopy missed seven as false negatives. Likewise, 67% of the 45 cases detected by qPCR were submicroscopic malaria infections, as microscopy missed 30 of them as false negatives. On the other hand, the hsRDT- based detection shows a false negative rate of 51.1% (23/45). These results reflect the superior advantage of the hsRDT over microscopy for diagnosing *P. falciparum malaria*, and a silently ongoing transmission of the disease among children in the study area.

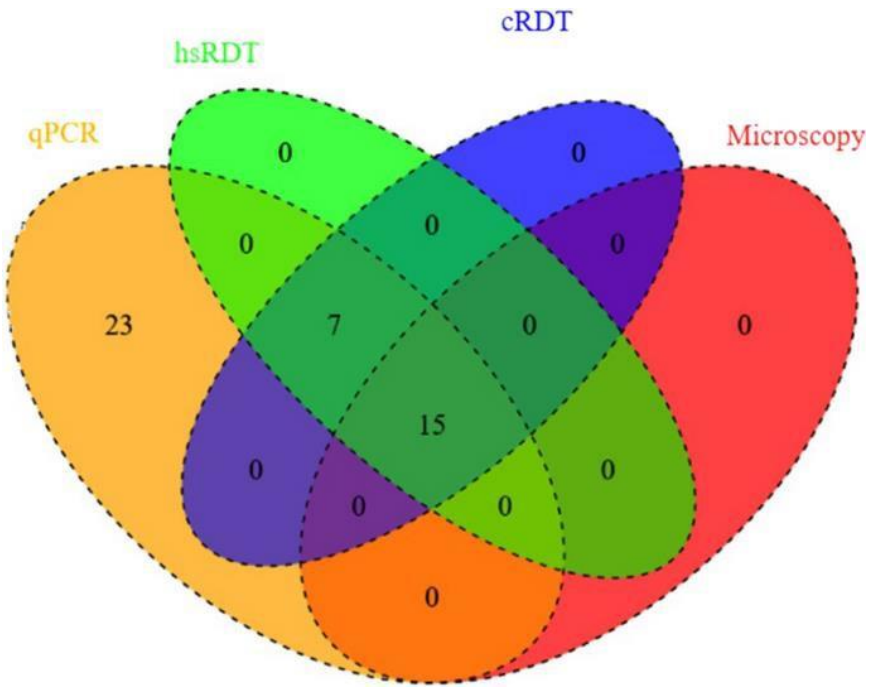


Figure 5. 4: Venn diagram showing *P. falciparum positivity* intersections or unique to the malaria diagnostic tool(s) in school children with asymptomatic malaria infection.

Asexual parasite density was determined using microscopic examination of Giemsa-stained thick blood smears using the microscope and ranged from 64 to 8080 parasites/µL (Table 5. 4).

Table 5. 4: Distribution of asymptomatic *P. falciparum density* by sex, age group, grade level, and place of residence

Characteristics		Parasite density /µL					
		< 200	201 - 500	501- 2000	2001 - 5000	5001 - 9000	p-value
Sex	Female	2	1	0	0	0	0.504
	Male	3	2	3	3	1	
Age (in years)	6-11	2	1	2	1	1	0 .710
	12 –15	3	2	1	2	0	
Grade level	1 – 4	2	1	2	2	1	0 .710
	5 – 8	3	2	1	1	0	
Residence	Semi-urban	2	0	0	0	0	0 .329
	Rural	3	3	3	3	1	
Altitude (masl)	<1600	2	1	3	1	0	0.306
	≥1600	3	2	0	2	1	

Statistically significant when p-value the chi square (X^2) test is < 0.05.

5.4 Discussion

As the Global Technical Strategy on Malaria 2016–2030 envisions a malaria-free world by 2030 with the aim of a 90% reduction in the global malaria burden (161) is approaching, the role of HRP2-based malaria RDT is paramount in monitoring and steering the success of the Strategy. The current study evaluated the field performance of the hsRDT for the detection of *P. falciparum HRP*2 antigen in whole blood from healthy school children with asymptomatic malaria. The results of this study confirmed the high sensitivity (100%) and specificity (99.28%) of hsRDT using microscopy as a reference method, which is consistent with the WHO prequalification report (395). However, the study participants in the report were symptomatic patients, and venipuncture blood was used for the analysis, as opposed to finger-prick blood samples collected from healthy school children in this study. The higher PPV (96.1%) and NPV (100%) in this this study is comparable with the study conducted in Saudi Arabia (399)] and Kenya (400)].

When qPCR was used as a reference, the hsRDT had sensitivity (48.89%) than that of microscopy (33.3%) and same specificity and PPV (100%). It also demonstrated a higher NPV (70.51%) and accuracy (74.4%) in correctly classifying children with asymptomatic malaria infection. The lower sensitivity in the current study is compared to the manufacturer's report (292, 395, 396) though they used a composite sensitivity (microscopy + qPCR). The difference could be attributed to variations in study population, source of specimen, and disease status. The sensitivity of hsRDT is also lower compared to a study conducted in western Kenya (60.3%) in asymptomatic school-age children (296). However, higher specificity and PPV were observed in the current study. The sensitivity of hsRDT in this study is also lower compared to a recently reported meta-analysis which estimated a pooled sensitivity at 56.1% in asymptomatic population and higher than that of cRDT (44.3%) in the report (293). A similar performance of the hsRDT as cRDT in this study is consistent with those from Mozambique and Uganda (401) where the diagnostic performances of uRDT and cRDT are comparable. A study from Uganda and Myanmar (402) using samples from the general population with asymptomatic malaria reported that hsRDT showed a tenfold improvement in detecting HRP2 compared to the cRDT. On the other hand, Landier and colleagues (295) in East Myanmar reported that the sensitivity of hsRDT was two-fold higher than that of cRDT. The sensitivity of hsRDT in our study is slightly higher than that performed in a low-transmission setting in Myanmar (44%) and lower than that performed in a high-transmission setting in Ugandan children (84%). However, the new kit showed higher specificity (100%) than the Uganda report (95%) and is consistent with a report from Myanmar. Interestingly, the same study reported that the cRDT had a sensitivity of 62% in Uganda and 0% in Myanmar(402). Existing evidences support these contradicting results, that might be attributed to differences in type and sensitivity of qPCR sensitivity (403) and parasite density and transmission settings (404). It is also important to recall that, mosquito bites are usually rare in low transmission settings, so that the parasite's main strategy is successful transmission with only a few bites, while the it becomes non-virulent (89, 353). However, in high transmission settings, the mosquito bite is so frequent and the priority of the parasite is surmounting the host immunity (353).

The outperformance of the new kit over the established method suggests that it can be considered a replacement for microscopy in a resource-poor rural setting. The results of the current study confirmed that the new kit offers no added value over the existing malaria RDT in the study

population. However, available evidences indicate that the performance of HRP2- based malaria RDTs could be affected by the parasite density (405, 406), HRP2/3 -gene deletions (407) and the prozone effect in PfHRP2 (408). Therefore, considering these points, in this study, the real comparison between the RDTs could have been compromised by all or any of these factors. In general, given that the cost and performance of the cRDT is comparable, the hsRDT can be a useful tool to diagnose asymptomatic malaria as part of the national malaria elimination programme.

In the current study, the proportion of infections detected by the RDTs was the same (2.2%, 22/994), higher than microscopy (1.5%, 15/994). Infection was higher in male school children and those from rural areas. This could possibly be due to male boys being more likely to play outdoors than female girls, and more mosquito bites in rural than urban areas. The proportion of detection of infection by RDTs [2.21% (22/994)] in this study is lower than a study conducted in Uganda (396) in which the hsRDT detected 24.9% (546/2,192) in school-age children. A closer look at this data show that microscopy missed 31.8% (7/22) of the infected children identified by RDTs, confirming the diagnostic advantage of the RDTs over microscopy in detecting low-density malaria infections. Likewise, microscopy missed 66.70% (30/45) of infected children identified by qPCR. This reveals that asymptomatic submicroscopic falciparum malaria infection was prevalent among school children in the study area. This is reinforced by previous work by Okell and colleagues (129) who reported that microscopy misses 20–50% of malaria infections in areas of low transmission and is responsible for all human-to-mosquito transmission. Hence, the results of this study challenge the importance of light microscopy for malaria control and elimination. Likewise, both RDTs missed 51% (23/45) of infected children identified by qPCR. As explained above, this RDT-negative/PCR-positive samples could be partially explained as (1) possible prevalence of HRP2/3-gene deletions in most samples or (2) due to low concentration of circulating HRP2 antigens which could not be detected and result in false-negative results (143) and in individuals with rheumatoid arthritis, cross-reactivity of rheumatoid factors with HRP2 results in false-positive results (409). At this point it should be noted that the conventional tool-based diagnosis underestimates the actual prevalence of falciparum malaria in the study population. In all cases, it was shown that there was silent and active transmission of the infection in the area.

Although the performance of both RDTs were identical in this study**,** differences in the intensity of the HRP2 positive band lines were observed. For example, some cRDT tests showed very faint positive Pf band lines that was difficult to declare, but all were clearly visible with the new RDT**.** These differences could be attributed either to the assumption of a low concentration of circulating HRP2 antigen in asymptomatic school children or the fluoresce material used. Briefly, both tests are lateral flow immunochromatographic kits that allow for a qualitative diagnosis based on the detection of HRP2 expressed by *P. falciparum* in the blood of infected individuals(410). Available evidence shows that the limit of detection (LOD) for HRP2 by most cRDTs is estimated at 800–1000 pg/mL when the parasitaemia is 100–200/µL (411) and the visibility of bands diminishes when the parasitaemia falls below 200 p/µL (412), indicating that parasite density and HRP2 concentration are directly related.

Another plausible piece of evidence in support of this observations is a study on the analytical performance of hsRDT (413), which demonstrated better detection of HRP2 at a tenfold lower LOD than most RDTs, suggesting that the new kit detects HRP2 better than cRDT in low-density parasitaemia. So far, there are only very few empirically published reports examining the HRP2 concentration and LOD of cRDT and HSRDT. For example, Jimenez and colleagues (411) compared both RDTs for their performance on samples with known HRP2 concentrations. The authors reported that the LOD of cRDT was 800 pg/L compared to 8 pg/L LOD for uRDT. Furthermore, in this study, the true comparison between both RDTs might have been compromised by the presence of HRP2 gene deletions in most of the samples, as the qPCR result shows.

5.5 Conclusions

The current study reports the diagnostic performance of the NxTek™ Elimination Malaria Pf Ag test in detecting *P. falciparum in* school children with asymptomatic malaria in Ethiopia. The hsRDT detected 7- fold more low-density *P. falciparum malaria* infections and provides a better measure of discriminating children with and without asymptomatic malaria (diagnostic value) than the malaria gold standard tool, microscopy. On the contrary, the new kit did not outperform the conventional RDT (SD Bioline Malaria Ag Pf. P.v) in the current study. The study provides significant numbers of asymptomatic submicroscopic *P. falciparum infections*. This active and

silent transmission of malaria in school-age children is an alarm for the malaria control programme in the Gomma District of the Jima Zone for appropriate interventions. The results of the current study also underscore the importance of molecular tools for screening asymptomatic populations to achieve malaria elimination goals.

CHAPTER SIX: UNRAVELING THE SPATIOTEMPORAL CLUSTERING OF MALARIA INCIDENCE: MODELLING A DECADE OF EPIDEMIOLOGICAL DATA IN JIMMA ZONE, SOUTHWEST OROMIA, ETHIOPIA



(Adapted from Biruksew *et al.* (2024)-under review: Submission ID: 26889577-307e-4112-a93e-4c0d13610bf7).

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Abstract

Background: Malaria remains a significant public health concern in Ethiopia. Understanding the historical backgrounds and future trends of malaria incidence is crucial for customizing local specific intervention strategies. The spatiotemporal patterns and putative modeling of future trends of malaria in the Jimma Zone have not been thoroughly investigated. This study aimed to model the spatiotemporal patterns and prediction of future malaria trends in Jimma Zone, Oromia, Ethiopia, Ethiopia from 2022 to 2026**.**

Methods: This study analyzed a decade of historical malaria data (2012-2021) from 15 districts of Jimma Zone. A District-level annual parasite incidence (API) and standardized incidence ratio (SIR) were calculated, and malaria risk maps were developed. Moran's I and Monte Carlo statistics were used for assessing spatial clustering of malaria incidence, while time series analysis with the Autoregressive Integrated Moving Average (ARIMA) model was used for analyzing the temporal patterns and predicted for the future trends**.**

Results: A total of 1,718,471 individuals were screened for malaria between 2012 and 2021, with 359,655 (20.93%) confirmed cases. *P. falciparum*, *P. vivax* and mixed infections accounted for 52.71%, 43.62% and 3.67%, respectively. Malaria incidence decreased dramatically by 96.13%, with the API falling from 87.97 per 1,000 population at risk in 2012 to 3.40 in 2021. It was revealed that there was random distribution of malaria transmission across districts. API and SIR maps identified potential risk areas and cold spots. The disease exhibited seasonal patterns, peaking from August to November (maximum in September) and from March to June. Despite a decline trend, malaria remains a potential threat in areas like Chora Botor and Shebe Sonbo. Forecasting with the ARIMA (0, 1, 3) x (1, 1, 0) model for 2022-2026 suggests stability in malaria incidence, with seasonal fluctuations but no significant trends**.**

Conclusion: Malaria incidence in Jimma Zone was significantly decreased from 2012 to 2021, reaching notably low risk levels by 2021. However, the disease persists in areas such as Chora Botor and Shebe Sonbo, indicating ongoing risk of transmission. The seasonal pattern of malaria underscores the need for targeted interventions during peak periods. Spatial autocorrelation analysis showed no distinct patterns, suggesting the necessity for continued surveillance in historically high-risk areas. While forecasts suggest a stable malaria cases, maintaining vigilance

and adapting interventions based on emerging trends is essential to sustain control progress towards elimination.

Key words*: Spatiotemporal, time series, ARIMA, Moran's I spatial autocorrelation I, malaria incidence, Ethiopia.*

6.1 Introduction

Malaria, a mosquito-borne infectious disease (414), remains a major global health issue, and causing millions of cases and hundreds of thousands of deaths each year(49) The disease is caused by five *Plasmodium* species: *Plasmodium* falciparum , *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*, with *P. falciparum* and *P. vivax* being the most threatening (31). Despite notable progress in malaria control, the challenge persists. The recent World Malaria Report indicates a concerning increase in malaria cases, with 5 million more cases reported in 2022, totaling an estimated 249 million cases across 85 endemic countries. Pakistan, Ethiopia, Nigeria, Uganda, and Papua New Guinea were identified as significant contributors to the increase. Disparities remain stark, as sub-Saharan Africa bears a disproportionate burden, accounting for more than 94% of global malaria cases and 95% of deaths in 2022. Tragically, children under five represent approximately 80% of these deaths. Although malaria mortality decreased steadily from 2000 to 2019, the COVID-19 pandemic disrupted services and led to a rise in deaths in 2020(49).

Ethiopia has made significant progress in reducing malaria cases over the last decade. However, recent data reveal a troubling surge in cases, with malaria incidence increasing by 32% from 2021 to 2022, rising from 46.3 to 60.9 cases per 1,000 population at risk REF???). This increase led to over 1.3 million reported cases in 2022. While the number of malaria-related deaths remains relatively low at 180, the predominance of *P. falciparum*, which accounts for 71.3% of cases, is concerning. *P. vivax* follows with 20.9%, while mixed infections account for 1.1%, and 6.7% of cases remain unidentified(299). The country's ambitious goal to reduce malaria morbidity and mortality by 50% from 2020 levels by 2025 involves eliminating indigenous cases in districts with an annual parasite incidence (API) <10 and preventing disease re-establishment in areas previously reported as malaria-free (415). With over 27% of the population at high risk of malaria(49, 297), the disease exhibits a clear seasonal pattern with distinct temporal and spatial dynamics.

Hence, malaria transmission in Ethiopia is highly seasonal, varying significantly with altitude and climatic conditions (237, 299). Areas below 2,000 meters are particularly vulnerable. The main transmission period coincides with the end of the primary rainy season (June to August), peaking between September and December. Stagnant water bodies formed during this period provide ideal breeding grounds for mosquitoes, leading to a surge in malaria cases. A secondary, less intense

peak often occurs during the shorter rainy season from March to June(240, 299). This seasonal pattern influences both short outbreaks and larger cyclic epidemics(241).

The complex interplay between female *Anopheles mosquitoes*, human hosts and *Plasmodium* parasites defines the ecological framework of malaria transmission, which is also influenced by climate change(416). Understanding the spatial and temporal dynamics of malaria is therefore essential for developing effective control strategies. Mapping disease incidence and forecasting future patterns using historical epidemiological data are crucial for this purpose(311, 417).

Advanced statistical methodology, such as ARIMA, play a pivotal role in understanding disease progression and forecasting future trends. These models, by analyzing historical malaria epidemiological data, help decipher patterns, enhance prediction accuracy, and guide targeted interventions(418-420). Besides, studies have explored both temporal and spatial malaria patterns using various spatial analysis tools[22,23], and ultimately guided targeted interventions. Global and local Moran's I models have been employed to assess spatial autocorrelation and understand clustering, dispersion, or random distribution of malaria incidence across geographic spaces(421-424). Given the seasonality of malaria, ARIMA models offer high predictive power(425) and have been utilized to predict risks numerous infectious diseases transmission(426, 427). Additionally, ARIMA's relative simplicity and stability make it effective for forecasting malaria trends(428).

While some studies in Ethiopia examined malaria's spatiotemporal distribution, comprehensive national-level analyses is not only limited, but also focused on specific localities(310, 429-434), although few studies have employed GIS and spatial statistics to understand the temporal spread of malaria across the country. A major challenge in understanding malaria dynamics in Ethiopia, especially in Jimma zone, is the limited availability of detailed published or unpublished data on the magnitude, temporal, and spatial patterns of malaria cases. This information deficit hampers our ability to track how malaria patterns evolve over time, complicating efforts to control the disease. Additionally, research on the distinct patterns of *Plasmodium* species in the region is scarce. Compounding this issue, studies employing time series analysis to assess past trends and predict future outbreaks are hardly available. We believe that addressing these critical gaps is imperative for generating the evidence needed to inform effective malaria control and safeguard public health at large.

This study, therefore, applied both ARIMA time series analysis and Moran's I spatial autocorrelation(435) to better understand malaria series cases in Jimma Zone to unravel the temporal and spatial dependencies of the observations and make prediction of its future patterns (436).

6.2 Methods

6.2.1 Study setting

The study was conducted in the Jimma Zone, the largest zone situated in the southwestern part of the Oromia Regional State (Fig. 6.1) in Ethiopia. Located at 7°13' to 8°56' north latitude and 35°49' to 38°38' east longitude, the zone borders the East Wollega Zone (north), East and Southwest Shawa Zones (northeast), Southern Nations and People's Region (south and southeast), and Illubabor Zone (west). Administratively, it comprises 20 rural districts and 2 urban centers: Jimma town (capital of Jimma zone) and Agaro town(313). As of July 2023, the recent estimate showed that Jimma Zone has a population of 3,638,103 with nearly equal number of males and females(314). The zone had a comprehensive healthcare system, with the public health facilities comprising 8 government hospitals (5 primary, 3 general), 122 health centers, and 513 health posts supported by about 1461 health extension workers. Additionally, the private sector contributed with 3 hospitals and 156 small and medium-sized clinics (315). Beyond its administrative characteristics, Jimma Zone is known for its rich natural resources, distinct climate and biodiversity sphere. Renowned for its coffee cultivation and production within the Oromia region, the zone also receives abundant rainfall, making it one of the wettest areas in the region. Annual precipitation reaches up to 1,500 mm (60 inches), with over 100 mm (4 inches) falling monthly between April and September. Jimma also experiences year-round warmth, with temperatures ranging from 9°C to 28°C. It rarely dips below 6°C or exceeds 31°C. The warmest period, lasting from December to April, sees average daily highs surpassing 28°C(316). Communicable diseases, including malaria, account for approximately 60-80% of health concerns in the Jimma Zone. Malaria is endemic, exhibiting seasonal variations linked to Jimma's climatic factors. These factors are believed to contribute to the disease's persistence in the region. Notably, local health facilities are currently reporting characteristics of malaria outbreaks within specific districts [Jimma Zone Health Office, unpublished](317).

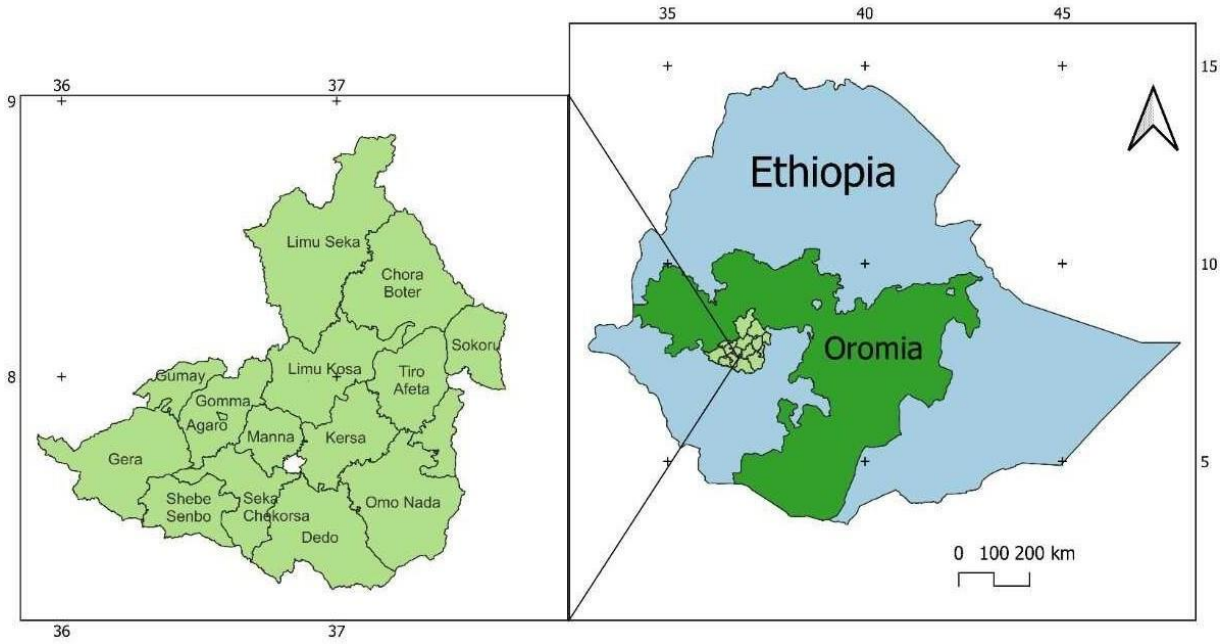


Figure 6. 1: Map of the study districts in the Jimma zone in the southwestern Oromia regional state, Ethiopia. Map created with QGIS3.36.2-Maidenhead (QGIS code revision 6d250527).

6.2.2 Study design

A retrospective analysis of ten-year malaria epidemiological data (January 2012 - December 2021).

6.2.3 Data sources

Monthly malaria case data from January 2012 to December 2021 were obtained from Jimma Zone Health Office, Disease prevention and control department. This dataset included information from all 20 districts within the zone. For each district, details such as name of the districts, year, month, total number of individuals screened for malaria (using both microscopy and rapid diagnostic testcard), the monthly confirmed positive cases, the *Plasmodium* species detected (*P. falciparum*, *P. vivax*, or mixed infections), and demographic details included (sex and age group: <5 and ≥5). These data followed the standardized malaria registry format of the zone, or Oromia region. Note that data for five districts were incomplete and excluded from the analysis, resulting in a total of 15 districts being included in the study.

Furthermore, to account for population at risk over the period, estimates of the annual total population for each district were obtained from the online repository of the Ethiopian Statistical Service population website(437). Spatial data like the polygon shapefiles of the district-level administrative boundaries of the Oromia Regional State were retrieved from the DIVA-GIS website(438). In accordance with WHO guidelines(111), a malaria case in study was defined as the presence of malaria parasites in the blood of an individual, confirmed either by microscopy or a RDT.

6.2.4 Statistical analysis

After entering and checking the data for completeness in Microsoft Excel, it was imported into R software version 4.4.1 for analysis.

Total malaria cases, trends, and seasonal analysis

To take into account the epidemiological aspects of malaria, the total malaria cases in the study districts were analyzed and the proportions of positive cases by age group and sex were also calculated. We also computed and graphed the monthly and annual proportions of malaria cases for each to examine the variation in malaria transmission over the specified time period. The number of reported malaria cases relative to the population at risk was then used to compute the annual malaria incidence.

Annual Parasite Incidence Rate (API) and Test Positivity Tate (TPR)

Based Based on the established epidemiological practices(439), this study employed two key metrics to examine the burden of the disease: annual parasite incidence (API) and test Positivity rate (TPR). Therefore, the API per 1,000 people at risk was estimated by dividing the number of confirmed malaria cases in a year by the population at risk and multiplying by 1,000 (440). The resulting value, expressed as the number of cases per 1,000 at-risk population, provides a crucial overview of the intensity of malaria transmission in the area studied. This standardized format allows comparisons of malaria burden in the study districts and is calculated as follows:

$$API = \frac{(Total\ no.\ of\ positive\ cases\ in\ a\ year)}{Total\ population} * 1000$$

Based Based on an API value of each district in a given year across time periods, we created a malaria risk map according to the current national malaria stratification (299) to identify districts with very low (API > 0-5), low (API 5-10), moderate (API > 10-50) or high (API > 50) risk of malaria.

The TPR also reflects the proportion of positive results per 100 suspected individuals. This was calculated by dividing the number of positive cases by the total number of tests performed, expressed as a percentage (441).

$$TPR = \frac{number\ of\ positive\ cases}{total\ number\ of\ tests} * 100$$

The standardized incidence ratio (SIR)

To comprehensively assess the variation in malaria incidence across the 15 districts and over time, the standardized incidence ratio (SIR) was calculated for each district-year combination. The SIR represents the ratio of the observed number of confirmed malaria cases (Yi) in a particular district (i) during a specific year (t) to the expected number of cases (Ei) in that district across over the periods. SIR was mathematically expressed as (442, 443):

$$SIR_{it} = \frac{y_{it}}{E_{it}},$$

where the expected count, Eit represents the total number of malaria cases that one would expect if the population of district i has the same risk as the zonal population during the same year. The expected number of malaria cases for each district (Eit) was computed as:

$$E_{it} = r_t^{(s)} n_{it};$$

Where $r_t^{(s)}$ is the overall crude malaria incidence in the Jimma zone (total number of malaria cases in a specific year divided by the total population of the zone during the same year); while n_{it} is the population of each district *i* during a year.

In general, an SIR = 1 indicates that the observed malaria incidence in a district is similar to the expected incidence based on the overall population. An SIR > 1 suggests a higher malaria risk in the district, while an SIR < 1 indicates a lower risk.

Spatial autocorrelation analysis, and identification malaria hotspots and coldspots areas

Spatial autocorrelation of malaria incidence was analyzed using Moran's I statistic in R version 4.4.1. This analysis examined how malaria incidence in one district relates to that in neighboring districts. The critical distance for the analysis was determined by a Global Moran's I analysis. The Global Moran's I statistic ranges from -1 to 1 and tests whether values at one location are independent of those at other locations. Positive values indicate positive spatial autocorrelation, negative values indicate negative autocorrelation, and zero indicates a random spatial pattern. Rejection of the null hypothesis suggests a non-random spatial distribution of malaria incidence and helps identify potential high or low incidence clusters (311, 444, 445). The Moran I model is expressed as:

$$Moran's\ I = \frac{n \sum_{i=1}^{n} \sum_{j=1}^{n} w_{ij}(x_i - \bar{x})(x_j - \bar{x})}{(\sum_{i=1}^{n} \sum_{j=1}^{n} w_{ij}) \sum_{i=1}^{n} (x_i - \bar{x})^2}$$

Before calculating Moran's I, the standardized Z(I) statistic was calculated to test the significance level of spatial autocorrelation (446). This also includes the expected value, which was defined as E (*I*).: $E(1) = \frac{-1}{n-1}$, and the theoretical variance of Moran's I was also given as $= \frac{I - E(I)}{\sqrt{Var(I)}}$,

where n is the number of districts; x_i and x_j are the incidence rates in districts i and j; $\bar{x}$ is the mean incidence rate in the entire zone; and w_{ij} is the spatial weight between districts i and j, and $\sqrt{Var(I)}$ is the variance of Moran's I. Finally, Rook's contiguity rule was applied to create a binary spatial weight matrix which was then created using Rook's contiguity rule, defining districts with a common boundary as neighbors (weight = 1) and all others as non-neighbors (weight = 0) (447). According to Moran's I statistic, I < 0 indicates negative autocorrelation. The closer it is to −1, the more spread out the distribution. An I = 0 means there is no autocorrelation, suggesting that the disease is randomly distributed. The significance of the observed spatial pattern was then assessed using a Monte Carlo test with a Z-statistic and a p-value. Rejection of the null hypothesis of random distribution and conclusion of statistically significant clustering or dispersion would be possible if the Z-statistic was outside a specified range (±1.96) with a p-value <0.05 (447).

Time series analysis and prediction of malaria trends from 2022 to 2026

A A times series (ts) is a sequence of data points collected/recorded at successive points in time, often at uniform intervals. In this context, a series of data points where each data point represents the total number of malaria cases (observed data) recorded in a specific month over a period of ten years. Thus, ts analysis involves examining this data to uncover patterns such as trends, seasonal effects and cycles in observed malaria cases over the decade. Ultimately, forecasting using methods like ARIMA to predict future values based on such historical data.

A time series is a sequence of data points collected at consecutive points in time, usually at equal interval of periods. In this context, it is a series of data points, where each data point represented the total number of malaria cases (observed data) collected in a given month over a ten-year period. Therefore, time series analysis in our context involved analysis of these data to reveal patterns such as trends, seasonal effects and cycles in observed malaria cases over the decade. Eventually, forecasts are made using time series model, ARIMA to predict future values based on such historical data (448). Prior to applying the ARIMA method, it was crucial to confirm whether time series was stationary, that means its mean and variance remained constant over time. To check this, we conducted a statistical test called the Augmented Dickey-Fuller (ADF), setting the statistical significance level at p-value < 0.05 to test the null hypothesis (H_0) that states that the time series has a unit root, implying non-stationarity. If the p-value falls below 0.05, we reject the null hypothesis, indicating that the series is stationary. If the p-value exceeds 0.05, the null hypothesis is not rejected, suggesting that the time series may need to be transformed to achieve stationarity. If the time series was non-stationary, we would apply differencing to stabilize the mean, typically starting with first differencing (d=1). After differencing, we recheck for stationarity. If stationarity is achieved, we reject H_0, and no further differencing is necessary, and proceed with the subsequent steps of the ARIMA modeling process(449).

The next step was model identification, which was primarily based on the identification of the autoregressive (AR) and moving average (MA) components and was carried out by examining the partial autocorrelation function (PACF) and autocorrelation function (ACF) plots of the differenced time series (Fig .5.10). For the AR component (p), the PACF plot was analyzed to

determine the lag at which the plot showed a clear increase outside the significant limits, followed by a rapid decay around zero, indicating the corresponding order. Similarly, the MA component (q) was determined from the ACF plot of the differenced series, where a significant spike at a particular lag, followed by a sharp drop, indications of the order of the MA term. After the series was differenced, these steps were required to guarantee stationarity, which in turn aided in choosing the AR and MA parameters for the ARIMA model(450, 451).

The diagnostic tests: After fitting the model, the residuals were examined to ensure that they resembled white noise. This included performing the Ljung-Box test to check autocorrelation in residuals, analyzing the ACF and PACF plots for the residuals, and assessing the histogram plot to determine whether the residuals assumed normality. In the end, three ARIMA models were compared using the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC). Finally, the model with the lowest AIC and BIC values was selected to balance fit and complexity(448). In the end, the final ARIMA model was used to generate forecasts from 2022 to 2026, including prediction intervals (at 95% CI) to account for uncertainties.

6.3 Ethics statement

This study was part of a main doctoral project entitled “Epidemiology malaria in the Jimma zone, southwestern Oromia, Ethiopia”, and was conducted with the ethical approval of the Institutional Review Board (IRB) of Jimma University (Ref.: IHRPED/550/2). Letter of approval was then obtained from the Jimma Zone Health Office.

6.4 Results

6.4.1 Epidemiological characteristics

A decade-long analysis of malaria epidemiological data from Jimma Zone showed both concern and optimism. This comprehensive study covered 75% of the zone's districts (15 out of 21), with all districts reported the presence of malaria. Over 1.7 million individuals (n =1,718,471) were screened for malaria using either microscopy or RDTs, resulting in an overall positivity rate of 20.93% over the years. As illustrated in Table 1, there was a dramatic decline in malaria case series over the past decade with a peak positivity rate of 32.87% in 2012 and the rate steadily fell to just 9.40% by 2021.

6.4.2 Total malaria cases series district level

A ten-year journey through the malaria landscape of the Jimma Zone reveals a story of progress and persistence. While the overall number of malaria cases has clearly declined, a closer look reveals notable disparities among the districts (Table 6. 1) although the decline wasn't uniform across all the districts. Some districts the once-heavily burdened significantly reduced malaria burden in recent years. As can be seen in Table, for instance, in 2012, districts like Gomma (while higher cases in 2013 than 2012), Sokoru, Limu Seka, Chora Botor, Tiro Afata, and Limu Kosa, which had over 12,000 malaria cases achieved a dramatic reduction with fewer than 1,000 or 500 cases reported in 2021. On the other hand, six districts (Agaro town, Dedo, Gera, Gummay, Omo Nada and Shebe Sonbo) showed fluctuating cases over time and generally showed increased cases between 2019 and 2021.

Table 6. 1: Annual distribution of malaria cases in the study districts of Jimma Zone, Southwest Oromia, Ethiopia (2012-2021).

	Annual total malaria cases										
District name	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**	**2020**	**2021**	Total
Agaro	2837	2414	3191	879	1240	994	340	235	883	353	13366
Chora botor	14800	7522	3627	2416	2083	933	721	1357	1068	910	35437
Dedo	3525	5652	4570	1436	987	1220	813	375	337	260	19175
Gera	1867	1598	1881	2121	874	599	302	386	430	351	10409
Gomma	11585	15804	9228	3718	1633	403	392	335	438	922	44458
Gumay	1086	3294	1632	562	502	183	247	204	239	137	8086
Karsa	8172	1848	1346	1404	442	210	306	285	342	352	14707
Limu kosa	13384	11449	5616	3107	1198	536	459	556	426	815	37546
Limu Seka	14255	8764	4355	1916	942	386	301	430	925	1067	33341
Manna	4993	2585	1963	1244	612	291	210	161	202	324	12585
Omo Nada	9230	4221	2224	1373	414	361	315	976	2133	858	22105
Seka Chokorsa	6168	3493	4007	1904	1194	165	383	145	162	96	17717
Shebe Sonbo	8388	6792	5525	3396	2112	744	745	473	855	1717	30747
Sokoru	15024	9814	3516	2923	1095	383	422	257	1062	523	35019
Tiro Afata	12410	6872	2895	1327	288	301	204	127	200	333	24957
Total	**127724**	**92122**	**55576**	**29726**	**15616**	**7709**	**6160**	**6302**	**9702**	**9018**	**359,655**

6.4.3 Sociodemographic characteristics of malaria patients

The distribution of malaria in under five children varied significantly over the period, averaging 12.56% over the years. The most substantial change occurred between 2012 and 2016, proportion of positivity was increasing from 5.37% to 16.11%. This shows infection was significantly increased by about 200% in this age group during the indicated periods. However, between 2016 and 2019, there was a prolonged malaria transmission in this age group before it attained maximum of more than 16. 60%, and generally increased by over 3.51% in 2020 and 2021. Nevertheless, those who were five years of age and older carried the heavy burden of the disease during same period, with an average of more than 87%, and typically showed a steadier trend. However, between 2012 and 2021, there was a minor decline, going from 94.63 percent to 83.49 %, or roughly 11% (Fig. 6.2.).

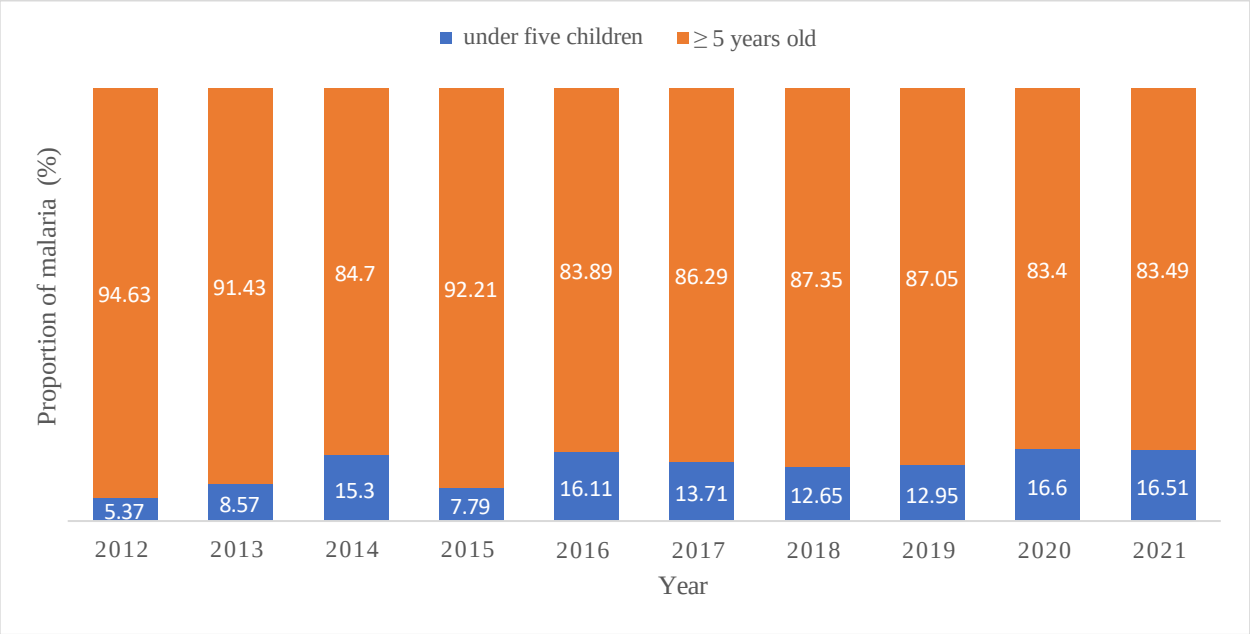


Figure 6. 2: Distribution of proportions of malaria by age group in Jimma Zone, Southwest Oromia, Ethiopia (2012-2021).

Figure 6.3 shows the distribution of the proportions of malaria cases by sex. Overall, the proportion of malaria infections in males was slightly higher 53.47% (192,312) than in females 46.53% (167,343). The highest proportion of males with malaria infection was recorded in 2013 at 56.87% and the lowest in 2015 at 40.13%. For males females, the highest and lowest proportions were 59.84% in 2015 and 43.13% in 2013, respectively. However, for males, the distribution fluctuated in 2012-2013, then decreased and increased between 2013-2015 finally from 2015 to 2021. For females, however, it was the other way around, it decreased between 2012 and 2015 and then increased from 2015 to 2021.

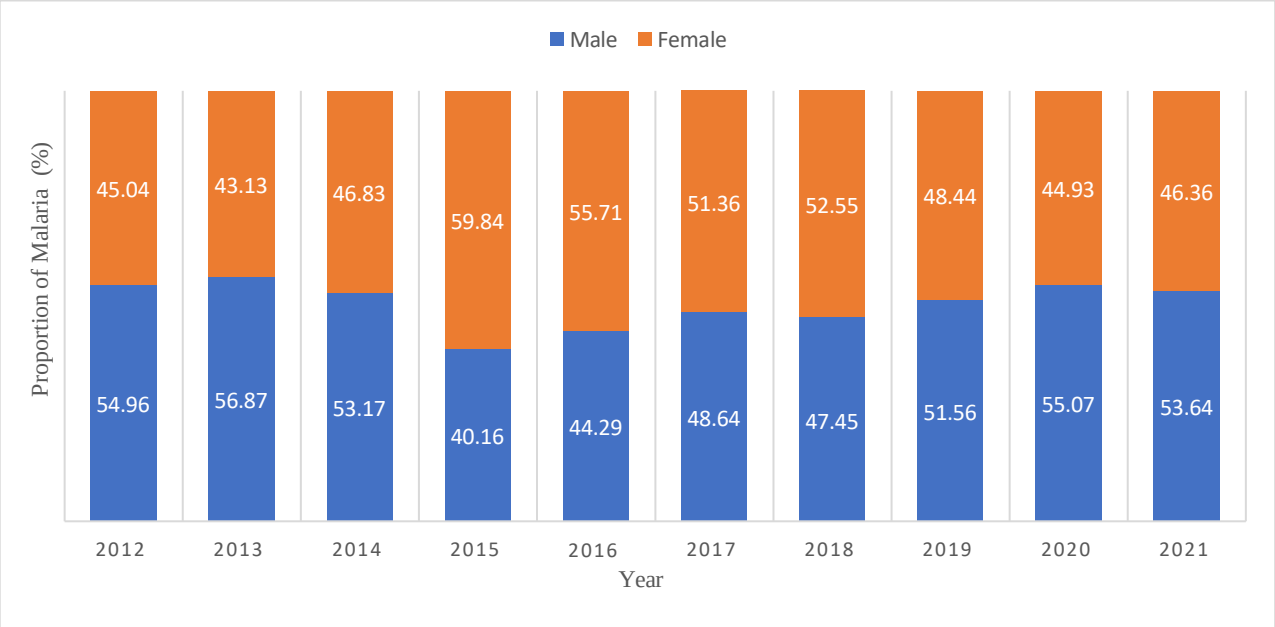


Figure 6. 3: Distribution of proportion of malaria by sex in Jimma Zone, Southwest Oromia, Ethiopia (2012-2021).

6.4.4 Distribution of Plasmodium species

Of the overall malaria case positivity rate of 20.93%, 52.71% (189,568) of the infections were caused by *P. falciparum*, followed by *P. vivax*, which accounted for 43.62% (156,877) of cases, while mixed infections accounted for 3.67 % (13,210), with *P. falciparum* generally being the predominant parasite species. *P. vivax* was dominant over *P. falciparum* only between 2012 and 2014, and then declined (Fig. 6. 4). Over the years, the proportion of *P. falciparum* showed fluctuations, starting at 53.99% in 2012 and peaking at 68% in 2021, an increase of 25.96% over the years. In particular, the highest values were recorded in 2020 (68.39%) and 2021 (68%), indicating a worrying upward trend in recent years.

In contrast, *P. vivax*, although significant, showed a decreasing trend starting at 42.05% in 2012 to 30.77% in 2021, decreased by 26.82%. The higher values in 2015 (49.77%) and 2016 (50.60%) indicate periods of increased prevalence before the overall decline. The proportion of mixed infections generally showed a steadily decreasing trend, starting from 3.96% in 2012 and

decreasing to 1.23% in 2021, a decrease of 68.94% with a higher proportion seen in 2014 was 4.07%.

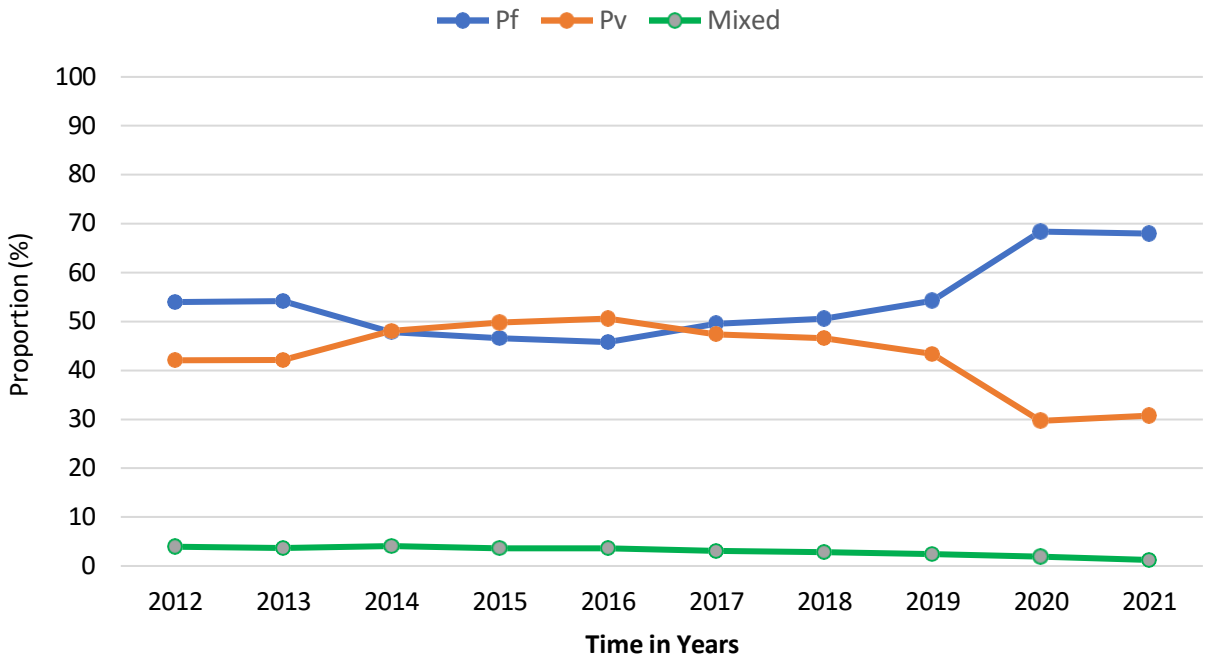


Figure 6. 4: Proportion of annual distribution of *Plasmodium* species in Jimma Zone, Southwest Oromia, Ethiopia (2012-2021).

6.4.5 Spatial analysis of annual parasite incidence (API) and malaria transmission risk map

The average decline in API over the period was 96.13%, from 87.97 in 2012 to 3.40 in 2021. It went down from 47.6 % in 2012 to 46.09% in 2013. The API decline year after year until it bottomed out in 2017 and 2019 and stabilized in a range of 2.55 to 4.06. In subsequent years, despite the general downward trend, there were minor fluctuations, with a slight increase to 4.19 in 2020 and a decrease to 3.40 in 2021. Further support for these declining trends was also found in the spatial distribution. An important example is the decrease in API observed for Chora Botor, where proportion reduced by 98.87 %, from 636.86 per 1,000 populations at-risk in 2012 to 7.19 in 2021. On the other hand, Agaro recorded a 91.63% drop in API from 83.85 in 2012 to 7.02 in 2021. There was a notable decrease of approximately 97.2% in Sokoru, with API dropping from

94.63 to 2.68 in 2021. Interestingly, a malaria transmission bottleneck was noted, with the incidence reaching 1.17 in 2019. Tiro Afeta also showed notable progress, reducing its API by 97.8% from 81.6 to 1.81. Limu Kosa recorded a 97.25% decline from 69.97 in 2012 to 1.92 in 2020, but an 84.4% increase to 3.54 in 2021.

The API of Limu Seka decreased by 91.77% between 2012 and 2019, from 65.19 to 1.70, but then increased somewhat after 2019. Shebe Sonbo likewise reduced API by 95%, from 64.62 in 2012 to 3.14 in 2019, although this accomplishment was eclipsed by a massive 248.09% spike to 10.93 between 2019 and 2021.

In light of this, we created a malaria risk map of the study districts (Fig. 6. 5), which illustrates a progressive shrinkage of malaria transmission areas over time. As a result, there were seven high API districts in 2012, including Chora Botor, Agaro Town, Gomma, Sokoru, Limu Kosa, Shebe Sonbo and Limu Seka. However, districts such as Toro Afeta and Limu Seka experienced a shift from high to moderate incidence levels, whereas Kersa had a low API. However, six districts such as Chora Botor, Agaro Town, Gomma, Sokoru, Limu Kosa and Shebe Sonbo continued to have higher incidence rates in 2013. In this context, Toro Afeta and Limu Seka shifted from higher to moderate incidence values, and Kersa had a low API.

It is interesting to note that there was a significant decline in malaria incidence from 2015 to 2021 and no district reported a high API value. Therefore, five of the 15 reported malaria cases (Gera, Gomma, Gumay, Limu Kosa and Sokoru) had low API levels. Importantly, in 2016 alone, about 50% of the district areas (Dedo, Kersa, Limu Seka, Manna, Omo Nada, Seka Chokorsa and Tiro Afeta) significantly reduced malaria incidence to a very low risk of malaria. A key example is 2019, when 93.33% of the study districts became very low malaria risk areas. Despite these laudable achievements, challenges remain in Agaro Town, Chora Botor and Shebe Sonbo, which continue to face moderate malaria burdens. In general, as the 2017 to 2021 map shows, very low malaria transmission has been achieved in over 86% of once heavily burdened areas (Table 6. 2).

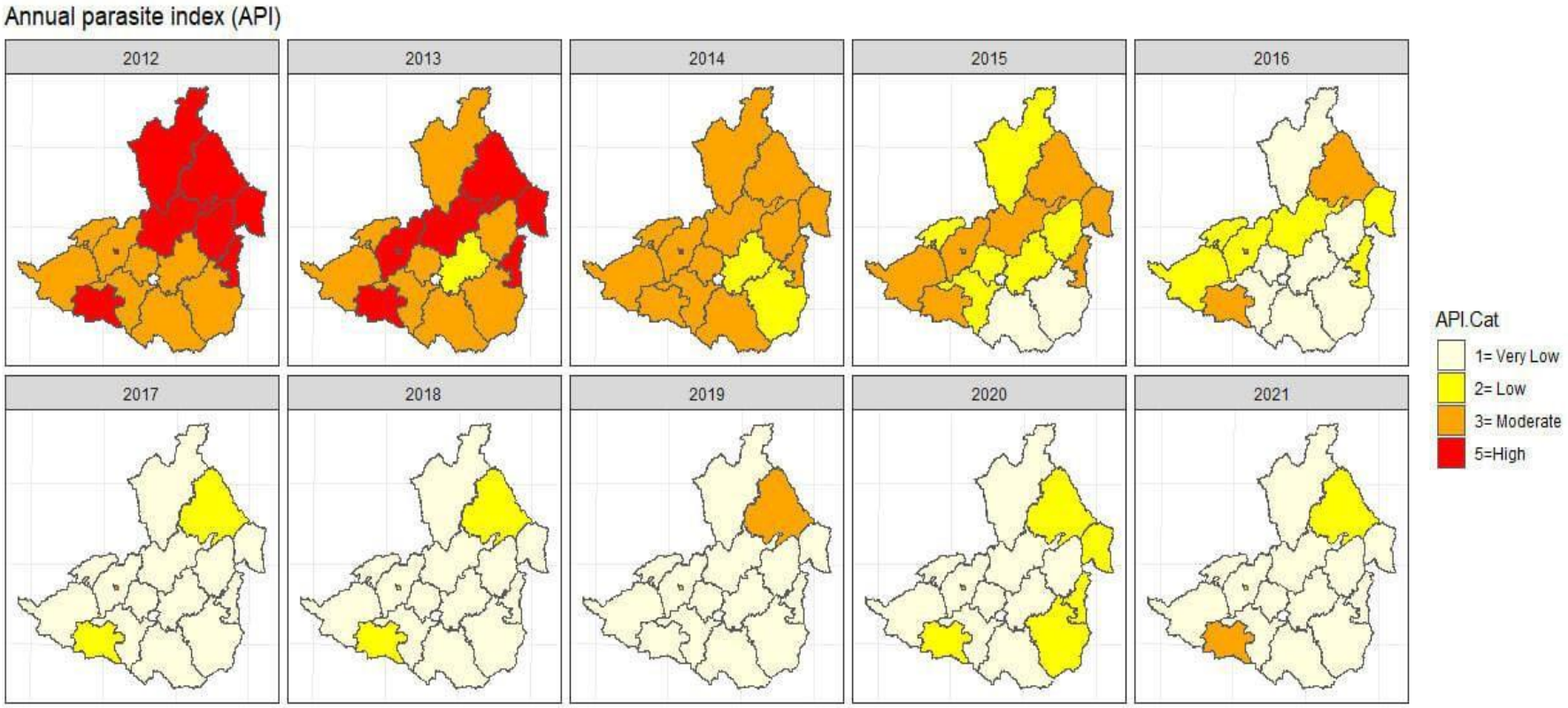


Figure 6. 5: Malaria risk map showing districts with very low, low, moderate and high malaria risk in Jimma zone, southwest of Oromia, Ethiopia (2012 – 2021).

Table 6. 2: Mean annual parasite incidence (API) in the study Districts of the Jimma Zone, Southwest Oromia, Ethiopia (2012-2021)..

	Year									
District	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021
Agaro town	83.85	71.14	89.49	23.47	31.65	24.19	7.96	5.16	18.44	7.02
Chora Botor	636.86	175.27	49.63	21.59	18.19	7.97	6.04	11.13	8.76	7.19
Dedo	10.68	16.79	13.27	4.07	2.74	3.31	2.16	0.98	0.88	0.87
Gera	14.91	12.43	13.96	15.37	6.19	4.14	2.04	2.56	2.84	2.23
Gomma	46.17	62.33	36.03	14.17	6.07	1.46	1.39	1.16	1.52	3.07
Gumay	15.39	45.87	22.33	7.5	6.53	2.32	3.06	2.47	2.88	1.59
Karsa	48.56	8.71	5.26	6.93	2.13	0.99	1.41	1.29	1.54	1.53
Limu Seka	65.19	39.38	19.23	8.26	3.97	1.59	1.21	1.7	3.65	4.05
Limu Kosa	69.97	59.3	28.79	15.53	5.84	2.55	2.13	2.52	1.92	3.54
Manna	29.45	14.99	11.19	6.93	3.33	1.55	1.09	0.82	1.03	1.59
Omo Nada	32.09	14.42	7.47	4.5	1.32	1.13	0.96	2.92	6.37	2.46
Seka Chokorsa	25.62	14.27	16.09	7.47	4.57	0.62	1.4	0.52	0.58	0.51
Shebe Sonbo	64.62	51.42	41.1	24.66	14.97	5.15	5.05	3.14	5.66	10.93
Sokoru	94.63	60.66	21.33	17.28	6.31	2.15	2.12	1.17	5.67	2.68
Tiro Afeta	81.6	44.38	18.37	8.22	1.74	1.78	1.18	0.72	1.13	1.81
mean	87.97	46.09	26.23	12.4	7.7	4.06	2.61	2.55	4.19	3.40

The spatial autocorrelation analysis of malaria incidence using Moran I models revealed no presence of any statistically significant clustering of malaria incidence in the Zone (Moran I statistic = -0.0240, P-value = 0.360). This result was further supported by a Monte Carlo simulation (P- value of 0.39 and a comparable statistic of -0.024). It is therefore evident that malaria incidence was randomly distributed (dispersed) across the study districts during the observed periods. Accordingly, the spatial incidence map (SIM) (Fig. 6.6) was created to provide a visual representation of the SIR across districts even in the absence of obvious spatial autocorrelation. However, this map shows variations in malaria incidence, with districts such as Chora Botor and Shebe Sonbo having higher SIR values.

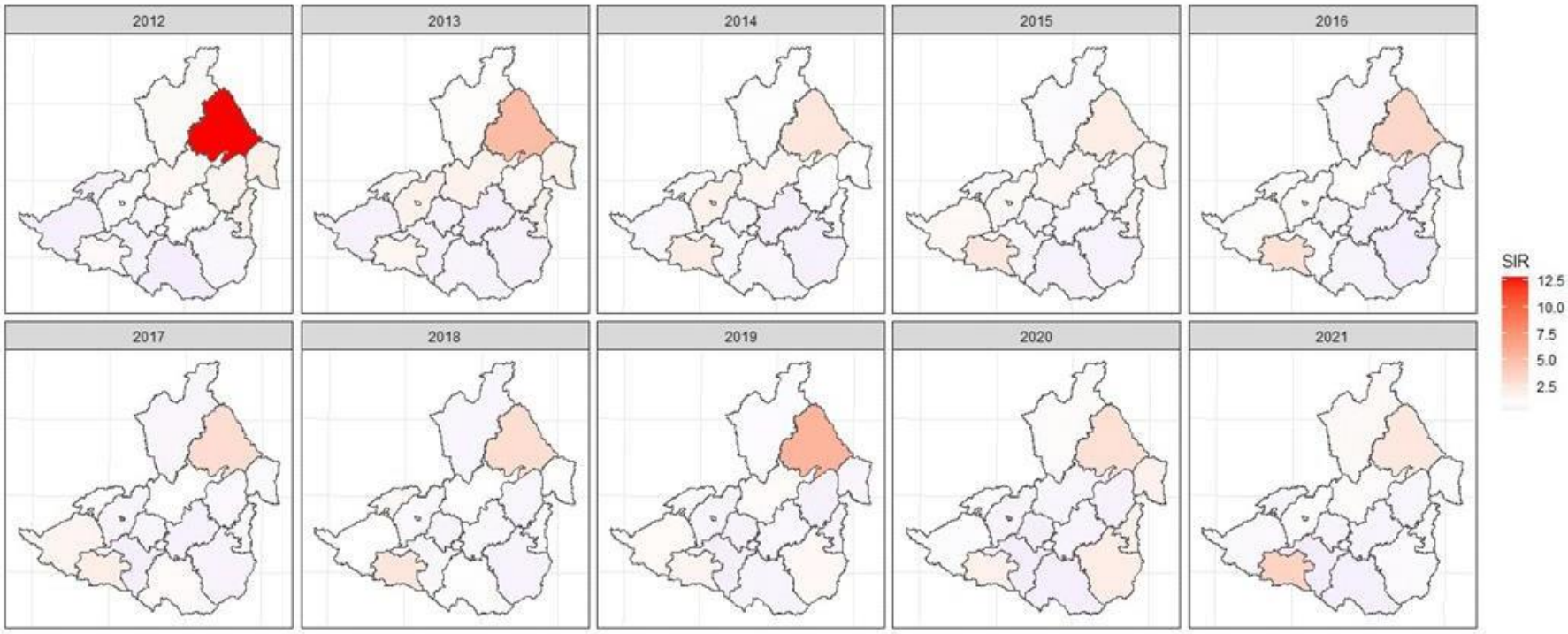


Figure 6. 6: Spatial Distribution of Standardized incidence ratio (SIR) of Malaria in 15 districts in Jimma zone, southwest Oromia, Ethiopia (2012-2021).

6.4.6 Temporal analysis: Seasonal patterns of malaria transmission

The monthly proportions of malaria cases followed a bimodal transmission pattern (Fig. 6.7), with pronounced peaks between August and November (with a maximum peak in September), and a second minor peak from March to June, representing the major and minor malaria transmission seasons, respectively. The inter-peak periods: the period between December and February generally shows lower malaria cases, suggesting low malaria transmission season.

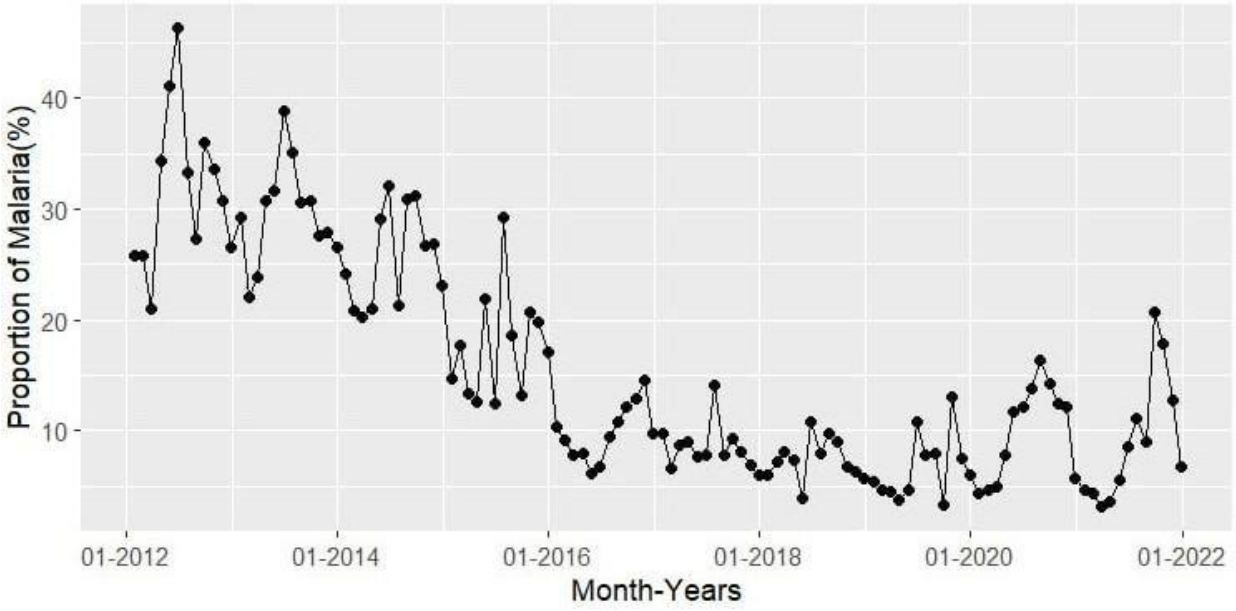


Figure 6. 7: Monthly trends of proportion malaria cases in Jimma Zone, Southwest Oromia, Ethiopia (2012-2021).

The proportions of the annual distribution of malaria (Fig. 6.8) also showed a clear decline in trend despite the fluctuations observed at different points in time. Overall, the proportion of malaria cases decreased significantly from 32.87% in 2012 to 9.40% in 2021, a reduction of 71.40%. This was particularly evident between 2014 and 2016, when it fell from 26.46% to 10.05%, a decrease of 62%. Between 2012 and 2014 it then fell by 19.54%. Between 2014 and 2016, there was a significant continuation of these declines, with the proportions dropping to 10.05%, a 62% decrease. Between 2016 and 2019, when the proportion of cases dropped from 42.5% to 5.8%, further achievement was made. But after 2019, a concerning pattern became apparent: the percentage of instances increased. It increased by 76.55% between 2019 and 2020, from 5.8% to

10.24%. However, there was a slight decrease between 2020 and 2021, with the proportion of cases falling to 9.4%, or an 8.2% decrease.

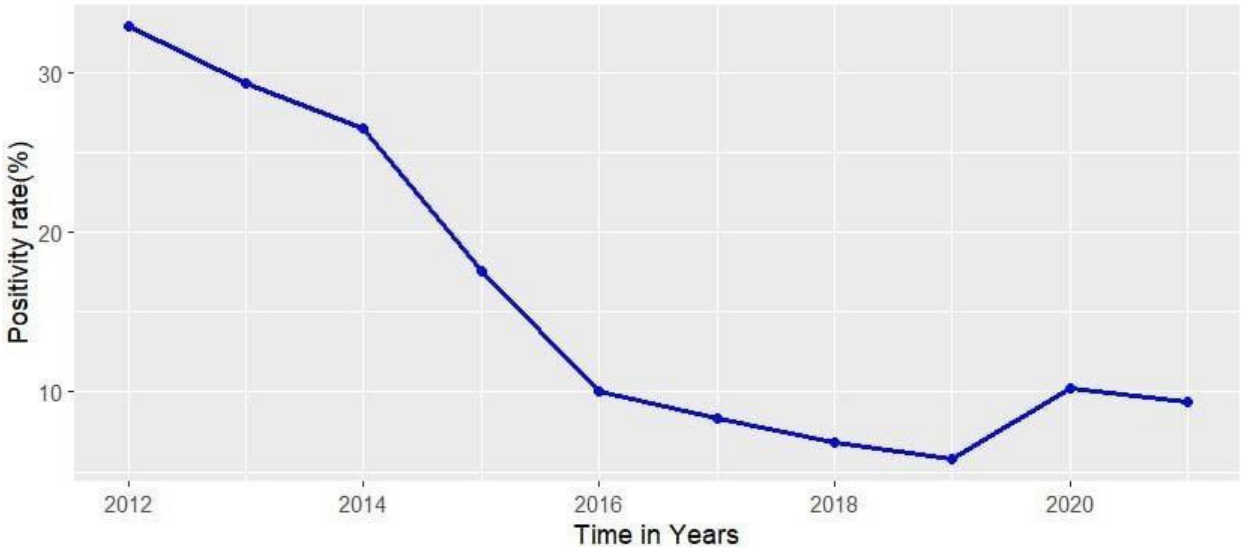


Figure 6. 8: Annual trends in Proportions of Malaria cases in Jimma Zone, Southwest Oromia, Ethiopia (2012–2021).

6.4.7 Time series and prediction of malaria trends using ARIMA Model

The monthly malaria case data converted into a time series (ts_data) showed that there were strong seasonal fluctuations in addition to that observed in Fig. 6.7, and the case series was found to be non-stationary, which was confirmed by the Augmented Dickey-Fuller test (ADF, p-value = 0.09214), where the null hypothesis of stationarity was not rejected. Since ARIMA models assume stationarity in the data, this non-stationarity made ts_data unsuitable for ARIMA modeling to predict the next five years (2022-2026). To overcome these challenges, we decomposed the ts_data into its components: the trend, which represents the long-term pattern; seasonality, which captures periodic fluctuations; and residuals, which include the unexplained variation (Fig. 6.9 A). A After applying a 1st order differencing, the series transformed into a stationary process (ADF test p-value = 0.01), where we failed to reject the alternative hypothesis of stationarity. This transformation allowed us to examine the decomposed components of the difference series (Fig. 6.9 B), which provided a solid basis for selecting appropriate time series models to achieve accurate forecasts.

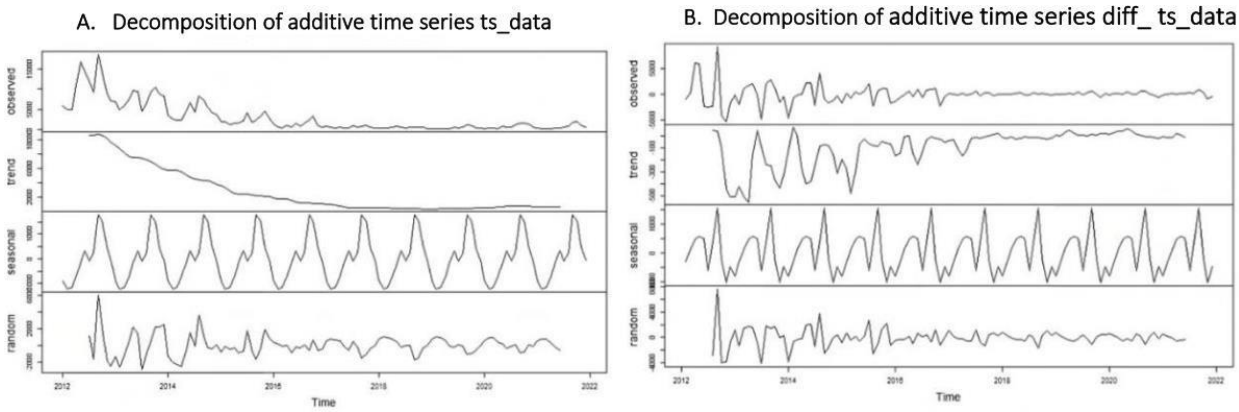


Figure 6. 9: Time series decomposition: (A) original ts_data exhibited non-stationary with trend, seasonal, and residual components. (B) Differenced time series demonstrated stationarity.

Based on the coefficients and standard errors of the parameters for the three ARIMA models (Table 6. 3), model one had statistically not significant coefficients and was simple (just three parameters). Model two, despite having a moderate number of parameters, produced statistically significant coefficients. Model three, with its more complex parameter structure, looked to be the least effective.

Table 6. 3: Coefficients and standard errors of the parameters of the three ARIMA models.

Model	**Model specification**	**Parameters**	**Coefficient**	**Standard error (s.e,)**
Model 1	ARIMA1 (1,1,1) x (1,0,0) [12]	Non-seasonal AutoRegressive (AR) (1)	0.5356	0.1039
		Non-seasonal Moving Average (MA) (1)	-0.9037	0.0403
		Seasonal AutoRegressive (AR) (1)	0.5742	0.1158
Model 2	ARIMA (0,1,3) (1,1,0) [12	Moving Average (MA) (1)	-0.4708	0.0988
		Moving Average (MA) (2)	-0.1768	0.0961
		Moving Average (MA) (3)	-0.1997	0.0997
		Seasonal AutoRegressive (AR) (1)	-0.2686	0.1228
Model 3	ARIMA (2,1, 4)	AutoRegressive (AR) (1)	-0.1794	0.0794
		AutoRegressive (AR) (2)	-0.9181	0.0687
		Moving Average (MA) (1)	0.1226	0.1159
		Moving Average (MA) (2)	0.6819	0.1824
		Moving Average (MA) (3)	-0.268	0.1522
		Moving Average (MA) (4)	-0.2035	0.1647

6.4.8 Model selection and evaluation

We compared three ARIMA models to determine the best fit based on specific criteria (see Table 6.4). The first model, ARIMA (1,1,1) x (1,0,0) [12], showed no seasonal differencing and had an AIC of 2096.08 and a BIC of 2107.2. The residual analysis for this model revealed marginally no significant autocorrelation (Ljung-Box test p-value = 0.07085) (Table 6.5).

The second model, ARIMA (0,1,3) x (1,1,0) [12], included one degree of seasonal differencing and demonstrated an AIC of 1886.47 and a BIC of 1899.84. The residuals of this model also showed no evidence of autocorrelation (Ljung-Box test p-value = 0.3009).

In contrast, the third model, ARIMA (2,1,4), selected based on visual observation of patterns in autocorrelation function (ACF) and partial autocorrelation function (PACF) plots (see fig. 6.10). This model was specified with P=2 (included 2 lag observations in the autoregressive component), d= 1 (applied first-order differencing to achieve stationarity), and q= 4 (incorporated four lagged forecast errors in the moving average component). Despite its selection based on these criteria, ARIMA (2,1,4) had higher AIC and BIC of 2110.8 and 2130.25, respectively. Additionally, the residuals of this model exhibited a statistically significant autocorrelation (Ljung-Box test p-value < 0.001).

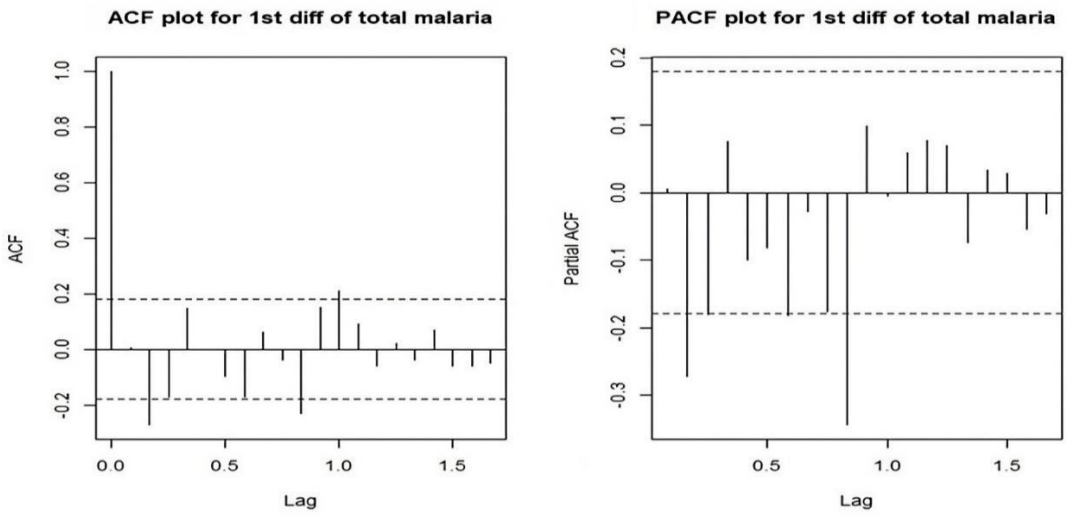


Figure 6. 10: The correlogram of the autocorrelation function (ACF) with 4 spikes (q) and partial autocorrelation function (PACF) with 2 spikes (p) for ARIMA model identification of I lag-differenced ts-data.

Therefore, the ideal model is the one characterized by the lowest AIC and BIC values, as these metrics provide a balance between model fit and complexity. A lower AIC or BIC indicates a model that not only fits the data well but also avoids excessive complexity while keeping the residuals free of significant autocorrelation. In our study, the AIC values for Model 1 and Model 3 were 10% and 10.63% higher compared to Model 2. Similarly, the BIC of these models were 9.84 and 10.80% higher than that of model 2. This indicates that Model 2 achieves the optimal balance between fit and complexity, making it the most efficient choice among the evaluated models. Therefore, the ARIMA (0,1,3) x (1,1,0) model was selected as the most parsimonious (Table 6. 3) and effective model for predicting malaria trends from 2022 to 2026 in the study districts (Table 6. 4).

Table 6. 4: Comparative evaluation and selection of ARIMA model based on information criterion, and Error measures.

Model	Model Specification	Information criterion		Error measures			
		AIC	BIC	RMSE	MAE	MAPE	MASE
1	ARIMA (1,1,1) (1,0,0)	2096.08	2107.2	1523.462	886.9096	41.086	0.674
2	ARIMA (0,1,3) (1,1,0)	1886.47	1899.84	1454.451	893.462	58.061	0.679
3	ARIMA (2,1, 3)	2110.8	2130.254	1600.04	980.15	56.804	0.991

Additionally, based on the error measures, the RMSE value of Model 2 was notably lower than both Model 1 and Model 3 (Table 6.4). Contrary to this, the MAE, MAPE, and MASE of model 1 were lower than those of model 2, However, we employed the Ljung-Box Q statistic to assess the adequacy of the residual autocorrelation for each model. As shown in Table 5. 5, Model 1 exhibited a p-value of 0.07085, suggesting marginal evidence of residual autocorrelation at the 10% significance level. Model 2 displayed a p-value of 0.3009, indicating no evidence of residual autocorrelation. Model 3, however, demonstrated a p-value of 0.00079, strongly suggesting the presence of residual autocorrelation. On the other hand, the error measures of the model 2 are smaller than those of model 3.

Table 6. 5: Comparison of the three models based on the Ljung-Box statistic (Q) with critical values for the residual test.

Chi-square distribution table values with h degrees of freedom(df) at three critical values						
Model	df	Ljung-Box Q	1%	5%	10%	P-value
1	21	31.17	38.93	32.67	29.6	0.07085
2	20	22.76*	37.56	31.41	28.41	0.3009
3	18	43.01	34.80	28.87	25.98	0.00079

***** Since the Ljung-Box statistic (22.757) in Model 2 is smaller than all the critical values in chi-square distribution table values with 20 df, we cannot reject the null hypothesis of the test (the residuals are independently distributed) and conclude that the data values are independent. We conclude that the residuals of our ARIMA model show no autocorrelation at the 1%, 5%, and 10% significance levels.

Model validation: Finally, we performed a model validation procedure that included both visual assessments and a statistical analysis using the Ljung-Box test (LBT) of the plots of the residuals. Visual assessments included residual, ACF and histogram plots from the fitted ARIMA (0, 1.3) x (1,1,0) [12] model. Consequently, the residuals had a mean close to zero, no clear pattern, and were randomly distributed, indicating the absence of systematic bias in the model's predictions. Furthermore, the ACF of the residuals revealed that all coefficients were within the confidence bounds, showing the absence of autocorrelations in errors terms. In a histogram plot, the residuals assumed an approximately bell-shape distribution, which implies they are normally distributed. These assessments were further confirmed statistically using the LBT, which corroborated the absence of autocorrelations in the residuals (LBT statistic = 22.757 and p-value = 0. 3009).

Overall, the outcome of the model validations provides strong evidence for its suitability for the forecasts in the whole procedures in time series analysis. As a result, an ARIMA (0,1,3) x (1,1,0) model was selected as the most parsimonious and effective model for predicting malaria trends from 2022 to 2026 in the study districts (Fig. 6.11).

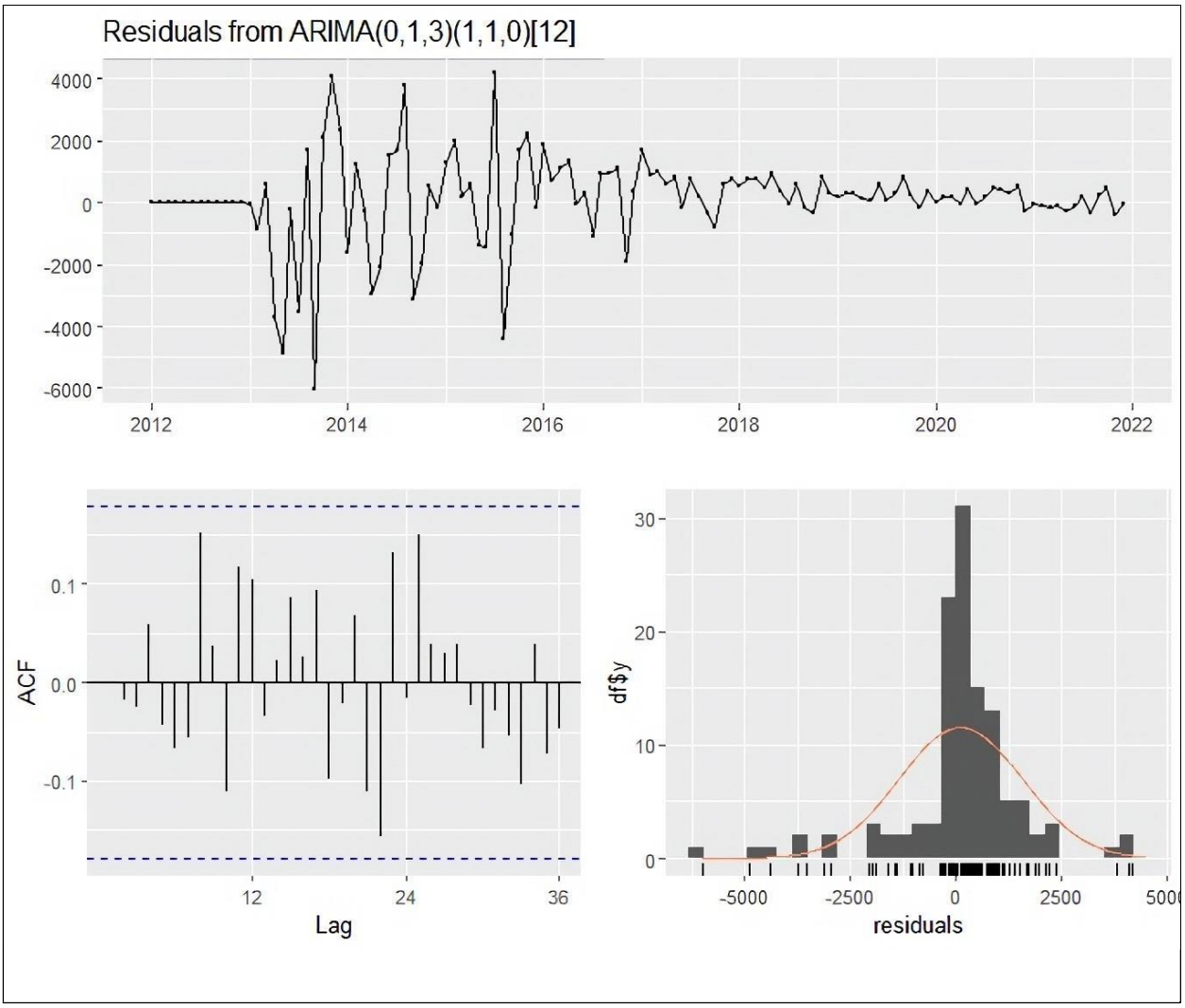


Figure 6. 11: The plots of the residuals from the fitted autoARIMA (0,1,3) x (1,1,0) [12] for malaria cases time series in Jimma zone, southwest Oromia, Ethiopia (2012-2021).

Therefore, we used this validated model to predict malaria cases from January 2022 to December 2026. Accordingly, our model generated the forecast diagram (Fig. 6. 12) that shows historical malaria data from 2012 to 2021, with black lines on the left. This shows a dramatic decline in malaria cases between 2012 and 2015, followed by a period of relative stability with minor fluctuations. Most importantly, the model automatically understood the time period of when the stationarity was achieved (between 2015 to 2021). Hence, the model used the values during this period as a staging and made predictions. Automatically, the model then generated malaria point estimates along the 95% confidence bounds (supplementary data 1). The blue line in the plot denotes the projected point estimates from 2022 to 2026. As a result, the model predicted that

malaria cases will continue to fluctuate around a stable mean, consistent with recent historical trends.

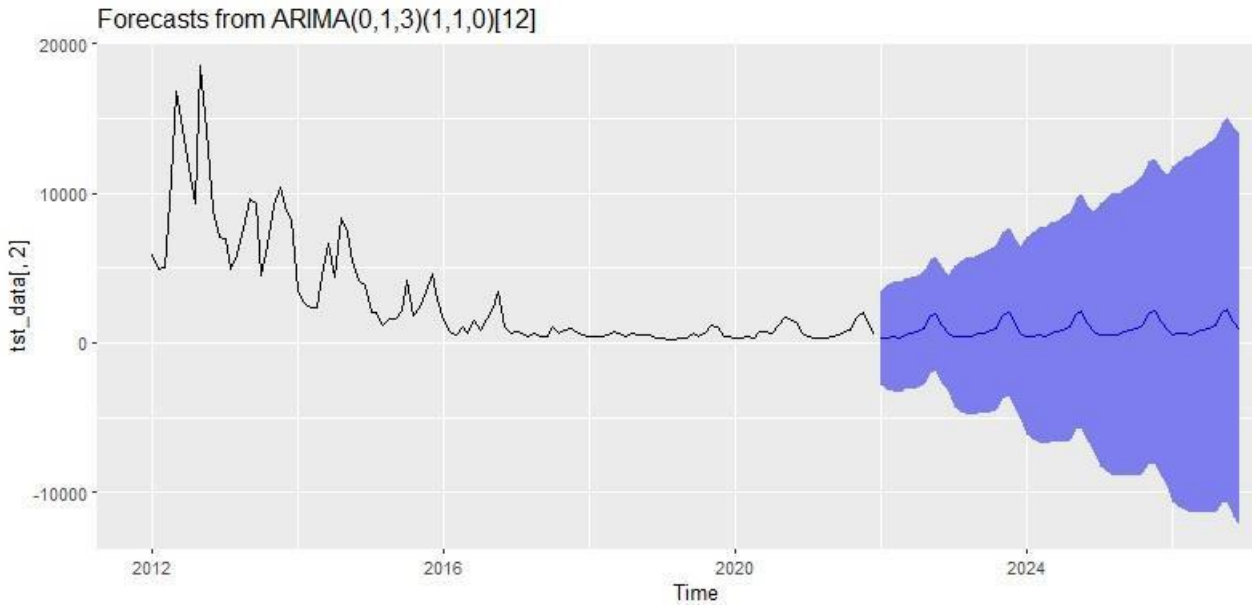


Figure 6. 12: Forecast plot of malaria cases using ARIMA (0,1,3) (1,1,0) x [12] model from 2022 to 2026 in Jimma Zone, Southwest Oromia, Ethiopia.

Malaria cases are expected to fluctuate starting in 2022. In January 2022, for example, the point forecast assumes approximately 325 cases, rising to a peak of 1,965 cases in October. This demonstrates a clear seasonal pattern, as lower predicted values are expected to occur in the first few months, implying a decrease in cases during this period. A peak of nearly 1,790 cases is expected in September 2022, with a subsequent decline in November and December. A closer look at the point estimate for 2023 reveals a moderate stabilization of malaria cases, though fluctuations persist. Monthly forecasts for 2023 range from 412 cases in January to a high of 2,050 in October. It is noteworthy that the anticipated September peak is consistent with previous year's pattern, indicating that maria transmission is likely to follow seasonal patterns. Ultimately, the projections showed a slight increase in point estimates from 2024 to 2026. Between January and October 2026, the point estimate ranges from 578 to 2,216, with a monthly average of over 500 cases. Finally, this upward trend indicates an overall increase in malaria incidence over time.

6.5 Discussion

This This study confirmed a significant decline in malaria positivity rate within the Jimma zone over the last decade. With over 1.7 million people screened for malaria over the period in the study districts revealed an overall proportion of malaria was 20.93%. There was a significant decrease from the peak proportion of malaria positivity of 32.97% in 2012 to 9.40% in 2021. However, the distribution of malaria and the observed decline in its incidence across the districts showed variability in space and time.

The results of the current study revealed that children under the age of five constituted small and fluctuating proportions of the total malaria burden over time compared to individuals above five years old. This finding is in agreement with the previous reports from Ethiopia(431, 452, 453) and South Africa(454). This could be due to factors such as effective prevention measures, improved healthcare and deployment of health extension workers in each village(297). Riley *et al.* (2001) hypothesized that children under five possess both innate immunity and physiological mechanisms that inhibit parasite growth. Moreover, the absence of primed T-cell responses required to initiate fever in this age group usually result in asymptomatic infections with lower parasite densities(455). These factors, in addition to other potential factors such as effective prevention measures, contribute to the lower malaria prevalence observed in this age group It follows that, distribution of the distribution sex showed that males appeared to be infected than females, which is consistent with a recent study from Uganda (456), a report from Oromia region of Ethiopia(431), and southern part of the country(453). This suggests that potential sex-based difference in susceptibility to malaria which could be attributed to differences in occupational such as agricultural activities(457), behavioral such as night outdoor sleeping(458) and activities(459), It has been suggested that behavioral factors such as alcohol consumption increase the likelihood of mosquito bites in males (460) and that physiological factors such as post-pubertal hormonal changes make males more susceptible to mosquito bites(461).

The present study found that *P. falciparum was* a predominant species followed by a significant proportion of *P. vivax* and a decreasing trend in mixed infections. This is in agreement with both the global and national trends. Globally, a higher proportion of malaria- related morbidity are caused by *P. falciparum* , with over 95% of the cases in Africa due to this parasite (49). Our

findings also consistent with national figures, where *P. falciparum remains* the predominant *Plasmodium* species which accounts for 71.3% of cases in Ethiopia. This dominance is followed by *P. vivax* at 20.9%, while mixed infections are less common, representing 1.1% of total cases (299). However, our finding of *P. falciparum* dominance is slightly higher than in a ten-year trend analysis of malaria cases around the Arjo-Didessa sugar developments sites in southwest Oromia, where *P. falciparum* accounted for 48% of cases and had a comparable positivity rate to *P. vivax* (42 .4%), while mixed infections in our study were lower than 9.6% in a reported from Arjo-Didessa(431). Likewise, the distribution of *Plasmodium* species in our study is consistent with studies reported from the western(462) and southern part of Ethiopia(453) and also a study that focused the national malaria survey(463), reported that *P. falciparum* accounted for 56% of the total infections, indicating its high dominance in Ethiopia(463).

The results of this study also showed a clear spatial pattern in malaria distribution. Notably, districts that previously had disproportionately high malaria burdens experienced a spatial contraction in transmission between 2012 and 2021 in Jimma zone. Over the period, the API decreased dramatically from 87.97 to 3.40 per 1000 at-risk populations, categorized the zone as a very low malaria risk area by 2021, which is consistent with recent national malaria stratification (299). Despite these successes, our malaria risk map shows that Shebe Sonbo and Chora Botor remain low and medium risk areas, respectively. However, this overall decline in malaria incidence in the Jimma zone is consistent with the nationwide figure recently reported by Teka *et al.* (2023). Their analysis of national malaria surveillance data shows a 62% decline in malaria incidence between 2014 and 2018 in Ethiopia(463). Nonetheless, the observed decrease in incidence could be attributed to the massive implementation of malaria control measures across the country(234), such as vector control, indoor residual spraying, the widespread deployments of health extension workers, and the expansion of healthcare facilities and community involvements. These measures might have greatly improved public access to diagnosis and treatment(464, 465). In contrast to these successes, between 2019 and 2020, the API increased from 2.55 to 4.19 per 1000 per population at risk, an increase of 64.31% and indicating a slight upward trend. This is likely due to disruptions in service delivery and malaria intervention activities during the global COVID-19 pandemic(466).

Additionally, SIR-based incidence map demonstrated malaria coldspot areas, and a spatial autocorrelation analysis using Moran's I also revealed a random pattern of malaria transmission in the Jimma zone that is local and spatially independent, with transmissions limited to localized areas. It can be implied as the occurrence of malaria in one district had no significant impact on the likelihood of incidence in neighboring districts over time. Thus, factors that might have contributed to this pattern could be localized environmental factors, such as water bodies and microclimates(467, 468) or vegetation coverages(376, 469, 470) that might have created breeding ground for mosquitoes, or effective local interventions such as targeted malaria control efforts like distribution of bed nets, indoor residual spaying, early diagnosis and treatment that might have been successful in containing the spread of the disease within localized areas(297, 299, 415).

Taken together, the lack of significant spatial clustering as indicated by Moran's I test suggests that malaria incidence is not spatially dependent across the studied districts. However, the SIM identifies areas with relatively high risk, such as Chora Botor and Shebe, which may still necessitate vigilance and targeted interventions. The data indicates that the period between 2018 and 2019 was the malaria bottleneck Jimma zone, implying that there could be a missed opportunity to eliminate malaria.

In the current study, the results of temporal analysis demonstrated distinct seasonal patterns of malaria transmission over the period. This study identified two malaria transmission seasons in the Jimma zone over the years: the high transmission occurred from August to November, peaking in September, following the main rainy season. A less intense transmission period was observed from March to July, with a peak in May and June, coinciding with a minor rainy season from April to June. This is due to the fact that water bodies create breeding ground for mosquitoes. This finding is consistent with the seasonal malaria transmission trends in Ethiopia(237, 299). As indicated elsewhere above, the annual proportions of malaria cases over the periods showed a general decline, with a concerning upward trajectory particularly between 2019 and 2020. In line with the Global Technical Strategy (GTS) for Malaria 2016-20230(471), the implementation of the malaria intervention across the country as well as the decentralized regional, zonal and local context of prevention and control efforts may have been significant contributing factors observed decline in malaria trends in the zone.

After accounting for the observed seasonal trend in malaria cases, three ARIMA models were tested, and ARIMA (0, 1, 3) x (1, 1, 0) [12] was determined to be the best fit. The forecast for the coming period anticipates a continuation of the recent stability, with no significant upward or downward trends expected. The slight fluctuations in the forecast values reflect the seasonality captured by the model. As a result, it predicts minor seasonal variations over the forecast period. However, as we project further into the future, the prediction intervals widen, indicating increased uncertainty—a common feature of time series forecasting that emphasizes the unpredictability of long-term predictions(449). The outcomes of the model's forecasts from 2022 to 2026 suggest that the series will remain relatively stable, with minor seasonal fluctuations and increasing uncertainty over time. That means, while the forecast is cautious and assumes that data values will remain roughly constant, the prediction intervals reflect the increasing uncertainty as time moves in to the future, which is the inherent limitation of the time series models(472).

Limitations of the Study**:** The malaria historical data obtained from the Jimma Zone health office, this study only included two age groups: which were children under five and individuals five years and older. This prevented the study from examining the significance of epidemiological insights in other age groups, such as school-age children (7–15 years old), people in reproductive age (15–49 years old), and the elderly (64+ years old). his prevented the study from examining the significance of epidemiological findings in other age groups, such as school-age children (7–15 years), people of childbearing age (15–49 years), and the elderly (64+ years). Another limitation was that the cases seen at private clinics weren't included in the historical malaria cases. The current current study also did not examine climate variables (temperature, precipitation, and relative humidity) as the potential drives of malaria transmission in the decline in malaria cases. as well were factors such as like intervention coverage and environmental characteristics that could offer important information about patterns of transmission that could help explain the drop in malaria incidence.

6.6 Conclusion

The outcomes of the various tools for analyses in this study could lead to the conclusion that, between 2012 and 2021 there was significant decline in malaria incidence in Jimma zone, resulting in a very low risk status in 2021. However, the disease remained a possible threat to public health, especially in regions like Cholar Botor and Shebe Sonbo, where transmission was historically higher. Of the two major *Plasmodium* species in the zone, *P. falciparum was* the predominant one, with a significant proportion of *P. vivax*. Coming to the pattern, malaria transmission was found to be highly seasonal, with higher cases occurring from August to November after the main rainy season and from March to June after the rainy periods. This pattern highlights the need for targeted interventions during these periods to effectively control the disease. Spatial autocorrelation analysis also showed that malaria transmission was both random and spatially independent over time, suggesting that there are no clear spatial patterns of transmission. Therefore, the authors of this work recommend that, in addition to consolidating the progress made, it is imperative to intensify surveillance efforts in historically high-risk districts such as Cholar Botor and Shebe Sonbo. It is recommended to preserve the current control measures while concurrently adapting strategies based on spatial data to ensure a prompt response possible emerging in transmission patterns. Although current malaria control measures appear to be effective, it is important to remain vigilant and monitor possible changes in transmission dynamics. Finally, the forecast indicates the persistence of current malaria burden in the study area in the near future. As we proceed further into the future, it is crucial to consider the inherent uncertainties in the model's predictions and thus, readers should interpret the findings of this study with caution.

CHAPTER SEVEN: GENERAL DISCUSSION

7.1 Introduction

Malaria is a serious threat to public health, particularly in the Jimma zone of the Oromia regional state in particular and Ethiopia at large. The zone appears to face unique challenges like significant number of individuals, such as schoolchildren who are asymptomatic but still infected with malaria parasites, the difficulties in diagnosing the disease, the distribution of parasite species, and the unique patterns of transmission. To inform a more targeted approach to intervention, gaining understanding of this intricate network of the epidemiological factors—especially the ones that have already been mentioned—is crucial. In order to address these pressing concerns, we conducted studies to investigate the epidemiology of malaria in the Jimma zone. In the discussion section that follows, we addressed critical research questions and provided thoughtful responses pertinent to the malaria epidemiology and evaluation of the new highly-sensitive malaria RDT presented in this PhD dissertation.

What proportion of apparently healthy schoolchildren harbor hidden malaria infections, and what factors are associated with these infections in the Gomma District?

In this study, we found a 5.03% overall prevalence of asymptomatic malaria infections diagnosed by qPCR among apparently healthy schoolchildren. However, microscopy and RDT revealed a lower prevalence, 1.51% and 2.2%, respectively, highlighting the limitations of the conventional diagnostic tools and the significance of molecular techniques for detecting low-circulating parasitemia. Of concern, 70% of the overall prevalence diagnosed by qPCR were submicroscopic infections missed by microscopy. The fact that although RDTs appeared to be more sensitive than microscopy, they still missed 56% of infections in this study. This is an additional roadblock that makes diagnosing malaria more difficult in resource-constrained areas like Ethiopia. *P. falciparum was* the most prevalent *Plasmodium* species, accounting for 90% of all asymptomatic infections, followed by *P. vivax* (6%), and co-infections (4%), showing a low species diversity in the Gomma district of the Jimma zone.

It is worth noting that in this study, neither microscopy nor the RDT detected both *P. vivax* or co-infections. However, these infections were only detected by qPCR. One of the unique features of

P. vivax is its low circulating parasitemia (473). Therefore, due to the fact that these conventional tools suffer from limitations with the detection threshold, such low-circulating parasites are suggested to be missed to detection (474). Similarly, studies suggested that the ability of an RDT to capture the glycolytic enzyme pLDH is directly linked to the parasite density, despite the enzyme being a short-lived antigen produced in small quantities. As a result of the low concentration of this antigenemia in circulation, the RDT might have missed it (190, 475, 476).

Hence, the findings of this study provide two critical implications for malaria elimination (policy implications):

First, the conventional malaria diagnostic tools significantly underestimated the true burden of asymptomatic malaria in schoolchildren. This underestimation arises because these tools, particularly microscopy, often misses low-density infections in asymptomatic cases. Results from a meta-analysis of 42 malaria prevalence studies support this finding; The researchers found that microscopy only detected half of the cases identified with PCR technique (206). Under such conditions, relying heavily on these tools can lead to far-reaching consequences, including delayed progress, inefficient resource allocation, and possibly even outbreaks caused by undetected transmission chains. Therefore, exploring more sensitive diagnostic techniques is imperative, especially in regions nearing elimination, such as asymptomatic infections in surveillance system, which may be critical for halting transmission and realizing a future free of malaria (477).

Second, the prevalence of asymptomatic malaria infections among subjects in the current study suggests that these children may act as potential reservoirs for the disease and silently contribute to local malaria transmission. This is supported by a study conducted by Gonçalves *et al.* (2017), which examined the human infectious reservoir for *P. falciparum* malaria in areas with varying transmission intensities. The study revealed that school-aged children contributed more to local transmission during the dry seasons than other age groups in Burkina Faso (478). This is also consistent with a systematic review by Okell *et al.* (2012) estimated that asymptomatic submicroscopic malaria infections accounted for 20–50% of ongoing transmission to community in low transmission settings (129). Further supporting evidence is an experimental study conducted in Ethiopia, which showed that 76.2% of the infectious reservoir was due asymptomatic microscopic infections, with symptomatic and submicroscopic cases contributing only 8.0% and

15.8%, respectively, to ongoing transmission of malaria(130). Taken together, regardless of variation in transmission settings, both microscopic and submicroscopic asymptomatic malaria infections have been shown to contribute significantly to ongoing malaria transmission (131).

The findings of this study can be explained in terms of existing knowledge (*theoretical assumptions, immunological perspectives,* and *empirical researches*). In the following sections, we have tried to make our best to mirror our findings in the eyes of those existing knowledge.

The phenomenon known as host-parasite co-evolution, in which the parasite evades immune detection and the host suffers no harm, was described by earlier theories (479). Regarding the *Plasmodium* parasite, it has been proposed that asymptomatic infections are necessary as a re-engineered strategy by the parasite to ensure its survival and enhance transmission while minimizing host damage, particularly as the intensity of transmission decreases. This equilibrium, which we refer to as *asymptomatism*, represents a harmonious relationship between the parasite and host (133, 480). That is, the parasites benefit by acquiring resources from the hosts without causing too much harm, while also optimizing transmission to mosquitoes when mosquito bites are scarce. The host, on the other hand, avoids the expenses associated with a serious illness. For example, a parasite that produces more gametocytes (transmission stages) may have a higher chance of being transmitted while causing less damage to its host. A parasite that causes more severe disease (produces more asexual stages) may have a lower transmission risk, while it also reduces the host's survival. This dynamic interaction is known as the transmission-virulence trade-off hypothesis (89, 133, 355).

According to this hypothesis, parasite strains must weigh their capacity for transmission against the harm (virulence or pathogenesis) they cause to their hosts in order to survive. As a result, extremely virulent parasites multiply quickly but before transmission can take place, they kill the host or cause them to seek medical attention. Conversely, less pathogenic parasite strains increase the chances of transmission by extending the host's life. Natural selection eventually favors parasite strains that reach the ideal balance. Consequently, this explains the cause of asymptomatic malaria infections, in which the parasites inflict little harm on the hosts, enabling them to live longer and be bitten by mosquitoes, thus guaranteeing transmission (481, 482). For example, *P. falciparum,* the deadliest malaria parasite, demonstrates such an interesting adaptation as a result

of this trade-off. Hence, the parasite gives priority to be transmitted in low-transmission environments by devoting more resources to the production of transmission stages to mosquitoes, the gametocytes. By maximizing its fitness for further spread, this tactic may be able to save the species from going extinct in places where mosquito bites are less common (483, 484). This highlights the remarkable adaptability and enduring presence of these ancient parasites throughout human history (485). In this context, transmission intensity refers to the rate at which new malaria cases occur in a population, and it can vary depending on factors such as season, vector abundance, and control measures (486).

On the other hand, our findings can also be viewed from an immunological perspective. The intensity of malaria is not a coincidence; rather, it is the outcome of a carefully regulated immunological tug of war. The pro-inflammatory cytokines (IFN-γ, tumor necrosis factor alpha (TNF-α), and IL-12) (487, 488) a and the anti-inflammatory cytokines (IL-10, IL-27, and tissue growth factor beta (TGF-β)) (488, 489) are the main actors behind this phenomenon. Consequently, the body produces an excess of pro-inflammatory cytokines due to immune system overstimulation, which activates immune cells and results in severe malaria (490). In contrast, increased production of anti-inflammatory cytokines, particularly IL-10, may impede parasite clearance, hinder the establishment of antiparasitic immunity, and prevent clinical malaria while promoting the development of asymptomatic malaria infections. It has therefore been demonstrated that increased production of anti-inflammatory cytokines was observed during repeated infections in children (491). The Frimpong *et al.'s* (2020) investigation into the relationship between pro- and anti-inflammatory cytokines and the onset of asymptomatic malaria in children under the age of 15 lends support to this. Their findings showed that, in comparison to children who were not infected, children with asymptomatic malaria had higher plasma levels of both cytokines. This cytokine balance contributes to the explanation of why these children did not display clinical malaria symptoms (492). Accordingly, Andolina et al.'s(2021) longitudinal study provided additional evidence of this phenomenon. In the end, they came to the conclusion that 83.8% of the human infectious reservoir was made up of microscopic asymptomatic infections, and that school-age children were the main source of transmission (58.7%). Notably, four children accounted for 62.2% of the infected mosquito population, indicating that these children were important contributors to the ongoing spread of malaria (120). On the other hand, it was suggested

that the absence of recurrent infections signifies a loss of immunity to the disease, which can result in clinical malaria (493).

In the end, the finding of our study can also be viewed through the lens of empirical research, which may clarify why some people—especially school-age children—remain asymptomatic while carrying the deadliest parasite species, *P. falciparum.* For example, Andrade et al. (2021) conducted a study in Mali to investigate how *P. falciparum managed* to bridge two transmission seasons that are frequently several months apart. During the dry season, *P. falciparum can* remain in the blood of asymptomatic people for months, according to their findings. The parasite then became avirulent and, most likely, keeping parasitemia levels below clinical and immunological surveillance. The authors concluded that low parasite density during the dry season was due to increased spleen clearance rather than decreased replication. This adaptation allowed the parasites to remain undetected and asymptomatic until conditions became favorable for transmission (122). However, recent scientific breakthroughs have discovered a hidden malaria life cycle within the spleen (123, 124). These studies discovered that the spleen contains significantly more live parasites than circulating parasites. In particular, it contained 98.8% and 93.1% of the live and total parasite biomass of *P. vivax* and *P. falciparum*, respectively. Similarly, *Nilson et al.* (2015) discovered another extravascular life cycle of malaria parasites in the bone marrow through a series of studies (44) that incubation appears to rewriting the malaria parasite life cycle

Furthermore, several studies have investigated the prevalence of asymptomatic malaria among schoolchildren elsewhere (262, 264, 274, 347, 348, 361, 368, 494-496). The prevalence of asymptomatic malaria in our study is marginally lower than the 6.8% reported from northern Ethiopia (264), but higher compared to the 1.62% reported from southern Ethiopia (348). Notably, both these comparative studies relied solely on conventional malaria diagnostic methods. Our findings diverge from the higher prevalence rates reported elsewhere, such as 22.4% reported from Rwanda (497), 26.4% in Tanzania (361), 59.6% in Nigeria (495), 28.73% in Benin and 38.1% in an island in Tanzania (496) schoolchildren.

In additionally, this study also examined key risk factors associated with asymptomatic malaria infection in schoolchildren. Accordingly, children who had anemia had a significantly higher risk of contracting the infection, which is supported by a previous school-based survey in Oromia

(274), Kenya (267), Ghana (347), Cameron (368), and Tanzania (361). In this regard, our study linked anemia with a higher risk of asymptomatic malaria infection suggesting that anemia could be as a consequence of underlying conditions that weakened the immune system and made children more susceptible to infection. Similarly, household factors also played a role: having a head of household over the age of 55 who engage in nighttime outdoor activities, as well as a lack of ITNs in the home, significantly increased the risk for schoolchildren. This association could be due to the fact that older household heads tend to be active outdoors at night. This could also be explained by increased exposure to infected mosquitoes feeding indoors at night, as nighttime is suitable for female *anopheline mosquitoes* to take blood, which might have increased the risk of bites for children (498, 499). Further to that, the presence of stagnant water near the home was identified as a significant environmental risk factor for malaria. This is consistent with our understanding of the existing knowledge that stagnant water bodies offer breeding sites for mosquitoes *(*500*)*. This is linked to a higher likelihood of children being exposed to infected mosquitos, which increases their risk of malaria infection. This is consistent with previous reports (501, 502). Strikingly, the result of this study revealed a link between a family history of malaria and a lower risk of infection in their children, indicating a possible protective effect. For this, we can draw two possible explanations: First: *Owing to behavioral adaptations:* Because of increased awareness, a family with previously experiences of malaria might have embraced preventive measures more readily (503). This could include using ITNs consistently, implementing other mosquito-protective measures, or seeking medical attention as soon as symptoms start. Such adaptations may lead to fewer mosquito bites and a lower risk of infection in their children The second factor could be the existence of partial immunity. Evidences have demonstrated that school-age children who have had multiple malaria infections had developed partially immune (504). However, this result of the current study is not consistent with the study conducted in northern Ethiopia (356).

What is the added diagnostic yield of hsRDT compared to conventional malaria diagnostic methods in detecting *P. falciparum infections* in asymptomatic schoolchildren?

Our study found that the hsRDT had a higher sensitivity (48.89%) compared to microscopy (33.33%) when qPCR was a reference method. It detected an additional seven cases of asymptomatic *P. falciparum* infections in schoolchildren that had been missed by microscopic examination. The findings align with a previous study that also reported higher detection rates with

RDTs compared to microscopy (505). This higher sensitivity of the hsRDT observed in our study is in agreement with the findings in western Kenya on asymptomatic school-age children (296). However, compared to the same study in western Kenya, the sensitivity of hsRDT in our study was lower while demonstrated higher specificity and PPV. This difference might be due to factors like parasite density in the study population or specific study design like a random selection of follow-up was used in the Kenya. Similarly, the sensitivity of the hsRDT in this study is lower than the average sensitivity (56.1%) of a systematic review that included eight studies on the RDT against qPCR as a reference (506). The inconsistency could be attributed to the fact that performer tests were compared on known concentrations of HRP2 in various samples in the review. However, when the reference method was a combination of microscopy and qPCR, the hsRDT manufacturer's information in the WHO requalification document (507) shows a sensitivity and specificity of 99.0% and 98.6%, respectively, showing higher test performance than the findings of our study. The discrepancy might arise from the manufacturer's assertions that tests were conducted on a mixed sample that was archived and came from various geographical and clinical backgrounds. On samples obtained from a low maria setting in Myanmar, however, test performance was assessed, and our results showed slightly higher levels of sensitivity and specificity (44 and 95.8%, respectively) than those stated by the manufacturer in the same document. The small sample size (493) they used seems to be the cause of the difference. It's worth mentioning here that in the current study, hsRDT and cRDT demonstrated identical test performances, defying expectations and consistent with previous findings in different clinical presentations (508-510). However, the sensitivity of hsRDT in this study is higher than elsewhere, (508-510).

Likewise, the hsRDT showed comparable performance to the cRDT (100% and 99.28% sensitivity and specificity, respectively) when both were evaluated using malaria microscopy as a reference. The sensitivity of the hsRDT in this study is comparable to the sensitivity reported in a systematic review and meta-analysis evaluating the diagnostic performance of uRDTs, Alere™ ultra-sensitive Malaria Ag *P. falciparum RDT* (now called hsRDT), was reported. This review found a pooled sensitivity of 50% and specificity of 100% in asymptomatic populations. However, the performances of the cRDT used in our study are superior to those in the review (290). In a similar vein, the new RDT demonstrated an enhanced PPV (100%) compared to the mean PPV (75%

)reported in the systematic review encompassing eight studies (506). This suggests that there were no false positive results and that every individual who tested positive with the new RDT was a true positive. Additionally, the NPV of the hsRDT in our study is marginally higher than that reported in the systematic review at 97.70% (506). This implies that a 97.70% probability of correctly identifying individuals without this condition compared to the reference method.

According to Cohen's Kappa, the current study demonstrated a moderate degree of agreement (64%) with qPCR in accurately classifying individuals as having or not having the infections. (511). Compared to microscopy, hsRDT showed a significantly higher diagnostic yield, detecting seven times more cases.

Using qPCR, the overall prevalence of *P. falciparum in* this study was 4.52. Consequently, the false-negative rates for microscopy and RDTs were 66.70% and 51.33%, respectively. This alarmingly high false-negative rates were undiagnosed by these tools when compared to qPCR, poses significant challenges to malaria control and elimination. This implies that a substantial portion of schoolchildren who had asymptomatic malaria had not been diagnosed, which could have resulted in missed or delayed treatment and a higher risk of developing a serious illness (512). Furthermore, relying heavily on these tools for malaria control and elimination, particularly among asymptomatic individuals, may thwart the efforts and allow the ongoing transmission (140). Microscopy, in particular, has long been considered the gold standard for malaria diagnosis, despite its acknowledged limited sensitivity in detecting malaria infections. During routine microscopic examinations in reference laboratories, parasite densities >10 parasites/µl of blood are detected (513). Furthermore, its sensitivity is significantly lower (in the range of 50-100 parasites/µl of blood) outside of research institutes and regional reference laboratories due to lower-quality reagents and a lack of strict guidelines for preparation, staining, and slide reading. Sensitivity is thus likely to drop even further in environments with limited resources and inexperienced microscopists (174).

Overall, the results of the current study demonstrated that the new kit does not yield more than the routinely used cRDT, even when both reference methods were used. It is important to note, though, that it has been suggested a number of factors may affect how well HRP2-based malaria rapid diagnostic tests perform. Hence apart from the LoD in such malaria RDTs, the r recently emerged

diagnostic challenges are due to resistance to diagnosis as biological threat to malaria control. These comprise low parasite density (as described else in the above sections), the possibility of HRP2/3 gene deletions being common which frequently has led to false-negative result (49).

A recent report from Ethiopia revealed that the widely used HRP2/3-based RDTs failed to detect approximately 9.7% of cases due to these deletions in the parasites (143). Additionally, Fola et al. (2023) reported that about 8.2% of parasites in Ethiopia carried both artemisinin resistance (K13 622I mutation) and diagnostic-resistance genes (pfhrp2/3 deletions) (144). Similar findings have been reported in Peru (145)**,** Ethiopia (146), Eritrea (147, 148), Kenya (149), Zambia (150), Uganda (151), and even in Germany (152) from a patient returning from Ethiopia. And the presence of some chronic diseases in some individuals that have circulated autoimmune antibodies linked to diseases like rheumatism (409).

Based on visual observations of both RDTs during our fieldwork, hsRDT consistently demonstrated stronger positive bands compared to the fainter ones produced by cRDT. This suggests that hsRDT may have greater sensitivity to a wider range of HRP2/3 antigen concentrations in blood samples. In contrast, cRDT exhibited reduced diagnostic capability at lower parasite densities, leading to negative results. This finding aligns with a study conducted in a low malaria transmission setting in Uganda, where hsRDT was significantly more sensitive than cRDT in samples with HRP2 concentrations between 10 and 1000 pg/ml (401). While the observed discrepancy in band intensity could be attributed to variations in circulating HRP2/3 concentrations, we cannot exclude the possibility of increased HRP2/3 gene deletions within the study population as a potential explanation for the identical diagnostic yields (514). Consistent with this, recent studies have revealed that *P. falciparum* evolves to evade diagnosis by malaria RDTs due to deletions of the HRP2/3 gene, leading to false-negative results in 9.7% of cases with HRP2-based malaria RDTs in Ethiopia (143, 515).

When using microscopy as a reference method, the hsRDT performed exceptionally well in detecting *P. falciparum.* With a sensitivity of 100%, it correctly identified all true positive cases. Furthermore, the high specificity (99.28%) indicates a low false-positive rate of 0.72%. This superior diagnostic yield is highlighted by a significant Kappa value of 81%, indicating a nearly perfect agreement with the microscopy. On the other hand, in this case, since the microscopy is

the god standard, individuals without the disease who test positive by hsRDT were seven (false positive).

It is also important to note that neither microscopy nor RDTs detected *P. vivax* and co-infections in this study. As described in the previous sections, the glycolytic enzyme pLDH is generally secreted in small amounts into the bloodstream as a short-lived antigen produced by all *Plasmodium* species, which appeared to limit test performance (190). Moreover, reticulocytes, which comprise only 1% to 2% of the human red blood cell population, are the host cell tropism for *P. vivax.* This could explain the low parasitemia in *P. vivax*-infected patients (516, 517). Even more strikingly, this makes it difficult to detect in asymptomatic populations with RDTs due to their LoDs.

What are the spatiotemporal patterns of malaria in Jimma Zone, over the past decade, and how do these patterns inform the forecast of its trends?

Between 2012 and 2021, over 1.7 million people were screened for malaria across all survey districts. The overall proportion of malaria cases was found to be 20.93%. This study confirms a significant decline in the malaria positivity rate in the Jimma zone over the past decade, with a decrease from a peak malaria positivity rate of 32.97% in 2012 to 9.40% in 2021. However, the distribution of malaria and the observed decline in its incidence varied significantly across different districts and over time. According to the age group, the result our study revealed that children under the age of five constituted small and fluctuating proportions of the total malaria burden over time compared to individuals above five years old. This fining is in agreement with report from Ethiopia (431, 452, 453), South Africa (454). This could be due to factors such as effective prevention measures, improved healthcare and deployment of health extension workers in each village (297). Riley *et al.* (2001) hypothesized that children under five possess both innate immunity and physiological mechanisms that inhibit parasite growth. Moreover, the absence of primed T-cell responses required to initiate fever in this age group usually result in asymptomatic infections with lower parasite densities(455). These factors, in addition to other potential factors such as effective prevention measures, contribute to the lower malaria prevalence observed in this age group. On the other hand, the male to female ratio of malaria infection was 1.15, indicates that males in the study population were slightly more likely to be infected with malaria compared to females, which is consistent with a recent study from Uganda (456), a report from Oromia region

of Ethiopia (431), and southern part of the country (453). This suggests that potential gender-based difference in susceptibility to malaria. The difference could be due to possible factors such as occupational exposure such as agricultural activities (457), behavioral factors such as night outdoor sleeping (458) and activities (459), beer consumption in males suggested to increase risk of contracting malaria due to high chance of mosquito bites (460) and physiological factors such as post pubertal hormone changes makes males prone to mosquito bites(461).

In terms of the distribution of parasite species, the current study found that *P. falciparum accounts* for the majority of malaria cases in the Jimma zone, with *P. vivax* accounting for a considerable proportion of cases and mixed infections showing a declining trend. This finding is consistent with global and national trends. Over 95% of malaria cases reported in Africa are due to this parasite (49). In Ethiopia, the parasite remains the major *Plasmodium* species, accounting for 71.3% of cases, followed by *P. vivax* at 20.9%, while mixed infections are less common, accounting for 1.1% of all cases (299). However, this result is slightly higher than that reported in a ten-year trend analysis of malaria cases around the Arjo-Didessa sugar development sites in southwest Oromia, where *P. falciparum accounted* for 48% of cases and had a positivity rate comparable to *P. vivax* (42.4%). In contrast, mixed infections in our study were below 9.6% (431). Similarly, the distribution of *Plasmodium* species in our study aligns with findings from studies in the western (462) and southern parts of Ethiopia (453) as well as a national malaria survey (463), well as a national malaria survey (463), which reported that *P. falciparum* accounted for 56% of total infections, indicating its high dominance in Ethiopia.

Next, the results of this study showed a clear spatial pattern in malaria distribution in the study districts. Notably, districts that once had disproportionate malaria burdens showed spatial shrinkage over time. Overall, API values fell by a factor of 96.13%, from 87.97 to 3.40 per 1,000 at-risk population. According to the latest national malaria stratification (299), this fall of API classifies Jimma zone to a very low malaria risk area (5<API< 3.40) by 2021. We created a malaria risk map based on the calculated API for all study districts. In support of the above findings, there is evidence of a shrinking malaria transmission area in the zone. That is, while in 2012 seven districts had a high API (≥50), in 2021 all districts exhibited a very low API, with the exception of Shebe Sonbo and Chora Botor, which remained classified as medium to low malaria risk areas. Overall, the results our study are supported by previous report by Teka *et al.* (2023),

which found a significant 62% reduction in malaria incidence across Ethiopia between 2014 and 2018 (463). The observed decline in the incidence could be attributed to the national wide implementation of comprehensive malaria prevention and control measures (234), including vector control interventions, indoor residual spraying, the widespread deployment of health extension workers, and the expansion of healthcare facilities and community participation. These measures might have significantly improved public access to diagnosis and treatment (464, 465). Despite notable progress in reducing malaria, incidence rose by 64.31%, from 2.55 to 4.19 per 1,000 people between 2019 and 2020. The rise in incidence post 2019 could be linked to two reasons: likely due to disruptions in malaria services caused by the COVID-19 pandemic (466), and possibly due to internal matters and civil unrest (463), which might have jeopardized the attentions of malaria control efforts.

Spatial autocorrelation analysis using Moran's I and simulations based on SIR data revealed a localized and random pattern of malaria transmission in Jimma. This indicates that malaria was confined to specific areas, as shown by the 'cold spots' on the map. The localized spread contributed to the overall reduction in malaria, with incidence in one district having minimal impact on nearby. Possible reasons for this pattern include localized environmental factors such as microclimates (467), water bodies (416) or vegetation (376, 469, 470) that could create mosquito breeding grounds, as well as effective local interventions like bed net distribution, indoor residual spraying, and early diagnosis and treatment (297, 299, 415). Taken together, the absence of significant spatial clustering as indicated by Moran’s I test suggests that malaria incidence is not spatially dependent across the studied districts. However, the Spatial Incidence Map (SIM) highlights areas with higher malaria incidence like Chora Botor and Shebe which may warrant targeted public health interventions.

Moving forward, the temporal analysis in the current study revealed seasonal patterns of malaria transmission over the period. Thus, the high transmission occurred from August to November and peaked in September after the main rainy season. A less intense transmission period was observed from March to July, with a peak in May and June, coinciding with a minor rainy season from April to June. This could be due to water bodies that might have created breeding grounds for mosquitoes. This finding is consistent with seasonal malaria transmission trends in Ethiopia (237, 299). As indicated elsewhere above, the annual proportions of malaria cases over the periods

showed a general decline, with a concerning upward trajectory particularly between 2019 and 2020. In line with the GTS for malaria 2016-20230 (471), the countrywide implementation of malaria intervention, along with decentralized regional, zonal, and local context to the prevention and control efforts, may have been significant factors contributing to the observed decline in malaria trends in the area.

After accounting for observed seasonality in malaria cases, we evaluated three ARIMA models and found that ARIMA (0, 1, 3) x (1, 1, 0) [12] provided the best fit. The forecast for the period from 2022 to 2026 indicates that the stability observed in recent years is likely to continue, with no significant upward or downward trends expected. The forecast reflects minor seasonal fluctuations, capturing the seasonality inherent in malaria transmission. However, as the forecast extends further into the future, prediction intervals widen, highlighting the increasing uncertainty typical of long-term time series predictions (449). Thus, while the ARIMA (0, 1, 3) model suggests relative stability with minor seasonal variations, the growing uncertainty in predictions underscores the challenges of forecasting malaria trends over extended periods.

7.2 Strength and limitations of the studies

One of the strengths of this PhD project was that data collection for the first and second objectives took place concurrently among schoolchildren at their respective schools. In this regard, exploring empirical evidence, as suggested by Brooker *et al*, recommends the practical advantages of using schools for malaria control, such as low costs, high compliance, and easier identification and selection of individual schoolchildren in schools, and as well as use of schools and school children as a complementary, inexpensive framework for planning, monitoring and evaluating malaria control particularly in Africa (275) and giant scholars like Cohee *et al*. showcased the importance of targeting schoolchildren and schools as a hub in this era of malaria elimination (142). As a result, the findings of these studies brought to the surface three critical elements necessary for the effective elimination of malaria: the diagnostic challenges of asymptomatic malaria infections, the strategic importance of targeting schoolchildren, and the potential use of schools as key intervention sites.

Another notable aspect of the project was its use of historical malaria data. The Jimma Zone Health Office collected monthly time series data for each district over the years. This approach enabled

us to use advanced statistical analyses such as trend analysis, spatial autocorrelation, and time series forecasting. Using these sophisticated methods, the project was able to provide insights that went beyond standard analyses. Importantly, this study represents the first comprehensive epidemiological examination of malaria in the Jimma Zone, providing invaluable historical perspectives and future trends that were not previously explored at this zonal level.

This project did not, however, come without limitations. Initially, although the qPCR apparatus utilized for the initial two goals was quantitative, it was not adjusted to yield quantitative molecular results, like the number of parasites per µl. As a result, the analyses of these studies did not address this aspect. The first study simultaneous collection of prevalence data and other risk factors, like anemia, was another drawback. Due to the simultaneous data collection, cause-and-effect linkages could not be established because the study was cross-sectional design. The malaria data registry format, which simply divided people into two age groups—under five and five or older—was another drawback. This broad classification limited the study to investigate the epidemiological aspects of malaria infection in greater detail across different age groups, limiting our understanding of age-specific patterns and trends.

7.3 General Conclusion

In conclusion, the significant number of asymptomatic malaria infections found in apparently healthy schoolchildren in this study suggests that disease transmission keeps going in the Gomma district. The qPCR analysis revealed that a substantial proportion of submicroscopic malaria infections were went undiagnosed microscopy (70%) and by RDTs (56%). This emphasizes the high risk of potential disease outbreaks and the critical need for improved malaria control measures. *P. falciparum was* prevalent in 90% of cases, followed by *P. vivax* (in 6%), and mixed infections (4%). This indicates that most schoolchildren had mono-infection with *P. falciparum*, the dominant parasite species among infected individuals. Therefore, it appears that the likelihood of parasite species complexes in such asymptomatic populations and setting with low malaria transmission is very low. Furthermore, risk factors that significantly increase the likelihood of asymptomatic malaria infections in schoolchildren, including the age of the household head, night-time outdoor activities of the household head, the absence of ITN, and the presence of stagnant water near houses. In contrast, family history of malaria was found to be a protective factor in

preventing children from becoming infected with asymptomatic malaria. The diagnostic yields of the hsRDT were higher compared to the gold standard for malaria diagnosis, the microscope, both in terms of sensitivity and detecting more asymptomatic cases when the qPCR was the reference method. However, it demonstrated identical diagnostic performance to the cRDT, when both microscope and qPCR were the reference methods.

Broadly speaking, between 2012 and 2021, the malaria incidence in Jimma zone was decreased by 96.13%, making it a very low-risk area. Additionally, 2019 was malaria bottleneck period in the zone; unfortunately, the disease then appeared to spiked from 2020 to 2021. Because of this, the disease still poses a significant public health threat, particularly in high-risk areas like Cholar Botor and Shebe Sonbo. *P. falciparum was* dominant species. Malaria transmission in the zone was seasonal dependent, mostly with significant peaks occurred from August to November, and March-June. Across the districts, malaria transmission was both random and spatially independent over time, suggesting that there are no clear spatial patterns of transmission. On the other hand, based on the observed data, the time-series forecasts strongly suggest that the current malaria burden is expected to remain stable for the period 2022 to 2026, provided that the current malaria control approaches remain in place. However, it is crucial to acknowledge the inherent uncertainties associated with such model-based predictions. Therefore, readers should interpret the results of this study with caution and consider the limitations of the model.

7.4 General recommendation

For the National malaria control programs (Policy): The current approach to malaria control, primarily focusing on clinical cases, falls short of achieving elimination goals. A holistic strategy is urgently needed, encompassing asymptomatic populations, particularly schoolchildren. Policymakers must expand intervention packages to include school-based programs that integrate malaria testing, treatment, and tracking, along with strengthen education on prevention and control measures.

The superior performance of hsRDT over microscopy in the current study highlights its potential for enhancing malaria control efforts, particularly in resource-limited settings where accurate and timely detection of low-density infections is critical. Equally important is the adoption of more advanced and sensitive diagnostic tools, which are crucial for successful malaria control and

elimination. Targeted interventions for asymptomatic malaria-infected schoolchildren can provide invaluable support to the NMCP in its efforts to control and eventually eliminate the disease.

In addition, a comprehensive examination of the spatiotemporal patterns of malaria is indispensable for identifying high-prevalence areas and peak transmission periods. Such insights are crucial for optimizing resource allocation and designing effective, locally tailored intervention strategies that maximize impact.

For Regional Health Bureau: Malaria transmission is driven by four key components: the parasite, the human host, the vector, and the environment. To effectively disrupt this cycle, it is essential to intervene in at least one of these critical components. Integrating malaria prevention into the primary school and targeting asymptomatic schoolchildren is an effective strategy, as children can help spread awareness and influence community behavior. Additionally, mapping malaria transmission patterns and identifying hotspots are vital for optimizing resource allocation and ensuring that interventions are applied where they will have the greatest impact. Such targeted efforts are crucial in reducing malaria prevalence and achieving long-term control and elimination goals.

Zonal Health offices: This study underscores the urgent need for the Zonal Health Office to strengthen malaria prevention efforts, particularly in areas identified as potential high-risk for transmission. It essential to actively engage the community in awareness campaigns that highlight critical risk factors, such as behavioral modification that discouraging nighttime outdoor activities, environmental interventions that promoting the elimination of mosquito breeding sites around residential areas, and universal access to ITNs that ensures such at-risk populations have access to insecticide-treated nets. Additionally, comprehensive historical malaria data must include all epidemiologically relevant age groups to enhance our understanding of transmission patterns.

For Scientific Scholars: Further research is needed to explore the broader context of school-based surveys on asymptomatic malaria infections. Longitudinal studies examining the cause-effect relationships between asymptomatic infections and the progression of malaria-related outcomes are particularly important to enhance our understanding of the disease dynamics. Additionally, we strongly recommend the development and deployment of rapid diagnostic tests (RDTs) that do not rely on HRP2-based detection of *P. falciparum,* given the increasing evidence of widespread prevalence of HRP2/3 gene deletions. Alternatives such as RDTs-based on pLDH or *Plasmodium*

aldolase may offer more accurate diagnostic tools for detecting *P. falciparum* infections in areas with high gene deletion rates.

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ANNEXES

1.1 Annex I. Curriculum vitae of the PhD candidate

europass



Abdissa Biruksew Hordofa

Date of birth: 28/07/1987 | **Nationality:** Ethiopian | **Gender:** Male |

Phone number: (+251) 911964174 (Mobile) | **Email address:** abdissa.hordofa@gmail.com | **Website:** https://www.ju.edu.et/cphms/node/521?q=node/515 | **Twitter:** https://twitter.com/abdissaburuksaa | **Skype:** abdissa Buruksa |

Address: Aba Jifar Road, 378, Jimma, Ethiopia (Work)

ABOUT ME

I'm a passionate scholar and educator currently advancing my knowledge as a PhD candidate in Tropical Diseases at Jimma University. My research delves into the Epidemiology of Malaria a in Jimma Zone, Southwest Oromia, Ethiopia. with a particular focus on understanding the prevalence and impact of asymptomatic malaria among healthy schoolchildren, reflecting my deep commitment to protecting vulnerable populations from the potential consequences of this silent infection in my home country. Before embarking on my PhD journey, in 2012, I earned an MSc in Medical Parasitology from Jimma University, solidifying my expertise in the field. Beyond research, I actively share my knowledge as an Assistant Professor the Medical parasitology course unit, School of Medical Laboratory Sciences at Jimma University. My academic path began with a BSc in Medical Laboratory Technology, also from Jimma University in 2008. My dedication to Medical Science is fueled by a profound aspiration to contribute to public health improvements in Ethiopia.

EDUCATION AND TRAINING

CURRENT Jimma, Ethiopia

PhD Candidate in Tropical and Infectious Diseases

11/09/2010 – 18/10/2012 Jimma, Ethiopia

MASTER OF SCIENCE IN MEDICAL PARASITOLOGY

22/09/2006 – 11/06/2012 Jimma, Ethiopia

BACHELOR OF SCIENCE IN MEDICAL LABORATORY TECHNOLOGY

WORK EXPERIENCE

18/10/2012 – CURRENT Jimma, Ethiopia

ASSISTANT PROFESSOR OF MEDICAL PARASITOLOGY JIMMA UNIVERSITY

SCIENTIFIC PUBLICATIONS OF MY PhD WORKS

1. Biruksew, A., Demeke, A., Birhanu, Z. et al. **Schoolchildren with asymptomatic malaria are potential hotspot for malaria reservoir in Ethiopia: implications for malaria control and elimination efforts**. Malar J 22, 311 (2023). https://doi.org/10.1186/s12936-023-04736-7
2. Biruksew, A., Demeke, A., Birhanu, Z. *et al*. *Diagnostic performance of NxTek™ Eliminate Malaria-Pf test for the detection of Plasmodium falciparum in school children with asymptomatic malaria*. Malar J 22, 112 (2023). https://doi.org/10.1186/s12936-023-04529-y.
3. Biruksew, A., Demeke, A., Birhanu, Z. *et al* . **Unraveling the Spatiotemporal Clustering of Malaria Incidence: Modelling a decade of epidemiological data in Jimma Zone, Oromia, Ethiopia**. Submitted to **Scientific Reports** Submission ID: 26889577-307e-4112-a93e-4c0d13610bf7.

CERTIFICATES OF TRANSFERABLE SKILLS AND IMPORTANT COURSES

1. ***Harvard University***: Verified certificate for successfully completed and received a passing grade in the course code PH425x: MalariaX: Defeating Malaria from the Genes to the Globe a course of study offered by HarvardX, Eleven weeks online learning initiative of Harvard University. Verified Certificate Issued November 14, 2023. Valid Certificate ID: 77fb2b27a088468dab023341df50b0f8.
2. ***Gene Drive Technology***: CERTIFICATE OF PARTICIPATION for the successful completion of Foundation Gene Drive Course held on 17th September 2023 at Addis Ababa, Ethiopia.
3. ***Hamburg University of applied Sciences, Germany***: Certificate of Participation on Education and Research Oriented Training in Health Sciences (EARTHS) Summer School on Scientific Training of Research Ethics Research Methods and Training Skills in Health Sciences- from 3rd June-28th June 2019.
4. ***Basic Bioinformatics, Techniques in Molecular Biology and LAMP***. June 19-30, 2017. Ethiopian Biotechnology Institute and Institute of Biotechnology; Addia Ababa university in collaboration with University of Calgary, Canada.
5. ***Certificate of Malaria Elimination Training Course***: offered by the WHO awarded on October 31, 2022.

SCIENTIFIC PRESENTATIONS ON NATIONAL AND INTERNATIONAL CONFERENCES

1. Oral presentation at the 14th Malaria Research Network of Ethiopia Symposium held from 9th - 10th May 2024 at Jigjiga University, Somali Regional State

2. Poster Presentation at the 9th Edition of the PAMCA Annual Conference & Exhibition Between September (17th - 21st) 2023 at Addis Ababa Ethiopia.
3. Oral presentation at 2nd Annual Health Research conference of Oromia Health Burau with theme:" Tackling health service delivery bottlenecks through evidence informed decision making" from September 01-02, 2023., Skylight Hotel, Addis Ababa, Ethiopia
4. Oral presentation at 34th Ethiopian Public Health Association annual conference, 2023 Addis Ababa
5. Poster presentation at 13th National Annual Symposium on Malaria Research, 2023 Hawassa University, Sidama Regional State, Ethiopia

PROFESSIONAL ASSOCIATIONS MEMBERSHIPS

1. Global Burden of Disease (GBD)-Since 2024
2. Ethiopian Red Cross Society- Since 2014
3. Ethiopian Medical Laboratory Association- since 2008
4. Ethiopian Public Health association- since 2006
5. Ethiopian Society for Tropical and Infectious Diseases- since 2016

ACADEMIC REFERENCE

Prof. Delenasaw Yewhalaw (PhD): Director, TIDRC, Jimma University.

Professor Delenasaw taught me the Medical Entomology and Malacology course at different levels of my academic career such as during my BSc in Medical Laboratory Technology, MSc in Medical Parasitology, and During my Ph.D. courses. Currently, Prof. Delenasaw is my main promoter while I'm pursuing my PhD in tropical and Infectious Diseases, working on Epidemiology of malaria in Jimma Zone at Jimma University. Email: delenasawye@yahoo.com, Phone (+251) 917804352

1.2 Annex II. Ethical Clearance

Jimma University Institute of Health

Institutional Review Board

Ref.No JIHRPE01/5502

Date: 15/10/2021

To: Abdissa Biruksew,

Subject: Ethical Approval of Research Protocol

The IRB of Institute of Health has reviewed your research project **"Epidemiology of Malaria and its socio-environmental determinants in Ethiopia"**

Thus, this is to notify that this research protocol has presented to the IRB meets the ethical and Scientific standards outlined in national and international guidelines. Hence, we are pleased to inform you that your research protocol is ethically cleared under the following strict conditions:

1. Any significant deviation from the methodological details indicated in the approved protocol must be communicated to the IRB before it has been implemented.
2. Approval shall be only for a period of twelve months. The principal investigator is required to submit an application for the renewal of the ethical approval.
3. The Committee must be notified, in writing, of any alteration to the project including unforeseen events/circumstances that might affect the acceptability of the approved protocol.
4. The Principal researcher is required to immediately notify the committee in the event of any adverse effects on participants or of any unforeseen events that might affect continued ethical acceptability or amendment to the original consent form.
5. The inability of the Principal Researcher to continue in that role, or any other change in research personnel involved in the project.

The IRB wishes you every success in your research.

IRB chairperson
Million Tesfaye, PhD
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1.3 Annex III: Participant questionnaire (English)

Annex 1:

Note: Form should be in duplicate – with a Participant identification number on each one (matching patient identification number). One copy stays with the test for Laboratory analysis of indicator and the other goes to the lab with filter paper for analysis of by qPCR.

A. Individual child's factors

1. Student's age (in years): _____
2. Student's sex:
   0. Female 1. Male
3. Student's Grade level: _____
4. Did the child sleep inside ITN the previous night?
   0. Yes 1. No
5. Does your child have a previous history of malaria?
   0. Yes 1. No
6. The Hb level(g/dl): _____

B. Household factors

7. Sex of the household head (HHH)
   0. Female 1. Male
8. Age group of the HHH (in years):
   1. < 34-18
   2. 35–44
   3. 45-54
   4. ≥55
9. Educational status of HHH
   1. Cannot read and write
   2. Grade 1–8
   3. Grade 9–12
   4. Tertiary education
10. Night-time outdoor activities of HHH
    0. Yes 1. No
11. Does any family member have a history of malaria infection?
    0. Yes 1. No
12. Household ITN ownership?
    0. Yes 1. No

C. Environmental factors

13. Presence of stagnant water around home (inspect)

    0. Yes 1. No

14. Family place of residency

    0. Urban 1. Rular 2. Semi-urban

15. Altitude of the household (in meter above sea level=masl)

    0. ≤ 1600 1. > 160

Participant questionnaire (Afaan Oromoo)

A. Individual child's factors

1. Umurii barataa (waggaadhaan): _____
2. Saala barataa:

   0. Shamarree 1. Dhiira

3. Kutaa barataa: _____
4. Halkan arbe mucaan keessan agoobara jala buleeraa/ bulteetti?

   0. Eeyye
   1. Lakki

5. Mucaan keessan Kanaan dura dhibee busaa/ woobaan qabamee beekaa?

   0. Eeyye 1. Lakki

6. The Hb level(g/dl): _____

B. Household factors

7. Saala abbaa mana bulchuu

   0. Dubartii 1. Dhiira

8. Garee umurii abbaa mana bulchuu (waggaadhaan):1. < 35

   1. 35
   2. –44
   3. 45-54
   4. ≥55

9. Sadarkkaa barumsaa abbaa mana bulchuu

    5. .Dubbisuufi barreessuu hin danda'u
    6. Kutaa 1–8
    7. Kutaa 9–12
    8. Sadrakaa ol'aanaa

10. Abbaan manaa halkan halkan amala gai bahuu qabaa?

    0. Eeyye 1. Lakki

11. Kanaan dura maatii keessan keessaa namni dhibee busaatiin qabamee beeku jira?

    0. Eeyye 1. Lakki

12. Agoobara manaa qabdu?

    0. Eeyye 1. Lakki

C. Environmental factors

13. Naannoo mana jireenya keessanii bishaan chiise jiraa (laalii mirkaneessi)

0. Yes
1. No

14. Family place of residency

0. Urban
1. Rular
2. Semi-urban

15. Altitude of the household (in meter above sea level=masl)

0. ≤ 1600
1. > 1600

1.4 Annex IV. Laboratory procedures

1.4.1 Procedures of the NxTekTM Eliminate Malaria P.f Test

Intended use

NxTek^{TM} eliminate malaria p.f is an ultra-sensitive, qualitative in vitro test for the detection of histidine- rich protein 2(HRP2) antigen of *P. falciparum* malaria in human whole blood. The test is intended for professional and the point-of-care testing to aid in the diagnosis of malaria infection to a general population including pregnant women.

TEST PRINCIPLE

NxTek™ eliminate malaria p.f test contains a membrane strip, which is pre-coated with immobilized poly-streptavidinon the test line and mouse monoclonal anti-chicken IgY on the control line. Two types of conjugates (mouse rFab fragment specific to HRP2 of p.f carboxyl modified latex conjugate and chicken IgY gold conjugate) move upward on the membrane chromatographically and react with biotinylated sheep rFab specific to HRP2 of *P. falciparum and* pre-coted mouse monoclonal anti-chicken IgY respectively. For a positive result, biotinylated sheep recombinant fragment antigen binding (rFab fragment) specific to HRP2 of *P. falciparum and* mouse rFab fragment specific to of p.f- carboxyl modified latex (CML) conjugate will form in the test line on the *p.f* region of the result window. Neither the test or control line are visible in the result window prior to applying the patient specimen. The control line is used for procedural control and shows only that the diluent has been applied successful and that the active ingredients of the main components on the strip are functional.

The general instruction

1. Check the expiration date. If the device is expired use another kit
2. Put on the glove, use the new glove for each patient
3. Open the test pouch and write the patients name or code on the test device
4. Open the alcohol swab and clean the patient's 4th finger. The alcohol must be dried before pricking or the test may not work

5. Pick the patient's finger with the sterile lancet. Wipe away the first drop of blood much sterile gauze or cotton
6. Use the inverted cup to collect the drop of blood
7. Add drowned specimen of blood into the round specimen well
8. Add 4 drops of assay diluent into the square assay diluent well
9. The control line should appear for all results. If it doesn't appear the result is considered invalid and the specimen should be refused using a new test kit.
10. Read the test result after 20 minutes. Do not read test results after 30 minutes. Which results from erroneous results

A. Procedures

1. Open the package and look for the following
   - ❖ Test devise with desiccant individual pouch
   - ❖ Assay diluent
   - ❖ Disposable inverted cup(5ml)
   - ❖ Sterile lancet
   - ❖ Alcohol swab
   - ❖ Instruction for use
2. First, carefully read the instruction for using NxTek^{TM} Eliminate malaria Pf test kit
3. Next, look at the expiration date on the back of the foil pouch. If the expiration date has passed, use another kit. To avoid false results, ensure that the assay diluent used is from the same kit as the new test devise.
4. Open the foil pouch and look for the following:
   - ❖ Result window
   - ❖ Specimen well
   - ❖ Assay diluent well
   - ❖ Then, label the devise with the patient identifier.

B. Test Procedures

I. Blood specimen by venipuncture

1. Using a micropipette draw whole blood: 5 ml specimen
2. Dispense 5ml of drawn whole blood specimen into the round specimen well
3. Dispense 4 drops of assay diluent into the square assay diluent well.

Caution: hold bottle vertically while dispensing. Do not let bottle nozzle touch devise in order to avoid cross-contamination. If you do not hold the bottle vertically, it can lead to erroneous results.

4. Read the test result after 20 minutes.

   Caution: do not read test results after 30 minutes. Reading after 30 minutes can yield erroneous results

II. Finger-pricked blood specimen with lancet

1. Clean the area to be lanced with an alcohol swab
2. Pick the lateral side of the finger with the sterile lancet provided. Then safely dispose of the lancet immediately after.
3. Wipe away the finger drop of blood with sterile gauze or cotton
4. Using a disposable inverted cup (5ml) provided, dip the circular end of a inverted cup into the blood specimen.
5. Dispense 5ml of drawn blood into round specimen well touching pad.
6. Dispense 4 drop of assay diluent into the square assay diluent well.

   Caution: hold bottle vertically while dispensing. Do not let bottle nozzle touch devise in order to avoid cross-contamination. If you do not hold the bottle vertically, it can lead to erroneous results.

7. Read the test result after 20 minutes

   Caution: do not read test results after 30 minutes; reading after 30 minutes can yield erroneous results.

C. Interpretation of results (see figure blow)

1. NEGATIVE: The presence of the result window of one colored line at "**C"** control line (violate) indicates a negative result.

2. POSITIVE: the presence within the result window of two-colored lines; one each at "C" control line (violate) and "P.f" test line (navy blue) indicates a positive result.

3. IN**VALID:** If the "C" control line (violate) is not visible within the result window after performing the test, the result is considered invalid. Instructions may not have been followed correctly or the test may have deteriorated beyond the expiration date. It is recommended that the specimen be restored using a new test devise.



Figure; Overview the NxTek^{TM} Eliminate Malaria P.f Test device

1.4.2 Annex V. procedure for capillary blood sample collection

Materials required

1. Disposable gloves
2. Automatic lancets
3. Alcohol (70%) and gauze
4. Clean microscope slides
5. Capillary blood sample
6. Pencil
7. Gloves
8. Waste and sharps disposal containers

Procedure

1. Label the slide with the student's unique code
2. Assemble the necessary materials and equipment
3. Identify the student
4. Select the finger either from the left or right hand
5. Prepare and clean the finger by swab using a cotton ball saturated with 70% alcohol
6. Allow the site to dry
7. Penetrating the finger with a sterile lancet and taking the blood
8. Place a small drop of blood 1.0 cm far from the frosted end of the slide
9. The spreading slide is placed in front of the drop of blood at an angle of about 30^{o} -40^{o} to the slide and then is moved back to make contact with the drop

10. The drop will spread out quickly along the line of contact with the slide
11. The spreader is advanced with a smooth steady motion so that a thin film of blood is spread over the slide
12. Allow the smear to air-dry

1.4.3 Annex VI: Preparation of Giemsa stock solution and working Solution

This Protocol was named "Malaria Microscopy Standard Operating Procedure (SOP)"– Mm-Sop-02 (178).

Preparation of Giemsa (0.76%) stock solution

For the purpose of quality blood film examination, 0.76% of Giemsa stock solution was prepared as follows:

1. A 3.8 g of Giemsa powder (AppliChem chemical synthesis service, product date 2018, Cat No. 51811-82-62, Germany) was weighed using digital balance and dissolved in 250ml of Absolute methanol (99.9 %) and 250ml of Glycerol anhydrous (AppliChem chemical synthesis service, Germany).
2. Glass beads were added to the solution for the purpose of frequent agitation to homogenize the solution.
3. The solution was heat at 60 ºC in the incubator for one week with frequent agitation.
4. Until used, the solution was kept for one moth at ambient temperature.
5. Date of preparation: 13/11/2021.
6. Place of preparation: Medical Parasitology laboratory, School of Medical Laboratory Sciences, Jimma University, Jimma; Ethiopia.

Standardization and optimization of the Giemsa working solution

For optimization and standardization of the working solution, it was compared to three commercially available Giemsa solutions:

- TAFLEN®: Giemsa stack solution (product date: Aug 2021, Exp: Aug:2023)
- Farmid Addis Ababa: Giemsa stock solution

From each stock solution, a 1:10 dilution was made in buffer solutions (made from one tablet in 100ml and another one tablet in 250ml distilled water) and distilled water alone.

The final solutions were filtered.

Then, for the three prepared solutions, stains were made on control slides (without parasites) to check the quality of the stains and to choose the best.

- Thick blood film- clear white blood cells cytoplasm stained blue and nuclear granules stained purple.
- Alcohol fixed thin blood film: to see platelets, white blood cells cytoplasm stained blue and nuclear granules stained purple.
- Red Blood Cells (RBCs): Size, shape, and hemoglobin-stained parts.

For all solutions, 20 minutes was set. Then the Quality of the preparations were poor.

Then, 30 minutes was set, AppliChem and Farmid Addis Ababa showed some promising.

After several trails, it was found out that, TAFLEN® was rejected as found to be not good based on the above criteria.

Then, AppliChem and Farmid Addis Ababa were continued to work with and without buffer solutions. Again, it was found out that use of Buffer solutions of different products was un necessary due the fact that it distorted our preparations (the pH).

Then, the two Giemsa working solutions prepared from distilled alone and control blood films were stained for 30 minutes.

Finally, Giemsa stain working solutions of AppliChem chemical synthesis service, product date 2018, Cat No. 51811-82-62, Germany and Farmid Addis Ababa were showed quality stains that showed both positive and negative control blood films.

For the purpose of our study, AppliChem chemical synthesis service, product date 2018, Cat No. 51811-82-62, Germany was chosen as a quality Giemsa preparation.

Final date for optimization/standardization approval: 21/2/2022: Medical Parasitology laboratory, School of Medical Laboratory Sciences, Jimma University, Jimma; Ethiopia

Supplies and Equipment

1. 3.8gm Giemsa powder
2. 250ml Glycerol
3. 250ml Methanol
4. Whatman filter paper

5. Measuring cylinders
6. Pasture pipettes
7. Containers
8. buffered water, pH 7.2
9. beaker or tube

Procedure for Giemsa stock solution preparation

1. weighed 3.8 Giemsa powder on a digital balance
2. Add 250ml absolute methanol
3. Add 250ml glycerin (after adding those materials shaking frequently)
4. Incubate at 60ºC incubator for one week with frequent shaking
5. Filtered by Whatman filter paper

Procedure for 10% Giemsa working solution preparation

1. Place 9 mL of distilled water into a beaker or tube
2. Filter the Giemsa stock solution through filter paper and transfer it to a 25 to 50 mL container
3. Add 1 mL of Giemsa stock solution
4. Use stain within 15 min of preparation, and discard unused stain

Procedure of Giemsa staining and examination

1. Place the air-dried smear film side up on a staining rack
2. Cover the smear with Giemsa stain and leave for 10 minutes
3. Place the Giemsa-stained smear film side up on a staining rack
4. Wait until the stained smear is dried
5. Add a drop of oil immersion on the slide and
6. Examine under 100x magnification power

1.4.4 Annex VII: Protocols for Molecular Assay

Protocol 1: DNA Extraction and Isolation

The Chelex-100 Resin-Saponin Method for Isolation of DNA from Dried Blood Spots (DBS) Filter Paper

Introduction

Chelex - chelating ion exchange resin has a remarkably high preference for copper, iron, and other heavy metals over monovalent cations like sodium and potassium. Its selective affinity for divalent than monovalent ions is roughly 5,000 to 1, and it has a very strong attraction for transition metals, even in the highly concentrated salt solution.

Principles: Chelex-100 resin during DNA extraction from Dried Blood Spots (DBS) prevents DNA degradation from degradative enzymes (DNases) and from potential contaminants that might inhibit downstream analyses. In general, the Chelex resin will trap such contaminants, leaving DNA in solution. The Chelex-100 resin arrests cations such as Mg2+, an important co-factor for DNase action, hence protecting the DNA from degradation (Ref). Saponin, on the other hand, is used to make the plasma membrane of Red Blood cells (RBCs) permeable. This allows small molecules and macromolecules to enter or leave it when DBS is incubated with saponin in an isotonic buffer like PBS. Hence, the procedure leaves parasite DNA in blood exposed to Chelex (518).

Purpose

The purpose of this protocol is to extract DNA from DBS for research use only.

Procedure I**:** adapted from references **(**398**,** 519**).**

Material and reagents

1. Microcentrifuge (>13000 rpm)
2. Water bath (95ºC)
3. Double distilled water (ddH_2O)
4. Eppendorf tubes (1.5 ml)
5. Falcon tubes (conical ended of 50 ml)
6. ¼ round hole puncher (manufactured in China, Catalogue no. BSN62895)
7. 10% saponin (Fluka Biochemika, Catalogue number: 47036, Sigma-Aldrich chemie Gmbh, Riedstr. 2, D-89555 Steinheim, 07329/970, Germany)
    a. Preparation (10% saponin): 1.0g of saponin power in 10 ml of double-distilled water in falcon tube)
8. 1*phosphate buffered saline, PBS (Catalogue number. 198886, expiry date 06/2022. Fisher Scientific. Jassen Pharmaceuticalaan, 2440 Geel-Belgium)
    a. Preparation: Dissolve one tablet in 200 ml ddH_2O

9. 20% Chelex-100(Sigma, Cat no. p44417, Bio-Rad, #412822)
    - Preparation (20% Chelex-100 solution):
        - ❖ Fill the Chelex up to 15 ml of falcon tube
        - ❖ Add ddH_2O up to 40 ml of the falcon tube

DNA Extraction procedures

1. Use the round hole puncher to cut approximately 3-5 mm pieces of the Dried Blood Blotted (DBS) filter paper and put into 1.5 ml labeled Eppendorf tubes and clean the puncher after every sample
2. Add 950 µl 1*PBS and 50 µl 10% saponin in the Eppendorf tubes containing the sample
3. Mix well in the thermomixer, and then incubate for 4ºC for > 4 hours or overnight
4. Centrifuge the mixture at 14000 rpm for 10 minutes at room temperature and discard the liquid content
5. Remove(wash) any remnants of saponin by adding 1ml (1000 µl) of PBS and then spine at 14000 rpm for 5 minutes
6. Discard the PBS and spine the tubes for 30 seconds, and remove the liquid by 200 µl pipet
7. Dry the DNA (on the filter papers) within the Eppendorf tubes left open for 15 minutes at room temperature
8. Add 150 µl of 20% Chelex suspension and 100 µl of ddH_2O to extract the parasite DNA by incubating the mixture at 95ºC in the water bath for 10 minutes, vortex the mixture every 2 minutes in the process of inoculating and extracting
9. Centrifuge the mixture at 14000 rpm for one minute
10. Transfer the parasite DNA into sterile Nunc tubes (*Trademark and Manufacturer??)* of 96-well S-plate or 0.5 ml tubes
11. Keep the DNA at -20 ºC for PCR.

Protocol 2: TaqMan probe detection of *Plasmodium* parasite species-specific detection

Procedure II: adapted from reference **(**344**,** 345**)**

Material and Reagents

1. Primer and Probes for both *P. falciparum* and *P. vivax*
2. qPCR master mix (PerfeCTa® qPCR ToughMix®, Low ROX ™, Quanta Bio vwr catalogue number: - 97065-968, Cost: $2485.19 for 50 ml)

3. PCR plate (Fisher Scientific catalogue number AB1400150. Cost: $284.87/case 150)
4. qPCR machine (QuantStudio3. Applied Bio system Multiplex Real-time PCR Instrument)

In summary:

Ref.	Species	Primer or Probe*	Sequence (5'-3')	Length(bp)
(344)	*P. falciparum*	F-F(forward)	TATTGCTTTTGAGAGGTTTTGTTACTTTG	221
		F-R(Reverse)	ACCTCTGACATCTGAATACGAATGC	
		Pf-fam (MGB)	ACGGGTAGTCATGATTGAGTT	
(345)	*P. vivax*	Pv-1 (Forward primer)	CGCTTCTAGCTTAATCCACATAACTG	142
		Pv-2(Reverse primer)	AATTTACTCAAAGTAACAAGGACTTCCAAG	
		Pv-probe (VIC-MGB)	CGCATTTTGCTATTATGT	

**Thermo Fisher Custom Primer and TaqMan Probe- MGBNFQ*

Multiplex Real-time quantitative (qPCR), QuantStudio3

Note that the standard molecular strength of each primer is 10 µM, and a probe is 2 µM.

1. **Prepare the following master mix in a 1.5 mL tube (Eppendorf tube):**

A. Preparation formula for primer working solution:

- Add 50 µl of each primer stock solution into 450 µl molecular grade water

B. Preparation formula for probe working solution:

- Add 10µl of the probe stock into 490 µl molecular grade water (ultrapure water that certified RNase, DNase, and Protease free)

C. Before starting wrap the tube in aluminum foil to protect sunlight.

D. After thawing, keep the qPCR MasterMix in the fridge if used frequently and store in a deep freezer that is not used for > 2 weeks.

E. Avoid frequent freeze-thaw of the probe. Once thawed, keep in the fridge for up to a month.

2. **Multiplex detection of *Pf* and *Pv***

Master Mix	1x(µl) - for one PCR well	100x (µl)- final volume
PerfaCTa(2x)	6.0	600
Pf-Fam (probe)	0.5	50
Pv-vic (probe)	05	50
F-F(Pf- forward primer)	0.4	40
F-R (Pf- Reverse primer)	0.4	40

Pv-1 (Pv- forward primer)	0.4	400
Pv-2 (Pv- Reverse primer)	0.4	400
DNA	2.0 µl	-
ddH_2O	1.4	140
Total volume	**12**	**1000**

3. Seal the plate with optical PCR foil

A. Use the plastic tool of fingers covering with tissue to press on the foil, in particular at the sides and corners after inserting in the Quant Studio qPCR machine

B. Centrifuge (use an empty plate for balance or mix using vortex for 30 seconds

4. qPCR program running

A. Label wells reading: - Probe Pf as FAM, Pv as VIC

B. Label Samples well and standard wells

C. Check the cycling conditions

Temperature (ºC)	Time	Number of cycles
50	2 minutes	
95	2 minutes	45
95	3 seconds	
60	30 seconds	

D. Confirm the total running time = one hour (60 minutes)

E. In the "Run" tab (computer connected-machine),

- ❖ select the instrument
- ❖ press starts
- ❖ Save the (your) experiment

1.4.5 Annex VIII: Log Book format for the [field] study of asymptomatic malaria infection in Schoolchildren

Date: _____

Code: _____

Log Book for The Study of Asymptomatic Malaria Infection in Schoolchildren

Brief details:

1. Student name: _____
2. Grade (Ganda): _____
3. Name of the primary school: _____
4. Gana (Kebele): _____
5. Family telephone number (if any) _____

Student characteristics					**Malaria test results**															
Age	sex	Grade	Body temp (°C)	Hg (g/dl)	cRDT [Pf [HRP-2], Pv[pLDH]]					hsRDT		Microscope					qPCR			
					Pf		Pv		Mixed	Pf		Pf		Pv		mixed	Pf		Pv	
					+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	-ve	+ve	-ve	+ve

Legend: Co-RDT: Conventional diagnostic test; Pf: P. falciparum; Pv: P. vivax; HsRDT: highly sensitive RDT, qPCR: Polymerase chain reaction; +ve: Positive; -VE: Negative