frontiers | Research Topics

Challenges for diagnosis, treatment and elimination of malaria

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Published in
Frontiers in Tropical Diseases
Frontiers in Public Health

ISSN 1664-8714
ISBN 978-2-8325-4843-1
DOI 10.3389/978-2-8325-4843-1

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Challenges for diagnosis, treatment and elimination of malaria

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Citation

Melo, G., De Sousa, T. N., Pucca, M. B., Viana, G. M. R., eds. (2024). *Challenges for diagnosis, treatment and elimination of malaria*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-4843-1

Table of contents

frontiers | Frontiers in Tropical Diseases

TYPE Editorial
PUBLISHED 18 April 2024
DOI 10.3389/fitd.2024.1394693

OPEN ACCESS

EDITED AND REVIEWED BY
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RECEIVED 01 March 2024
ACCEPTED 05 April 2024
PUBLISHED 18 April 2024

CITATION
Pucca MB, de Sousa TN, Cardoso de Melo GC and Viana GMR (2024) Editorial: Challenges for diagnosis, treatment, and elimination of malaria.
Front. Trop. Dis 5:1394693.
doi: 10.3389/fitd.2024.1394693

Editorial: Challenges for diagnosis, treatment, and elimination of malaria

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KEYWORDS

malaria, *Plasmodium*, eradication, malaria transmission, diagnosis, treatment

Editorial on the Research Topic
Challenges for the diagnosis, treatment, and elimination of malaria

Malaria is a major cause of death in many tropical and sub-tropical countries, presenting about 627,000 deaths and 241 million cases in the world. Malaria is still an important public health problem that needs to be more effectively controlled. Delays in diagnosis and treatment are responsible for most deaths in many countries. Moreover, in most malaria-endemic countries, the lack of resources is a huge barrier to reliable and timely diagnosis. It is a global priority to reduce the high malaria burden and to achieve long-term malaria eradication. In this Research Topic, constituted by 10 papers, we review and discuss the current challenges of malaria transmission, diagnosis, treatment, control, and future eradication.

Challenges for diagnosis, vaccines, and treatment

Vector-targeted interventions are highly effective in preventing malaria transmission and are an essential component of elimination strategies. The detection of *Plasmodium* infection in mosquitoes can be used to estimate exposure and transmission intensity and is a critical part of the management of malaria. Hugo et al. have successfully unveiled a groundbreaking diagnostic test designed for the rapid detection of *Plasmodium falciparum* in mosquitoes, which can achieve results in less than 30 min. This test is specifically tailored for low-resource settings, offering an invaluable solution to address the challenges posed by limited infrastructure. The methodology integrates a swift and uncomplicated sample preparation procedure with isothermal amplification by utilizing recombinase polymerase amplification (RPA). This is coupled with a subsequent lateral flow detection (LFD) step. The synergy of these elements enables the test to deliver efficient and timely outcomes. Remarkably, the developed RPA-LFD test exhibits analytical sensitivity which is at par with the gold standard,

PCR. This signifies its efficacy as a reliable tool for the surveillance of mosquito populations carrying the *P. falciparum* parasite. The rapidity, simplicity, and accuracy of this diagnostic method make it a promising asset in the ongoing efforts to monitor and control malaria in resource-constrained environments.

On the other hand, the study developed by Costa et al. aimed to describe the development of seven specific qPCR assays for the diagnosis of *Plasmodium vivax* and *P. falciparum*, targeting coding and non-coding mitochondrial genomic regions as well as evaluating the possible pitfalls associated with the development of these assays. Although qPCR assays with the tested mitochondrial targets reduced the occurrence of non-specific amplifications, they were not able to eliminate them, in addition to hindering the efficiency of specific amplifications. A Cq (quantification cycle) cutoff value could not exclude false-positive findings for most assays, except for PV_CYTB and PF_CYTB, which presented a cutoff value with good specificity. As noted, although mitochondrial targets are considered the most sensitive, they often lose specificity due to their high sequence conservation (*P. vivax* and *P. falciparum* have at least 90% of mtDNA conservation). Therefore, in the panorama of molecular assays with mitochondrial targets to identify *Plasmodium* sp., it is crucial to include a screening phase to evaluate the possibility of cross-reaction between species of the genus *Plasmodium* or even nonspecific amplification in a panel of samples free of human malaria.

In the realm of vaccination, the absence of an effective malaria vaccine stands out as a crucial gap in current strategies. This gap becomes even more pressing with the emergence of drug-resistant *P. falciparum* strains and the resistance of mosquitoes to insecticides, presenting formidable challenges to malaria treatment and elimination. The study of Waweru et al. aimed to bridge this gap by targeting the PfRh5 complex, a pivotal player in the erythrocyte invasion process. This complex comprises Pf-reticulocyte binding homolog 5 (PfRh5), Pf-interacting protein (PfRipr), Pf-cysteine-rich protective antigen (PfCyRPA), and Pf-P113 protein. Antibodies targeting these proteins have been proven effective in inhibiting parasite invasion, rendering them promising candidates for a blood-stage vaccine. However, the hurdle lies in the genetic polymorphisms within these genes, which pose potential obstacles to vaccine development. To unravel these complexities, the researchers conducted whole-genome sequencing of *P. falciparum* isolates from high-transmission regions in Kenya, with a specific focus on the PfRh5 complex. The study unveiled a total of 58 variants within the PfRh5 complex, with PfRh5 exhibiting the highest degree of polymorphism. Significantly, the Lake Victoria parasite population displayed low polymorphisms, suggesting the plausible candidacy of PfRh5 components for a malaria vaccine. These findings underscore the imperative for further exploration into the specific impacts of mutations on the parasite invasion process, offering valuable insights to propel the advancement of malaria vaccine development.

Malaria in pregnancy (MiP) presents a multitude of risks to the well-being of both mothers and their unborn infants. While the connection between severe pregnancy outcomes, including miscarriage and stillbirth, and MiP is firmly established, there is a pressing need for a more comprehensive understanding of adverse pregnancy outcomes and their prevalence in malaria-endemic regions. Acquiring such knowledge is crucial to evaluate the effectiveness of implemented strategies aimed at preventing MiP, notably the safety and efficacy of MiP vaccines. Berhe et al. reviewed the primary adverse effects associated with MiP and delineated the existing strategies to mitigate its impact. The authors underscore the significance of thoroughly assessing this information as a prerequisite to initiating clinical trials for MiP vaccines. This emphasis on pre-trial evaluation ensures a well-informed approach to vaccine development and implementation, thereby maximizing the potential for success in combatting the adverse effects of malaria during pregnancy.

Prevention and control

Malaria continues to be a major global health concern, particularly in resource-constrained settings, significantly impacting children under 5 years old. Long-lasting insecticide-treated nets (LLINs) are a key intervention endorsed by the World Health Organization (WHO) to combat malaria, which show a potential to reduce cases by 50%. In Ghana, where malaria is hyper-endemic, primarily caused by *P. falciparum*, the transmission is year-round, which peaks from June to October. Dako-Gyeke et al. lead efforts to combat malaria through mass LLIN distribution campaigns. Despite progress, challenges persist in achieving strategic plan targets, with identified barriers to LLIN use in various studies. In response, a community health advocacy team (CHAT) was collaboratively created in six Ghanaian communities, which aimed to promote LLIN use through a person-centered approach, thus leveraging the Community Health Planning and Services (CHPS) program. The qualitative study delves into the opportunities and barriers during the pilot implementation of CHATs, which involved 43 members across six communities in Ghana's Eastern and Volta regions. While CHATs effectively sensitized communities and positively influenced behavior change, the challenges included a lack of financial support for transportation and outreach activities.

Despite global efforts, regions like Djibouti and Ethiopia continue to report substantial transmission rates of malaria, which were exacerbated by disruptions from the COVID-19 pandemic. Moreover, studies across sub-Saharan Africa reveal varied knowledge, attitudes, and practices regarding malaria prevention. Factors such as education, income, age, and cultural beliefs may also influence prevention measures. In this context, the study by Addis and Wondmeneh focused on Ada'ar woreda district, in the pastoral region of Afar, Ethiopia, where malaria data is lacking. The research involved 422 households, revealing diverse knowledge, attitudes, and practices. Individuals with poor knowledge tend to practice inadequate prevention methods, and young adults exhibit suboptimal healthcare-seeking behaviors. The study highlights ongoing challenges in awareness and adherence to malaria control measures in the Afar region. The findings offer valuable insights for public health strategies in the Afar region, emphasizing the need for community-specific approaches to combat malaria.

Following the same context, Nigeria, which is celebrating 62 years of independence in 2022, faces a severe malaria burden, contributing significantly to the global caseload and mortality. The perspective article of Oboh et al. underscores that, despite numerous control initiatives, Nigeria consistently leads in both malaria cases and deaths. The diverse malaria transmission patterns across the country emphasize the need for tailored intervention strategies. To address this challenge, the authors advocate for a focused and research-driven approach, exploring vectorial capacity, insecticide susceptibility, hotspot identification, and the genetic makeup of *P. falciparum*. This targeted research has the potential to reveal crucial insights, including the migration of parasite populations. Achieving pre-elimination status demands prioritized efforts to comprehend the circulating *Plasmodium* strains, which will enable informed policy implementation for malaria transmission control in Nigeria.

Mozambique's National Malaria Strategic Plan targets 85% population protection through testing and treatment. However, the country faces challenges due to climatic vulnerability, frequent natural disasters, and susceptibility to climate change. The study of Armando et al. explores the spatial and temporal dynamics of malaria transmission, which integrate socioeconomic, climatic, and land use data. Analyzing data from 2016 to 2018 at the district level, the study employs a Bayesian framework to model malaria cases. The results reveal an increased malaria risk associated with higher temperatures and specific climatic conditions. Moreover, the study identifies lag patterns and establishes links between climate variables and malaria incidence. Notably, education level, access to electricity, and toilet facilities impact malaria risk. The findings provide valuable insights to design early warning systems and targeted prevention strategies to mitigate seasonal malaria surges in Mozambique, where the disease imposes a significant health burden.

In contrast, one focus of the study of Wang et al. was to present the epidemiological data of imported malaria cases in China from 2011 to 2019, i.e., before WHO has declared it to be malaria-free in 2021. This historical epidemiological pattern of imported malaria in China is of utmost importance to provide evidence-based data to prevent malaria re-establishment in this country. Prevention of re-establishment (POR) is understood as any strategy capable of preventing the emergence of malaria outbreaks/epidemics or avoiding the reestablishment of indigenous malaria in a malaria-free country. These findings revealed that the majority of malaria reported cases were from migratory volunteers, regardless of *Plasmodium* species, being imported cases mainly from West and/or Central Africa and Southeast Asia. Therefore, POR of malaria is a key strategy adopted by countries with malaria-free certification to successfully sustain the "malaria-free status."

Although the World Health Organization (WHO) has promoted "test and treat" guidelines since 2010, recommending that all suspected malaria cases be confirmed with a parasitological test, usually a rapid diagnostic test (RDT), prior to treatment, the compliance of this recommendation is not a reality, especially in malaria-endemic areas in developing countries. In these scenarios, febrile patients are presumptively treated as malaria without diagnostic confirmation. The state of the art of an observational study on private sector antimalarial sales in Uganda has been enumerated by Shelus et al. The main goal of this study was to expand the understanding and knowledge about the private sector malaria case management in Bugoye, western Uganda approximately 10 years after the Uganda Ministry of Health launched their "test, treat, and track" policy. Among the study's key findings, the authors noted that, of the 934 customers with suspected malaria who visited study drug stores during the data collection period, only 25% (233/934) purchased a RDT. Therefore, most cases used to be treated presumptively and possibly may not even have the malaria infection. This practice of irrational use of medicines can cause many organic disorders, in addition to contributing to the selection of resistant strains of *Plasmodium* sp. to antimalarials. In view of this, it is mandatory to adopt interventions in the field of pharmacovigilance, with the aim of ensuring rational use of medicines in the private sector of Bugoye, western Uganda.

Author contributions

MP: Writing – original draft, Writing – review & editing. TN: Writing – original draft, Writing – review & editing. Gd: Writing – original draft, Writing – review & editing. GV: Writing – original draft, Writing – review & editing.

Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

frontiers | Frontiers in Tropical Diseases

TYPE Perspective
PUBLISHED 18 November 2022
DOI 10.3389/fitd.2022.1074751

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Major Tropical Diseases, a section of the journal Frontiers in Tropical Diseases

RECEIVED 19 October 2022
ACCEPTED 04 November 2022
PUBLISHED 18 November 2022

CITATION
Oboh MA, Oyebola KM, Ajibola O and Thomas BN (2022) Nigeria at 62: Quagmire of malaria and the urgent need for deliberate and concerted control strategy.
Front. Trop. Dis. 3:1074751.
doi: 10.3389/fitd.2022.1074751

Nigeria at 62: Quagmire of malaria and the urgent need for deliberate and concerted control strategy

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Background: Sub-Saharan Africa (SSA) has disproportionately contributed the majority (95%) of all malaria cases and deaths for more than a decade (2010-2021) and Nigeria contributes the highest in global malaria cases and deaths in the last decade.

Main body: Despite several malaria control initiatives, why is Nigeria still the most endemic malaria country? Published reports have underlined possible reasons for the sustenance of malaria transmission. Malaria transmission pattern in the country is largely and remarkably heterogeneous, hence control measures must take this uniqueness into consideration when designing intervention strategies. Nigeria became 62 years post-independence on the 1st of October, 2022, therefore making positive impacts on all aspects of the country, especially in the health sector becomes imperative more than ever before. To achieve a pre-elimination malaria status, we propose the implementation of focused and calculated research strategies. Such strategies would be consciously geared towards understanding vectorial capacity, susceptibility to approved insecticides, identifying malaria hotspots, and deciphering the genetic structure and architecture of *P. falciparum* within and between groups and regions. This will provide insight into delineating the inter/intra-regional migration of parasite populations, amongst others.

Conclusion: With regard to malaria elimination, Nigeria still has a long way to go. There is a need for dedicated prioritization of research efforts that would provide a basic understanding of the *Plasmodium* parasite in circulation. Such information will support the implementation of policies that will drive down malaria transmission in Nigeria.

KEYWORDS

Nigeria, malaria, focused-research, heterogeneous, elimination.

Introduction

Malaria in Nigeria is predominantly caused by *Plasmodium falciparum* and the country experiences varied endemicity due to the different ecological zones across different states, supporting uneven parasite transmission. Moreover, of the estimated 241 million cases and 627,000 deaths reported in 2021, sub-Saharan Africa (SSA) contributes 95%, while Nigeria is responsible for 24% of the global cases and mortality within the same period (1). Nigeria, a country that is 62 years post-independence on the 1st of October 2022, continues to be the most overburdened in terms of morbidity and mortality. Since the last decade, Nigeria and the Democratic Republic of Congo have topped the number of malaria cases and deaths. However, Nigeria has taken the lead in contributing approximately a quarter (25%) of the total cases and deaths, except in 2017 (2), 2018 (3), 2019 (4), and 2020 (5), where it contributed 24%, 19%, 24%, and 23% respectively.

Main text

The Federal Ministry of Health, Nigeria through the National Malaria Elimination Program has instituted various control measures (6) amidst this gloomy report. The different control strategies being implemented include indoor residual spraying of wall surfaces with dichlorodiphenyltrichloroethane (DDT) and pyrethroids, distribution of long-lasting insecticide-treated nets, and larviciding in different parts of the country at different rates (6, 7). On the other hand, seasonal malaria chemoprevention, intermittent preventive treatment (in pregnant women and infants) with sulphadoxine-pyrimethamine and the use of artemisinin-based combination therapy (ACT) is being used to treat, and prevent malaria infections in susceptible individuals (8). Despite these control initiatives, however, Nigeria is still the most burdened malaria country. Published reports have underlined possible reasons discussed below responsible for the sustenance of malaria transmission.

Vector control interventions are sparingly contextualized to the different ecological zones of the country. In Nigeria, there are six ecotypes: Sahel, Sudan, Guinea savannah, Rainforest, mangrove forest, and freshwater. Peripherally, these ecological zones fall on the northern (Sahel, Sudan, and Guinea Savannah), and southern belts (rainforest, mangrove forest, and freshwater (9). Precipitation, which is a major driver of vector breeding and abundance varies significantly across these zones. For example, the Sahelo-Sudan-Guinea savannah has low precipitation (<2000 mm) (9, 10), while precipitation in the rainforest, mangrove forest and freshwater can be as high as 4000 mm per annum. An in-depth and adequate understanding of vector dynamics across these various zones would positively impact malaria control efforts in Nigeria.

In addition, several anopheline vectors abound in Nigeria from *An. gambiae s.l*, *An funestus*, *An. arabiensis* (11). However, some of these vectors can sometimes be geographically localized: for instance, *An. moucheti*, and *An. melas* has been found to be present and transmit malaria in the rainforest and mangrove forest ecotypes predominantly covering southern Nigeria, but absent in the guinea savannah (northwestern Nigeria) zone (10, 12).

Most studies have principally focused on the composition of vectors, sporozoites rate, entomological inoculation rate, biting behaviors, or insecticide resistance genes (11, 13, 14). The differences in the ecotypes present an opportunity to design vector-tailored control initiative that is peculiar to each zone: for instance, in the rainforest, mangrove forest, and freshwater zones where precipitation can be up to 4000 mm per annum, vector control initiatives such as larviciding would have to be implemented just before the rainy season (January –March in rainforest and mangrove forest; December –May in freshwater ecological zones) and not during the period of heavy rains when larvicides can be easily washed off. In addition, the behavior of ecotype-specific vectors and how they manifest when there is a change of season will be impactful in implementation of control strategies. Though a long-term goal, a deep and focused study to understand how each vector species contributes to, or evaluation of vector microbiome that can be employed to inhibit malaria transmission will provide valuable insights into vector capacity.

Malaria transmission pattern in Nigeria is largely and remarkably heterogeneous, hence control measures must take this uniqueness into consideration while designing intervention strategies. Healthcare accessibility differs between rural and urban areas, where it has been reported that individuals are more likely to embrace control measures, especially the use of insecticide-treated nets in urban as opposed to rural areas (15). Moreover, it has been reported that there is high probability of indiscriminate use of proscribed antimalarials (16–18) that could potentially contribute to drug-resistant parasites in circulation (19, 20). In addition, living in housing patterns that are conducive to mosquito breeding would strongly ensure continuous malaria transmission. Adding to the litany of factors ensuring the high and continuous transmission of malaria is the obvious change in climatic conditions. Different epidemiological and modeling studies (9, 21–23) have attributed a correlation between increased precipitation and temperature with vector abundance and hence malaria transmission. Some of the states in Nigeria sit along coastal lines where the excessive flow of water from dams in neighboring countries leads to flood; a situation that is almost an annual occurrence.

What course of action must then be taken to alleviate Nigeria from this perpetual malaria burden, and achieve a pre-elimination status? First, an increased political commitment at all levels of government, within and between various parastatals, is urgent and imperative. This will ensure that Nigeria, a signatory to the United Nations' sustainable development goal, which seeks to ensure healthy lives and promote well-being for all ages among other things, meets the World Health Organization 2030 Malaria Elimination Plan, or at least is on track to attaining a pre-elimination status. Therefore, adequate planning to prevent the

frequent flooding that occurs in some states must be instituted. Drainages along those coastal areas should be dredged to ensure the free flow of water. Additionally, structured and strategic research plans need to be established. Such will include continuous monitoring of vectorial capacity, and strengthening of ongoing vector susceptibility tests to insecticides in use (13), identifying hotspots of malaria transmission, understanding the genetic structure and architecture of *P. falciparum* within and between Nigerian groups, and geographical regions, delineating the inter/intra-regional migration of parasite populations. Furthermore, it will also be geared towards identifying the extent, dynamics, and heterogeneous nature of non-falciparum malaria transmission within the country. It is equally crucial for national policymakers to quickly align and identify with recent policies evidenced to reduce malaria morbidity within the country. The post-discharge malaria chemoprevention (PDMC) initiative is recommended to reduce hospital readmission and death in children who have been treated for severe malarial anemia (SMA). Children admitted for SMA do not regain full hematological function until 2-3 months. Hence, treatment of these children with approved ACTs 3- 6 months post-discharge has been found to be very effective in Kenya, Uganda (24), and Malawi (25). Data from modeling studies have also shown that PDMC can reduce malaria readmission by 37,000 annually in Africa (26). If implemented in Nigeria, PDMC may provide considerable protection against malaria and anemia in young children. As immunization is one of the most effective disease control strategies, it is crucial that the government through the Ministry of Health make prudent plans for the acquisition and deployment of the newly approved RTS S/ASO1 vaccine (27) to the most vulnerable group - children. The provision of an added 30% protection against malaria episodes will undoubtedly reduce the number of country-wide cases and ultimately mortality. Moreover, the approval of the promising R21/Matrix M malaria vaccine will provide an additional tool for prophylaxis. Due to reports of *P. falciparum* histidine-rich protein II gene deletion in some parts of the country (28), we recommend that a non-hrp2 pan-specific malaria rapid diagnostic test (mRDT) be deployed. This will provide the added benefit of being able to detect non-falciparum species that may be present even though their contribution to malaria in Nigeria is insignificant.

Conclusion

As Nigeria remains the highest global contributor of malaria cases and mortality, attaining the global malaria elimination 2030 target will require, on the one hand, a combination of various effective control strategies that will target the different factors sustaining malaria transmission in the various ecological zones; and on the other hand, a focused, calculated, and conscious research efforts to gain a better understanding of the parasite and their behavior to different control measures.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

MO conceptualized and wrote the first draft. KO, OA and BT provided critical review. All authors contributed to the article and approved the submitted version.

Acknowledgments

We acknowledge ongoing support and funding from the College of Health Sciences and Technology, Rochester Institute of Technology (BNT). MAO is supported through the American Association of Immunologists Intersect Fellowship Program for Computational Scientists and Immunologists.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Tropical Diseases

TYPE Original Research
PUBLISHED 01 March 2023
DOI 10.3389/fitd.2023.1102265

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Major Tropical Diseases, a section of the journal Frontiers in Tropical Diseases

RECEIVED 18 November 2022
ACCEPTED 08 February 2023
PUBLISHED 01 March 2023

CITATION
Waweru H, Kanoi BN, Kuja JO, Maranga M, Kongere J, Maina M, Kinyua J and Gitaka J (2023) Limited genetic variations of the Rh5-CyRPA-Ripr invasion complex in *Plasmodium falciparum* parasite population in selected malaria-endemic regions, Kenya. *Front. Trop. Dis* 4:1102265. doi: 10.3389/fitd.2023.1102265

Limited genetic variations of the Rh5-CyRPA-Ripr invasion complex in *Plasmodium falciparum* parasite population in selected malaria-endemic regions, Kenya

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The invasion of human erythrocytes by *Plasmodium falciparum* merozoites requires interaction between parasite ligands and host receptors. Interaction of *Pf*Rh5-CyRPA-Ripr protein complex with basigin, an erythrocyte surface receptor, *via PfRh5* is essential for erythrocyte invasion. Antibodies raised against each antigen component of the complex have demonstrated erythrocyte invasion inhibition, making these proteins potential blood-stage vaccine candidates. Genetic polymorphisms present a significant challenge in developing efficacious vaccines, leading to variant-specific immune responses. This study investigated the genetic variations of the *Pf*Rh5 complex proteins in *P. falciparum* isolates from Lake Victoria islands, Western Kenya. Here, twenty-nine microscopically confirmed *P. falciparum* field samples collected from islands in Lake Victoria between July 2014 and July 2016 were genotyped by whole genome sequencing, and results compared to sequences mined from the GenBank database, from a study conducted in Kilifi, as well as other sequences from the MalariaGEN repository. We analyzed the frequency of polymorphisms in the *Pf*Rh5 protein complex proteins, *Pf*Rh5, *Pf*CyRPA, *Pf*Ripr, and *Pf*P113, and their location mapped on the 3D protein complex structure. We identified a total of 58 variants in the *Pf*Rh5 protein complex. *Pf*Rh5 protein was the most polymorphic with 30 SNPs, while *Pf*CyRPA was relatively conserved with 3 SNPs. The minor allele frequency of the SNPs ranged between 1.9% and 21.2%. Ten high-frequency alleles (>5%) were observed in *Pf*Rh5 at codons 147, 148, 277, 410, and 429 and in *Pf*Ripr at codons 190, 255, 259, and 1003. A SNP was located in protein-protein interaction region C203Y and F292V of *Pf*Rh5 and *Pf*CyRPA, respectively. Put together, this study revealed low polymorphisms in the *Pf*Rh5

invasion complex in the Lake Victoria parasite population. However, the two mutations identified on the protein interaction regions prompts for investigation on their impacts on parasite invasion process to support the consideration of *Pf*Rh5 components as potential malaria vaccine candidates.

KEYWORDS

genetic variations, Rh5-CyRPA-Ripr invasion complex, malaria, vaccines, erythrocyte (RBC)

Background

The World Health Organization (WHO) estimates the latest global malaria health burden statistic at 627 000 deaths resulting from 241 million malaria infection cases (1). The scourge's heaviest health and economic burden is borne by the developing countries in Sub-Saharan Africa, where an estimated 90% of all malaria deaths occur, with children under five accounting for 78% of all deaths (2). The emergence of multi-drug resistance *Plasmodium falciparum (P. falciparum)* resistant strains and insecticide resistance mosquitos remains a significant challenge in treating and eliminating malaria (3, 4). The lack of an effective vaccine remains one of the most critical gaps in the strategies developed to eliminate *P. falciparum* malaria (5).

The development of an effective *P. falciparum* vaccine focuses on targeting pre-erythrocytic or erythrocytic stages for parasite development and malaria pathogenesis in humans or the parasite development within the mosquito vector (6). Symptoms elicited by parasite infection originate from the erythrocytic stage of malaria infection. At this stage, the merozoite invades the erythrocytes, where at initial recognition of the human erythrocytes, the merozoite orients itself such that the apical region comes to direct contact with the host's erythrocyte membrane, followed by irreversible attachment of merozoites to erythrocytes (7). Ring-like moving junction mediates complete parasite internalization to formation of an intracellular parasitophorous vacuole (8). The whole erythrocyte invasion process is mediated by multiple merozoite proteins mainly expressed on the surface, or in the apical organelles such as rhoptry and microneme (9). Since these proteins are essential for invasion and are exposed to host immune system, they are considered ideal targets for blood stage vaccines (BSV) (10–12). However, the exposure of candidate BSV antigens to human immune system during natural infections subjects them to selective pressure, that may result to high levels of polymorphisms (13). This presents a significant challenge for allele-specific immune responses as an ideal vaccine must be able to protect against multiple genetic variants of parasites (14, 15).

The *P. falciparum* reticulocyte binding homolog 5 complex (*Pf*Rh5) is a primary vaccine target for developing an effective malaria vaccine. The *Pf*Rh5 complex comprises four interacting proteins: *Pf*- reticulocyte binding homolog 5 (Rh5), *Pf*- interacting protein (*Pf*Ripr), *Pf*-Cysteine-rich protective antigen (CyRPA), and *Pf*-P113 protein (16). *Pf*Rh5 proteins bind to erythrocytes *via* the host receptor basigin, while the other three proteins interact within the complex to initiate erythrocyte invasion. *Pf*CyRPA binds directly to *Pf*Rh5, while *Pf*Ripr interacts with *Pf*CyRPA; thus, *Pf*CyRPA forms the contact sites for *Pf*Rh5 and *Pf*Ripr. Studies have shown that *Pf*P113 interacts with *Pf*Rh5 protein on the N-terminal, providing a releasable mechanism for anchoring *Pf*Rh5 to basigin (11, 17). The genes encoding for these proteins are highly maintained, as shown in gene knockout experiments suggesting they are vital for parasite survival (18, 19). Antibodies against *Pf*Rh5, *Pf*Ripr, and *Pf*CyRPA have been shown to inhibit parasite erythrocyte invasion in non-human primates and mice, while antibodies against *Pf*P113 protein have been associated with protection against clinical malaria *in vivo* (20, 21). These studies suggest that all proteins of the *Pf*Rh5 complex can be considered potential BSV targets.

Polymorphisms in all *Pf*Rh5 complex encoding genes could impede the development of an Rh5 malaria BSV. Like the *Pf*Rh5 complex, apical membrane antigen 1 (AMA1), once considered a potential malaria vaccine candidate is also essential for invasion. However, AMA1 is highly polymorphic, leading to allele-specific immune responses and limited efficacy in its Phase IIb trials (22). Investigation into polymorphisms on all members of the *Pf*Rh5 complex, their effects on the protein structure, and their association are significant considerations when designing a vaccine. Studies have demonstrated that *P. falciparum* parasites have a high within host genetic diversity in high transmission regions compared to low transmission settings (23, 24). This is due to the increased probability of recombination between genetically distinct variants in high transmission settings. This extensive genetic diversity is a major hindrance in malaria vaccine development as the host immune responses may fail to recognize all the variants of an antigen (25).

Here, to explore these questions, we analyzed the four genes of the *Pf*Rh5 complex by whole genome sequencing in a cross-sectional sample of parasites from two high malaria transmission regions in Kenya.

Methods

Sampling, DNA preparation, and whole genome sequencing

Parasite DNA was extracted from archived whole blood samples from patients recruited for a drug resistance surveillance study in

local hospitals on four selected islands (Mfangano, Takawiri, Kibuogi, and Ngodhe) in Lake Victoria, a coastal mainland (Ungoye) between July 2014 and July 2016. The study's approval was obtained from the Kenyatta National Hospital - University of Nairobi (KNH-UoN) ethical review committee (P609/10/2014) and the Mount Kenya University Ethics Review Committee (038/2014). Written consent was obtained from all the participants or guardians, and malaria cases were treated per the national malaria guidelines. The samples re-analyzed here were a subset of these studies which has been extensively described elsewhere (26, 27). Briefly, to increase the parasitemia, the field *P. falciparum* parasites were adapted for *in vitro* culture as previously described (28), and DNA was extracted from short-term cultures (1 month) at the schizont stage using QIAamp DNA mini kit (Qiagen, Valencia, CA). Paired-end sequencing libraries were prepared using Nextera XT DNA library preparation Kit according to the manufactures protocol. (Illumina, USA). Whole genome sequencing was performed on Illumina MiSeq technology (Illumina, USA) at 30X coverage generating reads of length 150 bps. These sequences are archived at the DDBJ BioProject, Accession number PRJDB12148. Quality control checks were performed using the FASTQC (Babraham Institute, UK) toolkit version 0.11.5.

Comparison of polymorphisms identified with other regions

For comparative analysis, we obtained previously reported whole genome sequences *P. falciparum* isolates collected from Kilifi, a malaria endemic region in coastal Kenya (29, 30). The mined sequences were generated from two drug trial studies that were conducted between 2005 and 2008, and the sequences deposited in the GenBank repository under accession numbers *Pf*Ripr: MW597717-MW597776, *Pf*Rh5: MW597550-MW597609, *Pf*CyRPA : MW597610-MW597716, and *Pf*P113: MW597459-MW597549.

We also accessed the catalogue of genetic variation in *P. falciparum*, of the global MalariaGEN database v6.0, for comparing and validating the SNPs identified from the Lake Victoria sample population. This dataset comprised of genomic variation records of 7,113*P. falciparum* samples from 28 malaria-endemic countries. The method used to retrieve the data was previously described by Amato et al., 2016. The dplyr v1.0.9 package (Wickham H, François R, Henry L, 2022) in R v4.2.1 was used to filter out the four genes of the *Pf*Rh5 complex using their PlasmoDB unique identifiers. SNPs identified were then filtered and analyzed.

Read mapping and coverage

Sequence reads were aligned against *Plasmodium falciparum* 3D7 reference genome (version 8.1) (https://plasmodb.org/common/downloads/release-46/Pfalciparum3D7/fasta/data/) using Burrows-Wheeler Alignment tool (BWA) (31) (http://bio-bwa.sourceforge.net) with default parameters. The resulting alignment was further processed with Samtools (32) and Picard v1.66 (33) to remove duplicates. SNPs were called using Genome Analysis Toolkit (GATK) HaplotypeCaller with the following parameters – genotyping mode DISCOVERY, –output mode EMIT_VARIANTS_ONLY, –stand_emit_conf 10, and –stand_call_conf 30. To improve the quality of variant calling, we further discarded genotyping calls with coverage of <5 reads. The resulting variant call format (VCF) files were then merged into one file using VCF tools (34).

Variant calling and analysis

The VCF file containing twenty-six samples that passed the quality test from read mapping analysis was used as the input file in VCF tools for variant analysis. High-quality SNPs in four target genes, *Pf*Rh5, *Pf*CyRPA, *Pf*Ripr, and *Pf*P113, were functionally annotated in the SNPEFF tool (35). Called variants were further analyzed in ARTEMIS software (36). MEGA 7 tool was used to perform multiple sequence alignment and translation of nucleotide sequences to amino acid sequences. Gene variants were identified by aligning the amino acid sequence reads to their corresponding 3D7 reference gene sequence. To test sensitivity of the above approach, we analyzed the sequences at different variant calling parameters to assess the impact of these settings on downstream analysis. Additionally, prior to variant calling, we performed base quality score recalibration to adjust the base quality scores of sequencing reads as well as local realignment around indels to reduce false-positive variant calls resulting from alignment artifacts.

Population genetics analysis

The population genetic tests for the neutral theory of evolution (37) and Tajima's D and Fu & Li's statistics and nucleotide diversity (Pi) were calculated using DnaSPv6.1 (38). Tajima's D tested departure from neutrality based on allele frequency distribution in each gene. Fu and Li's D test statistic calculated the variation between the observed number of singletons and the total number of mutations. Pi was used to test the genetic diversity of each gene of the *Pf*Rh5 complex within the parasite population from Lake Victoria region. The *P.falciparum* adenylosuccinate lyase gene, a house keeping gene and apical membrane antigen gene were used as control in this analysis. The sequences for these genes were obtained from Lake Victoria parasite population.

Protein structures

The structure of the Rh5-CyRPA-Ripr complex was retrieved from the Protein Data Bank (http://www.rcsb.org/) under the protein ID 6MPV. The datasets generated from this study were used to map the polymorphic sites of *Pf*Rh5 and *Pf*CyRPA protein structures on the Rh5-CyRPA-Ripr complex 3D structure in Pymol (The PyMOL Molecular Graphics System, Version 2.2.0, Schrödinger, LLC) to determine the location of the SNPs in the 3D protein structure and whether the SNPs were localized in the protein-protein interaction regions of the complex.

Results

Genetic variation in the *Pf*Rh5 complex genes

Whole genome sequence analysis data for the four genes of the *Pf*Rh5 complex were obtained from 26 samples from the Lake Victoria islands.A total of 45, 35, 25, and 3 Non-synonymous SNPs were identified within the *Pf*P113, *Pf*Ripr, *Pf*Rh5, and in the *Pf*CyRPA genes, respectively. The minor allele frequency in the four genes ranged from 0.7% to 24.06%. High-frequency alleles (>5%) were identified in codons Y147, H148, C203, S277, I410, and K429 of the *Pf*Rh5 gene and codons V190, M255, Y259, and A1003 of the *Pf*Ripr gene (Figure 1). Non-synonymous SNPs identified in *Pf*CyRPA gene at codons S25, D236, and V292 and *Pf*P113 gene at codons L17, E234, Q620, and Q857 occurred at low frequency (Figure 1).

Comparison of polymorphisms identified with the Kilifi population

A total of nine non-synonymous previously not observed from the Kilifi population were identified across the four genes in Lake Victoria isolates (39). *Pf*Ripr gene at codon Y226, F236, and T441, *Pf*Rh5 at codon S277, *Pf*P113 at codon L17, Q620, and Q857, and *Pf*CyRPA gene at codon S25 and V292 (Figure 1).

Comparison of polymorphisms identified with global MalariaGEN

We further explored the MalariaGEN data to establish whether the variants identified were also present in the global database. We also screened for variants missed due to differences in analysis methodologies. This analysis confirmed that most of the variants observed from the Lake Victoria population had been previously observed elsewhere and deposited in the global MalariaGEN database giving confidence in our analysis methodology on the probability of missing variants (Table 1).

Population genetics statistics

A sliding window approach was used to calculate the nucleotide diversity (*Pi*) and Tajima's D statistics. All four genes had a negative neutrality summary statistic. Analysis revealed that the *Pf*P113 gene was the most conserved relative to the other three genes of the *Pf*Rh5 complex and the positive control *Pf*ama1 gene, with a Tajima's D summary statistic of – 1.89 and a Pi of 0.00010 with 4 singleton mutations distributed along the 1578 bp nucleotide sequence. Relative to the negative control *Pf*adsl gene, the *Pf*Rh5 gene was the least conserved, with 25 non-synonymous polymorphisms distributed along the entire 2907 bp nucleotide sequence with a Tajima's D summary value of -0.56 and a Pi of 0.00109 (Figures 2, 3). The Fu & Li's statistics were not significant for *Pf*Rh5 and *Pf*Ripr genes. *Pf*CyRPA and *Pf*P113 gene had significant Fu & Li's values, $p < 0.05$. (Table 2)

Polymorphisms on the *Pf*Rh5 protein complex

The polymorphisms established from our dataset were mapped on the *Pf*Rh5 protein complex to show whether they occurred within known protein-protein interacting regions and the *Pf*Rh5 – basigin interaction region. The previously published Rh5-CyRPA-Ripr invasion complex (16) was superimposed with the basigin structure to show the interaction of *Pf*Rh5 with basigin. *Pf*Rh5 binds to basigin *via* His- 102 linker, α -2, α -4, and a disulfide loop

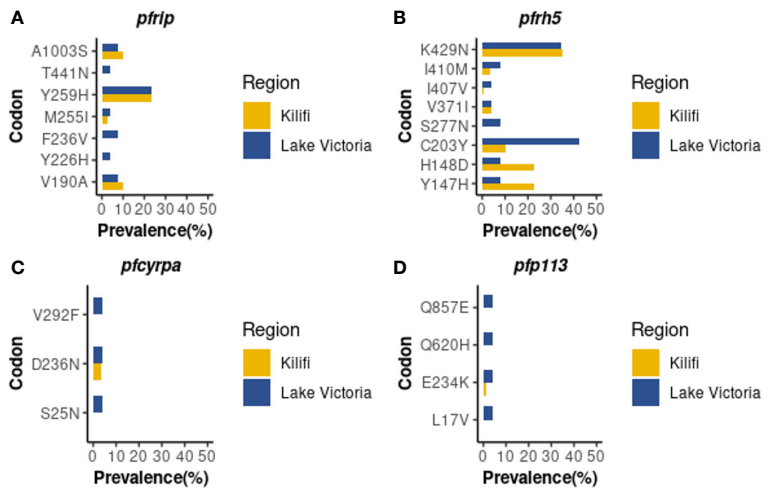

FIGURE 1
Prevalence of *PfRh5* complex polymorphisms. Frequency in percentage of single nucleotide polymorphisms identified across four genes of the Rh5 invasion complex **(A)** *Pf*Ripr, **(B)** *Pf*Rh5, **(C)** *Pf*CyRPA **(D)** *Pf*P113 in Lake Victoria (n=26) and Kilifi (n=68) populations.

(Cys345–Cys351) (40). The F350 and W447 *Pf*Rh5 residues stabilize binding by packing into basigin hydrophobic bonds. Only one SNP corresponding to codon 203 within the *Pf*Rh5-basigin interacting region was identified. *Pf*Ripr binds to *Pf*CyRPA blade 6 at amino acid residues 281 – 311. One mutation corresponding to this interaction region at codon 292 of *Pf*CyRPA was identified. No mutation corresponding to *Pf*CyRPA and *Pf*Rh5 binding regions was identified. Other polymorphisms were localized outside the protein interaction site (Figure 4).

Discussion

P. falciparum infects and replicates in human host erythrocytes leading to manifestation of clinical of malaria. The invasion process by invasive merozoites involves the interaction of *Pf*Rh5 protein and the basigin receptor localized on the erythrocyte membrane (41, 42). However, *Pf*Rh5 does not function alone. Upon secretion, it forms a heteromeric complex with two micronemal proteins, *Pf*Ripr and *Pf*CyRPA. *Pf*Ripr and *Pf*CyRPA proteins do not interact with basigin and have been shown to lack a membrane anchor (11). The rationale for developing a blood-stage malaria vaccine targeting the components of the *Pf*Rh5 complex has been supported by *in-vitro* and *in-vivo* studies in non-human primates. Antibodies raised against the *Pf*Rh5 proteins have been shown to block erythrocyte invasion by inhibiting its binding to basigin receptor (5, 40, 43–45). Genes coding for proteins of the *Pf*Rh5 complex are highly conserved in *P. falciparum*, suggesting their vital role in parasite survival (30, 46). Therefore, an *Pf*Rh5-complex-based vaccine would prove effective. In the present study, we identified the genetic variations of the proteins that make up the *P. falciparum* Rh 5 complex and determined the polymorphism's locus on the protein complex in the parasite population from the Mfangano, Takawiri, Kibuogi, and Ngodhe Islands of Lake Victoria in Western Kenya and compared with Kilifi and global databases. All genes of the *Pf*Rh5 complex were relatively conserved, and the negative population genetics statistic suggests the parasite population has limited potential to retain these mutations.

The observed negative Tajima's D statistics from the Lake Victoria population indicated an excess of rare variants and do

TABLE 1 List of high frequency SNPs identified in Lake Victoria and Kilifi parasite population.

Gene	Position	Ref	Alt	S/NS	Lake Victoria (n=26)	Kilifi (n=68)	MalariaGEN global dataset (n=7,113)
***pf*ripr**	*pfripr_190*	V	A	NS	7.7	10.3	+
	*pfripr_226**	Y	H	NS	3.8	0	+
	*pfripr_236**	F	V	NS	3.8	0	-
	pfripr_255	M	I	NS	7.7	2.8	-
	pfripr_259	Y	H	NS	23.1	23.6	+
	*pfripr_441**	T	N	NS	3.8	0	-
	pfripr_1003	A	S	NS	7.7	10.3	+
***pf*rh5**	*pfrh_147*	Y	H	NS	7.7	22.6	+
	pfrh_148	H	D	NS	7.7	22.6	+
	pfrh_203	C	Y	NS	42.3	10.3	+
	pfrh_277	S	N	NS	7.7	0	-
	*pfrh_371**	V	I	NS	3.8	4.1	+
	*pfrh_407**	I	V	NS	3.8	0.7	+
	pfrh_410	I	M	NS	7.7	3.4	+
	pfrh_429	K	N	NS	34.6	34.9	+
***pf*cyrpa**	*pfcyrpa_25**	S	N	NS	3.8	0	-
	*pfcyrpa_236**	D	N	NS	3.8	3.4	-
	*pfcyrpa_292**	V	F	NS	3.8	0	+
***pf*p113**	*pfp113_17**	L	V	NS	3.8	0	-
	*pfp113_234**	E	K	NS	3.8	1.4	+
	*pfp113_620**	Q	H	S	3.8	0	-
	*pfp113_857**	Q	E	NS	3.8	0	-

Polymorphisms identified in both populations and the global MalariaGEN dataset are indicated with a plus +, and singleton SNPs are indicated by an asterisk*. Ref refers to the 3D7 *Plasmodium falciparum* strain reference amino acid, while Alt refers to the amino acid variation. The SNPs are classified under S (synonymous or NS (non-synonymous) and frequency of SNPs is expressed as percentages.

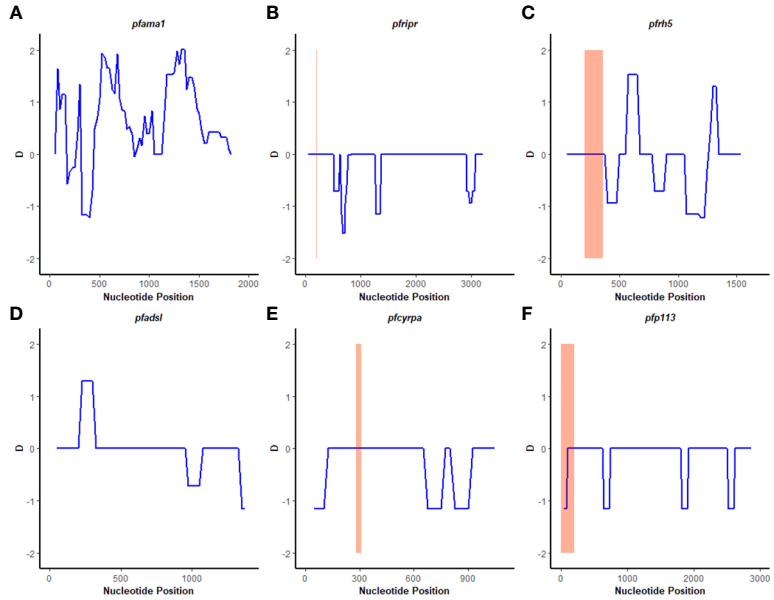

FIGURE 2
Tajima's D analysis. Sliding window analysis of Tajima's D test for neutrality for the four genes of the Rh5 invasion complex of twenty-six samples obtained from Lake Victoria region. **(A)** *Pfama1*, **(B)** *Pf*Ripr, **(C)** *pfrh5*, and **(D)** *pfadsl* **(E)** *pfcyrpa* **(F)** *pfp113* Tajimas' D was calculated in DnaSP v6.1 software with a window length of 100 and a step size of 25 bases. D values are plotted against the mid-point of window length. The highlighted region indicates the basigin – *Pfrh5* binding site and protein-protein interactions regions for *Pfripr*, *Pfcyrpa*, and *Pfp113*.

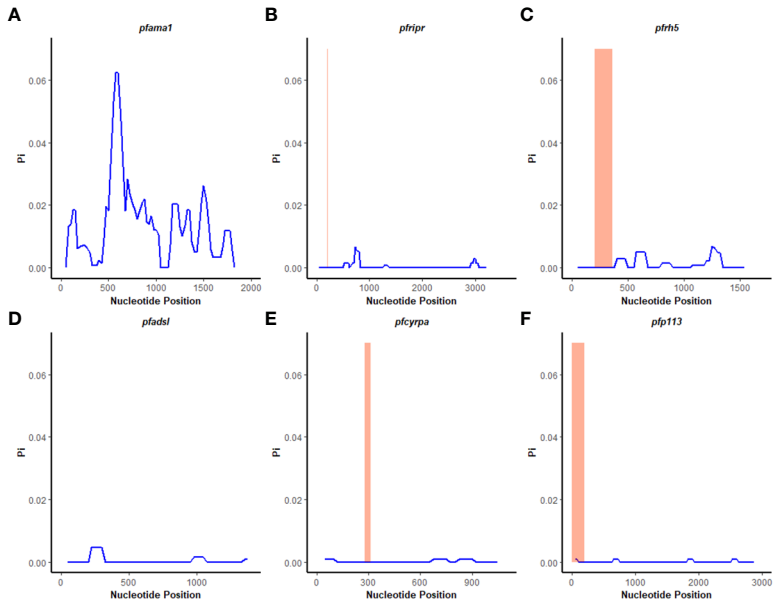

FIGURE 3
Nucleotide diversity analysis. Sliding window analysis of nucleotide diversity (Pi) per site to compare genetic diversity in four genes of the Rh5 invasion complex of twenty-six samples obtained from Lake Victoria region. **(A)** *pfama1*, **(B)** *pfripr*, **(C)** *pfrh5*, and **(D)** *pfadsl* **(E)** *pfcyrpa* **(F)** *pfp113*. Pi is nucleotide diversity calculated using DnaSP ver. 6.1 with a window length of 100 bases and a step size of 25 bases, plotted against the window length's midpoint. The highlighted region indicates the basigin – PfRh5 binding residues and protein-protein interactions regions for PfRipr, PfCyrpa, and PfP113.

not suggest balancing selection (30). Genes with a significant negative Tajima's D value indicate that the parasites population has a limited potential to retain polymorphisms, especially *Pf*P113 and *Pf*Ripr genes (47). These findings are consistent with previous studies of *P. falciparum* in the African population, which showed a majority of genes having a negative Tajima's D value, suggesting a historical parasite population expansion event (48, 49).

TABLE 2 Fu & Li's neutrality tests statistics based on Lake Victoria sample population.

Gene	No. of Samples	S	Fu & Li's D*	Fu & Li's F*
pfcyrpa	26	3	-2.58495*	-2.7088*
pfp113	26	4	-2.86849*	-2.99593*
pfrh5	26	8	0.10999	-0.10381
pfrirp	26	7	-1.11002	-1.37673
pfama1	26	38	4.28469	3.98709
pfadsl	26	3	-0.21602	-0.25135

S refers to segregation sites. Asterisk * indicates significance, p > 0.05.

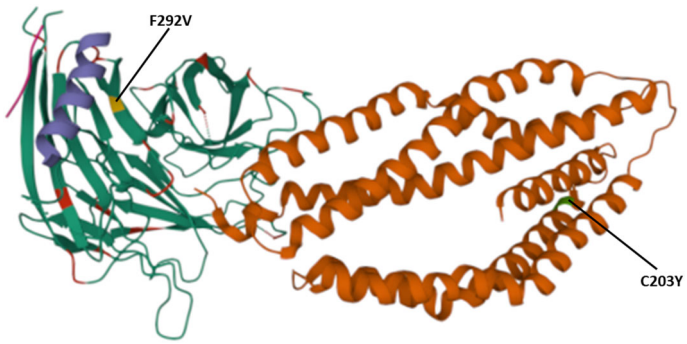

FIGURE 4
Location of polymorphisms on the 3D structure. Shows the SNPs identified that fall within the protein-protein and protein- basigin interaction regions of the Rh5 (brown), CyRPA (green) and Ripr (purple) protein complex. Polymorphic residues C203Y identified on Rh5 protein and F292V on CyRPA are highlighted in green and yellow, respectively. The Rh5 C203 mutation falls within the Basigin-Rh5 protein interaction region, while the CyRPA F292 mutation is located on blade 6, Ripr – CyRPA proteins interaction region.

In contrast to other merozoite antigens that are considered potential vaccine candidates such as Apical membrane antigen 1 (AMA1), merozoite surface protein 1 (MSP1), and merozoite surface protein 10 (MSP10) (50, 51), majority of polymorphisms of the *Pf*Rh5 complex components were rare variants and did not indicate balancing selection. Recent studies from Nigeria and Kenya reported one non-synonymous mutation on *Pf*Rh5 protein at codon C203Y (30, 52). We identified the C203Y mutation in the Lake Victoria population while mutations at codon Y147H, H148D, and K429N were reported in Kilifi samples and MalariaGEN global database as rare variants, which suggests a need for *P. falciparum* to maintain these mutations across various populations. Mutation at codon S277N observed from Lake Victoria isolates was not reported from the Kilifi population. Three singleton mutations at codons Y226H, F236V, and T441N of the *Pf*Ripr gene were identified in Lake Victoria. Among the three polymorphisms, only the mutation at codon Y226H was reported in MalariaGEN global dataset. The mutations were, however, absent from the Kilifi populations.

P. falciparum population from Uganda identified 16 SNPs in the *Pf*Ripr gene (53). Among the SNPs on P*f*Ripr gene identified in our study, three were also observed in Uganda, where a negative population statistic on these variants was reported (53). Considering the geographical proximity between Uganda and Lake Victoria islands, the common variants across the two study sites should be investigated to determine if they affect the functionality of the *Pf*Rh5 complex. Mutations on *Pf*CyRPA S25N, V292F, and *Pf*P113 gene L17V, Q620H and Q857E were identified only in Lake Victoria isolates.

We identified two mutations at the basigin-*Pf*Rh5 interaction region and *Pf*CyRPA-*Pf*Ripr proteins interaction regions. The mutation C203Y on *Pf*Rh5 protein was located on the Rh5- basigin interface, while mutation V292F located on blade 6 of *Pf*CyRPA protein which is the region of interaction with *Pf*Ripr. Studies have demonstrated that recombinant *Pf*Rh5 with the C203Y mutation binds to basigin with the same affinity as the wild type (54).

The components of the *Pf*Rh5 complex are located in different subcellular locations; thus, the complex only forms during erythrocyte invasion when they are secreted from the rhoptries or micronemes (42). Field studies have demonstrated the *Pf*Rh5 complex components exhibit low immunogenicity suggesting the antigens are under limited immune pressure (55). This could explain the limited high-frequency and rare variants observed in this study, as the parasite has a limited need to acquire mutations to escape host immune responses.

Put together, developing an effective malaria vaccine remains a priority among strategies to eliminate and eradicate the disease. One major hindrance to achieving this is the emergence of polymorphisms within the various vaccine antigen targets leading to allele-specific immune responses. Among the *Pf*Rh5 complex,

*Pf*Rh5 is the most advanced vaccine target. However, the presence of low-frequency mutations raises concerns about immune system evasion. This study recommends functional assay studies to investigate the immunological and biological relevance of the identified mutations.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ddbj.nig.ac.jp/, PRJDB12148.

Ethics statement

The study's approval was obtained from the Kenyatta National Hospital - University of Nairobi (KNH-UoN) ethical review committee (P609/10/2014) and the Mount Kenya University Ethics Review Committee (038/2014). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

HW, BK, and JG conceived and designed the study; JG provided the analysed data sets; HW performed the bioinformatic and statistical analysis, under supervision of JOK and BK; HW and BK wrote the first draft of the manuscript, and the final version included edits from all authors. The final manuscript was read and approved by all authors.

Funding

This work was supported by the Royal Society of Tropical Medicine and Hygiene (RSTMH) small grants 2019 (HW). BK is an EDCTP Fellow under EDCTP2 programme supported by the European Union grant number TMA2020CDF-3203. JG received support from the African Academy of Sciences. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

We wish to acknowledge the Mount Kenya University research team for their insights in the improvement of this work.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Public Health

TYPE Original Research
PUBLISHED 28 March 2023
DOI 10.3389/fpubh.2023.1140405

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Infectious Diseases: Epidemiology and Prevention, a section of the journal Frontiers in Public Health

RECEIVED 09 January 2023
ACCEPTED 06 March 2023
PUBLISHED 28 March 2023

CITATION
Shelus V, Mumbere N, Mulogo EM, Barrington C, Baguma E, Muhindo R, Herrington JE Jr, Emch M, Maman S and Boyce RM (2023) Private sector antimalarial sales a decade after "test and treat": A cross-sectional study of drug shop clients in rural Uganda. *Front. Public Health* 11:1140405. doi: 10.3389/fpubh.2023.1140405

Private sector antimalarial sales a decade after "test and treat": A cross-sectional study of drug shop clients in rural Uganda

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Background: The World Health Organization has promoted "test and treat" guidelines for malaria since 2010, recommending all suspected malaria cases be confirmed with a parasitological test, typically a rapid diagnostic test (RDT), prior to treatment with antimalarial medications. However, many fevers at private drug shops in Uganda continue to be treated presumptively as malaria without diagnostic testing.

Methods: The purpose of this study was to document private sector malaria case management in rural Uganda through a cross-sectional survey of drug shop clients in Bugoye sub-county. Drug shop vendors ($n = 46$) recorded information about sales interactions with clients reporting fever or requesting antimalarials and collected capillary blood samples from clients who purchased medications without an RDT. We estimated the proportion of clients who purchased an RDT, adhered to the RDT result, and received antimalarials without having laboratory-confirmed malaria.

Results: Most drug shops were unlicensed (96%) and sold RDTs (98%). Of 934 clients with suspected malaria who visited study drug shops during the data collection period, only 25% bought an RDT. Since some clients reported previous RDTs from the public sector, 40% of clients were aware of their malaria status at the drug shop. Among those with negative tests, 36% still purchased antimalarials. Sixty-five percent of clients who purchased an antimalarial without an RDT subsequently tested negative.

Conclusions: Despite national guidelines, drug shop clients who purchase antimalarials from drug shops in Bugoye are often not tested to confirm a malaria diagnosis prior to treatment. Most clients treated presumptively with antimalarials did not have malaria. Interventions are needed to improve malaria case management and rational drug use in the private sector.

KEYWORDS

malaria case management, malaria diagnosis, drug shops, private health sector, rational drug use

1. Introduction

Malaria remains a leading cause of death in low-income countries, despite considerable investment and effort toward elimination (1, 2). Globally, there were an estimated 241 million cases and 627,000 deaths attributed to malaria in 2020, with 96% of deaths occurring in sub-Saharan Africa (3). In Uganda, *Plasmodium falciparum* malaria is the leading cause of morbidity and mortality among all ages and is responsible for more than one quarter of inpatient deaths in children under 5 years of age (4). Malaria places a substantial burden on the Ugandan health system, accounting for 20–50% of pediatric outpatient visits (5), and has a negative socio-economic impact, with households spending up to 25% of their income on malaria prevention and treatment (4).

Early diagnosis and prompt treatment with antimalarial medications, specifically artemisinin-based combination therapy (ACT), are the primary strategies to prevent severe or fatal malaria complications (6–8). However, the non-specific symptoms of malaria, such as fever, headache, and myalgia, complicate clinical diagnoses (9). While malaria is often equated with a fever, other causes, particularly viral illnesses, account for most presentations (10–12). Therefore, the World Health Organization (WHO) has promoted "test and treat" guidelines since 2010, recommending that all suspected malaria cases be confirmed with a parasitological test, typically a rapid diagnostic test (RDT), prior to treatment (4, 6, 13). RDTs have proven effective for the timely and accurate diagnosis of malaria in many low-resource settings (14, 15).

Regardless of these policies, many fevers continue to be treated presumptively as malaria without confirmatory testing (16). Malaria overtreatment may occur if: (i) no diagnostic test is performed and fever is treated presumptively; (ii) the test result is negative but antimalarials are taken anyway; or (iii) the test result is falsely positive (17). Overtreatment wastes limited resources, delays treatment for other illnesses, and masks the true causes of disease within a population (9, 18, 19). Inappropriate antimalarial use also raises concerns about parasite resistance (9, 18, 19). *P. falciparum* has developed resistance to many previously used antimalarial medications (20), and artemisinin-resistant strains of *P. falciparum* have recently been reported from Uganda and Rwanda (21, 22).

In many low- and middle-income countries a substantial proportion of health services are provided outside the public healthcare system (23). In rural Uganda, where access to public health facilities is limited, private drug shops are commonly the first point of care for fever and an important source of antimalarials (24–27). Drug shops in Uganda can legally sell antimalarials and RDTs, but many shops are not licensed or regulated, and clients may purchase antimalarials without a confirmed malaria diagnosis (26, 28, 29). While RDT availability has increased recently, a 2015 national survey found less than one third of febrile drug shop clients received an RDT (30). Family or friends sometimes seek treatment on behalf of someone with suspected malaria, making it impossible to administer an RDT at the drug shop (30). Even when RDTs are used, clients do not always adhere to the results, purchasing antimalarials despite a negative test (26, 30).

The purpose of this study was to improve understanding of private sector malaria case management in Bugoye, western Uganda ~10 years after the Uganda Ministry of Health launched their "test, treat, and track" policy. To achieve this goal, we documented malaria diagnostic and treatment practices at drug shops, and then assessed the proportion of clients purchasing an RDT prior to the purchase of antimalarials, adhering to RDT results at the drug shop, and receiving antimalarials without having laboratory-confirmed malaria.

2. Materials and methods

2.1. Study setting

Bugoye is a malaria-endemic sub-county in rural western Uganda. The sub-county consists of 35 villages and has a population of ~42,000 (31). More than 80% of households rely on subsistence farming for their livelihood (32). The geography is characterized by deep river valleys and steep hillsides with elevations up to 2,000 meters. The tropical climate allows for year-round malaria transmission interspersed with semi-annual peaks after the rainy seasons (33). Malaria prevalence from the most recent Malaria Indicator Survey in the mid-western region of Uganda, which encompasses Bugoye, was estimated at 18% among children under 5 years of age (34), although more recent studies in the sub-county demonstrate substantial geographic heterogeneity (35).

Public health services in Bugoye are available from six level II health centers staffed by nurses and midwives, two level III health centers staffed by clinical officers, and community health workers (CHWs) who treat pneumonia, diarrhea, and malaria in children under 5 years of age. Level IV health facilities, staffed by physicians, are only available outside the sub-county. Given the remote nature of many villages in Bugoye, private drug shops play a large role in antimalarial distribution. Of nearly 4,000 encounters for antimalarials previously documented in 1 month, 53% sought care from drug shops, compared with 39% from health centers and 7% from CHWs (36). While several patient safety concerns emerged related to the type and dosage of antimalarials administered, no data was collected on the use of RDTs.

2.2. Study design

We conducted a cross-sectional survey of drug shop clients. Vendors recorded information about sales interactions with clients reporting fever or requesting antimalarials and collected capillary blood samples from clients who purchased medications without an RDT. Samples were subsequently transported to a laboratory and tested for malaria. Outcomes of interest included the proportion of clients who: purchased an RDT at the drug shop, purchased antimalarials after a negative RDT, and purchased an antimalarial without an RDT and subsequently tested negative for malaria.

2.3. Drug shop identification

A community sensitization meeting was held to discuss the objectives and methods of the study with local leaders and CHWs and enlist their help in identifying all the drug shops in their

respective areas of the sub-county. The study team then visited the identified drug shops to provide information about the study and assess interest in participation. Drug shops were eligible to participate, regardless of licensing status, if they (i) were in Bugoye sub-county, (ii) sold any medications with an antimalarial effect, and (iii) vendors were willing to be trained on study procedures. Data on the professional background of vendors, years of operation, and the cost and type of RDTs sold was collected during initial drug shop visits (Supplementary material). Licensing status was verified with registration records at the Kasese District Health Office. Participating drug shops were divided into four groups based on geographic proximity, with all groups including a trading center and the surrounding villages. Each group completed training and data collection before the next group started training to reduce the logistical burden of collecting case report forms and blood samples over a relatively large geographic area.

2.4. Vendor training

Prior to implementation, participating vendors received a detailed study manual and completed a 90-min training on study procedures, including instruction on determining client eligibility, assigning ID numbers, completing data collection forms, obtaining informed consent, and collecting blood samples. A laboratory technician from Bugoye Health Center III demonstrated procedures for blood sample collection at each training. Finger-prick blood samples are widely employed to diagnose malaria within this setting, as they are required when using RDTs. Therefore, vendors who sell and administer RDTs were already familiar with this type of blood sample collection. Trainings were conducted by the study team in Ihukonzo, the local language. In accordance with national guidelines on COVID-19, all trainings were conducted outside with masks and social distancing. After the training, vendors collected data for 2 weeks. This data collection period was chosen based on budget and feasibility considerations, using previous antimalarial sales tracking in Bugoye to estimate client volume (36). At the end of data collection, vendors received a one-time stipend of 30,000 Ugandan Shillings (∼$8.50) and a certificate for their participation.

2.5. Data collection and measures

The study was conducted from July to September 2021. All clients who visited participating drug shops during the data collection period reporting fever or purchasing antimalarials for themselves or another individual were eligible to participate. Drug shop vendors completed a paper data collection form for each eligible client. Data collection forms (Supplementary material) included information about the sales interaction (date, time), client demographics (village, age, sex, pregnancy status), brief clinical history (days of illness, symptoms), and medication purchases. Drug shop vendors recorded the results of RDTs purchased and performed at the drug shop, as well as any recent, client-reported results from RDTs conducted elsewhere (e.g., health center or CHW).

If the individual with fever (hereafter referred to as the "index client") was present, did not purchase an RDT at the drug shop, and provided informed consent, vendors collected finger-prick blood samples (∼0.2 mL) into Ethylenediaminetetraacetic acid (EDTA) microtainers (37). If a "surrogate client" was at the drug shop to purchase medications for someone sick at home, no blood sample was collected. Vendors were provided with all materials necessary to safely collect blood samples and dispose of waste. As a public health preventive measure, beginning with group two, clients who provided a blood sample were given a bar of soap to wash their hands. Blood samples were stored at the drug shop in insulated stainless-steel bottles packed with ice for up to 72 h.

2.6. Laboratory procedures

Blood samples from clients who did not purchase an RDT at the drug shop were tested for *P. falciparum* at Bugoye Health Center III by study staff using one of two RDTs: the CareStart Malaria Pf (HRP2) Ag RDT or the SD Biosensor Standard Q Malaria P.f. Ag test, based on local market availability. These RDTs detect the histidine-rich protein II antigen of *P. falciparum, are similar to kits used in routine clinical practice, and have* received pre-qualified status from WHO (38). RDTs were performed in accordance with the manufacturers' instructions and prior to the expiration.

2.7. Statistical analyses

Data was entered using REDCap electronic data capture tools hosted at the North Carolina Translational and Clinical Sciences Institute at the University of North Carolina at Chapel Hill (39). Data cleaning and statistical analyses were conducted in SPSS 28 (IBM Corp.) and SAS 9.4 (SAS Institute, Cary, NC). The primary outcome was the proportion of drug shop clients who purchased an antimalarial without an RDT result and subsequently tested negative for malaria. Secondary outcomes were the proportion of drug shop clients: seeking treatment for themselves vs. others, purchasing an RDT at the drug shop, knowing their malaria status (from RDTs conducted at the drug shop or elsewhere), adhering to the RDT results, and purchasing antimalarials or antibiotics. These outcomes were adapted from a systematic review of interventions introducing RDTs into private medicine retail outlets (40). We employed log-binomial regression modeling to estimate crude and adjusted risk ratios for having a known malaria status at the drug shop. Explanatory variables included client demographic factors, illness history, drug shop and vendor characteristics, RDT cost, visit date and time, and data collection group. Multivariate models included all explanatory variables.

3. Results

3.1. Drug shop characteristics

Forty-six eligible drug shops were identified in 20 villages, and all shops participated in the study. Half of the drug shops (n = 23, 50%) were concentrated in the three largest trading centers in

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TABLE 1 Characteristics of drug shops that provide malaria treatment in Bugoye sub-county, Uganda.

	N = 46	
	n	%
	med (IQR)	
Location*		
Small trading center	23	50.0
Large trading center	23	50.0
Years of operation		
<1 year	9	19.6
1–5 years	19	41.3
5–10 years	8	17.4
10+ years	10	21.7
Licensing status†		
Unlicensed	44	95.6
Licensed	2	4.3
Vendor training		
Nursing assistant	32	69.6
Nurse or midwife	14	30.5
Availability of RDTs		
Yes	45	97.8
No	1	2.2
Malaria RDTs in-stock‡		
Yes	28	60.9
No	18	39.1
Malaria RDT cost	2,000 UGX§ (IQR: 2,000, 3,000)	

*Large trading centers were defined by the presence of a weekly market day.
†License to operate a drug shop issued by the National Drug Authority.
‡On the day of initial drug shop visits in May, June, or July 2021 (prior to data collection).
§Approximately 0.57 USD.

Bugoye (Table 1). Drug shops had been operating for a median of 3 years (IQR: 1.8, 8.0), nearly all ($n = 44$, 96%) without a license from the Uganda National Drug Authority. Most drug shop vendors ($n = 32$, 70%) were trained as nursing assistants, which does not meet the necessary qualifications to operate a drug shop in Uganda. Nearly all drug shops sold RDTs (n=45, 98%), at a median price of 2,000 Ugandan Shillings (IQR: 2,000, 3,000) or ~$0.57 US Dollars. More than half of shops had RDTs in stock during the initial visit ($n = 28$, 61%). Eight types of RDTs were used with the most frequent being SD Bioline, SD Biosensor, Carestart, and First Response. All examined RDTs were valid based on the printed expiration date.

3.2. Drug shop clients

During the data collection period, 934 clients visited drug shops in Bugoye reporting fever or requesting antimalarials. Many clients ($n = 410$, 44%) came to the drug shop outside normal business hours (i.e., 9 a.m. to 5 p.m.), including 28% in the evening and 24% over the weekend. Drug shop clients were evenly split by sex and had a median age of 21 years (IQR: 12, 32) (Table 2). Few clients were among the highest risk groups for severe malaria outcomes—only 11% of clients were under the age of five, while 4% reported being pregnant. The median length of time clients had been sick prior to coming to the drug shop was 3 days (IQR: 2, 4), most commonly with fever ($n = 832$, 89%), headache ($n = 747$, 80%), and joint or muscle pain ($n = 560$, 60%).

In most cases, the index client came to the drug shop to purchase their own medications ($n = 662$, 71%), while 29% of clients purchased medications for others. If a surrogate client was at the drug shop ($n = 272$), it was often a parent seeking treatment for their child ($n = 125$, 46%). Surrogate clients also included siblings ($n = 56$, 21%), spouses ($n = 38$, 14%), children ($n = 34$, 13%) and friends ($n = 8$, 3%). Index clients present at the drug shop were significantly older (24.5 ± 15.8) than those who sent a surrogate (21.1 ± 14.9), $t_{(915)} = -3.067$, $p = 0.002$. There were no significant differences in presence of the index client at the drug shop by sex, days of illness, or visit day and time.

Approximately one quarter of all clients ($n = 239$, 26%), and 36% of index clients present at the drug shop, purchased an RDT at the drug shop (Figure 1). Additionally, 20% of clients reported prior RDT results from a health center IV ($n = 4$, 2%), health center III ($n = 49$, 29%), health center II ($n = 75$, 44%), CHW ($n = 21$, 12%), or another drug shop or private clinic ($n = 20$, 12%). In total, 40% of clients were aware of their malaria status while purchasing medications. Of those with a test result, 54% tested positive for malaria ($n = 202$), while 46% tested negative ($n = 171$).

Most clients purchased an antimalarial ($n = 741$, 80%), specifically artemether/lumefantrine ($n = 499$, 53%), sulfadoxine/pyrimethamine ($n = 118$, 13%), quinine ($n = 76$, 8%), artesunate ($n = 34$, 4%), dihydroartemisinin/piperaquine ($n = 19$, 2%), and chloroquine ($n = 1$, <1%). The majority were oral antimalarials, though 6% of clients ($n = 60$) purchased intravenous antimalarials (artesunate or IV quinine). Additionally, 41% of clients purchased antibiotics, commonly amoxicillin ($n = 240$, 26%), ampicillin ($n = 58$, 6%), and erythromycin ($n = 51$, 6%). Twenty-eight percent of clients purchased both antimalarials and antibiotics at the drug shop (Figure 2).

Among clients who knew they were positive for malaria at the drug shop, most purchased antimalarials ($n = 189$, 94%) and/or analgesic/antipyretic medications ($n = 167$, 83%), though 40% purchased both antimalarials and antibiotics (Table 3, Supplementary material). Clients who tested negative at the drug shop or another location commonly purchased analgesic/antipyretic medications ($n = 158$, 92%) or antibiotics ($n = 119$, 70%), but more than one third purchased antimalarials despite their negative result ($n = 62$, 36%), and 22% purchased both antimalarials and antibiotics ($n = 38$). Most clients with an unknown malaria status at the drug shop purchased antimalarials ($n = 490$, 87%) and/or analgesic/antipyretic medications ($n = 505$, 90%). Approximately one third of clients who did not test for malaria at the drug shop purchased antibiotics ($n = 182$, 32%), and one quarter purchased both antibiotics and antimalarials ($n = 147$, 26%).

10.3389/fpubh.2023.1140405

TABLE 2 Characteristics of clients seeking malaria treatment from drug shops in Bugoye sub-county, Uganda*.

	All clients, $N = 934$		Index client present at drug shop, $N = 662$		Surrogate client present at drug shop, $N = 272$	
	n	%	*n*	%	*n*	%
Sex of client						
Male	448	48.0	305	46.1	143	52.6
Female	485	52.0	356	53.9	129	47.4
Missing	1		1			
Age of client						
Under 5 years	96	10.5	73	11.2	23	8.6
5–14 years	194	21.2	104	16.0	90	33.8
15–49 years	566	61.7	428	65.7	138	51.9
50–69 years	50	5.5	35	5.4	15	5.6
70+ years	11	1.2	11	1.7	0	0.0
Missing	17		11		6	
Individual at drug shop						
Person who is sick	662	70.9	662	100.0	0	0.0
Family member or friend	272	29.1	0	0.0	272	100.0
Days of illness						
0–1 days	129	14.5	83	13.1	46	17.9
2–3 days	478	53.6	332	52.3	146	56.8
4–5 days	179	20.1	139	21.9	40	15.6
6–7 days	79	8.9	60	9.4	19	7.4
More than 1 week	27	3.0	21	3.3	6	2.3
Missing	42		27		15	
Presenting symptoms						
Fever	832	89.1	582	87.9	250	91.9
Headache	747	80.0	536	81.0	211	77.6
Joint or muscle pain	560	60.0	413	62.4	147	54.0
Cough	357	38.2	252	38.1	105	38.6
Nausea or vomiting	204	21.8	158	23.9	46	16.9
Shivering/chills	132	14.1	102	15.4	30	11.0
Fatigue	113	12.1	88	13.3	25	9.2
Diarrhea	97	10.4	68	10.3	29	10.7
Day of drug shop visit						
Weekday	695	75.6	503	77.3	192	71.6
Weekend	224	24.4	148	22.7	76	28.4
Missing	15		11		4	
Time of drug shop visit						
Morning	314	34.7	230	35.9	84	31.7
Afternoon	342	37.7	244	38.1	98	37.0
Evening	250	27.6	167	26.1	83	31.3
Missing	28		21		7	

*Data collected between July 12 and September 22, 2021 from 46 drug shops in Bugoye sub-county. Drug shop clients were eligible to participate in the study if they presented during the data collection period reporting fever or requesting antimalarial medications.

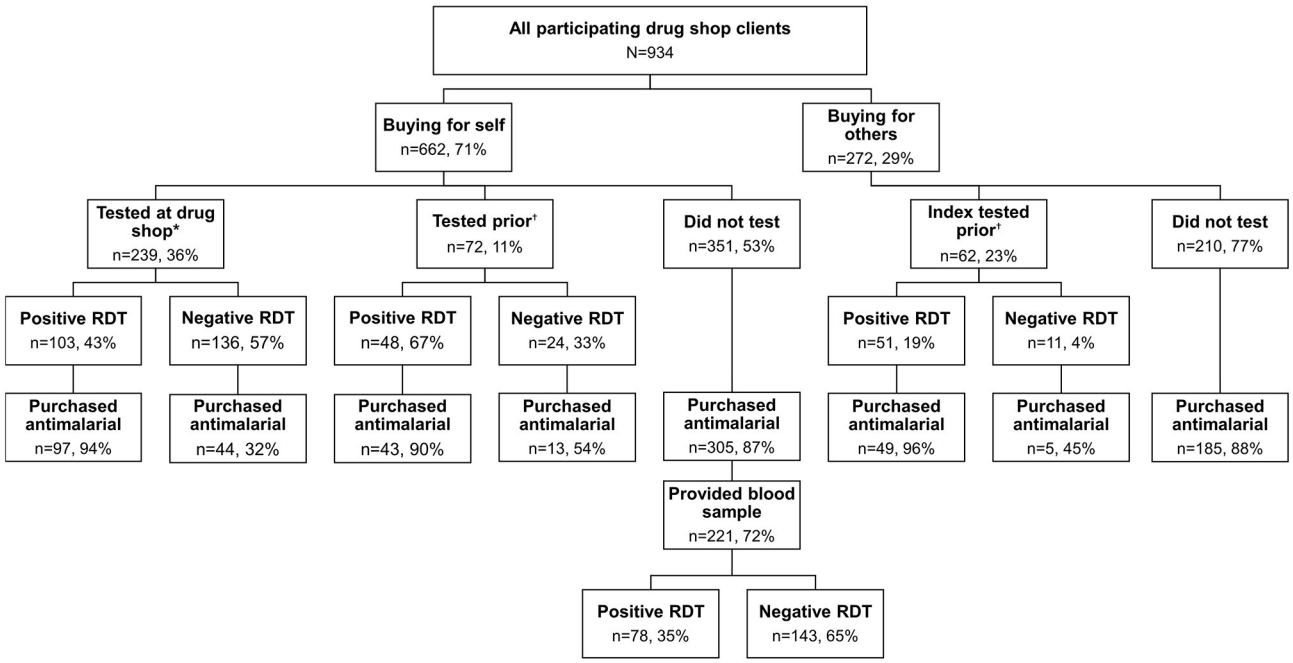

FIGURE 1
Use of malaria rapid diagnostic tests among clients seeking malaria treatment from drug shops in Bugoye.

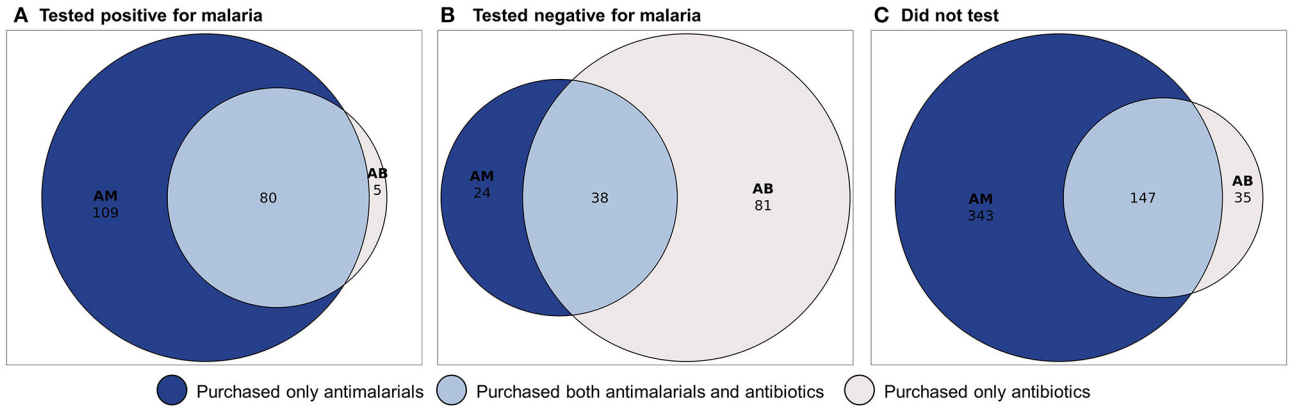

FIGURE 2
(A–C) Antimalarial and antibiotic purchases of drug shop clients by malaria test status.

Blood samples were collected from 253 index clients with an unknown malaria status at the drug shop, out of 351 eligible clients (72%). There were significant associations between consenting to blood sample collection and sex [$X^2_{(1)} = 6.09$, $p = 0.014$], as well as study group [$X^2_{(3)} = 127.6$, $p < 0.001$]. Women and clients from later study groups were more likely to provide a blood sample than men or clients from study group one. There were no significant differences by age or days of illness. Among 221 clients treated presumptively for malaria (i.e., unknown status at the drug shop and purchased an antimalarial), 65% tested negative based on RDTs conducted on collected blood samples (Figure 1). Nearly all blood samples from clients with an unknown status who did not purchase an antimalarial ($n = 32$) tested negative ($n = 31$, 97%).

3.3. Predictors of rapid diagnostic test uptake

Client demographic characteristics, vendor qualifications, RDT cost, and time of visit were not significant predictors of having a known malaria status at the drug shop (Table 4). Days of illness, drug shop location, and shop years of operation predicted RDT use in univariate models, but significance was not maintained in multivariate models. Study group and day of visit were associated with RDT use in both univariate and multivariate models. Clients who came to the drug shop on a weekday were 1.25 times as likely (95% CI: 1.02, 1.54) to know their malaria test status than clients who came over the weekend. Compared to study group 3 (Katooke trading center), clients in study group 1 (Bugoye trading center)

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TABLE 3 Medication purchases of clients seeking malaria treatment from drug shops in Bugoye sub-county, Uganda by known test status at the drug shop*.

	Tested positive for malaria, $N = 202$		Tested negative for malaria, $N = 171$		Did not test, $N = 561$	
	n	%	*n*	%	*n*	%
Type of medication purchased						
Antimalarial†	189	93.6	62	36.3	490	87.3
Antibiotic‡	85	42.1	119	69.6	182	32.4
Analgesic/Antipyretic§	167	82.7	158	92.4	505	90.0
None	2	1.0	10	5.8	7	1.2
Purchased both antimalarial and antibiotic medications	80	39.6	38	22.2	147	26.2

*Includes both RDTs conducted prior to drug shop visit and RDTs conducted at the drug shop.
†Antimalarials included artemether/lumefantrine (Coartem, Lonart), sulfadoxine/pyrimethamine (Fansidar), quinine (in any form—tablet, IV, or syrup), artesunate, dihydroartemisinin/piperaquine (P-Alaxin, Duo-Cotecxin), and chloroquine.
‡Antibiotics included: amoxicillin, ampicillin, erythromycin, metronidazole, co-trimoxazole, ciprofloxacin, ceftriaxone, gentamicin, benzylpenicillin, penicillin V, amplicox, cefalexin, tinidazole, azithromycin, and chloramphenical.
§Analgesic/Antipyretics included: paracetamol and combinations (Panadol, Curamol, Painex, Dynapar, Ibupar, Action, Kamadol, Metopar), ibuprofen, diclofenac, piroxicam, tramadol, aspirin, indomethacin, meloxicam, and amitriptyline.

were 1.88 times as likely (95% CI: 1.40, 2.52), and clients in study group 2 (Ibanda trading center) were 2.43 times as likely (95% CI: 1.82, 3.24) to know their malaria status at the drug shop.

4. Discussion

While the Uganda Ministry of Health has adopted the WHO guidelines of "test, treat, and track" for all suspected malaria cases, this study demonstrates that despite widespread RDT availability, individuals purchasing antimalarials from drug shops were often not tested to confirm a malaria diagnosis (60%). When RDTs were conducted, clients who tested negative sometimes still purchased antimalarials (36%). Qualitative research in this setting found that these antimalarial purchases stemmed from vendor and client distrust in negative RDT results, and fear or uncertainty about treatment next steps for conditions other than malaria (41).

The low RDT use at drug shops in our study is especially concerning because vendors and clients could not distinguish between malaria and other causes of fever, and most clients treated presumptively did not have malaria (65%). This confirms malaria overtreatment at drug shops in Bugoye sub-county is substantial, with potential consequences for individual health, economic status, and population-level parasite resistance.

While previous interventions in Uganda reduced malaria overtreatment by introducing RDTs into drug shops (42–45), these tests are readily available in Bugoye sub-county. These programs also relied on the provision of free or subsidized RDTs. However, our study results suggest that this strategy alone would be insufficient to improve practices. It was common in Bugoye for surrogate clients to seek treatment for another person-—30% of index clients were not present at the drug shop, a finding aligned with previous studies in Uganda (30, 46). If the sick individual is at home, increased availability and lower cost of RDTs at drug shops will not increase their use. Furthermore, antimalarial purchases after testing negative suggest interventions that promote trust and understanding of RDT results are needed.

Our analysis did not identify many modifiable predictors of RDT use. Clients visiting drug shops on weekends, when public health facilities are closed, were less likely to receive an RDT. This suggests longer and more flexible operating hours at public facilities could increase the proportion of drug shop clients with a known malaria status. Differences by study group may be explained by the timing and location of data collection. Study groups were chosen based on geographic proximity, and access to health centers and socio-economic status (not measured in this study) vary across the sub-county.

This study also raises concerns about the types of medications sold at drug shops. While the most common antimalarial purchased by drug shop clients in our study was artemether/lumefantrine (53%), the first-line ACT in Uganda (47), clients also purchased antimalarials no longer recommended in Uganda due to high levels of resistance, such as sulfadoxine/pyrimethamine (13%) and quinine (8%) (48–50). Clients also purchased both antimalarials and antibiotics (28%), regardless of RDT purchases and results. Antibiotic misuse fuels resistance, with global implications for the effective treatment of infectious diseases (51).

Poor malaria case management at drug shops in Bugoye sub-county may be related to inadequate training and experience, which can be reflected in low vendor knowledge (52, 53). Most drug shops in Bugoye were unlicensed and operated by vendors without the necessary qualifications to operate a drug shop in Uganda, a trend consistent across sub-Saharan Africa (54). Despite concerns, drug shops are a reliable source of essential medications. While services and medications from the public sector are free, drug shops are appealing because they have convenient locations and hours, short wait times, and infrequent drug stockouts (24, 55). Given the important role drug shops play in community health, and the continued persistence of malaria as a leading cause of morbidity and mortality, interventions are needed to improve malaria case management in the Ugandan private sector.

10.3389/fpubh.2023.1140405

TABLE 4 Estimated risk ratios from univariate and multivariate log binomial regression modeling of having a known malaria RDT status* among clients seeking malaria treatment from drug shops in Bugoye sub-county, Uganda†.

	Univariate regression			Multivariate regression		
	RR	95% CI	*p*-value	aRR	95% CI	*p*-value
Study group						
Group 1—Bugoye	1.93	1.46, 2.55	<0.0001	1.88	1.40, 2.52	<0.0001
Group 2—Ibanda	2.59	1.98, 3.38	<0.0001	2.43	1.82, 3.24	<0.0001
Group 3—Katooke	Ref.			Ref.		
Group 4—Kisamba	1.19	0.83, 1.70	0.3398	1.15	0.76, 1.74	0.5024
Sex of client						
Male	Ref.			Ref.		
Female	1.09	0.93, 1.27	0.3085	1.06	0.91, 1.25	0.4448
Age of client‡						
5 years	0.97	0.79, 1.19	0.7794	1.29	0.95, 1.72	0.1027
15 years	0.98	0.84, 1.14	0.7794	1.24	0.95, 1.61	0.1096
30 years	0.99	0.92, 1.07	0.7794	1.17	0.94, 1.46	0.1517
45 years	Ref.			Ref.		
Days of illness‡						
1 day	Ref.			Ref.		
3 days	1.06	1.04, 1.08	<0.0001	1.15	0.94, 1.42	0.1820
7 days	1.19	1.12, 1.27	<0.0001	1.23	0.99, 1.54	0.0672
Drug shop location§						
Small trading center	Ref.			Ref.		
Large trading center	1.43	1.22, 1.67	<0.0001	1.08	0.88, 1.34	0.4585
Drug shop years of operation‡						
1 year	Ref.			Ref.		
5 years	1.09	1.02, 1.16	0.0076	1.16	0.93, 1.44	0.1889
10 years	1.21	1.05, 1.39	0.0076	1.21	0.93, 1.58	0.1531
Vendor training						
Nursing assistant	Ref.			Ref.		
Nurse or midwife	1.04	0.88, 1.24	0.6381	1.04	0.86, 1.25	0.6919
Malaria RDT cost‡						
2,000 UGX#	0.93	0.80, 1.08	0.3167	0.99	0.76, 1.30	0.9662
3,000 UGX¶	Ref.			Ref.		
Day of drug shop visit						
Weekday	1.26	1.03, 1.54	0.0269	1.25	1.02, 1.54	0.0339
Weekend	Ref.			Ref.		
Time of drug shop visit						
Morning	1.06	0.86, 1.31	0.5809	0.98	0.78, 1.21	0.8233
Afternoon	1.22	1.00, 1.48	0.0512	1.12	0.91, 1.37	0.2971
Evening	Ref.			Ref.		

*Includes both RDTs conducted prior to drug shop visit and RDTs conducted at the drug shop.
†From 934 clients, data from 840 with no missing values were used in the adjusted analysis.
‡Age of client, days of illness, drug shop years of operation, and malaria RDT cost were modeled as continuous variables. RRs were calculated from select, illustrative values.
§Large trading centers were defined by the presence of a weekly market day.
#Approximately 0.57 USD.
¶Approximately 0.85 USD.

4.1. Limitations

This study may overestimate RDT use. Evaluating drug shop practices without influencing vendor or client behavior is a recognized challenge for studies with private medicine retailers, because behavior may change when individuals are aware they are being monitored (42, 56). Vendor data collection was chosen over direct observation or exit interviews to minimize research participation effects. However, vendors may have been more likely to stock or promote RDTs, or insist on adherence to results because of study participation. Additionally, prior RDT results were self-reported by clients and subject to social-desirability bias. This study also relied on vendors to accurately record information. Data collection in groups allowed for more control over data quality and forms were reviewed triweekly. No systemic issues in reporting were detected and completeness was high, with missing values <5%. Finally, blood samples were only obtained if the index client was present and provided consent, and the data collection period was short. Therefore, data may not provide a complete representation of malaria diagnostic and treatment practices at drug shops in Bugoye. Nevertheless, estimates provide a useful starting point to understanding the magnitude of the problem, a prerequisite to proposing solutions.

5. Conclusion

This study is the first to quantify RDT use and malaria overtreatment at drug shops in Bugoye sub-county. Since drug shops provide a substantial percentage of antimalarials (>50%), future interventions to improve malaria case management at drug shops could increase rational drug use, reduce unnecessary spending, and delay the development of parasite resistance to ACTs. The consequences of malaria misdiagnosis disproportionately affect the poor and vulnerable, contributing to a cycle of disease and poverty (18). Therefore, efforts to improve fever case management and diagnostic practices at private drug shops could have tangible social and economic benefits. Since challenges related to malaria overtreatment and the quality of care at drug shops are not unique to Bugoye, findings could be relevant for other low- and middle-income countries.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the University of North Carolina Office of Human Research Ethics (20-3019), Mbarara University of Science and Technology Research Ethics Committee (MUST-2021-55), and the Uganda National Council for Science and Technology. The patients/participants or participants' legal guardian/next of kin provided their written informed consent to participate in this study.

Author contributions

VS, EM, CB, and RB contributed to conception and design of the study. NM was responsible for all project administration. VS, NM, and EB trained drug shop vendors. VS conducted the analysis and wrote the original draft of the manuscript. All authors contributed to manuscript revisions and approved the submitted version.

Funding

This work was supported by the US Department of State, Bureau of Educational and Cultural Affairs [Fulbright-Fogarty Award in Public Health E0636820 to VS], the Doris Duke Charitable Foundation [Caregivers at Carolina Award 2015213 to RB], and the National Institutes of Health [K23AI141764 to RB].

Acknowledgments

The authors thank the entire team at MUST-UNC and Bugoye Health Center III for their support of this research, especially Franklin Kule and Ronnie Ndizeye for their assistance with training and laboratory questions, and Shem Bwambale. We would also like to acknowledge the Village Health Team members who helped us identify and recruit drug shops, the drug shop vendors in Bugoye who collected data for the study, and the clients who provided blood samples.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2023.1140405/full#supplementary-material

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frontiers | Frontiers in Public Health

TYPE Original Research
PUBLISHED 01 June 2023
DOI 10.3389/fpubh.2023.1162535

OPEN ACCESS

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RECEIVED 09 February 2023
ACCEPTED 28 April 2023
PUBLISHED 01 June 2023

CITATION
Armando CJ, Rocklöv J, Sidat M, Tozan Y, Mavume AF, Bunker A and Sewes MO (2023) Climate variability, socio-economic conditions and vulnerability to malaria infections in Mozambique 2016–2018: a spatial temporal analysis.
Front. Public Health 11:1162535.
doi: 10.3389/fpubh.2023.1162535

Climate variability, socio-economic conditions and vulnerability to malaria infections in Mozambique 2016–2018: a spatial temporal analysis

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Background: Temperature, precipitation, relative humidity (RH), and Normalized Different Vegetation Index (NDVI), influence malaria transmission dynamics. However, an understanding of interactions between socioeconomic indicators, environmental factors and malaria incidence can help design interventions to alleviate the high burden of malaria infections on vulnerable populations. Our study thus aimed to investigate the socioeconomic and climatological factors influencing spatial and temporal variability of malaria infections in Mozambique.

Methods: We used monthly malaria cases from 2016 to 2018 at the district level. We developed an hierarchical spatial–temporal model in a Bayesian framework. Monthly malaria cases were assumed to follow a negative binomial distribution. We used integrated nested Laplace approximation (INLA) in R for Bayesian inference and distributed lag nonlinear modeling (DLNM) framework to explore exposure-response relationships between climate variables and risk of malaria infection in Mozambique, while adjusting for socioeconomic factors.

Results: A total of 19,948,295 malaria cases were reported between 2016 and 2018 in Mozambique. Malaria risk increased with higher monthly mean temperatures between 20 and 29°C, at mean temperature of 25°C, the risk of malaria was 3.45 times higher (RR 3.45 [95%CI: 2.37–5.03]). Malaria risk was greatest for NDVI above 0.22. The risk of malaria was 1.34 times higher (1.34 [1.01–1.79]) at monthly RH of 55%. Malaria risk reduced by 26.1%, for total monthly precipitation of 480mm (0.739 [95%CI: 0.61–0.90]) at lag 2months, while for lower total monthly precipitation of 10mm, the risk of malaria was 1.87 times higher (1.87 [1.30–2.69]). After adjusting for climate variables, having lower level of education significantly increased malaria risk (1.034 [1.014–1.054]) and having electricity (0.979 [0.967–0.992]) and sharing toilet facilities (0.957 [0.924–0.991]) significantly reduced malaria risk.

Conclusion: Our current study identified lag patterns and association between climate variables and malaria incidence in Mozambique. Extremes in climate variables were associated with an increased risk of malaria transmission, peaks in transmission were varied. Our findings provide insights for designing early

warning, prevention, and control strategies to minimize seasonal malaria surges and associated infections in Mozambique a region where Malaria causes substantial burden from illness and deaths.

KEYWORDS

malaria vulnerability, DHS, Mozambique, INLA, Bayesian, climate variability, spatio-temporal, DLNM

1. Introduction

Malaria is a critical public health problem in sub-Saharan Africa causing significant morbidity and mortality (1), especially among children under 5 years (2, 3), pregnant women (4, 5), HIV infected individuals (6), low socioeconomic households (5, 7–13), households without access to Insecticide-treated nets (ITNs) (14) and non-compliant users of ITNs (5). Mozambique has high rates of under-five malaria mortality (7, 15) and is the fourth out of six countries that accounted for more than half of all malaria cases and deaths worldwide in 2019, corresponding to 4% of the global burden of cases and deaths (1). The country had the second highest prevalence of malaria in Eastern and Southern Africa, estimated at 17.2% in 2019 (1). Malaria is endemic in Mozambique, and the entire population is at risk (16, 17). In 2020, Malaria was estimated to account for approximately 26% of all outpatient consultations with over 11 million cases diagnosed in public health facilities and communities (18). Frequent natural disasters have likely contributed to increases in malaria transmission in recent years (19).

Malaria cases are rising in Mozambique and regional differences exist. For example, Gaza province, Maputo province, and Maputo City have reported reductions in cases, in contrast to increases in Manica, Cabo Delgado, Zambezia, and Nampula provinces. The national malaria incidence was estimated at 368 cases per 1,000 population in 2020 (18). Malaria prevalence in rural areas was double relative to urban areas (20). The 2018 Malaria Indicator Survey (MIS) showed considerable variation in average malaria prevalence among children under 5 years at the provincial and country wide levels at 1–57, and 39%, respectively (21). Several factors affect malaria transmission dynamics, from climatic conditions to social-economic factors (8, 22, 23). Climatic factors such as temperature and precipitation affect the life cycle and breeding of mosquito vectors that transmit malaria (22). The predominant malaria vector species are *Anopheles gambiae* and *Anopheles funestus* in Mozambique—accounting for 90% of all malarial infections (7). Malaria transmission varies significantly depending on the natural environment, climatic conditions, locally dominant malaria vector species, and structural vulnerability factors including behavioral, social, economic conditions and malaria control interventions (24).

Abbreviations: DHS, Demography Health Survey; INLA, Integrated nested Laplace approximation; NDVI, Normalized Different Vegetation Index; DLNM, Distributed lag nonlinear modeling; RH, Relative humidity; ITNs, Insecticide-treated nets; ENSO, El-Niño-Southern-Oscillation; IOD, Indian Ocean Dipole; IPCC, Climate change; MIS, Malaria Indicator Survey.

Preventive measures including ITNs, prophylactic antimalarial drugs and indoor residual spraying (IRS) are used in Mozambique to curb malaria infections. Mozambique's 2017–2021 National Malaria Strategic Plan aims to provide at least 85% of the population with adequate protection against malaria which includes provision of testing to all suspected cases, treatment to all confirmed cases according to existing national malaria treatment guidelines (21). Targets have been set for malaria elimination in areas of low and very low transmission through appropriate interventions (25).

Mozambique is geographically prone to natural disasters and highly vulnerable to climate change. Increased frequency and intensity of extreme weather events over the past 60 years has increased population susceptibility to malaria infection (26). The coolest months fall between June to August, and the dry season occurs between May to October (27, 28). The warmest and wettest months range from December to February, when malaria transmission is the highest (29, 30). Precipitation anomalies occur on different spatial and temporal scales with varying intensity and frequency, providing suitable breeding sites for malaria vectors. Temperature affects the development of anopheles mosquitoes and their biting rates (22). Precipitation and temperature variation over the country are affected by weather patterns at the South Indian Convergence Zone (31), Intertropical Convergence Zone (30, 31), subtropical high-pressure systems, and semi-permanent anticyclones, namely the Mascarene High and St. Helena tropical cyclones (27, 32–34), El-Niño-Southern-Oscillation (ENSO), and Indian Ocean Dipole (IOD) among others (35–37). These factors are associated with above or below normal precipitation or temperatures and affect malaria morbidity through effects on transmission dynamics (29, 30, 38–41).

Our current study investigated factors influencing the spatial and temporal variation in malaria transmission in Mozambique by leveraging socioeconomic, climatic and land use data. We sought to identify malaria vulnerability indicators, and the lag times between climate events and the highest risk of malaria transmission to inform development of a malaria early warning system in Mozambique.

2. Materials and methods

2.1. Setting

Mozambique is positioned at longitudes 30.12° and 40.51° East and latitudes 10.27° and 26.52° South (Figure 1) covering an area of 783,000 km^2 of which 4,500 km^2 is designated as a maritime area with a coastline stretching 2,700 km (26).

Mozambique has a population of approximately 30 million people, with <60% living in coastal areas including lowlands with sandy beaches,

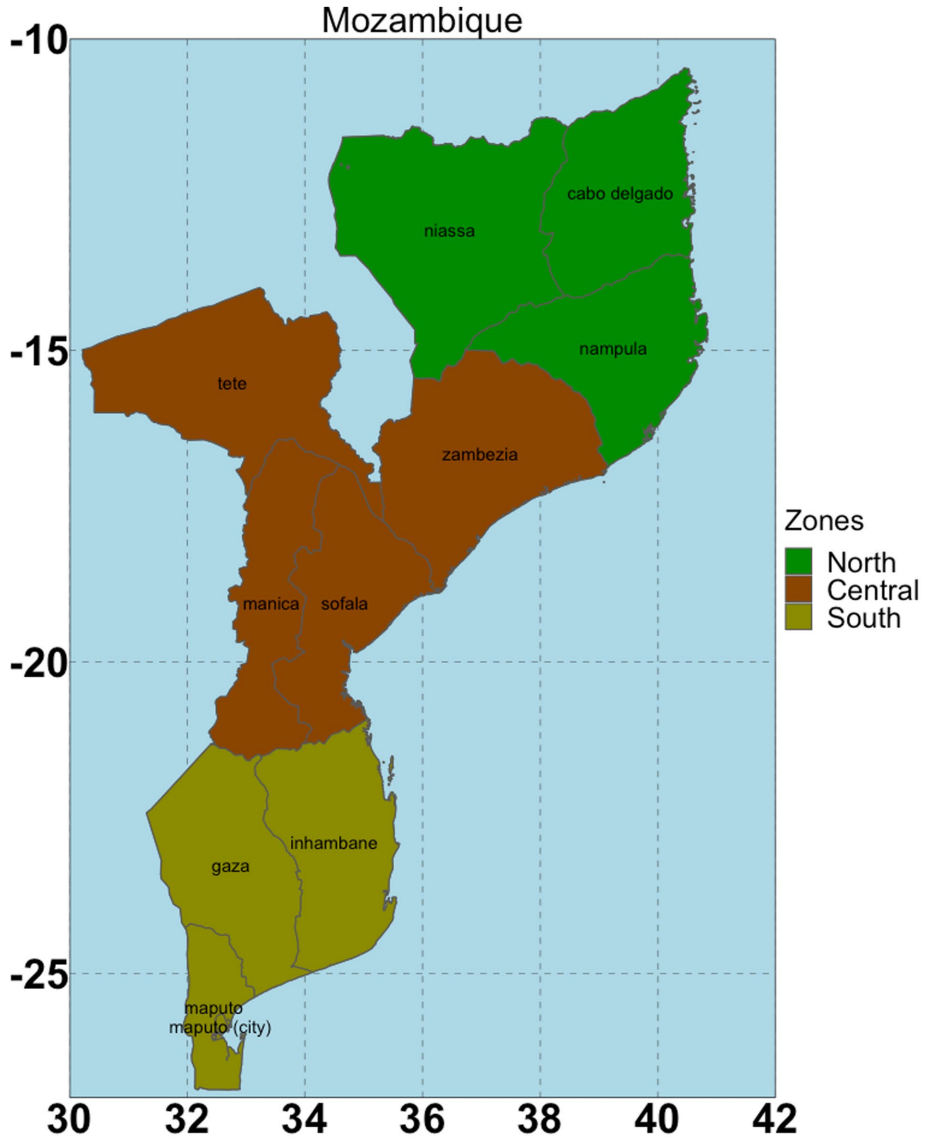

FIGURE 1
The study area with three geopolitical zones (North, Central and South).

estuaries, and mangroves (26). The AR6 Intergovernmental panel on climate change (IPCC), in its report assessed that climate change will adversely affect the health of people in coastal regions (42), owing to their dependence on local resources such as rain-fed farming and fishing.

2.2. Data and model

We used data on weekly malaria cases from the Mozambique Ministry of Health disease surveillance system (43), between 2016 and 2018. Weekly malaria incidence data were first aggregated to monthly temporal resolution for 159 districts, and then combined. Socio-economic data were extracted from the 2015 DHS and 2018 MIS-both nationally representative population-based household surveys (20). Variables from the MIS and DHS surveys with known association to malaria transmission were included considering their availability and the extent of missing values.

We included the following variables from DHS: wealth index derived from household asset ownership, number of children under five, type of residence, ITN use and ownership, indoor residual spraying, type of toilet facilities, radio, mobile and television ownership, housing conditions, number of sleeping rooms, sharing of toilet with other households, number of households sharing toilet, number of mosquito bed nets, mother's education level, doctor to population ratio, and number of health facilities per population. DHS and MIS variables were aggregated from individual to district level by computing proportions of selected variable level (Supplementary Table S5).

We retrieved daily climate data including precipitation, minimum and maximum temperature (T_{min} and T_{max}), relative humidity (RH), and Normalized Different Vegetation Index (NDVI) from NCEP-reanalysis II (44) with a spatial resolution of 0.25° × 0.25° (45) from 2016 to 2018, and aggregated to monthly temporal resolution. Means were computed for T_{min}, T_{max}, RH, and NDVI, and cumulative totals

for precipitation. We used gridded population data from WorldPop (46) as the denominator in computation of malaria incidence rates.

Each climate variable was included as a non-linear term in the model. We computed crossbasis functions following the distributed lag non-linear methodology developed by Gasparrini et al. (47). This is a flexible approach that allows simultaneous modeling of the lag and exposure-response relationships of the variables. The cross-basis for the lag and nonlinear dimensions were modeled using natural cubic splines with equally spaced knots. We considered lags up to 6 months (48). Centering values were chosen through graphical analysis of exposure response relationship, and risk comparisons are based on these reference values. The chosen reference values for T_{mean}, NDVI, RH and Precipitation were 18°C, 0.2, 70% and 120 mm, respectively.

Using a Bayesian disease mapping approach, we accounted for spatial dependence among neighboring districts in Mozambique. The Bayesian model consisted of three components; the data model (distribution of data given the parameters), the process model (underlying spatial patterns), and the parameter model (prior specification of the model parameters) (49, 50). We assumed—in the data model—that malaria cases followed a negative binomial distribution, used to account for overdispersion in data (48).

We implemented the spatio-temporal extension of the spatial Besag-York-Mollie (BYM) model for the spatial process, which is the Conditional Auto-Regressive (CAR) convolution model with two random effects, one spatially structured and one unstructured random effect (51, 52).

2.3. Model selection

The DHS variables were included one at a time in the base model, controlling for spatial and temporal covariance. The significant variables based on the 95% credible interval were then included in a joint multivariate model. Backward elimination was used to select the DHS variables that were included in the final model.

Selected DHS variables were combined with environmental crossbasis terms as the final model specification (Equation 1). Relative risks (RR) were predicted for different values of climate variables.

$$\log(Y_i) = \propto + u_i + v_i + \gamma_t + f(X_j, lagdf, vardf) + \beta_k X_k \quad (1)$$

$$Y_i \sim NBin$$

where Y_t represents the malaria cases; $\propto$ the intercept; u_i the spatially-structured random effect (for smoothing among adjacent areas), the set of neighboring districts, and the number of neighboring districts for a specific district i; v_i the unstructured random effect component, which was modeled using as a Gaussian process and which allowed for extra heterogeneity in malaria case counts due to unobserved (and spatially unstructured) risk factors; γ_t the temporally structured effect of the month, was modeled dynamically using a random walk of order 1 (RW1) to capture the seasonal patterns; $\boldsymbol{f(X_j, lagdf, vardf)}$ is the crossbasis function of climatic variable t and its lag dimension with vardf and lagdf degrees of freedom, respectively, controlling for the ***k*th socio-demographic** covariate, $\boldsymbol{X_k}$ with coefficient β_k.

A Bayesian approach is attractive for modeling complex longitudinal count data but requires specification of the prior distributions for all the random elements of the model. In the case of hierarchical models, this involves choosing priors for the regression coefficients and the hyperparameters. Two classes of prior distributions, informative and non-informative, are typically used in Bayesian modeling. While informative prior distributions are used when substantial information on the model parameters is available from previous studies, non-informative prior distributions facilitate Bayesian inference when little is known about the parameters beyond the data included in the analysis (53). In this analysis, we used default prior specifications in INLA.

We used Integrated Nested Laplace Approximation (INLA) in R for Bayesian inference (50). INLA is a deterministic algorithm for Bayesian inference and designed for latent Gaussian models and spatial models. Bayesian estimation using the INLA methodology takes much less time than standard Bayesian computations methods using Markov Chain Monte Carlo Methods (MCMC) (54, 55).

3. Results

3.1. Malaria cases and environmental variables

A total of 19,948,295 malaria cases were reported in Mozambique between 2016 and 2018. The reported malaria incidence rates were 189.3, 259.2, and 252.2 per 1,000 population in the years 2016, 2017, and 2018, respectively. The mean malaria caseload across the country was 554,119 per year over this period. The year 2018 had the highest national average of 614,083 cases per year, as shown in Table 1.

Over the study period, temperature varied slightly in the study area with a mean maximum temperature of 29°C and a mean minimum temperature of 19°C. The mean annual precipitation across the country were 827, 1,080, and 952 mm in the years 2016, 2017, and 2018, respectively (Supplementary Table S1). The mean monthly concentration of green vegetation varied less across the years ranging from 0.23 to 0.25, with the highest mean monthly NDVI of 0.25 recorded in 2017 (Table 1).

During the study period between 2016 and 2018, annual mean maximum and minimum temperatures varied from 24.3 to 32°C, and 14.5 to 22.7°C, respectively (Supplementary Table S1), while the annual monthly mean average temperature ranged between 19.5 and 26.8°C (Supplementary Table S1). The annual average temperature steadily decreased from coastal areas into the inland. The coldest temperatures were observed in Manica and Niassa provinces (Figure 2), probably due to the prevailing winds of the western areas bringing cold air mass from the orographic areas during the warm half-year from November to April. The warmest temperatures were observed from December to March across the country with a peak in December (Supplementary Figures S2, S5–S7), while the coldest temperatures were recorded between June and July (Supplementary Figures S2, S5–S7).

As shown in Figure 2, the southern part of Mozambique received lower precipitation from 2016 to 2018 while the central and northern parts of the country averaged a higher annual precipitation. The monthly spatial climatology of precipitation over

10.3389/fpubh.2023.1162535

TABLE 1 Summary of (annual monthly) malaria cases and environmental variables, Mozambique, 2016–2018.

Variables	Year	Mean (*SD*)	Min	Median	Max
Malaria incidence rate (per 1,000 population)	2016	15.77 (5.87)	5.461	14.746	24.296
	2017	21.6 (5.43)	14.121	23.245	28.257
	2018	21 (5.14)	14.061	21.838	30.278
Malaria cases	2016	434947.17 (162000.82)	150,600	406660.5	669,996
	2017	613326.92 (154135.29)	400,947	660018.5	802,350
	2018	614083.83 (150271.81)	411,075	638434.5	885,185
Min temperature	2016	18.99 (3.21)	14.593	19.503	22.746
	2017	18.77 (2.62)	14.996	19.379	22.183
	2018	18.75 (2.55)	14.91	18.664	21.94
Max temperature	2016	28.7 (2.6)	24.371	29.155	32.065
	2017	28.67 (1.76)	26.044	28.827	31.006
	2018	28.59 (1.89)	24.443	29.145	30.887
Relative humidity (%)	2016	74.07 (10.15)	58.862	74.371	88.113
	2017	73.23 (9.76)	60.032	71.119	88.542
	2018	73.57 (9.42)	61.292	73.305	88.336
Precipitation (mm)	2016	68.98 (88.9)	0.783	10.871	257.005
	2017	90.07 (112.21)	1.445	21.045	294.725
	2018	78.99 (94.52)	2.801	22.752	241.461
NDVI	2016	0.24 (0.05)	0.154	0.233	0.298
	2017	0.25 (0.05)	0.154	0.251	0.336
	2018	0.23 (0.07)	0.128	0.226	0.343

NDVI, normalized difference vegetation index; Min, minimum; Max, maximum; SD, standard deviation.

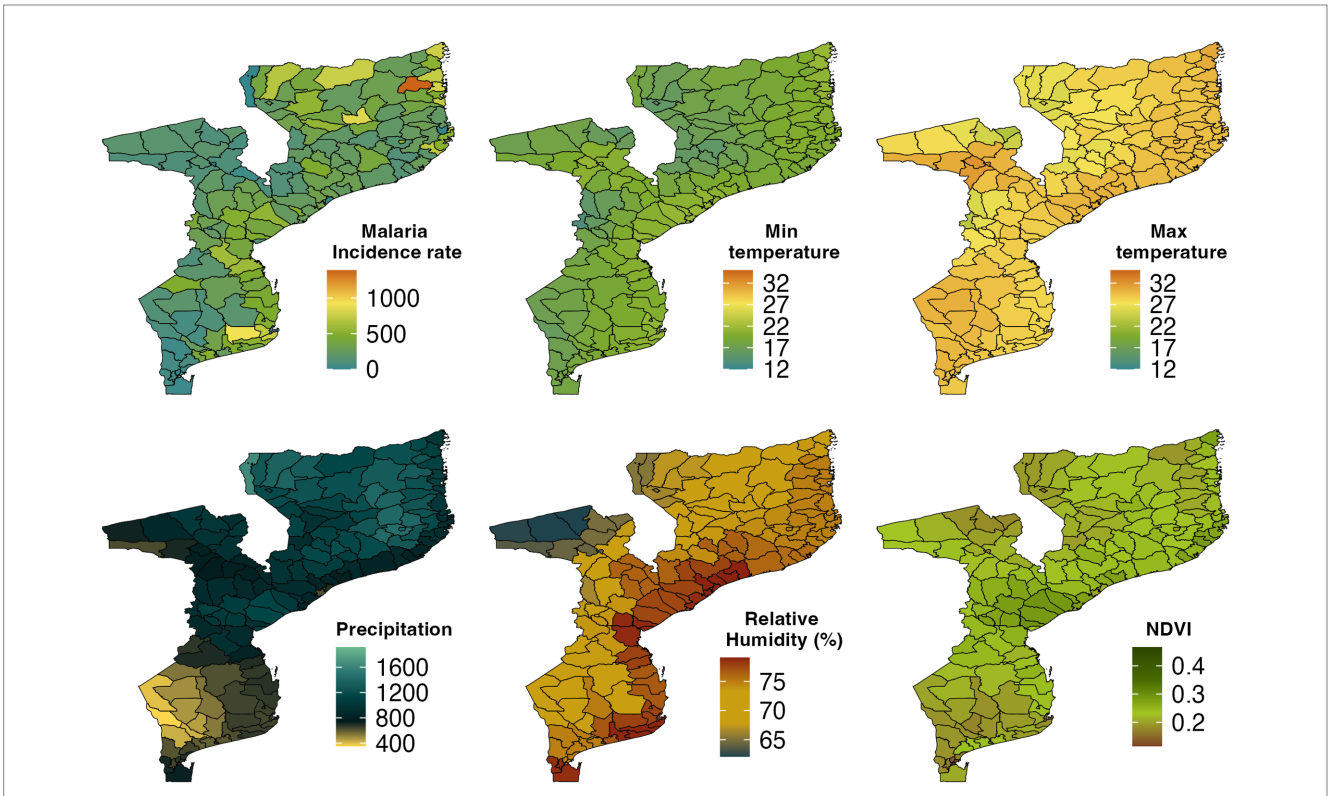

FIGURE 2
Spatial distribution of malaria incidence rate per 1,000 population, Minimum temperature, Maximum temperature, Precipitation, Relative humidity, and Normalized difference vegetation index (NDVI) by district in Mozambique, 2016–2018.

Mozambique in 2018 showed that the highest amount of precipitation was recorded in January followed by February (Supplementary Figure S10). The driest months were June, July, and August during which the entire country received less than 50 mm precipitation.

The monthly mean NDVI ranged from 0.13 to 0.34 between 2016 and 2018 (Supplementary Table S1). In Figure 2, we observed that Central province and some parts of Inhambane, Gaza, and Nampula provinces had the highest NDVI, while the southern and northern parts of the country had the lowest NDVI. Manica and Sofala provinces were the greenest areas followed by Zambezia, Inhambane, and Tete provinces whereas the least green areas were Cabo Delgado, Niassa, Gaza, Maputo City, and Maputo provinces. The southwest corner of the country had an NDVI less than 0.2 between June and August and in the northeast part of the study area between September and October.

We also examined RH over Mozambique (Figure 2). In the southern, central, and northern parts of the country, RH was above 74% in 2018, while in Niassa, Tete, Manica, and Gaza provinces, it was below 74%. Figure 2 shows that RH decreased from coastal areas to the inland. The monthly mean RH during the period from December to April was over 80% in most areas over the country (Supplementary Figure S8).

3.2. Descriptive summaries for DHS socio-economic indicators

The description of the household-level DHS indicators, which were aggregated to district level for the analysis, are given in Supplementary Table S6. About 78.0% of the households were located in rural areas within the districts. The mean proportion of households characterized as poor was 46.2% with some districts registering as high as 96.4% of households as poor. About 20.8 and 35.6% of the households had electricity and radio, respectively. However, mobile phone ownership was high at 62%. The average proportion of households reporting no education was low at 26.8%, though some districts reported as high as 92.3%. In terms of malaria control, 89.4% of the households reported having mosquito nets, although the proportion of dwellings sprayed in the last 12 months was low at 14.1%, with few children reported to be sleeping under ITNs at only 2%.

3.3. Model results

3.3.1. Temperature

Figure 3A displays the overall relationship between mean monthly temperature and malaria risk in Mozambique. We observed an

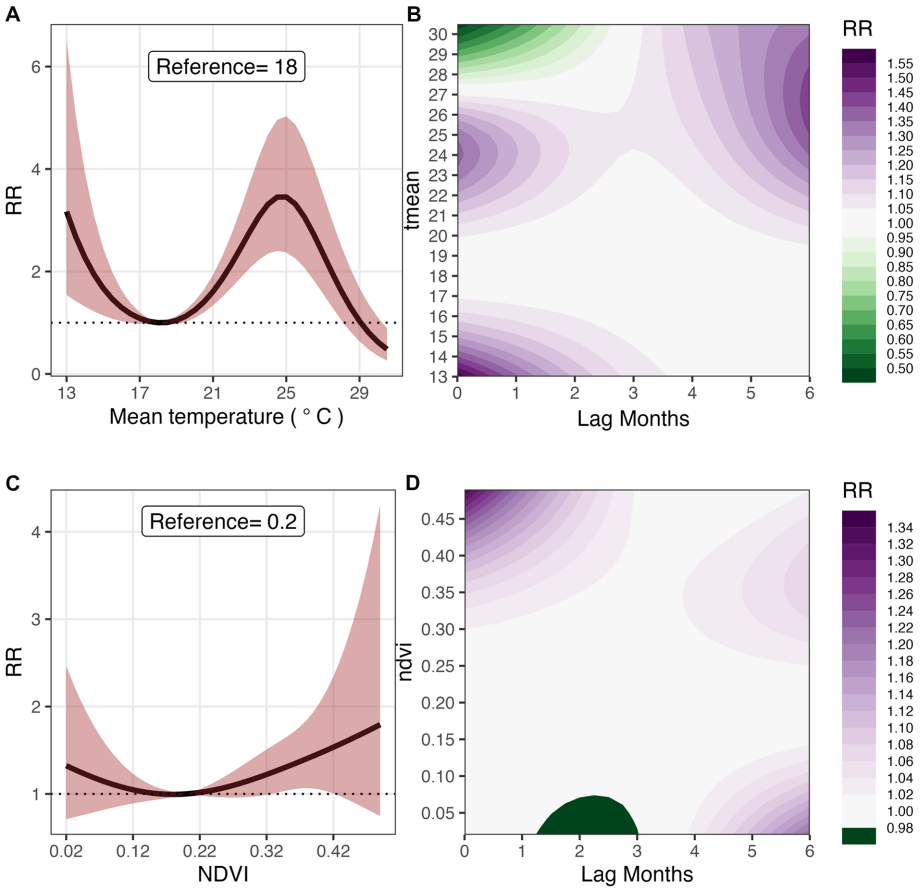

FIGURE 3
Overall effect and 3D Contour plots of T_{mean} **(A,B)**, and NDVI **(C,D)** on malaria risk at lags 0–6 months in Mozambique, 2016–2018. The reference values for T_{mean} and NDVI, are 18°C and 0.2 respectively.

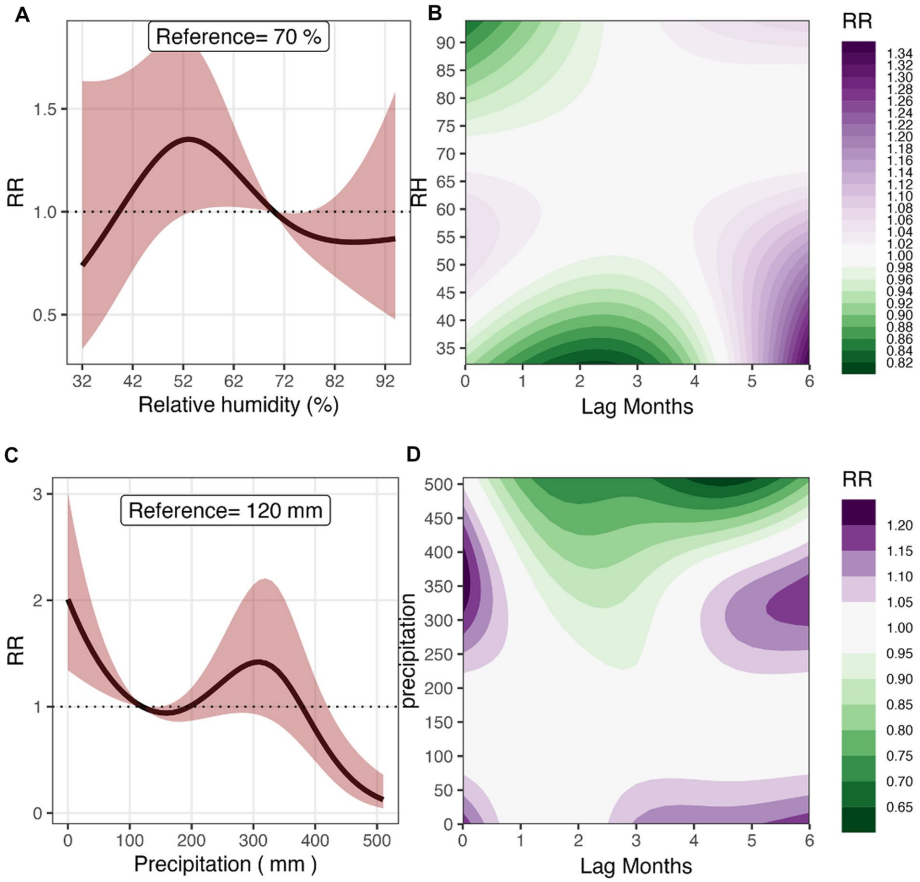

FIGURE 4
Overall effect and 3D Contour plots of relative humidity **(A,B)**, and precipitation **(C,D)** on malaria risk at lag times 0–6 months in Mozambique, 2016–2018. The reference values for RH and Precipitation are 70% and 120mm, respectively.

elevated risk for temperatures values between 20 and 29°C compared to the reference of 18°C; for example, at temperature value of 25°C malaria risk was 3.45 times higher (RR 3.45 [95% CI: 2.37–5.03]) compared to the reference. At higher mean temperatures, malaria risk plummeted, and temperature even became protective; for example, at 30°C, malaria infection risk reduced by up to 52% (RR 0.48 [95% CI: 0.26–0.89]). We also observed an increasing relative risk of malaria for temperatures below the reference value. Figure 3B shows the contour plot depicting the lag-response relationship between mean temperature and relative risk of malaria. For temperature between 21 and 26°C, the relative risk of malaria was higher at shorter lags of 1–3 months. At a monthly mean temperature of 26°C, malaria risk was 12.2% higher at a lag of 1 month (RR 1.12 [95% CI: 1.03–1.22]). The protective effect of higher temperatures occurred at much shorter lags.

3.3.2. Normalized difference vegetation index (NDVI)

Figure 3C shows the exposure-response relationship between NDVI and malaria risk. Compared to the reference value of 0.2, malaria risk was significantly higher for NDVI above 0.22. Specifically, at a monthly mean NDVI of 0.34, malaria risk was 28.0% higher (RR 1.28 [95% CI: 1.02–1.60]), and at 0.42, it was 53.5% higher (RR 1.53 [95% CI: 1.01–2.34]). Figure 3D shows the lag-response relationship between NDVI and malaria risk. At NDVI above 0.3, we observed significantly shorter lag patterns; for example, at NVDI of 0.49, malaria risk was the highest (19.9%) at one-month lag (RR 1.199 [95% CI: 1.02–1.41]). A significantly higher risk is observed at a lag of 5 months for an NDVI of 0.32 (RR 1.04 [95% CI: 1.00–1.08]).

3.3.3. Relative humidity

In Figure 4A, the exposure-response relationship between RH and malaria risk is shown. In comparison to the reference value of 70.0% for RH, malaria risk was the highest (34.3%) at 55% (RR 1.34 [95% CI: 1.01–1.79]). For RH greater than the reference value, we observed a decrease in risk though the association was not statistically significant. Figure 4B shows the contour 3D plot of exposure-lag response surface for RH and malaria risk. For RH between 50.0 and 60.0%, we observed much shorter lags but at lower RH, we saw much longer lags; for example, malaria risk increased by 30.1% at an RH of 38%, (RR 1.301 [95%CI: 1.08–1.55]) at a lag of 6 months. Higher RH was found to be significantly protective at shorter lags; for example, at an RH of 90%, malaria risk was decreased by 8.4%, (RR 0.916 [95% CI: 0.84–0.99]) at a lag of 1 month.

3.3.4. Precipitation

Figure 4C shows the relationship between cumulative precipitation and malaria risk. The risk was significantly higher at precipitation less than 100 mm in reference to the relative risk at a

TABLE 2 The relative risks of malaria infections in relation to household socio-economic indicators and doctor to population ratio.

Variables	RR	95% CI	
Proportion sharing toilet (%)	0.957	0.924	0.991
Proportion with electricity (%)	0.979	0.967	0.992
Proportion with no education (%)	1.034	1.014	1.054
Number of doctors per 1,000 pop	1.04	1.006	1.075
Proportion with mosquito net (%)	1.066	1.046	1.086

monthly total precipitation of 120 mm. For example, at a monthly total precipitation of 10 mm, there was an 87% increase in risk compared to the reference rainfall (RR 1.87 [95% CI: 1.30–2.69]). The risk increased at higher precipitation levels from 100 mm to 300 mm and then plummeted but not significantly. At precipitation levels of 400 mm and above, we observed a significant protective effect on malaria risk; for example, at a precipitation level of 490 mm, malaria risk reduced by 81.8% (RR 0.18 [95% CI: 0.07–0.45]). Figure 4D shows the exposure-lag response surface of monthly total precipitation and malaria risk at lags 0–6 months. We observed a shorter lag of 1 month for a high monthly total precipitation between 300 and 400 mm, and both shorter and longer lags over 3 months for lower total precipitation levels. For example, at a lag of 5 months, a total precipitation of 340 mm resulted in a 12.4% increase in malaria risk (RR 1.12 [95% CI: 1.01–1.25]) while at 50 mm of precipitation, the risk increased by 5.9% after 3 months (RR 1.06 [95% CI: 1.00–1.12]). The protective effect of a higher precipitation was also observed at much shorter lags, for example at a precipitation of 480 mm, malaria risk reduced by 26.1% (RR 0.739 [95% CI: 0.61–0.90]) at a lag of 2 months.

3.4. Socio-demographic factors

Table 2 shows the influence of socio-economic factors on malaria risk after controlling for the environmental factors. The districts with high proportion of households with electricity had significantly lower risk of malaria. Specifically, malaria risk decreased by 2.1% for every unit increase in the proportion of households with electricity (RR 0.979 [95% CI: 0.97–0.99]). Similarly, malaria risk was lower in districts with low proportions of individuals who share a toilet facility (RR 0.957 [95% CI: 0.924–0.991]). Malaria risk increased by 3.4% for every unit increase in the proportion of uneducated population (RR 1.034 [95% CI: 1.014–1.054]).

4. Discussion

In summary, in this study we identified temperatures of between 25 and 29°C to be associated with high malaria risk with shorter lagged associations of 1 month. Higher NDVI values above 0.2 were also associated with elevated risk of malaria with lags ranging from 1 to 5 months. The optimal relative humidity (RH) range for malaria risk was 50–60%, with shorter lags for lower RH and longer lags for RH within the optimal range. Lower monthly rainfall totals were associated with higher risks of malaria at lags of one to 3 months compared to wetter conditions associated with lowers risks with much shorter lags. We also showed that low educational level was associated with high risk of malaria, while owning a radio significantly lowered malaria risk.

We combined INLA Bayesian modeling and distributed lag nonlinear modeling approaches to explore the non-linear lagged exposure-response relationships between climate variables and risk of malaria infection in Mozambique controlling for socio-demographic factors and spatial–temporal covariance. The flexible DLNM approach allowed us to capture both the nonlinear exposure-response functions and their lag dimensions in assessing the relationship between climate variables and malaria incidence (56, 57). The INLA Bayesian approach has previously been used to investigate the association between malaria and climatic variables in other settings (50, 58–61).

From our findings, mean temperature was positively associated with malaria incidence, which is consistent with study done in Vietnam (62), China (63), and Thailand (64). At temperatures value above 20°C, malaria risk is higher at lags 4 to 6 months. At temperatures between 27 and 30°C, the risk is lower at shorter lags of 0–2 months. In Western Kenya, temperatures above 28°C were observed to be positively associated with malaria risk after 2 months (22). In Swaziland, malaria transmission risk increased when temperature was above 25°C with the effect pronounced at a 2-month lag (61). The association between temperature and the incubation period of malaria parasites and malaria transmission are well-known (65). High temperatures increase the biting rate of malaria vectors and expand malaria transmission geographically and temporally (66). The optimal temperature for malaria transmission ranges between 20.9 and 34.2°C (65–70), consistent with findings of this study which identified a range of 20–29°C. This temperature range favors parasite development and vector survival, resulting in increased malaria risk.

NDVI reflects the amount and the vigor of vegetation coverage over a certain area. Changes in the spatial distribution of NDVI can be primarily explained by geographical and climatic factors, such as precipitation. In areas with low precipitation, where water is a limiting factor for vegetation growth, seasonal NDVI is closely linked to precipitation. Overall, NDVI was found to be positively associated with malaria morbidity in our study. Similar observations were made in studies in Ivory Coast (71), Nigeria (72), and Uganda (56). Our findings showed that at an NVDI value of 0.49, malaria risk was higher after 1 month. In contrast, in Western Kenya (22), NDVI above 0.4 was found to be negatively associated with malaria.

Study done in Cameroon (73) found that RH is the most important climatic variable that determines the number of malaria cases. Other study showed a strong and significant effect of RH during the pre-transmission season on malaria burden in India and also indicates that RH is a critical factor in the spread of malaria (74). Our findings showed higher RH to be negatively associated with malaria morbidity, which is consistent with previous studies done in Korea (75), Indonesia (76). At RH values between 50 and 60%, the risk of malaria was higher at lags of 5–6 months and lower at lags of 1–4 months. Study conducted in China found that for RH values between 68.57 and 80.57 the risk of malaria was higher at lags of 1–5 months

(77). In Iran, RH was also found to be the most important climatic driver of malaria infections (78). RH influences mosquito survival as insects are highly susceptible to desiccation. An increase in RH may be associated with heavy precipitation when temperatures are increasing, since moisture evaporating from the land surface in warm conditions is prevented from escaping by the arrival of clouds. Near the land surface, high RH leads to an increase in mosquito survival and host-seeking behavior. These factors are associated with variation in RH and are linked to malaria morbidity within an optimum RH range of approximately 60–80% (38). Our results are within the optimum RH range to malaria transmission.

The results from this study showed that malaria transmission was significantly associated with precipitation over the study area at a one-month lag. Malaria risk was negatively associated with precipitation above 300 mm which could be associated with flooding which destroys the mosquito habitat. In South-West China, precipitation value of 26 mm found to be positively associated with malaria infection after 2–4 months (77). While for a study done in Brazil, Guyana and Venezuela showed that malaria infection decreased by 1.6% per 1 cm increase in 6 months lagged precipitation (79). In Indonesia, a 1 mm increase in precipitation was associated with a 0.08% increase of malaria infection at lag of 3 months (76). Precipitation provides suitable habitats for mosquito breeding and is thus considered to be a dominant factor in driving malaria transmission (38). Unsurprisingly, studies conducted in Senegal (80), Ethiopia (81), Paraguay and Argentina (82), and Equador (83) showed that precipitation was the major determinant of malaria transmission. This may be explained by geographical and topographic conditions of an area. In addition, heavy precipitation or storms may destroy the breeding grounds of mosquitoes and interfere with the development of mosquito eggs or larvae (84).

Our findings showed that there was a significant decrease in malaria infection in households with electricity, which is consistent with other studies (85). Some studies suggested that households who share a toilet have a greater risk of malaria (86, 87). We found that less educated individuals were more vulnerable to malaria infection, which is consistent with earlier studies (5, 12, 13). Interventions and prevention measures plays a crucial role in the management and control of malaria infections. We only assessed ITNs and indoor residual spraying on the risk of malaria as these were the only control/prevention measures in the DHS and MIS datasets used in this study. However we know that malaria infections can be managed by the use of antimalarial drugs and prevented through the use of protective measures against mosquito bites (88), e.g., use of repellants and treated mosquito nets (89–91).

Treatment of malaria with an effective antimalarial in endemic settings is one of the key strategies of malaria control and prevention (92, 93). Artemisinin-based combination therapy (ACT) has been the recommended by the World Health Organization for the treatment of uncomplicated malaria in Mozambique since 2006, with artemether-lumefantrine (AL) and amodiaquine–artesunate (AS-AQ) as the first option (94, 95). In Mozambique, antimalarial drugs such as artemether-lumefantrine (AL) were observed to have therapeutic efficacy of 97.9% (95% CI 95.6–99.2%) to malaria infection, while for amodiaquine-artesunate (AS-AQ) were observed to have therapeutic efficacy of 99.6% (95% CI 97.9–100%) to malaria infection (94). In Tanzania, AL were observed to have therapeutic efficacy of 98% which is the WHO recommended threshold and remain well tolerated in the country (96). The therapeutic efficacy of AL in Ethiopia was 98.6% (95% CI 92.3–100) for malaria infection, which suggests the continuation of AL as the first-line antimalarial drug for the treatment of uncomplicated plasmodium falciparum malaria in Ethiopia (97). Abacassamo et al. assessed the clinical efficacy and parasitological response of Plasmodium falciparum to antimalarial drugs, he found that the therapeutic efficacy of 91.6% of amodiaquine (AQ) was better than that of 82.7% of sulphadoxine–pyrimethamine (SP) and 47.1% of chloroquine (CQ) to malaria infection (98). The therapeutic efficacy of AL and CQ in Ethiopia was 100% (95% CI 96–100) and 98%(95%CI: 95–100) for malaria infection, respectively, (99). Assessment of antimalarial therapeutic efficacy is needed to guide policies and practices (100, 101). The development of an effective malaria vaccine is, therefore, essential for mitigating malaria infections on vulnerable population. Currently, more than 2.3 million doses of malaria vaccine have been administered in three Sub-Sahara countries namely Ghana, Kenya and Malawi (102) though the efficacy is only at 39% (102).

The lagged association between environmental covariates and malaria incidence could aid in the development of a malaria early warning system to guide planning and control of malaria transmission. For example, precipitation and sea surface temperature monitoring has been used in issuing malaria early warnings in Botswana with great success in reducing malaria incidence (103). Similarly, a study in South Africa showed that seasonal climate forecasts could be used in a malaria early warning system with high prediction skill providing lead times of up to 16 weeks for planning (104).

In this study we showed that suitable temperatures of 21–26°C provided leads time of 1–3 months, higher rainfalls also provided shorter leads time of 1 month, but longer lead times for drier conditions of up to 6 months. Both Higher NDVI and relative humidity values also provided shorter lead time of 1 month. Combining all these lagged climatic covariates into an early warning system could provide lead times of 1–3 months for planning. Seasonal climate forecasts can potentially be utilized with this model to provide early warnings for malaria in Mozambique.

The major strength of this study is the combination of INLA Bayesian framework and DLNM framework to estimate the unbiased lag-exposure response functions between climatic factors and malaria risk by robustly adjusting for spatial–temporal covariance and socio-economic indicators. However, the study also had some limitations with the included socio-economic data. We only included the 2018 survey that covers the analysis period (2016–2018), assuming the values were similar in the previous years which may not be true. In addition, the aggregation of individual DHS covariates over large spatial units (the districts) may have masked the association between socio-economic indicators and malaria risk. Thus, interpretation should be made considering these limitations.

5. Conclusion

This study indicates that climate and socioeconomic variables influence the incidence and distribution of malaria in Mozambique. Temperature,precipitation, NDVI, and RH play a role in influencing

malaria cases at specific lag periods. The results of the study support the need to identify malaria vulnerability indicators to further support malaria control and efforts including combining climate variables, environmental conditions, regional spatial stratification, socioeconomic factors, public health interventions related to malaria transmission, and also reinforces the applicability of the use of climate services for risk mapping of malaria in areas where climate data is not routinely available. Achieving the targeted reductions in malaria infections in Mozambique will require a multidisciplinary effort, innovative approaches for malaria prevention and sustained political commitment at national, province, and district levels, as well as continued investment in malaria control and elimination efforts.

Vulnerability mapping should be carried out to identify areas with high malaria risk using climate variables. Climate variables such as temperature, NDVI, RH and precipitation should be used in identifying vulnerable areas. The identified lagged patterns can be used in the development of a climate-based early warning systems to strengthen malaria prevention in Mozambique. More research is needed to identify how to incorporate the identified vulnerability indicators and lagged associations into a malaria early warning system in Mozambique and assessing the forecast accuracies.

This study has relevance for achieving the Sustainable Development Goals (SDGs): (i) Ensuring healthy lives and wellbeing for all; on strengthening capacity for response to health risks (ii) Improving education, awareness-raising and human and institutional capacity on climate adaptation, impact reduction. Achieving the SDGs will require focusing on the poorest and most vulnerable populations as those are the most affected by malaria, ensuring no one is left behind. Ending malaria by 2030 requires a reference like the one presented here for planning, monitoring, and evaluation of malaria control efforts.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: The malaria datasets analyzed during the current study are available from the corresponding author on reasonable request. Requests to access these datasets should be directed to CA, cjarmando.jose@gmail.com.

Ethics statement

The study was based on secondary registries of surveillance data and no personal data was used, and thus no ethical approval was required.

Author contributions

CA and MSe wrote the manuscript. CA, JR, MSe, YT, AM, and AB conceived, designed the study, reviewed, and revised the manuscript. CA, MSi, MSe, JR, and AM contributed to data collection and statistical analysis. All authors contributed to writing the article and approved the submitted version.

Funding

This research was supported by the Swedish International Development Agency (SIDA).

Acknowledgments

CA is appreciative of the PhD scholarship by the Swedish International Development Agency (SIDA). All authors thank their respective institutions for supporting the research.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2023.1162535/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Mean and Standard deviation of Random Spatial effect Mozambique by year 2016–2018.

SUPPLEMENTARY FIGURE S2
Monthly seasonal patterns of **(A)** normalized different vegetation index, **(B)** relative humidity, **(C)** mean temperature, **(D)** malaria cases and **(E)** precipitation from 2016–2018.

SUPPLEMENTARY FIGURE S3
Scatter plot of malaria cases with **(A)** normalized different vegetation index, **(B)** relative humidity, **(C)** mean temperature and **(D)** precipitation.

SUPPLEMENTARY FIGURE S4
Seasonal Map of malaria cases by district in Mozambique 2018.

SUPPLEMENTARY FIGURE S5
Seasonal Map of minimum temperature by district in Mozambique 2018.

SUPPLEMENTARY FIGURE S6
Seasonal Map of mean temperature by district in Mozambique 2018.

SUPPLEMENTARY FIGURE S7
Seasonal Map of maximum temperature by district in Mozambique 2018.

SUPPLEMENTARY FIGURE S8
Seasonal Map of Relative Humidity by district in Mozambique 2018.

SUPPLEMENTARY FIGURE S9
Seasonal Map of Normalized different vegetation index (NDVI) by district in Mozambique 2018.

SUPPLEMENTARY FIGURE S10
Seasonal Map of precipitation by district in Mozambique 2018.

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frontiers | Frontiers in Public Health

TYPE Original Research
PUBLISHED 28 June 2023
DOI 10.3389/fpubh.2023.1203095

OPEN ACCESS

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RECEIVED 10 April 2023
ACCEPTED 16 May 2023
PUBLISHED 28 June 2023

CITATION
Wang X, Xu W, Luo F, Lin K, Zhang T, Yao L, Zhang X, Zhang J, Auburn S, Wang D and Ruan W (2023) Increasing incidence of *Plasmodium ovale* and persistent reporting of *Plasmodium vivax* in imported malaria cases: an analysis of 9-year surveillance data in four areas of China.
Front. Public Health 11:1203095.
doi: 10.3389/fpubh.2023.1203095

Increasing incidence of *Plasmodium ovale* and persistent reporting of *Plasmodium vivax* in imported malaria cases: an analysis of 9-year surveillance data in four areas of China

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Background: This study aimed at exploring the epidemiological pattern of imported malaria in China before malaria elimination in 2021, to provide evidence-based data for preventing malaria re-establishment in China.

Methods: Nine-year surveillance data on imported malaria in four provincial-level administrative divisions (PLADs) (Anhui, Chongqing, Guangxi, and Zhejiang) between 2011 and 2019 were thoroughly collected and analyzed.

Results: A quite stable trend in imported malaria cases between 2011 and 2019 was observed. In total, 6,064 imported patients were included. *Plasmodium falciparum* was the most frequently reported species (4,575, 75.6%). Cases of malaria were most frequently imported from Western Africa (54.4%). We identified an increasing trend in *P. ovale* and a persistence of *P. vivax* infections among the cases of malaria imported from Western Africa. Most patients (97.5%) were 20–50 years old. Among imported malaria infections, the main purposes for traveling abroad were labor export (4,914/6,064, 81.0%) and business trips (649, 10.7%). Most patients (2,008/6,064, 33.1%) first visited county-level medical institutions when they sought medical help in China. More patients were diagnosed within 3 days after visiting Centers for Disease Control and Prevention (CDCs) or entry–exit quarantine facilities (EQFs) (1,147/1609, 71.3%) than after visiting medical institutions (2,182/3993, 54.6%).

Conclusion: Imported malaria still poses a threat to the malaria-free status of China. County-level institutions are the primary targets in China to improve the sensitivity of the surveillance system and prevent the re-establishment of malaria. Health education should focus on exported labors, especially to Western and Central Africa. Increasing trend in *P. ovale* and persistence of *P. vivax* infections indicated their underestimations in Western Africa. Efficient diagnostic tools and sensitive monitoring systems are required to identify *Plasmodium* species in Africa.

KEYWORDS

imported malaria, China, Western Africa, migrant workers, surveillance, medical visit pattern

1. Introduction

Malaria is a parasitic disease with a long history in humans and is common in tropical and subtropical regions. According to the World Malaria Report (1), the disease affected an estimated 241 million people and caused 627,000 deaths in 2020. Of all these cases of malaria, most occurred in the World Health Organization (WHO) African Region, which accounted for 95% of reported cases, followed by the WHO Eastern Mediterranean Region (2.3%) and the WHO South-East Asian Region (2%). In response to the great efforts of the WHO Global Malaria Program and its partners, the malaria burden has decreased substantially over the past decade. Between 2010 and 2020, the total number of cases of malaria in the 21 countries that participated in the WHO "eliminating countries for 2020" (E-2020) initiative was reduced by 84% (2). Cases of and deaths from malaria have also fallen sharply in other regions.

China was one of the countries involved in E-2020 (2). According to the criteria of the Global Technical Strategy for Malaria 2016–2030 and under a country-led and country-owned endeavor, China has been certified a malaria-free country in 2021, after the implementation of an integrated strategy for malaria control, coupled with the country's socioeconomic and environmental development. Since 2017, all cases of malaria reported in China have been imported malaria, and imported malaria poses a non-negligible threat to China's malaria-free status. Most of these cases came from the African Region, with some from South-East Asia. Under the Belt and Road Initiative, and with the increasing globalization, labor exportation, and business cooperation, the reintroduction of local malaria transmission in currently malaria-free areas is highly possible. Besides continued surveillance of the distribution and insecticide resistance of malarial vector mosquitoes (3), the rapid identification of imported malaria and its timely treatment before the parasite is transmitted to mosquitoes are also essential in preventing the re-establishment of local transmission (4).

During the period in which malaria was eliminated, China instituted a nationwide malaria-specific surveillance system, and cases of malaria were reported in a real-time, web-based manner (5). A network of national reference laboratories was also created, facilitating case detection, with the blind assessment of samples. Local annual team-based microscopy competitions also maintained a capacity for the rapid and accurate diagnosis of malaria. However, as the number of imported cases of malaria has gradually increased, China's malaria-free status is potentially threatened. To understand the epidemiological characteristics of the imported malarial species, and the timeliness of their diagnosis and treatment, we retrospectively analyzed malaria surveillance data on cases of imported malaria in four provincial-level administrative divisions (PLADs) in China (Anhui province, Chongqing Municipality, Guangxi Zhuang Autonomous Region, and Zhejiang Province) reported between 2011 and 2019, which have relatively high numbers of imported malaria cases. This information should allow the development of further preventive and control strategies to avert the potential reintroduction of locally transmitted malaria and of more sophisticated health education.

2. Methods

2.1. Data source

Data on the malarial infections in the study area were obtained from the Information System for Parasitic Disease Control and Prevention (ISPDCP) (Supplementary Figure S1). The basic information collected for further analysation including sex, age, occupation, residence, and details of the malarial illness, such as the date of symptom onset, the date of diagnosis, and the date of treatment. After a patient was first diagnosed with malaria, the case should be reported into the ISPDCP within 24 h. Briefly, the county-level CDCs are responsible for case confirmation and investigation, to collect the relevant details of the patient, including travel history, dates of departure from and arrival back to China, countries visited, and purpose of travel. Laboratory reviews are preliminarily done by CDCs at county (sometimes municipal) level using microscopy method and/or rapid diagnostic test (RDT), and finally reviewed by provincial CDCs, using microscopy, RDT and PCR techniques. All the epidemiological information and malaria species will be submit to the ISPDCP, and also included in our analysis.

A total of 6,126 cases of imported malaria in the four PLADs studied were downloaded from the ISPDCP. Sixty-two were excluded because of incomplete information, and 6,064 cases were finally included.

2.2. Case definition

The case definition of malaria in China was based on a national guideline. The diagnosis was made based on the clinical symptoms, travel history, microscopy test, rapid diagnostic test and PCR. An imported malaria case was defined as "a malaria infection traced to an origin in a malaria-endemic area outside China and within 1 month after returning from the endemic area," according to the surveillance scheme of the National Malaria Elimination Program. Imported malaria cases would be laboratory-diagnosed, have a travel history to malaria-endemic areas outside of China during the local malaria transmission season, and the disease onset occurred < 1 month after returning to China during the local transmission season.

2.3. Data analysis

Statistical analyses were performed with R Project version 3.2.5[1] and Microsoft Office Excel 2019 (Los Angeles, CA, United States). Two different reviewers pooled the data from the four PLADs studied and reviewed the database independently,

1 http://cran.r-project.org

deleting cases with missing data on important indicators. The data are described with descriptive statistics. Bar plots and pie plots were used to visualize the numbers and proportions of malaria cases. Box and whisker plots were used to describe the temporal pattern of imported malaria cases. We used kernel density estimation to create smooth curves with which to assess the temporal changes in the numbers of malaria cases over time. Simple linear regression was used to test for linear temporal trends. A difference was considered significant at $p < 0.05$. All reported p values are two-tailed.

3. Results

3.1. General description

Among the 6,064 cases of imported malaria detected in 2011–2019, 1,088 (17.9%) were reported in Anhui, 267 (4.4%) in Chongqing, 3,107 (51.3%) in Guangxi, and 1,602 (26.4%) in Zhejiang. Only 10 cases were in non-Chinese subjects.

3.2. Imported malaria trends and seasonality

As shown in Figure 1, from 2011 to 2019, the annual number of cases of imported malaria was relatively stable, except in 2013. In that year, the confirmed cases of malaria peaked at 1670, because there was a dramatic increase in patient numbers in Guangxi (Figure 1B). In contrast, the numbers of cases ranged from 350 to 721 in all other years studied. The other three provincial areas hold a relatively stable trend of imported malaria cases however variance can also be discovered (Figure 1B). The numbers of cases ranged from 66 (in year 2012) to 191 (in year 2013) in Anhui, 22 (in year 2011) to 36 (in year 2016) in Chongqing, and 110 (in year 2011) to 238 (in year 2016) in Zhejiang.

The peak of imported malaria cases was detected in June (Supplementary Figure S2), and the *Plasmodium* species distribution in the malaria patients differed slightly by month. *P. falciparum* malaria seemed to peak in June, *P. vivax* between April and September, whereas *P. ovale* and *P. malariae* had a relatively stable pattern (Supplementary Figure S3).

3.3. Countries and regions of origin

The 10 most frequently reported countries of origin of imported malaria accounted for 78.2% (4,740/6,064) of the total cases. Among these countries, four were in Western Africa (Ghana, Nigeria, Angola, and Côte d'Ivoire), with 3,322 (54.8%) reported cases and four were in Central Africa (Cameroon, Democratic Republic of the Congo, Republic of the Congo, and Republic of Equatorial Guinea), with 1,173 (19.3%) reported cases. Myanmar from Southeast Asia (140, 2.3%) and Ethiopia from Eastern Africa (105, 1.6%) were the other two countries rounding up the top 10 origins of imported malaria cases to the area under study in China. Among individual countries, Ghana was the source of most infections, accounting for 34.5% (2095/6064) of the total cases (Figure 2 and Supplementary Figure S4).

According to the WHO Malaria Report 2021, the source countries could be classified into eight regions (Table 1). Specifically, 3,298 (54.4%) cases in this study were imported from the West African Region, followed by Central Africa (1,888, 31.1%) and countries with high transmission rates in Eastern and Southern Africa (458, 7.6%). Cases from South-East Asia accounted for 3.8% (228/6,064) of the total imported cases.

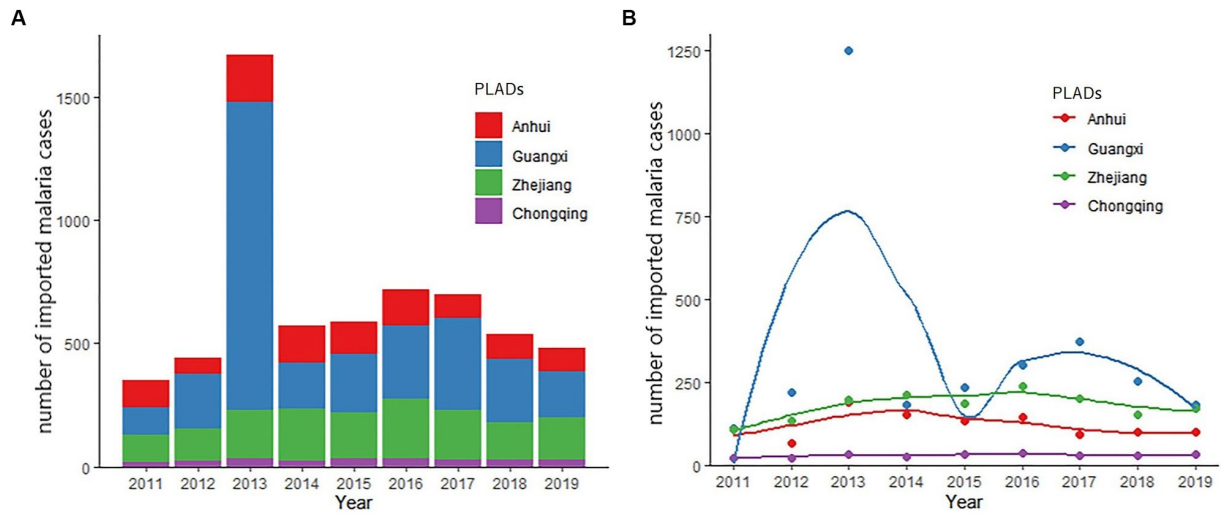

FIGURE 1
Trends in imported malaria in the four studied PLADs, 2011–2019. **(A)** Number of the total imported malaria cases from 2011 to 2019. **(B)** Trends of imported malaria in the studied PLADs. The smooth curves were created using kernel density estimation to describe the temporal changes in the numbers of malaria cases over time.

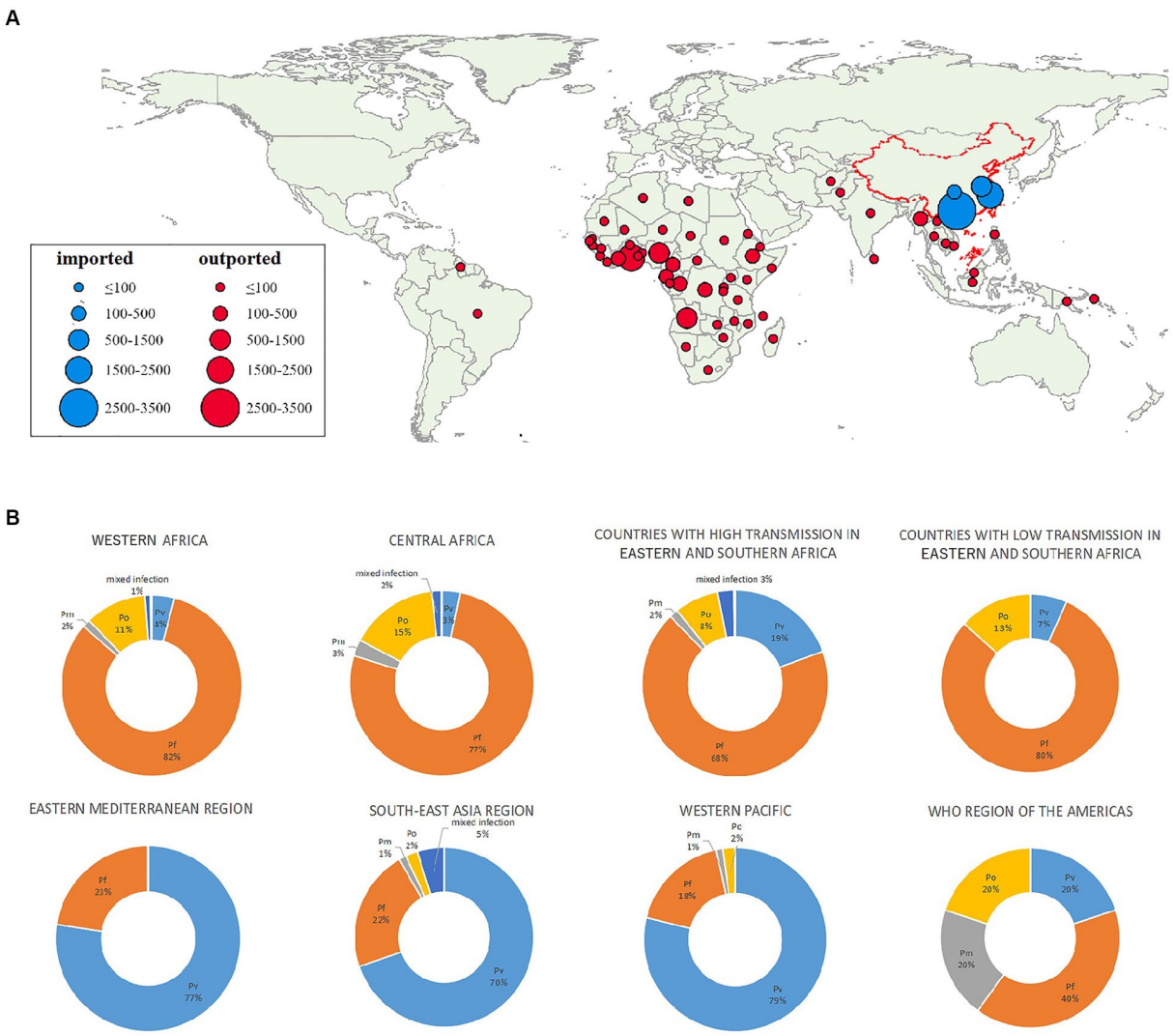

FIGURE 2
Geographic distribution and species of malarial infections by country and subregion. **(A)** Origins of malaria cases by country. **(B)** Percentages of infections caused by each species of *Plasmodium* by region. *Pf, P. falciparum*; *Pv, P. vivax*; *Pm, P. malariae*; *Po, P. ovale*.

3.4. Species of malaria parasites

Out of 6,064 cases recruited during the study period, the etiological agents of 11 cases were not identified at the species level. Among the remaining 6,053 cases, *P. falciparum* was most frequently reported (4,575, 75.6%), followed by *P. ovale* (703, 11.6%), *P. vivax* (574, 9.5%), *P. malariae* (103, 1.7%), and mixed infections (88, 1.5%). An increasing trend in the proportion of *P. ovale* ($\beta = 0.03$, $p = 0.003$) and a downward trend in the proportion of *P. vivax* ($\beta = -0.02$, $p = 0.007$) were detected with simple linear regression (Supplementary Figure S5).

Most *P. falciparum* infections (2,714, 59.3%) were imported from Western Africa, followed by Central Africa (1,447, 31.6%). Most *P. vivax* cases reported were acquired in South-East Asia (159/574, 27.7%), Western Africa (134/574, 23.3%), or countries with high transmission rates in Eastern and Southern Africa (89/574, 15.5%). Most *P. ovale* infections were acquired in Western Africa (363/703, 51.6%) and Central Africa (293/703, 41.7%). Similarly, 41.6% (47/113) of the *P. malariae* infections were acquired in Western Africa and 47.8% (54/113) in Central Africa (Table 1 and Figure 2).

3.5. Age distribution, sex ratio, and main purposes for traveling

Ages of the patients with imported malaria ranged from 0 to 71 years, of whom the median age of was 40 years (interquartile range, *IQR*: 31–47). Most patients (97.5%) were 20–59 years old (Table 2). The median age of female patients (35 years, *IQR*: 28–45) was generally lower than that of male patients (42 years, *IQR*: 31–47) in each year studied, except 2013 (Supplementary Figure S6B). Age of imported cases increased significantly by simple linear regression ($\beta = 0.583$, $p < 0.001$), and the median age increased by 5 years in that period, from 37 years in 2011 to 42 years in 2019 (Table 2).

Males accounted for 95.7% of all the patients with imported malaria. The male to female sex ratio (MFSR) was 17.93. Among all the age groups, this ratio was highest in patients aged ≥60 years

TABLE 1 Source regions of imported malaria by species in the four studied PLADs, 2011–2019.

Malaria species	Central Africa	Countries with high transmission in east and southern Africa	Countries with low transmission in east and southern Africa	Eastern Mediterranean	South-east Asia	West Africa	Western Pacific	Region of the Americas
P. vivax	60 (10.5)	89 (15.5)	1 (0.2)	55 (9.6)	159 (27.7)	134 (23.3)	71 (12.4)	1 (0.2)
P. falciparum	1,447 (31.6)	313 (6.8)	12 (0.3)	16 (0.3)	50 (1.1)	2,714 (59.3)	16 (0.3)	2 (0)
P. malariae	54 (47.8)	7 (6.2)	0 (0)	0 (0)	3 (2.7)	47 (41.6)	1 (0.9)	1 (0.9)
P. ovale	293 (41.7)	35 (5)	2 (0.3)	0 (0)	5 (0.7)	363 (51.6)	2 (0.3)	1 (0.1)
Mixed infection	32 (36.4)	13 (14.8)	0 (0)	0 (0)	11 (12.5)	32 (36.4)	0 (0)	0 (0)
NA*	2 (18.2)	1 (9.1)	0 (0)	0 (0)	0 (0)	8 (72.7)	0 (0)	0 (0)
Total	1888 (31.1)	458 (7.6)	15 (0.2)	71 (1.2)	228 (3.8)	3,298 (54.4)	90 (1.5)	5 (0.1)

*NA: Not available. Origin countries were classified into eight regions according to WHO malaria report of 2021. Eleven cases were not included due to their unavailable origin country. Central Africa: Angola, Burundi, Cameroon, Central African Republic, Chad, Congo, Equatorial Guinea, Gabon, Zaire. West Africa: Algeria, Benin, Burkina Faso, Republic of the Gambia, Ghana, Guinea, Guinea-Bissau, Ivory Coast, Liberia, Mali, Mauritania, Niger, Nigeria, Senegal, Sierra Leone, Togo. Countries with high transmission in East and Southern Africa: Ethiopia, Kenya, Madagascar, Malawi, Mozambique, Rwanda, United Republic of Tanzania, Uganda, Zambia, Zimbabwe. Countries with low transmission in East and Southern Africa: Comoros, Eritrea, Namibia, South Africa. Eastern Mediterranean: Afghanistan, Djibouti, Libya, Pakistan, Somalia, Sudan. WHO Region of the Americas: Brazil, Guyana. Western Pacific: Cambodia, Laos, Malaysia, Papua New Guinea, Philippines, Solomon Islands, Vietnam. Southeast Asia: India, Indonesia, Myanmar (Burma), Sri Lanka, Thailand.

TABLE 2 Demographic characteristics of malaria patients in the four studied PLADs, 2011–2019 (*n*/proportion or %).

Variable	Categories	2011	2012	2013	2014	2015	2016	2017	2018	2019	Total
Gender (*n*, %)	Male	329 (94)	423 (95.7)	1,635 (97.8)	545 (95.3)	558 (94.9)	684 (94.9)	665 (95.4)	507 (94.1)	457 (94.4)	5,803 (95.7)
	Female	21 (6.0)	19 (4.3)	36 (2.2)	27 (4.2)	30 (5.1)	37 (5.1)	32 (4.6)	32 (5.9)	27 (5.6)	261 (4.3)
Male to female sex ratio (MFSR)		15.7	22.3	45.4	20.2	18.6	18.5	20.8	15.8	16.9	22.2
Age (*n*, %)	≤20	4 (1.1)	12 (2.7)	15 (0.9)	3 (0.5)	3 (0.5)	4 (0.6)	3 (0.4)	6 (1.1)	3 (0.6)	53 (0.9)
	20–29	95 (27.1)	84 (19.0)	359 (21.5)	113 (19.8)	120 (20.4)	127 (17.6)	99 (14.2)	86 (16.0)	68 (14.0)	1,151 (19.0)
	30–39	115 (32.9)	144 (32.6)	513 (30.7)	163 (28.5)	169 (28.7)	203 (28.2)	199 (28.6)	162 (30.1)	131 (27.1)	1799 (29.7)
	40–49	106 (30.3)	152 (34.4)	603 (36.1)	209 (36.5)	224 (38.1)	254 (35.2)	249 (35.7)	163 (30.2)	155 (32.0)	2,115 (34.9)
	50–59	24 (6.9)	46 (10.4)	168 (10.1)	71 (12.4)	65 (11.1)	122 (16.9)	132 (18.9)	106 (19.7)	112 (23.1)	846 (14)
	≥60	6 (1.7)	4 (0.9)	13 (0.8)	13 (2.3)	7 (1.2)	11 (1.5)	15 (2.2)	16 (3.0)	15 (3.1)	100 (1.6)
Purpose of travelling overseas (*n*, %)	Labor export	239 (68.3)	351 (79.4)	1,556 (93.1)	450 (78.7)	453 (77)	546 (75.7)	553 (79.3)	409 (75.9)	357 (73.8)	4,914 (81.0)
	Business trip	42 (12.0)	10 (2.3)	70 (4.2)	76 (13.3)	79 (13.4)	99 (13.7)	109 (15.6)	84 (15.6)	80 (16.5)	649 (10.7)
	Tourism	1 (0.3)	1 (0.2)	2 (0.1)	0 (0)	4 (0.7)	4 (0.6)	4 (0.6)	3 (0.6)	3 (0.6)	22 (0.4)
	Study	3 (0.9)	1 (0.2)	0 (0)	0 (0)	1 (0.2)	3 (0.4)	2 (0.3)	0 (0)	1 (0.2)	11 (0.2)
	Others	65 (18.6)	79 (17.9)	43 (2.6)	46 (8.0)	51 (8.7)	69 (9.6)	29 (4.2)	43 (8.0)	43 (8.9)	468 (7.7)
Total		350	442	1,671	572	588	721	697	539	484	6,064

(MFSR: 34.8) and in those aged 50–54 years (MFSR: 31.4) (Supplementary Figure S6A). The ratio peaked in 2013, when a large number of gold miners returned to Shang-ling, Guangxi.

The main purposes for traveling abroad were labor export (4,914/6,064, 81.0%) and business trips (649, 10.7%). Other reasons included tourism (22, 0.4%), study (11, 0.2%), and other (468, 7.7%) (Table 2). The proportion of business trips tended to increase in recent years.

3.6. First medical visit

Among all the cases of imported malaria, the accurate diagnosis rate at first visit was 74.9% (4,540/6064) between 2011 and 2019. Most patients (2,008/6,064, 33.1%) chose county-level medical institutions for their first medical visit when symptoms developed (Table 3). However, Centers for Disease Control and Prevention (CDCs)/Entry–exit quarantine facilities (EQFs) presented the highest accurate diagnosis rate on the first visit (1,578/1609, 98.1%), followed by provincial-level (466/534, 87.3%), county-level medical institutions (1,734/2,008, 86.4%) and municipal medical institutions (579/716, 80.9%). Town-level medical institutions (159/481, 33.1%) and village-level clinics/private clinics (20/254, 7.9%) had relatively low accurate diagnosis rates at the first visit (Table 3).

3.7. Days from visit to diagnosis

Most cases of imported malaria were diagnosed at CDCs/EQFs (2,235/6,064, 36.9%) and county-level medical institutions

10.3389/fpubh.2023.1203095

TABLE 3 Medical institutions first visited by patients with imported malaria, and the rates of accurate first diagnosis in four PLADs, 2011–2019 (*n*/proportion or %).

Year	CDCs/ entry-exit quarantines	Provincial medical institutions	Municipal medical institutions	County-level medical institutions	Town-Level medical institutions	Village clinics/ private clinics	Others	Total
2011	11 (3.1)	59 (16.9)	23 (6.6)	14 (4)	13 (3.7)	5 (1.4)	225 (64.3)	350
Accurate diagnosis at first visit (%)	11 (100)	59 (100)	22 (95.7)	12 (85.7)	7 (53.8)	1 (20)	0 (0)	112 (32)
2012	39 (8.8)	32 (7.2)	33 (7.5)	70 (15.8)	27 (6.1)	11 (2.5)	230 (52)	442
Accurate diagnosis at first visit (%)	39 (100)	30 (93.8)	24 (72.7)	49 (70)	3 (11.1)	0 (0)	3 (1.3)	148 (33.5)
2013	859 (51.4)	84 (5)	108 (6.5)	450 (26.9)	117 (7)	52 (3.1)	1 (0.1)	1,671
Accurate diagnosis at first visit (%)	849 (98.8)	68 (81)	82 (75.9)	413 (91.8)	51 (43.6)	6 (11.5)	0 (0)	1,469 (87.9)
2014	151 (26.4)	65 (11.4)	84 (14.7)	187 (32.7)	56 (9.8)	27 (4.7)	2 (0.3)	572
Accurate diagnosis at first visit (%)	147 (97.4)	56 (86.2)	69 (82.1)	146 (78.1)	17 (30.4)	0 (0)	1 (50)	436 (76.2)
2015	136 (23.1)	62 (10.5)	89 (15.1)	208 (35.4)	63 (10.7)	29 (4.9)	1 (0.2)	588
Accurate diagnosis at first visit (%)	133 (97.8)	57 (91.9)	74 (83.1)	183 (88)	22 (34.9)	3 (10.3)	0 (0)	472 (80.3)
2016	130 (18)	79 (11)	114 (15.8)	305 (42.3)	55 (7.6)	38 (5.3)	0 (0)	721
Accurate diagnosis at first visit (%)	124 (95.4)	71 (89.9)	94 (82.5)	267 (87.5)	13 (23.6)	2 (5.3)	0 (0)	571 (79.2)
2017	127 (18.2)	46 (6.6)	98 (14.1)	326 (46.8)	73 (10.5)	27 (3.9)	0 (0)	697
Accurate diagnosis at first visit (%)	125 (98.4)	39 (84.8)	80 (81.6)	283 (86.8)	24 (32.9)	1 (3.7)	0 (0)	552 (79.2)
2018	92 (17.1)	57 (10.6)	77 (14.3)	238 (44.2)	39 (7.2)	34 (6.3)	2 (0.4)	539
Accurate diagnosis at first visit (%)	86 (93.5)	47 (82.5)	59 (76.6)	205 (86.1)	12 (30.8)	4 (11.8)	0 (0)	413 (76.6)
2019	64 (13.2)	50 (10.3)	90 (18.6)	210 (43.4)	38 (7.9)	31 (6.4)	1 (0.2)	484
Accurate diagnosis at first visit (%)	64 (100)	39 (78)	75 (83.3)	176 (83.8)	10 (26.3)	3 (9.7)	0 (0)	367 (75.8)
Total	1,609 (26.5)	534 (8.8)	716 (11.8)	2008 (33.1)	481 (7.9)	254 (4.2)	462 (7.6)	6,064
Accurate diagnosis at first visit (%)	1,578 (98.1)	466 (87.3)	579 (80.9)	1734 (86.4)	159 (33.1)	20 (7.9)	4 (0.9)	4,540 (74.9)

(2,001/6,064, 33.0%). The percentage of malaria patients diagnosed at CDCs/EQFs declined, whereas those diagnosed at county-level medical institutions increased (Supplementary Table S1).

Among all 6,064 reported cases, most of the reported cases were diagnosed in 1 ~ 3 days by medical institutions (2182/3993, 54.6%) or CDCs/EQFs (1147/1609, 71.3%), respectively. A quarter of them were diagnosed in 1 day (23.9% by medical institutions and 26.3% by CDCs/EQFs) (Supplementary Table S2).

4. Discussion

China was declared malaria-free on June 30, 2021 (6). This remarkable achievement was the result of a dedicated effort to prevent malaria over many years, and has become an important monument of malaria elimination in the Asia–Pacific region. However, imported malaria will still pose a threat to China's malaria-free status until global malaria eradication is finally achieved. There are still malaria vectors widely spread in China. *An. sinensis*, the most widely spread malaria vector in China which is, distributes all over the country. *An. lesteri*, *Anopheles minimus s.l.* and *An. dirus s.l.* are the three other major malaria vectors, sparsely distribute in limited mountainous forest areas, mainly in Southern China (3). In 2011, 20 indigenous cases of malaria were reported in Greece, although it was officially announced malaria-free in 1974 (7, 8). Similarly, a history of malaria reintroduction has been reported in Mauritius (9), Armenia (10), and Sri Lanka (11). Although the reintroduction of malaria into these countries is partly attributable to internal conflicts, migrant workers, visitors, and refugees, the failure to establish prevention of re-establishment (POR) strategies or their poor implementation has had deadly consequences (12). Therefore, in this exploratory epidemiological analysis, we have comprehensively examined the

cases of malaria imported into four different PLADs of China between 2011 and 2020, their epidemiological characteristics, and their changing patterns, to offer important information on POR and to protect this momentous achievement.

The cases of imported malaria in three of four provincial areas were quite stable in the years studied, except 2013, when a large number of Chinese gold prospectors were expelled by the Ghana government and returned to Shanglin city in Guangxi Zhuang Autonomous Region within a short period (13). Since 2006, >10,000 inhabitants have traveled abroad from Shanglin for gold mining, most of them to Ghana. This event demonstrates that local governments must be prepared for such unusual, dramatic increases in imported malaria, including provisions for patient diagnosis, epidemiological investigations, treatment, and mosquito surveillance, because the timely detection and treatment of new infections is critical in preventing the re-establishment of local transmission.

In this study, malaria infections were most frequently seen in oversea laborers, who have travelled, lived and worked in malaria endemic countries or territories. Different from European countries where malaria patients are usually travelers and migrants (14), these people have travelled for a longer period of time, also the demographic characteristic and occupation could result in additional risk. According to published studies, most of these laborers are farmers, who are generally poorly educated and unaware of the risk of malaria and personal protection measure to avoid mosquito bites (15). Health education and personal protection equipment are recommended for laborers, especially at the primary health care level. We have also observed an increasing trend of median age of imported malaria. Because older travelers are at higher risk of malaria-related morbidity and mortality than younger people (16), attention should be paid on changes in the age distribution of imported malaria cases. Greater awareness of mosquito protection measures and chemoprophylaxis, and health education before traveling are required.

Similar to other epidemiological studies in other parts of China (17, 18), a weak seasonal peak in June was observed. A possible explanation is that the busy agricultural work during May–September in China requires migrant workers returning to their hometown (19, 20). However, other studies have identified no specific seasonality in imported malaria, so this issue warrants further research.

Sub-Saharan Africa was the main source region for imported malaria in China. Patients were most frequently infected with *P. falciparum*, especially in sub-Saharan African regions. With the high prevalence and relatively high virulence of *P. falciparum*, the importance of its timely diagnosis and effective treatment should not be neglected. Intravenous artesunate for severe malaria and oral artemisinin-based combination therapies for uncomplicated malaria are the first-line treatments for all disease caused by *Plasmodium* species in humans (21). However, evidence of resistance to artemisinin is not only found in the Greater Mekong Subregion, but also been found in sub-Saharan African countries, such as Rwanda (22) and Uganda (23). Though both were Eastern sub-Sahara African countries, the artemisinin resistance still posed a great challenge in the treatment and control of imported *P. falciparum,* and thus, not only for those returning from GMS but also for those returning from African countries, continuous molecular surveillance of *pfk13* mutation in *P. falciparum* are necessary.

Two observations in the Africa Region are noteworthy. About 4% of malaria cases imported from WHO West Africa region involved *P. vivax*, which is inconsistent with the consensus that *P. vivax* infections are uncommon in Western Africa because of the prevalence of the Duffy-negative genetic status. We also noted that during the study period, the number cases of imported *P. ovale* malaria exceeded the number of *P. vivax* infections reported in the African Region, and increased significantly from 2011 to 2019. Our observations are supported by growing evidence suggesting that a Duffy-negative status is no longer a barrier to *P. vivax* infection (24, 25). Our data suggest that *P. vivax* and *P. ovale* infections are underestimated in Africa because these species are not routinely identified in most countries. Besides, though we did not detect any case infected with *Plasmodium knowlesi* (*P. knowlesi*) in the four studied areas from 2011 to 2019, the expanding prevalence of *P. knowlesi* infection in Southeast Asia should never be ignored for the control and elimination of malaria. *P. knowlesi* infection occurs in forested areas where monkeys and humans coexist (26). The majority of *P. knowlesi* malaria infections cause mild clinical manifestations, with an estimated 6%–9% of severe cases and increasingly frequently reported asymptomatic infections in the last decade (27). PCR methods are important to confirm *P. knowlesi* infection, since the morphology of rings of *P. knowlesi* in thin blood film smears is similar to *P. falciparum*, as well as trophozoites and schizozoites similar with *P. malariae.* In China, a network of reference laboratories, including a national laboratory and provincial laboratories in CDCs, have been built since 2013. PCR is used in reference laboratories to identify and double check the species responsible for malaria infections. However, the large number of imported *P. vivax* and *P. ovale* cases is also a challenge in China, because their less-typical symptoms or even asymptomatic presentation require effective diagnostic tools and sensitive monitoring systems, not only in CDCs but also in medical institutions.

In this study, we found that most malaria patients first visited county-level institutions when seeking medical help in China, and that nearly a third of imported infections were diagnosed and treated at county-level institutions. However, the rate of correct diagnosis at county-level institutions was lower than that at CDCs/EQFs and provincial-level institutions. Our study also showed that CDCs/EQFs had a quicker response to patients with malaria than medical institutions, insofar as more patients were diagnosed within 3 days of visiting a CDCs/EQFs than within 3 days of visiting a medical institution. Based on our data, medical institutions, especially county-level institutions, should be the primary targets of measures to improve the sensitivity of the surveillance system in China.

This study had several limitations. First, no information on the severity or mortality of the malaria reported was included in our analysis. Second, the number of travelers returning to China from various countries was not obtained, so we could not estimate and compare the incidence of malaria imported by the country of origin. Third, the specific origins of malaria within the source countries were not investigated, which limited further interpretation of the data.

5. Conclusion

In this study, we thoroughly analyzed the surveillance data on malaria imported into four PLADs of China between 2011 and 2019. We detected

a quite stable trend in imported malaria cases, suggesting that imported malaria still poses a threat to the malaria-free status of China. Our analysis also demonstrated that county-level institutions should be the primary targets of measures to improve the sensitivity of surveillance systems in China and to prevent the re-establishment of malaria. We also observed an increasing trend in *P. ovale* and the persistence of *P. vivax* infections in Western Africa throughout these years, indicating that the prevalence of *P. ovale* and *P. vivax* is underestimated in Western Africa. Efficient diagnostic tools and sensitive monitoring systems are required to identify the *Plasmodium* species prevalent in Africa.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: The datasets presented in this article are not readily available because the ISPDCP is not a publicly available data repository. Requests to access these datasets should be directed to DW, wangdq@nipd.chinacdc.cn.

Author contributions

DW and WR conceived the study. XW, WX, FL, KL, and TZ designed and carried out the analysis. XZ, FL, and JZ prepared the datasets. LY, SA, DW, and WR advised on the analysis. XW and WX wrote the manuscript. XW, WX, FL, KL, TZ, LY, XZ, JZ, SA, DW, and WR contributed to the interpretation of results. All authors contributed to the article and approved the submitted version.

Funding

This work was funded by grants from the Major Health Science and Technology Projects in Zhejiang Province (Grant No. WKJ-ZJ-2119) and Medical Research Program of Zhejiang Province (Grant No. 2020PY038 and 2022KY723).

Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2023.1203095/full#supplementary-material

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frontiers | Frontiers in Tropical Diseases

TYPE Original Research
PUBLISHED 25 July 2023
DOI 10.3389/fitd.2023.1204195

OPEN ACCESS

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RECEIVED 11 April 2023
ACCEPTED 26 June 2023
PUBLISHED 25 July 2023

CITATION
Costa GL, Alvarenga DAMd, Assis GMPd, Aguiar ACC, Louzada J, Pereira DB, Pina-Costa Ad, Hirano ZMB, Moreira SB, Pissinatti A, Brasil P, Daniel-Ribeiro CT, Sousa TNd and Alves de Brito CF (2023) Malaria mitochondrial diagnosis: challenges and pitfalls.
Front. Trop. Dis 4:1204195.
doi: 10.3389/fitd.2023.1204195

Malaria mitochondrial diagnosis: challenges and pitfalls

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Background: High-copy genomic sequences could be used as PCR targets for the detection of *Plasmodium* infections, providing increased sensitivity over single- or low-copy genes. Mitochondrial genomes of malaria parasites are present in multiple copies in a single mitochondrion, and each parasite has many mitochondria. Here, we describe the development of seven species-specific qPCR assays for the diagnosis of *Plasmodium vivax* and *Plasmodium falciparum*, targeting coding and non-coding mitochondrial genomic regions.

Methods: The optimization of the qPCR protocols involved a gradient of annealing temperatures and concentrations of primers and probes, as well as the inclusion of PCR additives/enhancers [e.g., dimethyl sulfoxide (DMSO), glycerol, bovine serum albumin (BSA)] to improve the specificity of qPCR amplification.

Results: Non-specific amplification of other *Plasmodium* species and of human targets was observed in different levels for all assays. Regardless of the late Cq values for most non-specific amplifications, the application of a cutoff value did not completely exclude false-positive amplification, compromising the specificity and also the sensitivity of the assays.

Conclusions: Therefore, although mitochondrial targets have higher sensitivity, they frequently lose specificity due to their high levels of sequence conservation. A screening to evaluate the cross-reaction between *Plasmodium* species and the non-specific amplification of human malaria-free samples must be performed for *Plasmodium* mitochondrial assays.

KEYWORDS

malaria, *Plasmodium*, diagnosis, quantitative PCR, mitochondrial DNA

1 Introduction

Malaria remains an important public health problem in many tropical and subtropical countries, despite the efforts that are currently being undertaken toward controlling the disease around the world. In 2021, 247 million human malaria cases and 619,000 deaths were reported worldwide (1). A rapid and accurate malaria diagnosis is crucial for the effectiveness of the disease control. Microscopy of Giemsa-stained blood smears is the most widely used approach for malaria diagnosis due to its low cost and relatively simple procedure, despite its poor sensitivity (2). Molecular approaches, such as polymerase chain reaction (PCR), are more specific and sensitive, and have improved the capacity to diagnose submicroscopic infections, defined as a low density of *Plasmodium* parasites in blood. This is of paramount importance for *Plasmodium vivax* infections, as this species generally presents with lower levels of parasitemia than *P. falciparum* due to its preferential invasion of the reticulocyte, rather than the mature red blood cell. Moreover, *P. vivax* is more difficult to diagnose and control because of its early gametogenesis, which allows transmission even before symptoms appear (3). In the context of malaria elimination, the detection of asymptomatic carriers of the infection is highly relevant, since they present a silent reservoir for ongoing transmission (4).

Nested PCR based on the 18S rRNA gene is largely used for molecular-based malaria diagnosis (5). Quantitative PCR (qPCR) has been increasingly implemented as it provides fast results in high-throughput screening. It is highly sensitive and specific, and allows parasite quantification (6–8). PCR sensitivity is greatly influenced by the copy number of the target molecule; therefore, a target with a low copy number limits the detection capability of these assays, particularly for low parasitemia. The *Plasmodium* mitochondrial (mt) genome is an ideal target for PCR, because it has higher copy number per parasite (20–150 copies) than single-copy targets or than the 18S rRNA gene (4–8 copies), thus allowing a greater sensitivity (9–14). The genus *Plasmodium* has one of the smallest mt genomes in the form of a tandemly repeated linear element of 6 kb (15). This genome encodes only three genes: cytochrome c oxidase subunit 1 gene (*cox1*), cytochrome c oxidase subunit III gene (*cox3*), and cytochrome b gene (*cytb*).

Despite the high sensitivity of mtDNA-based assays, in this study we describe the pitfalls related to the development of malaria diagnosis assays by qPCR targeting the mt genome of *P. vivax* and *P. falciparum*. Seven species-specific assays were designed to amplify regions of the *Plasmodium* mt genome (i.e., coding and non-coding) by qPCR. Because all our assays showed some level of non-specificity, we strongly recommend that the mt assays for *Plasmodium* species diagnosis undergo a screening of different *Plasmodium* species samples to access this potential issue.

2 Methods

2.1 *Plasmodium vivax* and *Plasmodium falciparum* samples

DNA of *P. falciparum* and *P.vivax* was obtained from blood samples of patients previously diagnosed as having a single infection by well-trained microscopists, and PCR using ribosomal and non-ribosomal targets (5, 16). One hundred and eleven blood-positive samples were used from patients from different regions of the Brazilian Amazon infected with *P. vivax* and *P. falciparum* or stored at the biorepository of Laboratory of Malaria at Instituto René Rachou (Supplementary Table 1). Parasite density was determined as the number of asexual parasites observed per 200 white blood cells on a thick smear and was estimated by assuming a leukocyte count of 8,000 per μL. Parasitemia ranged from 90 to 16,950 parasites/μL for *P. falciparum*-infected individuals, and from 30 to 17,100 parasites/μL for *P. vivax*-infected individuals. The inclusion criteria were mild malaria or asymptomatic, more than 5 years old, absence of pregnancy, and a signed informed consent form.

The collection of human samples for DNA extraction was performed in accordance with relevant guidelines and regulations. All participants and/or their legal guardians provided written informed consent. Ethical and methodological aspects of this study were approved by the Ethical Committee of Research on Human Beings from the IRR (N° 2.243.058), in accordance with the Brazilian National Council of Health (Resolutions 196/96 and 466/12). The INI-Fiocruz Ethical Board approved the study concerning the patients from the Atlantic Forest (number 0062.0.009.000-11).

DNA extraction from blood samples was performed using a QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany) or Gentra® Puregene® Blood Kit (QIAGEN, Chatsworth, CA, USA),

using 300 µL of blood and a final volume of 50 µL, in accordance with the manufacturer's instructions. Both blood and extracted DNA material were stored at –20°C.

2.2 Other *Plasmodium* species and uninfected samples

Seventeen blood samples were included from patients from the Atlantic Forest area diagnosed as being positive for *Plasmodium simium* by PCR analysis (17, 18). Samples from 26 non-infected individuals (i.e., human negative controls tested with distinct malaria PCR protocols) from a non-transmission malaria area (i.e., Belo Horizonte, Minas Gerais, Brazil) were included as negative controls in the assays.

Thirteen *P. simium* and 15 *P. malariae/P. brasilianum* DNA samples obtained from free-living and captive non-human primates (NHPs) from the Brazilian Atlantic Forest were included; these samples were stored at the biorepository of the Laboratory of Malaria (18–20). Samples from 12 NHPs from areas without malaria transmission, negative in distinct PCR protocols (5, 18), were included as negative controls in the assays. All samples were processed to obtain DNA, as mentioned above, and stored at –20°C. NHPs were diagnosed by only PCR (17, 18), as their low parasitemia hampers the diagnosis by light microscopy. The Brazilian government (Ministry of Environment) authorized the capture, handling, and collection and transport of biological samples from NHPs (SISBIO numbers: 43375–4/2015, 54707–137362–2 and 52472–1; and INEA license 012/2016012/2016). This study was approved by the Institutional Ethics Committee of Animal Use (CEUA license L037/2016).

2.3 Primer design and qPCR assay optimization

qPCR assays for the diagnosis of *P. vivax* and *P. falciparum*, targeting coding and non-coding mt genome regions were designed. Whole-mt sequences of *P. falciparum* (GenBank accession number: AY282930.1), *P. malariae* (GenBank accession number: AB354570.1), and *P. vivax* (GenBank accession number: PvP01, GCA_900,093,555.1) were aligned using ClustalW software in the BioEdit package. For primer design, regions of mt genome that contained polymorphisms between *Plasmodium* species, preferentially in the 3′ ends, were used to ensure assay specificity. The primer design followed the optimal primer recommendations, such as: (i) a primer length around 18–30 bases; (ii) a GC content > 40 (whenever possible, because of the high A/T content of *Plasmodium* genomes); (iii) a melting temperature between 50°C and 62°C; and (iv) an absence or reduced regions of secondary structure, intra-primer homology (self-dimer), or inter-primer homology (primer dimers). Primers/probes with the highest stringency in the parameters of the Primer Express™ software (Applied Biosystems™) and Oligo (Molecular Biology Insights, Inc) were chosen (Supplementary Figure 1). Probes for the *cox1* gene assays contained minor groove binders (MGBs) and for the *cytb* gene and non-coding regions assays, two different quenchers were used: an internal quencher, ZEN, and a Black Hole Quencher™ at the 5′ end.

For the qPCR assays, the optimum concentrations of the primers and probes were defined using a concentration gradient (0.3–0.9 µM of each primer and 0.15–0.25 µM of each probe) (Supplementary Table 2). A gradient of annealing temperatures (52°C–63°C) was also tested. To improve the specificity of qPCR amplification, PCR additives/enhancers [e.g., dimethyl sulfoxide (DMSO), glycerol, bovine serum albumin (BSA)] were used in different concentrations (21) (Supplementary Table 2). All assays used TaqMan™ Universal PCR Master Mix (Applied Biosystems), and the reactions were standardized on 384-well plates using QuantStudio® 12K Flex Real-Time PCR System (Applied Biosystems) for the *cox1* gene assays or the ViiA™ 7 Real-Time PCR System (Applied Biosystems) for the *cytb* and non-coding regions assays. All results were analyzed using QuantStudio Real-Time PCR Software (Applied Biosystems) or QuantStudio 12K Flex Real-Time PCR Software (Applied Biosystems).

Positive and negative (no-DNA) controls were used in each round of amplification. The positive controls in the qPCR assays used DNA from *Plasmodium* species previously diagnosed by well-trained microscopy and/or by other molecular tests (nested PCR and qPCR) (5, 16): (i) *P. falciparum* DNA strain 3D7, which was maintained in the Laboratory of Malaria at IRR, (ii) DNA extracted from the blood of patients with high parasitemia for *P. vivax*, (iii) DNA of *P. simium* from a NHP (*Alouatta guariba clamitans*—MB), and (iv) DNA of *P. brasilianum* from the MR4 Malaria Research and Reference Resource Center [American Type Culture Collection (ATCC), USA].

To prevent cross-contamination, the DNA extraction and reaction mix preparation were performed in "parasite DNA-free rooms" distinct from each other. Each of these separate areas had different sets of pipettes and all procedures were performed using plugged pipette tips. Furthermore, DNA extraction was performed twice on different days, and for each sample with non-specific amplification, two or three independent PCR reactions were performed using the same conditions (i.e., with reagents and thermal cycler).

2.4 Statistical analysis

A cutoff value for each assay was established by the receiver operating characteristic (ROC) curve given by MedCalc for Windows, version 20.123 (MedCalc Software, Ostend, Belgium), which considered the positive Cq values of specific and non-specific amplifications. The Cq values from positive samples were considered "true positives", whereas for the negative samples (i.e., negative for the species to be tested) were considered "true negatives". These samples were previously diagnosed by molecular tests with ribosomal (5) and non-ribosomal targets (16). Thus, the combined results of these two molecular tests were used as the reference to estimate the sensitivity and

specificity of each mt assay. From each cutoff value, the true- and false-negative/-positive samples were determined using the R package, as well as the sensitivity and specificity values. The optimal value for the cutoff was determined as a specificity of ≥ 90%.

The overlap in the Cq values between specific and non-specific amplifications for each assay was established as the percentage of samples showing similar Cq values of non-specific and specific amplifications, that is, overlap occurred when Cq values between specific and non-specific amplifications superimposed each other and included the same Cq data (Cq min. non-specific < x < Cq max. specific).

3 Results

3.1 Design and analysis of *Plasmodium* mitochondrial qPCR assays

Seven TaqMan-based qPCR assays were designed for the detection of *P. falciparum* and *P. vivax*. Coding genes and non-coding regions of the *Plasmodium* mt genome were targeted in the assays: *cox1* gene (PV_COX1_1; PV_COX1_2), *cytb* gene (PV_CYTB, PF_CYTB), and non-coding regions (PF_NC_1; PF_NC_2; PV_NC) (Figure 1 and Supplementary Figure 1).

Although the mt genome is highly conserved among *Plasmodium* species, the sequence alignments of the amplicon, primers, and probes of each assay showed the presence of some single-nucleotide polymorphisms (SNPs) used to distinguish the different *Plasmodium* species (particularly at the 3′ ends of the primers) (Supplementary Figure 1).

3.2 Specificity of amplification for mt *Plasmodium* assays

A wide screening to verify the cross-reaction between the *Plasmodium* species or amplification using human malaria-free samples was performed in all *Plasmodium* mt assays. A panel of 153 *Plasmodium* samples, which had been diagnosed by well-trained microscopists and/or had been previously assayed with ribosomal and non-ribosomal targets and samples from 26 healthy volunteers, were used. Non-specific amplification was observed in all assays for other *Plasmodium* species and/or human negative control samples, as described below. The geometric mean of parasitemia was 2,528 parasites/μL (CI_{95} 1,914–3,338) for *P. vivax* samples and 1,697 parasites/μL (CI_{95} 1,009–2,854) for *P. falciparum* samples used in the assays.

For the *cox1* gene assays (PV_COX1_1; PV_COX1_2), optimization included different primer and probe concentrations and DNA quantities. To improve the specificity of qPCR amplification, PCR enhancers (DMSO, glycerol, BSA) were also evaluated. For both cytochrome oxidase 1 assays, no non-specific amplifications with human malaria-free samples (0/15) were observed. The *Plasmodium vivax* PV_COX1_1 assay was tested with 40 *P. vivax* samples, all of them amplified (Cq mean = 29.0; range = 20.6–38.2) and did not cross-react with any of 30 *P. simium* samples. However, non-specific amplification was identified in five out of nine (55.5%) reactions using well-characterized *P. falciparum* samples (with known parasitemia and confirmed by other PCR protocols to exclude co-infection) amplified with a Cq mean value of 39.2 (range = 36.6–40.8) (Supplementary Table 2). To reduce non-specific amplification, the probe PV_COX1_2 was redesigned

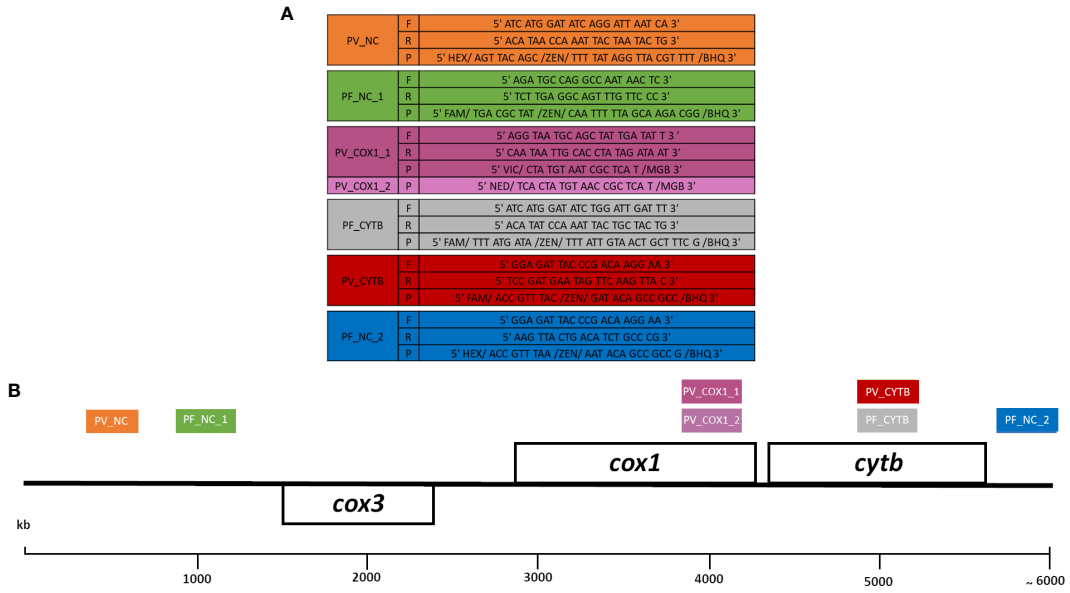

FIGURE 1
Sequences of primers and probes **(A)** and schematic representation of amplicons **(B)** from each assay in the *Plasmodium* mitochondrial genome. Seven assays were developed targeting the *cox1* gene (PV_COX1_1; PV_COX1_2), the *cytb* gene (PV_CYTB, PF_CYTB), and the non-coding regions (PV_NC, PF_NC_1; PF_NC_2). Each assay is represented by colored boxes, and the positions are based on the *P. falciparum* mitochondrial genome sequence (accession number NC_037526.1). Primers (F: forward; R: reverse) and probes (P) sequences of each assay are shown in the same color of boxes. PF and PV correspond to *P. falciparum* and *P. vivax*, respectively.

by including nucleotides spanning a more polymorphic region. Using the new probe, 49/50 (98%) *P. vivax* samples were amplified correctly and did not cross-react with any of the 20 *P. simium* samples or with the 15 *P. malariae/P. brasilianum* samples. Nevertheless, 8 out of 38 (21.0%) reactions showed non-specific amplification using *P. falciparum* samples, even using the enhancer DMSO (Supplementary Table 2). The Cq mean values were 42.3 (range = 35.3–44.6) and 32.4 (range = 24.2–42.0), for non-specific and specific amplifications, respectively (Supplementary Table 2).

The assay targeting the non-coding region, PV_NC, was tested with 19 *P. vivax* samples, all of them amplified (Cq mean = 23.0; range = 18.0–32.4). Non-specific amplification was identified in 8 out of 29 (27.6%) reactions using well-characterized *P. falciparum* samples (Cq mean = 37.4; range = 35.2–39.9) and in 1 out of 56 (1.8%) reactions using human uninfected controls (Cq = 39.3) (Supplementary Table 1).

For the PV_CYTB assay, which uses the *cytb* gene as target, 22 out of 23 (95.6%) *P. vivax* samples were amplified correctly (Cq mean = 30.6; range = 27.0–37.4). However, non-specific amplification was identified in three out of six (50%) reactions using *P. falciparum*, *P. simium*, or *P. brasilianum* (Cq mean = 34.8; range = 32.7–38.8) and in 8 out of 68 (11.8%) reactions using human uninfected controls (Cq mean = 34.5; range = 30.7–38.6) (Supplementary Table 2).

In the assay targeting the *P. falciparum cytb* gene (PF_CYTB), 33 out of 48 (68.7%) reactions using *P. falciparum* samples (Cq mean = 23.8; range = 14.7–33.3) were amplified correctly. However, non-specific amplification was identified in five out of six (83.3%) reactions using *P. vivax*, *P. simium*, or *P. malariae/ P. brasilianum* (Cq mean = 38.3; range = 38.0–38.4) and in 29 out of 104 (27.9%) reactions using human uninfected control (Cq mean = 37.2; range = 36.1–39.8) (Supplementary Table 2). To reduce non-specific amplification, different DMSO concentrations were used: 1%–5% without significant improvement for specificity (data not shown).

For the PF_NC_1 assay, based on different regions of the non-coding mt genome, 12 out of 13 (92.3%) reactions using *P. falciparum* samples were amplified correctly (Cq mean = 24.9; range = 19.3–29.7), while 7 out of 18 (38.9%) and 18 out of 42 (42.9%) reactions showed non-specific amplification for *P. vivax* (Cq mean = 35.0; range = 29.2–37.4) and human uninfected control samples (Cq mean = 35.3; range = 34.6–38.7), respectively (Supplementary Table 2). For the PF_NC_2 assay, also based on the non-coding region, 10 out of 14 (71.4%) reactions using *P. falciparum* samples were amplified correctly (Cq mean = 20.9; range = 12.7–34.6), whereas two out of nine (22.2%) *P. vivax* reactions (Cq mean = 34.7; range = 33.4–36.0) and 9 out of 43 (20.9%) reactions using human uninfected control (Cq mean = 36.3; range = 33.0–39.7) showed non-specific amplification (Supplementary Table 2).

A cutoff value for each assay was stablished by the ROC curve considering the positive Cq values of specific and non-specific amplifications. From each cutoff value, the true and false negative/positive samples were determined, as well as the sensitivity and specificity values (Figure 2). The Figure 2A shows the accuracy for each assay. Comparing all assays, PV_CYTB presents the best accuracy for *P. vivax* detection, with 95% of the sensitivity and 96% of the specificity. The PF_CYTB assay was the only *P. falciparum* assay with a specificity of > 90%. For these two assays with best results for each *Plasmodium* species, the cutoff was

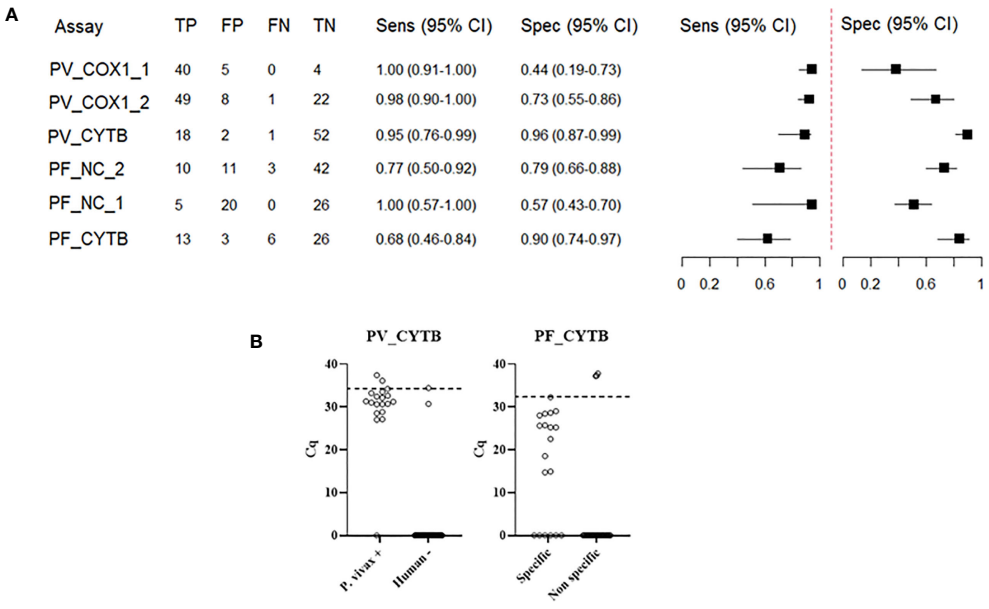

FIGURE 2
Determination of accuracy of each assay. **(A)** Sensitivity (Sens) and specificity (Spec) estimate for each assay. PV_NC assay was not represented here because of the low number of samples analyzed. TP = true positive; FP = false positive; FN = false negative; TN = true negative **(B)** Distribution of Cq values for specific and non-specific amplifications of different samples and the cutoff value for PV_CYTB and PF_CYTB assays, with a specificity of > 90%. To estimate the sensitivity and specificity of each assay, we defined the combined results of two other molecular tests targeting ribosomal and non-ribosomal genes as a reference (4).

not able to totally discriminate the Cq values for specific and non-specific amplifications (Figure 2B).

3.3 Optimization of protocols to increase assay specificity

Initially, to minimize the non-specific amplification, a set of parameters was modified, such as the concentration of primers/probes in each assay, the denaturing and annealing conditions, and the number of cycles. The primer concentrations varied from 0.3–0.9 µM and from 0.15–0.25 µM for probes. The annealing temperature tested varied from 52°C–63°C. An incremental annealing temperature in progressive cycles was also tested (Supplementary Table 2). These modifications decreased the number of non-specific amplifications in some cases, but did not eliminate all of them. However, the Cq values for the specific amplifications increased, showing that changes in annealing temperature and primer/probe concentrations interfered with assay sensitivity (Supplementary Table 2). A variety of PCR additives/enhancers (DMSO, glycerol and BSA) has also been used to increase the specificity, yield, or consistency of reactions (Supplementary Table 2). The reduction of non-specific amplification was notable in most of the samples; however, the use of these additives hampered the efficacy of specific amplifications. For the PV_COX1_2 assay, 5% of DMSO, glycerol, or BSA was individually tested, and only DMSO was able to reduce non-specific amplifications. Nevertheless, the use of additives also had an impact on the specific amplifications, increasing the Cq values (Supplementary Table 3). Different concentrations of DMSO and DNA were also tested. The best combination to avoid non-specific amplification without interference with specific amplification was using 3.5% DMSO and 2 µL of DNA (Supplementary Table 3). Then, a large panel (n = 30) of well-characterized *P. falciparum* samples were screened using this combination of DMSO, and eight samples were amplified, with a mean Cq of 42.3 (range = 35.3–44.6) (Supplementary Table 2). None of the adopted strategies eliminated the non-specific amplification. For the PF_CYTB, all concentrations of DMSO tested (1–5%) were not able to eliminate the non-specific amplification (using 52 samples, including *P. vivax* and human uninfected control, data not shown). In a single experiment with 1% DMSO, 3 out of 29 human uninfected control samples were non-specifically amplified for *P. falciparum*, with a Cq mean of 37.4 (range = 37.2–37.8), whereas 13 out of 19 *P. falciparum* samples were amplified specifically (Cq mean = 24.5, range = 14.7–32.3). Once again, the use of DMSO hampered the efficiency of specific amplifications, and it explains the decreased sensibility of PF_CYTB assay, which amplified only 68% of specific samples (Supplementary Table 2).

4 Discussion

In the past decade, improved nucleic acid amplification techniques have established increasingly high standards in diagnosis sensitivity using multi-copy target genes. Mitochondrial (mt) genomic sequences can provide alternative PCR targets for the detection of malaria infections, offering increased sensitivity over single- or low-copy targets such as the 18S rRNA genes (4). We evaluated the specificity of seven different assays targeting the mt genome of *P. vivax* and *P. falciparum*. To test assays' specificities, a screening with *Plasmodium* samples, which were well characterized with known parasitemia and confirmed by molecular protocols with ribosomal (5) and non-ribosomal targets (16) to exclude co-infection, was performed. Here, the exclusion of co-infections was essential to truly access the specificity of the assays.

After an exhaustive evaluation with different qPCR assays targeting polymorphic sequences in coding and non-coding regions of mt genomes to distinguish *Plasmodium* species, the non-specific amplification of other *Plasmodium* species or human DNA was observed for almost all assays. Different studies have demonstrated the use of the *Plasmodium* mt genome as a useful target for malaria genus-specific diagnoses using conventional PCR (8), nested PCR (15), and qPCR (22). However, only a few studies have demonstrated a species-specific malaria diagnosis, based on loop-mediated isothermal amplification (LAMP) (23), PCR (24), and qPCR (4, 25, 26). It is not clear in some published reports whether cross-reactivity between *Plasmodium* species or with human malaria-free samples was evaluated. Even though most studies have screened a large number of samples, they lack information about assay specificity, such as cross-reactivity tests and melting curve analyses for qPCR tests using DNA intercalating dye (4, 25–28).

Determining which Cq cutoff value discriminates between positive and negative amplifications should be based on the PCR efficiency of the assay. According to MIQE guidelines (The Minimum Information for Publication of Quantitative Real-Time PCR Experiments, a guideline that describes the minimum information necessary for evaluating qPCR experiments) (29), Cq values of >40 are uncertain because of the implied low efficiency and generally should not be reported; however, the use of arbitrary Cq cutoff values is not ideal, because they may be either too low (eliminating valid results) or too high (increasing false-positive results). In this study, to differentiate between specific and non-specific amplifications based on Cq values, a cutoff value was defined for each assay by applying the ROC curve analysis. The optimal value for the cutoff was determined as a specificity of ≥ 90%. Although the cutoff value determination was not able to completely exclude false-positive results for most assays, it was possible to select a cutoff value with a good specificity value for two of them (the PV_CYTB and PF_CYTB assays). Cytochrome b has been used for many authors as a target for genus *Plasmodium* or species-specific diagnoses, based on DNA intercalating dye or using probes (9, 30–32). Haanshuus et al. (22), comparing different quantitative PCR methods, showed similar sensitivity: the lowest was for a 18S rRNA protocol and the highest was for their *cytb* SYBR assay (22). The dilemma of cutoff value determination consists in a trade-off between sensitivity and specificity. The correct identification of *Plasmodium* species is of paramount importance and is one of the major challenges in malaria diagnosis. Misdiagnosis of the *Plasmodium* species, particularly in areas with transmission of

more than one *Plasmodium* species, such the Amazon region, may have a significant negative impact on the effectiveness of treatment and prognosis of the disease. On the other hand, a good sensitivity is also relevant to diagnose low parasitemia, preventing individuals from remaining untreated and at risk of greater disease severity. Moreover, in the context of malaria elimination, detection of asymptomatic carriers able to maintain the transmission is crucial (4).

To improve the sensitivity and specificity of the assays, a set of parameters was modified, such as time and temperature of the denaturing and annealing, the number of cycles, and the concentration of primers and probes in each assay. These modifications decreased the number of non-specific amplifications but did not eliminate all of them. A variety of PCR additives and enhancers was used to increase the specificity of PCR reactions such as DMSO, glycerol, and BSA. DMSO has been proven to considerably enhance both the specificity and the efficiency of DNA polymerization (21). Several polyhydroxyl alcohols are also potent PCR enhancers, such as glycerol, which improves PCR specificity (21). The addition of bovine serum albumin (BSA) to PCR reactions is often beneficial for its ability to scavenge and neutralize several contaminants that inhibit *Taq*, including hemin and iron chloride (21). They interfere with hydrogen bonding, thus facilitating strand separation, lowering DNA melting temperature (TM), and consequently improving the specificity of primer binding (21). The beneficial effects of additives are often template and primer specific and must be determined empirically. Herein, the PCR additives tested reduced but did not eliminate non-specific amplifications; besides, the use of these additives hampered the efficiency of specific amplifications.

We observed that *P. vivax* assays had the best sensitivity and specificity values when compared with *P. falciparum* assays. *P. falciparum* parasites have about 20 mt genomes per ring stage; however, with sequestered late stages, the gain in sensitivity from using a mt marker rather than nuclear markers is potentially limited in *P. falciparum* assays (9). On the other hand, *P. vivax* parasites have late stages present in peripheral blood with multiple replicating mt genomes; then, a substantial template multiplication factor can be expected. Thus, the gain in sensitivity from targeting the mt genome might be greater for *P. vivax* than for *P. falciparum* (4). This is of great importance for *P. vivax* infections, as this species generally presents lower levels of parasitemia due to its preferential invasion of the reticulocyte (33). Alternatively, other multi-copy genes could be used, particularly for *P. falciparum*, such as the varATS or pfr364 genes (14, 34, 35). This study has some limitations, particularly that a standardized mt target was not included for comparison with our new studied targets and the same samples panel was not tested in all assays.

5 Conclusion

The *Plasmodium* mt genome is an attractive target for PCR-based detection of malaria parasites. However, as *P. vivax* and *P. falciparum* have at least 90% of conservation mtDNA, the design of species-specific primers and probes is a challenge (36). The high degree of conservation of the *Plasmodium* mt genome may be due to structural constraints on the genome. Thus, a rigorous testing including the screening of a panel of well-characterized samples should be performed to verify the existence of non-specific amplifications in *Plasmodium* mt assays.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethical Committee of Research on Human Beings from the IRR (number 2.243.058) and INI-Fiocruz Ethical Board for patients from Atlantic Forest (number 0062.0.009.000–11). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Brazilian government (Ministry of Environment), which authorized the capture, handling, and collection and transport of biological samples from NHPs (SISBIO numbers. 43375-4/2015, 54707-137362-2 and 52472-1, and INEA license 012/2016012/2016). This study was approved by the Institutional Ethics Committee of Animal Use (CEUA license L037/2016).

Author contributions

CB, TS, DA, and GC conceived the idea and participated in the study design; DA, AA, JL, DP, AP-C, ZH, PB, CD-R, SM, and AP were responsible for sample collection. GC, DA, and GA extracted human and NHP DNA and performed the previous molecular diagnosis; GC and DA performed qPCR assays and data analysis; and CB, TS, DA, and GC wrote the manuscript. All authors contributed to the article and approved the submitted version.

Funding

The study was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Grant nos. 457274/2014-0, 310477/2017-4), the Secretaria de Vigilância em Saúde (SVS) of the Ministry of Health (Grant nos. IOC-017-FIO-17 and IOC-028-FIO-18, the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) (Grant no. CBB-APQ-02620-15, and Fiocruz Inova Grant for generation of Knowledgment (VPPPCB-007-FIO-18-2-11130) the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES; Finance code 001). Scholarships from CNPq (CB, TS, GC) and SVS/MS (DA) are also acknowledged. CD-R also receives a fellowship from the FAPERJ, as a "Cientista do Nosso

Estado". The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

The authors thank the PDTIS sequencing facilities of Fiocruz for use of the Real-Time PCR Facility (RPT09D) at René Rachou Institute, the team of the Primate Center of Rio de Janeiro (CPRJ/INEA), and Secretaria de Vigilância em Saúde (SVS) of the Ministry of Health.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The author CD-R declared that they were an editorial board member of Frontiers at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fitd.2023.1204195/full#supplementary-material

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frontiers | Frontiers in Public Health

TYPE Original Research
PUBLISHED 31 July 2023
DOI 10.3389/fpubh.2023.1133151

OPEN ACCESS

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RECEIVED 28 December 2022
ACCEPTED 07 July 2023
PUBLISHED 31 July 2023

CITATION
Dako-Gyeke P, Hornuvo R, Glozah FN, Asampong E, Tabong PT-N, Nwameme A, Chandi GM, Peprah NY, Gittelman D and Adongo PB (2023) Pilot implementation of community health advocacy teams to improve the effectiveness of long-lasting insecticide net distribution through both campaigns and continuous channels in Ghana: a qualitative study of opportunities and barriers to implementation.
Front. Public Health 11:1133151.
doi: 10.3389/fpubh.2023.1133151

Pilot implementation of community health advocacy teams to improve the effectiveness of long-lasting insecticide net distribution through both campaigns and continuous channels in Ghana: a qualitative study of opportunities and barriers to implementation

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Introduction: In Ghana, the National Malaria Elimination Programme (NMEP) distributes long-lasting insecticide net (LLIN) to households for free through the periodic point mass distribution (PMD) campaign and continuous distribution to populations most vulnerable to malaria. It is known that the existence of effective and functional community-based groups could influence positive behaviours regarding health interventions promoted through health campaigns. However, there is no evidence of functional community-based groups that aim to improve the effectiveness of LLIN distribution campaigns by transitioning into primary healthcare delivery. This study aimed to explore the opportunities and barriers to the pilot implementation of co-created community health advocacy teams (CHATs) to improve the effectiveness of LLIN distribution through both campaigns and continuous channels in Ghana.

Methods: A qualitative research approach was used among 43 CHAT members across six communities in the Eastern and Volta regions of Ghana. The CHAT constitutes significant community actors whose roles are centred on key elements of community/social mobilisation and capacity building, all nested in social and behaviour change communication (SBCC) strategies. The CHATs were pilot implemented in all study communities for 4 months after which we identified opportunities and barriers during implementation. CHAT members participated in six focus group discussions which were audio recorded, transcribed verbatim, and analysed thematically using the NVivo 13.

Results: CHATs were instrumental in sensitising community members through SBCC strategies. Moreover, there were changes in the behaviour of community

members who were receptive towards and participated in CHAT activities. Community members were accurately informed about malaria (e.g., causes and preventive measures). However, the CHAT experienced barriers during implementation, including a lack of financial support to aid in transportation, organisation of meetings, and outreach activities. Additionally, the level of participation by CHAT members in activities and the medium of communication among members were key areas of concern.

Conclusion: The CHATs would be instrumental in promoting LLINs' use during and after PMD campaigns through community outreaches. It is therefore necessary to provide resources to support their operations and a good network to address communication barriers. Finally, continuous capacity strengthening of CHAT members by the NMCP is important.

KEYWORDS

community health advocacy team, implementation research, intervention, long-lasting insecticide net, malaria, Ghana

1. Introduction

Malaria is a public health concern with nearly half of the world's population at risk of infection, and the major cause of morbidity and mortality in many resources constrained settings especially for children under 5 years (1). The World Health Organisation (WHO) has recommended long-lasting insecticide-treated nets (LLINs) as a core intervention in all malaria-endemic settings. The LLIN is estimated to reduce malaria cases by 50% (2). To reduce the global burden of malaria by 90% by 2030, WHO advises universal coverage with effective vector control utilising LLINs and indoor residual spraying (IRS) for all persons in malaria-endemic areas (1).

Malaria is a parasitic and infectious disease caused by *Plasmodium*. The parasite is transmitted through the bite of an infective female *Anopheles* mosquito during a blood meal from one person carrying the parasite to the other. The main vectors of malaria in the country are *Anopheles gambiae* complex and *Anopheles funestus* group. In Ghana, malaria is mainly caused by the *Plasmodium falciparum* parasite, which is responsible for >85% of malaria cases. The other malaria parasites are *Plasmodium malariae* and *Plasmodium ovale*. Because the malaria parasite is found in the red blood cells of an infected person, malaria can also be transmitted through blood transfusion, organ transplant, or the shared use of needles or syringes contaminated with blood. Malaria may also be transmitted from a mother to her unborn infant before or during delivery ("congenital" malaria). Malaria is hyper-endemic in Ghana with transmission occurring year-round, and the peak transmission occurring between June and October (rainy season).

The Ministry of Health (MoH) in Ghana oversees healthcare organisations in Ghana and this includes public, private, or traditional ownership in the country. The Ghana Health Service (under the MoH) is a public service body that provides and supervises public healthcare in the country. It has eight directorates that include the National Malaria Elimination Programme, regional and district health administration, and subdistrict health administration, which includes Health Centres and Community-based Health Planning and Services (CHPS).

The National Malaria Elimination Programme (NMEP) in Ghana is responsible for mass LLIN distribution campaigns by engaging and involving stakeholders at all levels (national, regional, district, sub-district, and community) (3). In accordance with Ghana's Malaria Strategic Plan (2021–2025), the mass LLIN distribution campaign seeks to protect at least 80% of the population at risk with effective malaria prevention interventions through household registration (90%) and distribution (90%) in target regions (4). Over the years, the NMCP together with its partners continues to scale up the LLIN ownership through point mass distribution (PMD). As part of efforts to achieve universal coverage of LLINs, continuous distribution of LLINs to the population most vulnerable to malaria (i.e., pregnant women, mothers of children under 5 years, and primary school children) is done through antenatal care clinics (ANC), child welfare clinics (CWC), and schools. From 2010 to 2012, there was a nationwide LLIN door-to-door mass distribution and the hang-up campaign, which was followed by another mass distribution campaign in 2018. Despite progress in overall LLIN ownership, the challenge remains to reach the NMCP strategic plan target of 80% usage among pregnant women and children under 5 years. Moreover, the 2019 Ghana Malaria Indicator Survey shows that 67% of Ghanaian households have access (percentage of the population that could sleep under an LLIN if each LLIN in the household were used by up to two people) to LLINs, but only 43% of Ghanaian household population slept under a net the night before the survey (3). This indicates that a relatively large number of people have not used the LLIN despite the distribution campaign. Although these campaigns have exposed a large proportion of Ghanaians to LLINs, they may not have led to desired health-related behaviours (i.e., sleeping in LLINs every day).

Various studies have documented barriers to LLIN use, which include inadequate distribution of nets per household, limited social and behaviour change communication (SBCC) activities to support distribution, lack of malaria education on the proper use of LLINs, and complaints of nets being distributed to communities with little or no information on their

relevance for malaria prevention (5–7). Furthermore, LLINs are not used following complaints of burning sensation or itching from sleeping under the net and inconvenience due to heat. At the community level, LLINs are sometimes inappropriately used for gardening/fencing, fishing, crop farming, and processing of farm produce (6, 7). The inability to hang LLINs due to housing type and sleeping places has been observed in other communities. Barriers that health workers experience include a lack of community mobilisation training, inadequate personnel, lack of follow-up, involvement, and supervision (8, 9).

To achieve national LLIN access and use targets, innovative social interventions that facilitate behaviour change may be needed both during and in follow-up to campaigns (10). Social innovation is described as a collaborative approach that generates ideas to improve community or hospital delivery systems (11). Social purpose emphasises engaging concerned communities within which innovative approaches fulfilling both social and health concerns will be distributed (11). Such community-based programmes allow the government, health agencies, social actors, and individuals to work closely with populations impacted by diseases, especially infectious conditions. The Community-based Health Planning and Services (CHPS) in Ghana is a national-level programme that aims to provide accessible, equitable, efficient, and high-quality healthcare (12). The CHPS programme is considered a pragmatic strategy for achieving universal health coverage of a basic package of essential primary health services. The CHPS concept involves the provision of door-to-door primary healthcare services to community members by trained nurses known as community health officers (CHOs) and has proven to be successful in providing maternal, reproductive, and child health services in communities where they are much needed (12–14). CHOs provide antenatal care, family planning, health education, outreach clinics for delivery of child welfare services, and school health services. Some community health workers (e.g., health volunteers and community health nurses) are involved in household registration and distribution of LLINs during the PMD campaigns, after which they are remunerated for their work. These community health workers may not necessarily be the ones mandated to engage in LLIN promotion and use both during and after campaigns.

In order to use a person-centred approach to promote LLIN use which leverages CHPS and ensures community involvement, ownership, and sustainability of the LLIN mass distribution campaigns, a community health advocacy team (CHAT) was co-created in six Ghanaian communities (15). The terms of reference of the CHAT are generally based on NMCP's key elements of the campaign at the sub-district level (e.g., household registration, training, SBCC, and logistics). Specifically, the CHAT members should be equipped with skills in community mapping; promoting correct LLIN use, maintenance, and repurposing; leadership and supervision, record-keeping, and interpersonal and persuasive communication. This study explores the opportunities and barriers to the pilot implementation of co-created CHAT in Ghana. The goal is to transition the community-level LLIN ownership and use promotional functions provided during the PMD Campaigns with ongoing LLIN promotion post-campaigns for continuous distribution under the Community Health Planning and Services (CHPS) programme.

2. Materials and methods

2.1. Study design

This study used a qualitative research approach to explore the opportunities and barriers experienced by CHAT members in a pilot implementation of the intervention. A total of six districts (one community per district) across two regions in southern Ghana participated in this study. These were communities in districts where the 2021 PMD campaigns of LLINs were ongoing. These communities were selected to avoid possible biases concerning community engagement (i.e., communities that are yet to be involved in registration and distribution activities for the 2021 PMD campaign) by ensuring that components align with the timelines of the National Malaria Elimination Programme (NMEP) and the funder. The study was also conducted in districts with the highest malaria prevalence as reported in the District Health Information System: Ho West (Tsito-−90%), Ho (Takla Hokpeta-−75%), and Agortime Ziope (Kpetoe-−100%) in the Volta Region; and Birim South (Apoli-−94%), Achiase (Achiase-−94%), and Abuakwa North (Kukurantumi-−93%) in the Eastern Region (Data source: DHIMS 2). At the time of the study, continuous/routine LLIN distributions in schools and antenatal care clinics were ongoing in these communities.

2.2. Population and sample

The study population comprised adult men and women from communities within the selected districts across two regions in southern Ghana (Eastern and Volta Region). The sample consisted of 43 members (18 women and 25 men) of the CHAT from six communities in the two regions (Figure 1).

2.2.1. The community health advocacy team

The community health advocacy team (CHAT) was co-created through the participatory learning in action technique using participatory workshops (PWs) which is a practical approach. This approach involves adaptive research strategies that enabled diverse groups and individuals to learn, work, and act together in a co-operative manner, to focus on issues of joint concern, identify challenges, and generate positive responses in a collaborative and democratic manner. This was done by using the findings from the initial phases of the project (i.e., desk review, focus group discussions (FGDs), key informant interviews (KIIs), and baseline surveys) (15). The participatory workshops involved various stakeholders (i.e., project investigators, NGO representatives, school health education programme coordinators, ANC nurses, disease control officers, district health management teams (DHMTs), CHOs, community leaders, and opinion leaders). Findings from the PWs suggested the establishment of a CHAT can be instrumental in facilitating and improving the effectiveness of LLIN distribution campaigns within communities in Ghana.

A CHAT consists of nine members who are influential in their communities: health officers, religious leaders, school health education programme coordinators, assemblymen/women, community information officers, representatives from any of

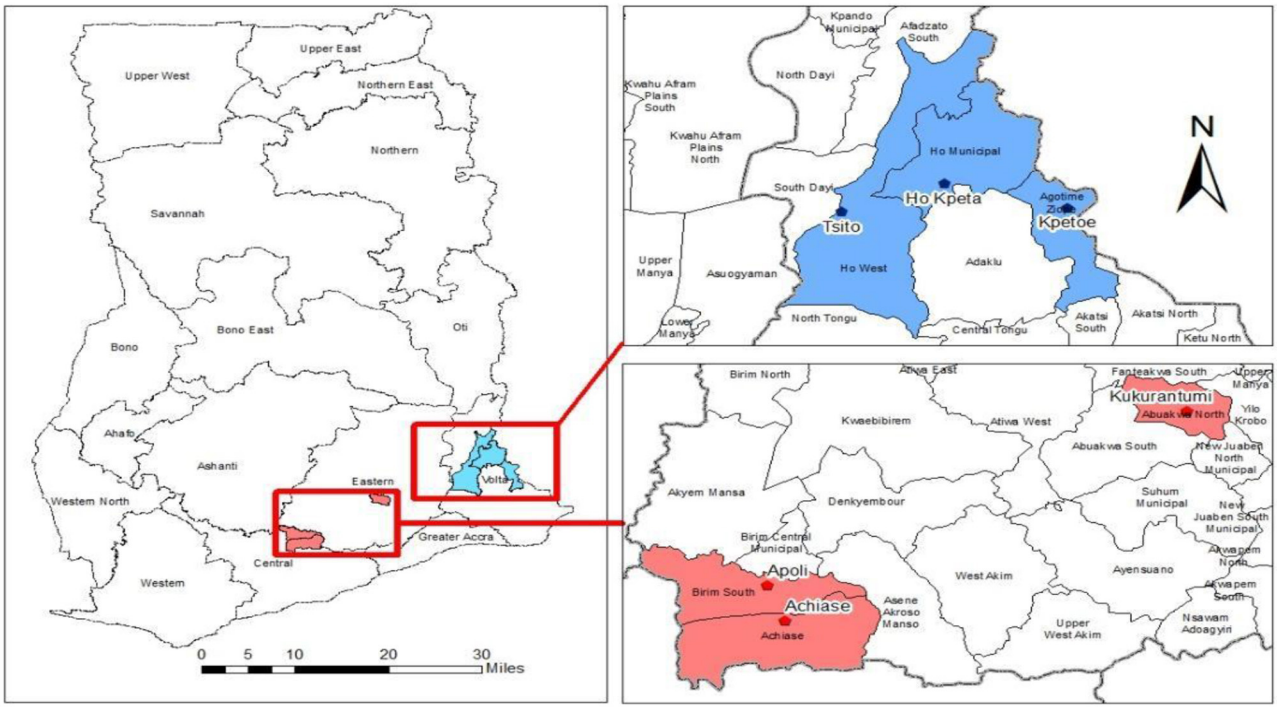

FIGURE 1
Location of the six districts in the Eastern and Volta Regions of Ghana.

the security services, community-based organisations, and traditional authorities.

The CHAT members were trained by officials from the NMCP and project investigators as part of their capacity-strengthening efforts. They were trained in key elements of the NMCP's campaign (i.e., training, registration, SBCC, logistics, distribution, and supervision) and skill-enhancing strategies in leadership, communication, and community mapping, as well as record-keeping competencies. This training provided CHAT members with the capacity to carry out malaria education and prevention activities as well as the promotion of net use within communities and primary healthcare levels during and after LLIN campaigns. Specifically, CHAT members are expected to support PMD for LLIN campaigns at the community level, as well as provide support on the continuous distribution of LLINs through the school-based, antenatal, and child welfare clinics at the community level, development of context-based social and behavioural change communication (SBCC) strategies on malaria prevention and regular use of LLINs, sensitise the community on the proper use of LLINs and its maintenance, support with the management of LLINs logistics and accountability, and support other community-based health campaigns. All stakeholders agreed that the CHAT would meet quarterly to discuss implementation progress and re-strategizing as needed.

After successful training of the CHAT, a total of six community health advocacy teams, one in each of the six districts, were inaugurated and out-doored (an introduction of CHAT to community members for the first time) at an organised community durbar (an outdoor community gathering, where members of the community are present to discuss issues of community importance). These community durbars included traditional leaders, community members, religious leaders, opinion leaders, representatives from the Ghana Health Service (Regional Deputy Director of Public Health (DDPH), District Director(s), regional and district malaria focal persons, CHAT members, project investigators, and representatives from the NMCP) in the six study sites.

2.2.2. Long-lasting insecticidal net

The NMEP is responsible for reducing malaria morbidity and mortality in Ghana and has, over the years, carried out several malaria prevention interventions such as PMD of LLINs (Figure 2). The distribution and the use of LLINs are core interventions for preventing malaria infection in malaria-endemic countries, including Ghana. LLINs provide protection against mosquito bites, repel, and kill mosquitoes, thereby reducing the transmission of malaria parasites and decreasing malaria risk at the individual and community levels when high coverage is achieved (Figures 3, 4). Mosquito nets can be obtained mainly during PMD campaigns; however, as part of targeted continuous distribution programmes, LLINs are distributed through antenatal care (ANC), child welfare clinics (CWC), and primary schools. LLIN can be used for up to 3 years or after 20 washes.

FIGURE 2
School children receiving treated mosquito net in Ghana. Source: Malaria consortium.

FIGURE 3
Mother and child sleeping under treated mosquito net. Source: WHO Africa.

2.3. Data collection

A total of six focus group discussions (FGDs) were organised to explore the opportunities and barriers of the CHAT intervention after four months of pilot implementation in all six study communities. Each FGD included members of the CHAT, with a total of 43 participants in all six FGDs. Some CHAT members were playing key roles at a traditional function during the time of data collection and hence could not participate. All participants were contacted with the assistance of the conveners of the team and an arrangement was made for the FGDs to be conducted. The FGDs were conducted by trained qualitative research assistants using a designed implementation stage FGD guide within a relaxed and convenient atmosphere while observing all COVID-19 protocols. The interviews were conducted by experienced research assistants who have been trained in public health and with several years of conducting qualitative research and interviews. Saturation was achieved during interviewing as similar themes emerged repeatedly in the course of the interviews. Informed consent was sought from all participants and FGDs were audio-recorded. Each FGD lasted approximately one hour.

2.4. Data analysis

All audio-recorded FGDs were transcribed verbatim and augmented with researchers' field notes made through observations and during FGDs. A codebook was developed based on the research objectives. The codebook development involved qualitative experts from the project team who reviewed the various components of the codes to ensure they aligned with the datasets.

FIGURE 4
Demonstration of LLIN use, care, and maintenance. Source: Ghana Health Service.

The data resulting from transcriptions were evaluated, coded, and analysed using the thematic analysis method, employing both deductive and inductive processes as described by Braun and Clarke (16). The data were analysed thematically and managed using the NVivo software version 13. The initially developed codebook was revised throughout the coding process to include emerging codes. The consolidated criteria for reporting qualitative studies (COREQ): 32-item checklist was used to guide the process.

2.5. Ethical consideration

Ethical clearance was obtained from the Ghana Health Service Ethics Review Committee (GHS-ERC: 002/06/21) before the commencement of all data collection. All research assistants received specific training before data collection as per the study's training protocol.

Before beginning, all study participants provided written informed consent after reviewing the study aim, procedures and benefits, and their rights as participants. The information and consent documents for participants were written in simple English. However, for better comprehension, research assistants were present during the informed consent process to explain any questions that the participants do not understand. Those consenting to participate either signed or placed a thumbprint on an informed consent form. All participants were assured that the information they provided would be handled confidentially and research findings would be reported with complete anonymity.

3. Results

The findings of the study are presented under the following headings: sociodemographic characteristics of participants, CHAT implementation opportunities [increase LLIN use and malaria prevention (malaria control interventions) through CHAT educational and SBCC activities, community participation in CHAT activities], and CHAT implementation barriers.

3.1. Sociodemographic characteristics of participants

A total of 43 participants comprising 18 women and 25 men, aged between 23 and 73 years, were involved in the focus group discussions. Participants were the CHAT members from the selected districts in the Eastern Region (Kukurantumi, Achiase, and Apoli) and Volta Region (HoKpeta, Tsito, and Kpetoe) of Ghana. Table 1 presents the socio-economic characteristics of the participants (*NB: R = Respondent/Participant*).

3.2. CHAT implementation opportunities

CHAT implementation opportunities were assessed both during the campaign and afterwards in continuous distribution mode. These opportunities include sensitisation on the use of LLIN and malaria prevention, as well as community participation in CHAT activities were explored.

TABLE 1 Sociodemographic characteristics of participants.

Characteristic of participants	Number of participants
Region	
Eastern Region	27
Volta Region	16
Total	**43**
Sex	
Female	18
Male	25
Total	**43**
Age	
20–29 years	4
30–39 years	17
40–49 years	8
50+ years	14
Total	**43**
Educational Level	
Primary	2
JHS/Secondary/Middle School	10
Tertiary	31
Total	**43**
Marital Status	
Single	15
Married	28
Divorced/Widowed/Separated	
Total	**43**

3.2.1. Sensitisation on the use of LLIN and malaria prevention

Findings from the study revealed that the CHAT was able to promote LLINs use and sensitise community members on malaria prevention strategies during and after the 2021 mass LLIN distribution campaign. The CHAT members provided education on malaria prevention to various target groups such as mothers of children under five years, primary school children, and household members. For instance, the CHAT provided mothers of children under 5 years, at the CHPS level, with education on the proper use of LLINs and malaria prevention:

> *"We organised and have Child Welfare Clinic (CWC) every time, there we demonstrated to the mothers how to use the net. Because we got to know[sic] most of the kids are coming to the hospital with malaria, so[sic] we saw that the malaria cases are still going high with the kids now. It's[sic] no more with the adults much like that. So, we came to demonstrate to them how the mosquito net is being used." (R5, Tsito, Volta Region)*

> *"CWC. We all do that because the volunteer also helps in the organisation of the people. We entreat the pregnant woman to sleep under the mosquito net. We talk to them about the causes of malaria and that sleeping under the treated net will help prevent malaria." (R3, HoKpeta, Volta Region)*

In addition, the CHAT also engaged school children and community members during religious gatherings, and this appears to be a good platform because school children are likely to spread information from the school to members of their households. Moreover, as Ghanaian communities are largely religious, it is a good platform to communicate health messages. For instance, some participants highlighted that

> *"For the school, I talk to the kids about mosquito nets and I ask them if their parents are using them and their response is always yes. I think they were given just last year so according to them, they are using it. So far, so good." (R3, Tsito, Volta Region)*

> *So far, we have been to schools to give education about the prevention of malaria." (R2, Kukurantumi, Eastern Region)*

> *"After our inauguration, it was almost in the festive season, that is Christmas, and the New Year was approaching, so we decided to take that opportunity to meet the large crowd" [at various churches]. (R5, Apoli, Eastern Region)*

> *"We educate them. When we go to church after we close, we also remind them on the use of the mosquito net and their children under 5 years should also sleep under and also after worship on Wednesdays." (R4, HoKpeta, Volta Region)*

Home visitation is another effective way to communicate health messages to achieve desired behaviour changes. The findings further revealed that the CHAT engaged community members on a one-on-one basis during home visits to educate community members on the need to use the mosquito nets, so as to achieve the intended purpose:

> *"As a community volunteer, when I am walking within the community and see they are using the net for the wrong purpose, I talk to them to use it the right way, sleep under it in order to prevent malaria." (R2, HoKpeta, Volta Region)*

The study also showed that CHAT members employed various SBCC channels in delivering malaria messages to community members. Mediums, such as the community information centres (CIC) and banners, were mentioned. For instance, some participants mentioned that

> *"We use the CIC, the local information centres. We have radio stations too-[sic] we engage them and durbars as well. Any gathering we get, we chip in and say something as well." (R4, Kukurantumi, Eastern Region)*

> *"When we go, we educate with the SBCC materials as we said; our posters are showed[sic] to them as they see and they remember." (R6, Apoli, Eastern Region)*

> *"With CIC Representative, you know he is into the broadcasting and as a member, he took that one up and you know we don't pay again to him and it's the service to the community. So, every Wednesday, they do come for the early morning program. That is at 5:30 so by then people will wake up and listen to worship and after that, they come in mostly about the mosquito net issue and the prevention of malaria... every Wednesday. Every Wednesday they do it unless maybe if[sic] when there is no resource person on Wednesday." (R1, Tsito, Volta Region)*

Use of social media platforms could have been a faster way of promoting malaria prevention messages. However, some participants indicated that poor network access prevented use of social media in some communities.

> *"You see, there is no network here, so those who live here will have difficulty in downloading and watching the video but those of us in Ho can easily view it." (R3, Hokpeta, Volta Region)*

3.2.2. Community participation in CHAT activities

The findings also revealed that there have been perceived changes within the various communities concerning malaria prevention and LLIN use that can be attributed to the malaria education activities that the CHAT has been engaged in. For instance, some CHAT members mentioned that there has been a perceived reduction in malaria cases within the communities:

> *"In fact, in my place, it has been a long time now that I have heard of malaria. Even in my room, my children are using it, so that one is out. So, as we said, before we can see the actual result, it will be the statistics. But for me, we believe that even when you ask people. It's just the young ones who are reluctant in using it. But older adults and women are using it." (**R1,** Tsito, Volta Region)*

In addition, there has been a positive reception from community members towards CHAT education activities on malaria prevention:

> *"In schools, knowing the presence of this group, they are paying more attention. The misuse of the nets is not good for both teachers and pupils. Also, we told them that there is a committee, like the security personnel, to cheque on the misuse of the net…As we went to some school[sic, the children came asking individually; if the net is torn and if it is used otherwise, they also will be caught?[sic] The madam continued that they will be interrogated about when they were given and the number of times it has been used that will show whether it is due to be thrown away or not. So, they are now serious about the dump of the nets." (R3, Achiase, Eastern Region)*

> *"When we go for gathering and we announce that we are coming and you see lots of the community members seated and during funerals and the outreach we had too, they were very attentive and will do what we say." (R2, Kpetoe, Volta Region)*

Active participation in CHAT activities may have helped change in behaviour among community members, as they observed improved understanding of malaria prevention and LLIN use.

> *"The education we have been doing has been valued because when we visit the back of some houses, there is now neatness. Those we educated are now aware and now everyone is tidying their surroundings up because dirt brings malaria so what we have been educating has been valued and the elders have seen it will help and their expenses will be cut short because a single mosquito bite can cause serious loss of income before you get healed[sic]. So, if the mosquito net can solve that problem, anytime, everyone should try and use the mosquito net. Malaria is reducing and we hope that it won't be long for it to be curbed." (R2, Achiase Eastern Region)*

> *"Yes, we have got many moments as we visit churches and gatherings where we have our health talk, misunderstandings about the[sic] malaria are being discussed as to the cause of malaria as some are misinformed that malaria is from the sun." (R5, Apoli, Eastern Region)*

> *"Some also said that malaria is brought by unripe mangoes so some people with these misconceptions, we speak to them to inform them that it is mosquito bites that cause it." (R8, Apoli, Eastern Region)*

> *"What we can say is, OPD cases attendance has increased. So, with that, we can justify by saying that, maybe because of the sensitisation going on home management and those things are reducing and they are now visiting the facilities to seek treatment." (R6, Kpetoe, Volta Region)*

3.3. CHAT implementation barriers

Some barriers that the CHAT faced within the four months after their inauguration were discussed during the discussions. For instance, some members said that the medium of communication and active participation by members at CHAT meetings to plan and strategise activities have been a major challenge:

> *"We were using the WhatsApp page, and some people may not even have the data and even at Takla, the network is not all that good so we have to be calling everyone. So, it is very difficult." (R2, HoKpeta, Volta Region)*

> *"Also, like the heads, every position has its duty to undertake like 'Madam A', is part of us (CHAT). Like for the Friday[sic] she didn't go to the farm. Saturdays, there are funerals, and on Sundays people go to church. She was supposed to go to the farm yesterday but there was another meeting she was supposed to attend so she wasn't able to [sic] join us (CHAT meeting). And for me too, sometimes I have other tasks to take so I have to stop (the CHAT meeting) and attend the other meeting. So, these are the challenges we are facing." (R2, Apoli, Eastern Region)*

> *"Yes, when you call for a meeting, not even a meeting, when you post something on the page, for people to respond to it, it becomes a problem. It is as a result [of lack] of motivation; that the motivation level has fallen. So, people feel like, I need to go somewhere to make (some money) than to have attention here so that is it...So I think being frank with you[sic], it will help all of us." (R3, Kpetoe, Volta Region)*

Moreover, the need for some form of support in terms of transportation to undertake outreach activities, especially in hard-to-reach communities was mentioned by members.

> *"Yes, going for programmes, there are some costs that come with it such as the transportation and maybe feeding. All these are on the individuals and sometimes, the Madam will have to come in by buying water for the people. So, these are barriers and we need to fix them." (R3, Kpetoe, Volta Region)*

> *"The main challenge now is our means of transport unless like we were going round, I was the one who bought petrol to all the six churches to the other places. Also, in other places, we let them meet in a congregation, then I pick them up. Currently, the earth has also been challenging where you take someone out for hours and you don't give them [sic]chance at their work, it feels bad though this is just the beginning of the team but after that, everyone can return to their workplaces after success. So, our challenge is as we are planning to visit another town like Aprade and other places, we need means for[sic] transport to support as[sic] like getting us petrol so we don't charge anything- we can just go and come back." (R4, Achiase, Eastern Region)*

> *"…and like places like Yaw Agbo, going there, we have to take the motorbike and most of us don't have the motorbike, so unless we hire and that also sometimes it's hard to." (R3, Apoli, Eastern Region)*

> *"Like today as we were going to the school, we paid the fare of the transport. The last time we went, we were supposed to go in a group but already some were members so we had to pay for the fare." (R6, Kukurantumi, Eastern Region)*

Similarly, participants expressed the need for the provision of refreshment during outreach activities as most of the outreach activities take long hours and they need some water or food during these outreaches.

> *"There is no addition really, but if you can be supporting us small, small- at least water will do." (R4, Hokepta, Volta Region)*

> *"… and probably if we get something small like lunch or minerals, though they are adults but they know what they are doing so they are not demanding anything. So, they need something small to support them like small snacks like biscuits, that is our challenge." (R4, Achiase, Eastern Region)*

> *"Also, we would be needing water and snacks so that inclusive will help because some from morning do not eat when going for education in the other towns. So at least, water should be provided." (R7, Achiase, Eastern Region)*

The need for some financial support in carrying out outreach activities was also mentioned.

> *"Our main problem is the financial aspect where resources are needed like inviting professional resource personnel to come educate us, we need money to invite them too. Sometimes we volunteer and take on such tasks…we also plan to involve other members like the midwives and some doctors so they can join in the education. But afterwards, we have to pay them, which might turn into another challenge." (R2, Kukurantumi, Eastern Region)*

In addition, some CHAT members reported the need for further instruction on transmitting messages to communities on malaria prevention and correcting LLIN misuse. Notably, some indicated that

> *"I once met a lady like that, upon probing, she said, they are very old mosquito nets that are over 7 years old and are worn out and can't be used, that is why, she used them for gardening/fencing. And I couldn't say anything again because 7 years is a long time. So such people too, what do we do to them[sic]?" (R3, Hokpeta, Volta Region)*

> *"I will say we need some motivation (e.g., capacity building) in relation to the net distribution. Some people complain about the net. Let's say they are five (5) in the house and maybe only two (2) were able to get the nets so the rest explain to them that okay maybe there was distribution in the various schools so when*

> *the child gets it in the school, they can use it at home. So, we need to know it and explain [sic]to them so that every household can get it." (R1, Kpetoe, Volta Region)*

4. Discussion

This study explored the opportunities (LLINs use and malaria prevention strategies, community participation in CHAT activities) and implementation barriers to the pilot implementation of the co-created CHAT. These teams seek to integrate the LLIN distribution campaigns with the CHPS programme to promote community LLIN ownership and use both during and beyond campaigns in Ghana. This study has yielded important findings that will help provide the CHATs with the necessary support to effectively perform their roles and address barriers. The findings can also inform further scaling-up of the CHATs across Ghana.

The study showed that CHAT members engaged different groups of the population during their malaria prevention sensitisation. The CHAT members employed SBCC strategies to educate them on the continuous use of the treated mosquito nets. As evidence suggests, the use of SBCC can improve malaria prevention and treatment behaviours (10, 17). The employment of various mediums (e.g., community information centres) and platforms (e.g., child welfare clinics, schools, religious gatherings, and door-to-door visits) serves as a means of reaching a larger population with malaria prevention messages which will help contribute to achieving sustainable outputs and impacts concerning malaria control in Ghana (18–21).

Community reception is key to achieving desired health behaviours (20). The study revealed that community members were receptive to the CHAT members whenever they were carrying out malaria sensitisation activities. Moreover, community members became better informed about the causes of malaria, malaria prevention strategies, as well as the use of LLINs for the intended purpose. Community members were also taught how to properly hang the nets regardless of housing style or sleeping places. Possible positive changes in community behaviour are very important as they address some barriers to LLIN use that have been realised in earlier studies such as limited use of SBCC activities, lack of continuous malaria education (5, 22–24), knowledge gap at the community level on malaria prevention, inability to hang LLINs in many household types and sleeping places, and the misuse of nets (6, 7). Access to accurate information about malaria can promote increased use of LLINs and reduce the gap between LLIN access to and use eventually contributing to the reduction in malaria morbidity and mortality.

Although the pilot implementation of the CHAT has achieved many successes, it encountered several barriers. Most of these barriers revolved around financial support in carrying out voluntary activities. Although the CHAT has been set up as a voluntary team without any remuneration, there were situations where they needed financial resources to carry out LLIN and malaria sensitisation activities. Some of these activities include transportation to neighbouring communities or hard-to-reach areas of communities as well as providing refreshments after community outreach activities, as they usually spend long hours during these outreaches. These findings are consistent with other studies conducted in other parts of Africa, where a lack of financial support is reported to impede the delivery and sustainability of health volunteer work (25–27).

5. Limitation

Although this exploratory study of the opportunities and barriers in the pilot implementation of the CHAT provides some useful lessons, the four-month duration of the pilot implementation is relatively short to unravel all possible lessons. There is a need for further scale-up beyond the six pilot districts in Ghana to assess its effectiveness and impact with respect to LLINs promotion and use. Moreover, due to the seasonality of malaria transmission in Ghana (i.e., during rainy seasons—April to June and September to November), the season within which the CHAT was implemented could also influence the opportunities and barriers realised; hence, there is the need for long-term implementation of the CHAT to effectively observe the seasonal variations of opportunities and barriers of CHAT in promoting LLIN use and malaria prevention.

6. Conclusion

The community health advocacy teams have a great promise to sustain community LLIN promotion activities both during PMD campaigns and afterwards in relation to the primary healthcare system in Ghana. In order for this to happen, there is the need to address barriers to the effective functioning of the CHATs, including the provision of financial support to aid transportation, the provision of refreshments and support for their outreach activities, the provision of financial motivation to increase their level of participation in CHAT activities, and the provision of good network access to address their communication barriers. Finally, CHAT members require continuous capacity building, especially in use of SBCC to promote LLIN access and use, to most effectively support the PMD and to transition LLIN distribution to routine or continuous channels through CHPS.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ghana Health Service Ethics Review Committee. The patients/participants provided their written informed consent to participate in this study.

Author contributions

PD-G, FG, EA, PT, and AN were in charge of conceptualisation, data curation, formal analysis, methodology, and original draft. RH supported with data analysis, methodology, and drafted the

manuscript. PA and DG reviewed and revised the final draft. All authors participated in designing the study with NP and GC providing technical support. All authors contributed to writing the manuscript and approved the final draft.

Funding

This work was supported, in whole or in part, by the Bill and Melinda Gates Foundation (Grant Number INV-01076 to the Task Force for Global Health's Health Campaign Effectiveness Program). Under the grant conditions of the foundation, a Creative Commons Attribution 4.0 Generic License has already been assigned to the author accepted manuscript version that might arise from this submission.

Acknowledgments

We would like to thank all stakeholders: members and staff from the Ghana Health Service (GHS) and the National Malaria Elimination Programme (NMEP), the Volta and Eastern Regional Directors of Health Services, the District Health Directors of Ho West, Ho, Agortime Ziope, Birim South, Achiase, and Abuakwa North districts, CHAT members, as well as community members, who committed time to share experiences and provide data for this study. They are also grateful to the field staff for their meticulous work during data collection.

Conflict of interest

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

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frontiers | Frontiers in Tropical Diseases

TYPE Mini Review
PUBLISHED 14 August 2023
DOI 10.3389/fitd.2023.1229735

OPEN ACCESS

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RECEIVED 26 May 2023
ACCEPTED 12 July 2023
PUBLISHED 14 August 2023

CITATION
Berhe AD, Doritchamou JYA and Duffy PE (2023) Malaria in pregnancy: adverse pregnancy outcomes and the future of prevention.
Front. Trop. Dis 4:1229735.
doi: 10.3389/fitd.2023.1229735

Malaria in pregnancy: adverse pregnancy outcomes and the future of prevention

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Malaria in pregnancy (MiP) poses a dangerous health risk to both mothers and their fetuses, causing severe outcomes such as preterm delivery, intrauterine growth restriction, miscarriage, stillbirth, and neonatal and maternal death. *Plasmodium falciparum* infected erythrocytes sequester in placental intervillous spaces causing placental malaria (PM), eliciting inflammatory responses associated with severe sequelae. Current MiP prevention strategies have improved pregnancy outcomes, but serious morbidity and mortality persist. Vaccines to prevent MiP and PM are under development and are expected to improve pregnancy outcomes. To prepare for safety and efficacy trials of these vaccines, the incidence of adverse pregnancy outcomes including those caused by MiP should be documented at clinical sites. This review summarizes reported key adverse pregnancy outcomes attributable to MiP, providing important baseline context to define measurable safety and efficacy endpoints for malaria vaccine trials in pregnancy.

KEYWORDS

malaria, pregnancy, vaccine, perinatal death, pre-term birth (PTB), miscarriage, stillbirth, low birth weight (LBW)

Introduction

Despite progress in reducing malaria globally, malaria in pregnancy (MiP) remains a pervasive issue in endemic areas. According to the World Health Organization (WHO), malaria infection occurred in 32% of pregnancies from 38 moderate-to-high transmission African countries in 2021 (1). Exposure to malaria during pregnancy, even in women with pre-existing immunity, poses risks to both the mother and her fetus, as infection increases the risk of maternal death, severe maternal anemia, and fetal and neonatal death (2, 3). While pregnant women and children under age 5 are most vulnerable to severe malaria complications, poor pregnancy outcomes are often missed as malaria-related events, and generally not included in annual malaria burden reports such as malaria-related infant mortality estimates (4). Further, it has been demonstrated that malaria-endemic regions, such as countries in sub-Saharan Africa and South Asia, have pregnancies with the highest

risk of death for the newborn but the lowest availability of data on adverse birth outcomes. This lack of data, particularly lack of national administrative data, poses difficulties in establishing baseline rates of adverse outcomes in regions where interventions are needed most (5). Current efforts to develop vaccines that prevent MiP will benefit from defined and measurable safety and efficacy endpoints including malaria-related adverse birth outcomes, such as embryonic, fetal, and neonatal deaths occurring from conception to the weeks following birth. In this review, we briefly review the pathogenesis of placental malaria and existing tools for prevention, and then focus on MiP-related pregnancy outcomes including miscarriage, stillbirth, perinatal death, preterm birth (PTB), and low birth weight (LBW) (Figure 1) and discuss their confounding or contributing factors, as a context to plan for future MiP vaccine trials.

Pathology of placental malaria

While adult residents of malaria-endemic regions typically enjoy immunity acquired over frequent exposures to malaria parasites, malaria poses a new risk to pregnant women. Women are particularly susceptible to malaria in their first pregnancy due to placental sequestration of *Plasmodium falciparum* infected erythrocytes. Parasites with binding affinity to the placental receptor chondroitin sulfate A (CSA) are responsible for this placental sequestration, defining a placental malaria (PM) syndrome (6). Nulligravidae lack immunity to CSA-binding parasites, but malaria-exposed women acquire protective antibodies to PM over successive pregnancies. These protective antibodies, including antibodies against the variant surface protein VAR2CSA, block parasite adhesion to CSA in the placenta (7). VAR2CSA is a distinctly structured member of the PfEMP1 family of variant surface antigens and mediates parasite adhesion to CSA in the placenta (8). The sequestration of parasite-infected erythrocytes within the intervillous spaces of the placenta elicits an inflammatory infiltrate and placental pathology that are associated with poor pregnancy outcomes.

Placental parasitemia may contribute to these adverse outcomes in a number of ways. Healthy pregnancies are characterized by immunomodulatory phenomena and a predominant Th2 immunity, while inflammatory responses to PM including Th1 cytokines can alter this balance and contribute to sequelae (9, 10). For example, increased inflammatory responses in the *P. falciparum* infected placenta can lead to oxidative stress and apoptosis of placental cells (11). This stress on the placenta can have deleterious effects on embryo development and parturition. Placental structure can also be compromised, wherein women with MiP display decreased transport villi, increased placental lesions, and syncytial knotting (12, 13). Further, a Doppler ultrasound study in Kenya demonstrates that *P. falciparum* infection is associated with abnormal uterine artery blood flow (14)

Current strategies to prevent malaria in pregnancy

Diagnosis of placental malaria

Diagnosing malaria early during infection is important to prevent disease sequelae but is not easily accomplished. Many women carry parasitemia with few or no symptoms, owing to systemic immunity acquired during a lifetime of exposure. The microscopic detection of parasites on blood smears is the most widely used method to detect placental malaria due to long-standing clinical practice. However, parasites are often not detected on a peripheral blood smear, even when relatively large numbers of parasites are sequestered in the placenta (15). Thus, an individual with placental malaria may produce negative test results while infected, leaving them undiagnosed and untreated. For pregnant patients, diagnosis with rapid diagnostic tests (RDTs) serves as an alternative. This immunochromatographic antigen test reports sensitivities above 90%, making it potentially more effective than microscopy or clinical systems of detection (16). In addition, polymerase chain reaction (PCR) techniques have significantly helped to unravel the true burden of infection in pregnancy by detecting sub-microscopic infections that also contribute to poor pregnancy outcomes (17, 18).

Vector control strategies

Vector control strategies for MiP target the *Anopheles* mosquito to prevent transmission of *Plasmodium* parasites to pregnant women. WHO currently recommends deployment of insecticide-treated nets (ITNs) for pregnant women in endemic regions (19).

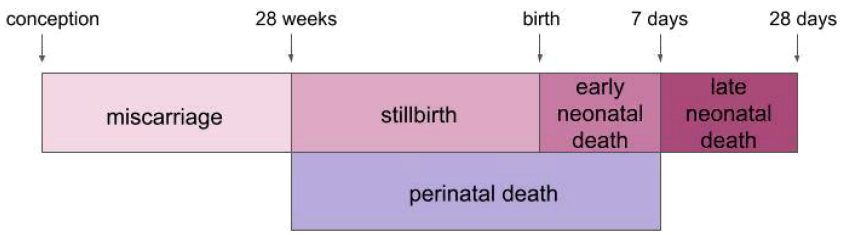

FIGURE 1
Timeline of adverse outcomes throughout pregnancy. Miscarriage, stillbirth, perinatal death, early neonatal death and late neonatal death are represented according to their timing during or after pregnancy.

Indoor residual spraying (IRS) is typically recommended in malaria-endemic regions but raises safety concerns, as insecticide residues have been found in breast milk and prenatal exposure to insecticides may impact human neurodevelopment (20, 21).

Genetic modification of mosquitoes provides options for more permanent vector control, through sex-specific sterilization, species replacement with genetically modified mosquitoes resistant to parasite infection, and gene drive approaches to spread disadvantageous genetic traits throughout mosquito populations (22). While potentially powerful, gene-based mosquito control strategies for malaria have not been widely implemented, due to many factors including uncertainty of ecological impact, genetic variance among *Anopheles* species, and difficulty of wide-scale implementation.

Intermittent preventive treatment in pregnancy

In addition to vector control strategies, WHO strongly recommends intermittent preventive treatment in pregnancy (IPTp) with sulfadoxine-pyrimethamine (IPTp-SP) in areas with moderate-to-high malaria transmission (23). IPTp-SP is administered to pregnant women up to once a month after the first trimester. While proven to significantly reduce several malaria-related adverse outcomes, IPTp is not recommended in the first trimester due to teratogenicity concerns, leaving early pregnancies at risk of malaria infection (24, 25). Alternatives to SP have also been explored in studies that investigated mefloquine (26) or dihydroartemisinin-piperaquine in both HIV-negative and -positive women (27, 28). The challenge for malaria control in early pregnancy is compounded by the difficulty in reaching women during this period, as women in endemic regions typically consult for antenatal care at mid-pregnancy or later (29). For instance, attendance to antenatal clinics in the first trimester ranges from 12% in Malawi to 15% in Kenya (30). Of note, parasites infecting women in the first trimester of pregnancy can display a placenta-binding phenotype. These early infections have been associated with adverse outcomes such as LBW (31–33), highlighting the importance of new tools like vaccines that can provide durable protection from before conception through the postpartum period.

Recommendations for antimalarials in the first trimester have been stymied due to concern of these drugs causing birth complications. WHO recommends quinine therapy for uncomplicated *P. falciparum* malaria in the first trimester despite ample evidence of superior efficacy by artemisinin-containing combination therapies (ACTs) (34). In animal models, artemisinin derivatives have been shown to have teratogenic and embryotoxic effects in early pregnancy (35). However, these toxic effects have not translated to discernible toxicity in humans as a meta-analysis of twenty studies found that treatment with artemisinin during pregnancy was not associated with miscarriage (35). Another meta-analysis of 30,618 pregnancies found no difference in the risk of miscarriage when comparing the use of artemisinin and quinine during the first trimester (36). Considering the ample safety evidence, it may be advantageous to regularly implement ACTs to combat adverse pregnancy outcomes such as miscarriage until vaccines for MiP are available.

Development of vaccines against malaria in pregnancy

Vaccine candidates in clinical trials

Women in endemic areas naturally acquire resistance to PM over successive pregnancies, and this is associated with acquisition of functional antibodies against placenta-binding parasites and the surface antigen VAR2CSA (37). High levels of antibodies against VAR2CSA have been associated with improved pregnancy outcomes, and VAR2CSA is the leading candidate for a placental malaria vaccine (38). PAMVAC and PRIMVAC are two VAR2CSA-based vaccine candidates tested in humans that have demonstrated a good safety profile and induced functional variant-specific but not variant-transcending antibodies (39, 40). More work must be done to develop a placental malaria vaccine effective against a wide range of VAR2CSA-expressing parasite variants, a major challenge given the extensive sequence variation of the protein (41).

While not specifically designed to prevent placental malaria, *P. falciparum* sporozoite (PfSPZ) Vaccine is an attenuated whole malaria sporozoite vaccine entering the early stages of development in pregnant women. In clinical trials with non-pregnant African adults, PfSPZ Vaccine has been remarkably well-tolerated and showed significant efficacy against *P. falciparum* infection (42, 43). Thus, the protective efficacy of PfSPZ Vaccine in preventing malaria infection warrants further consideration for trials in pregnant women (44). RTS,S/AS01 and R21/Matrix M™ are recombinant subunit virus-like-particle vaccines that target the same sporozoite surface protein (circumsporozoite protein, CSP) and prevent clinical malaria in African children. These are the only licensed malaria vaccines to-date; RTS,S (45) was recommended by WHO in October 2021 (46), and R21 was first approved for use in Ghana (47), then Nigeria (48), each with the indication to prevent clinical malaria in children aged 5 months to 3 years. These vaccines might also be assessed for benefits against pregnancy malaria. While less explored for MiP, monoclonal antibodies against malaria present another potential intervention. In phase 2 clinical trial, monoclonal antibody CIS43LS has demonstrated protection against *P. falciparum* infection of Malian adults (49).

Safety and efficacy endpoints for trials in pregnant women

Contemporary information on adverse pregnancy outcomes is needed to monitor safety and efficacy of MiP vaccines during development and implementation. Vaccine trial safety endpoints include number and grade of adverse events in study subjects. In

general, safety endpoints for vaccines being tested in pregnant women classify adverse pregnancy outcomes as an adverse event (AE) that can dictate study progress. Maternal, fetal or neonatal death in particular are serious adverse events (SAEs) that can warrant halting a study, so documenting their pre-existing frequencies will be key to ensure safety while mitigating unwarranted delays in trial progress. In malaria endemic regions where adverse pregnancy events occur at high frequencies, baseline rates of AEs should be established before trial initiation. Baseline rates of adverse events provide safety boards with the information necessary to assess whether a vaccine is increasing the incidence of such events.

One such study has been conducted in Mali with a cohort of women of child-bearing age who were monitored monthly for pregnancy *via* hCG testing. Women who became pregnant were followed thereafter to track their pregnancy outcome, thus providing data regarding the incidence of AEs across all trimesters including miscarriage, stillbirth, preterm delivery, and small for gestational age (50). Such data exemplify the types of information that can serve as benchmarks for efficacy and safety endpoints during vaccine trials. These endpoints are routinely used to assess the safety of any vaccine tested in pregnant women, but are also caused by malaria and therefore can be measured to assess vaccine efficacy.

Region-specific documented ranges of outcomes can provide reference for researchers to assess how vaccine candidates reduce or contribute to fetal or neonatal mortality, and these data can be used to create consensus on trial endpoints globally. Since rates for poor pregnancy outcomes can vary by site due to local factors, documentation of background rates of poor outcomes at a trial site is warranted. This is particularly relevant for early phase trials with small cohort sizes in which a single or few adverse events may be difficult to interpret and could prematurely halt trials. Well-documented background rates will provide an evidence base to more confidently assess the relationship of severe adverse events to the study product. This is especially important considering the relatively high frequency of adverse pregnancy outcomes that occur in malaria-endemic regions, whether due to malaria or other causes.

Pregnancy-specific endpoints require clear definitions that specify factors including timing of gestation during AEs and occurrence of congenital anomalies (44). Further, measurements of these outcomes should be thoroughly standardized so that data can be accurately synthesized across sites. For instance, precision of scales and gestational age assessment tools make a significant impact on reported rates of low birthweight and gestational age respectively (44). Creating vaccine endpoints for MiP requires a nuanced approach that considers multiple metrics with an understanding that the occurrence of AEs does not automatically disqualify a vaccine from being safe and effective. *P. falciparum* parasitemia is always treated in pregnant women owing to the strong association with poor pregnancy outcomes and is a relatively frequent event with which to assess vaccine efficacy. Thus, baseline rates of parasitemia in pregnant women are useful to calculate sample sizes for example for Phase 2 trials that assess efficacy.

Malaria-related adverse pregnancy outcomes

Miscarriage

Miscarriage is defined here as the death of a fetus during the first two trimesters, or before 28 weeks of pregnancy, per WHO's definition. However, the definition of the gestational age of viability varies (between 24 and 28 weeks) and it has been general practice that pregnancy losses below that age are considered miscarriages. Preventing miscarriage is important for women and their future pregnancies as previous miscarriage is associated with an increased risk of PTB, fetal growth restriction, and other obstetric complications in subsequent pregnancies (51). Miscarriage is one of the lesser studied complications of MiP, partly due to low attendance at antenatal clinics early in pregnancy in endemic regions (29). In a study conducted on the Thai–Myanmar border, it was found that a single episode of symptomatic or asymptomatic *falciparum* or *vivax* malaria in the first-trimester can increase the risk of miscarriage (52). Incidence of miscarriage was highest in women infected with *falciparum* malaria with 16% of pregnancies resulting in miscarriage compared to 11% of women with *vivax* infection and 9% of noninfected women. In a cohort in Mali, 43 of 358 pregnancies resulted in miscarriage (12%) with 65.1% of these miscarriages occurring within the first trimester of pregnancy (50). This study did not evaluate malaria infection but demonstrates the burden of miscarriage in the first trimester of pregnancy in a malaria-endemic region of Mali. Additional risk factors for miscarriage in infected women include severe malaria [adjusted odds ratio (aOR) 3·63, 95% CI 1·15–11·46] and increased parasitaemia [aOR 1·49 (1·25–1·78) for each ten-fold increase in parasitaemia] (Table 1) (53).

Stillbirth

An estimated 2.6 million stillbirths occur annually, with 98% in low- and middle-income countries, and prenatal malaria is a major cause (54). An estimated 20% of stillbirths in sub-Saharan Africa are attributable to *P. falciparum* malaria in pregnancy (55). Pregnancy losses above the gestational age of viability are stillbirths, and here are defined as occurring from 28 weeks of pregnancy onward. Malaria-induced stillbirth may be caused by a myriad of sequelae that develop during infection of the placenta including impaired placental perfusion, maternal anemia, and preterm labor (56, 57).

In an observational study in Mali, malaria infection predicted increased risk of stillbirth (adjusted hazard ratio 3.87, $P = .03$) (18). In a meta-analysis of nineteen different countries, *P. falciparum* detected and treated during pregnancy was also associated with stillbirth (OR 1·47 [1·13-1·92], but to a lesser extent than untreated infection (OR 1·95 [95% CI 1·48-2·57]) (55). *P. vivax* malaria increased the odds of stillbirth when detected at delivery (OR 2·81 [0·77–10·22]), but not when detected and treated in pregnancy (OR 1·09 [0·76–1·57]). The timing of infection is also of interest; in Uganda, malaria within two

TABLE 1 Factors associated with increased risk of adverse birth outcomes in MiP.

		Miscarriage	Stillbirth	Perinatal Death	IUGR	Preterm Birth	Low Birth Weight
Timing of infection	Early pregnancy	Among all gravidae at Thai-Burmese border (McGready et al., 2012), and Benin (Briand et al., 2016)			Among primigravidae in Congo [Griffin et al., 2012], and Benin [Briand et al., 2016]	Among primigravidae in Malawi (Elphinstone et al., 2019)	Pooled population in sub-Saharan Africa and the Western Pacific (Cates et al., 2017) All gravidae in Burkina Faso (Valea et al., 2012; Cottrell et al., 2007) Primigravidae in Benin (Huynh et al., 2011)
	Late pregnancy		Among all gravidae in Uganda (Beaudrap et al., 2013)		Among all gravidae in Malawi (Kalanda et al., 2006) and in Benin (Briand et al., 2016)	Among all gravidae in Uganda (Beaudrap et al., 2013)	All gravidae in Malawi (Kalilani-Phiri et al., 2013) and Burkina Faso (Cottrell et al., 2007) Pooled population in sub-Saharan Africa and the Western Pacific (Cates et al., 2017)
Maternal factor	Severe maternal anemia		Among all gravidae in Ghana (Yatich et al., 2010)		Among all gravidae in Malawi (Kalanda et al., 2006)	Based on metanalysis among all gravidae (Xiong et al., 2000) Among all gravidae in Cameroon (Tako et al., 2005)	Among primigravidae in Papua New Guinea (Brabin et al., 1990) Among all gravidae in Benin (Bodeau-Livinec et al., 2011)
Severity of infection	Symptomatic malaria	Among all gravidae at Thai-Burmese border (McGready et al., 2012)	Among all gravidae at the Thai-Myanmar border (Moore et al., 2017a) and in Sudan (Taha & Gray, 1993)	Among all gravidae at the Thai-Myanmar border (Moore et al., 2017a) and in Sudan (Taha & Gray, 1993)	Among all gravidae in Uganda (Beaudrap et al., 2013)	Among all gravidae in Mali (Gaoussou et al., 2022)	Among primi- and secundi- gravidae in Tanzania (Schmiegelow et al., 2013)
	Hyper-parasitemia	Among all gravidae at Thai-Burmese border (McGready et al., 2012)			Among all gravidae in Benin (Ibhanesebhor & Okolo, 1992)	Among all gravidae in Benin (Ibhanesebhor & Okolo, 1992)	Among primigravidae in Papua New Guinea (Brabin et al., 1990) Among all gravidae in Benin (Ibhanesebhor & Okolo, 1992)
Malaria Endemicity	Low-to-intermediate malaria endemicity		All gravidae (Moore et al., 2017b)				
	Malaria endemic countries	Among all gravidae (Ahmadal-Agroudi et al., 2017)		Among all gravidae (van Geertruyden et al., 2004)		Among all gravidae (Lawn et al., 2023)	

weeks of delivery was found to be associated with a two-fold greater risk of stillbirth (OR 2.15 [1.04-4.46]) (Table 1) (58). In Sudan, the risk of stillbirth was significantly increased among women who reported malaria infection in the first and second trimester of pregnancy (OR 1.4 [1.1-1.9]). However, antenatal records of pregnant women from the Thai-Burmese border suggest that an episode of malaria in the first trimester does not predispose a pregnancy to later stillbirth (53). Thus, it is not completely clear how malaria in the first trimester is related to stillbirth, and whether this varies across parasite strains or populations.

Preventative measures contribute toward the reduction of stillbirth and related complications as IPTp and ITNs can reduce

stillbirths by 22% (59). Antenatal care visits are also important; in areas of high malaria transmission in Malawi, stillbirth was associated with fewer than five antenatal care visits (aOR 3·1 [1·4–7·0]) (60). Nevertheless, these useful tools do not adequately address the MiP problem: in a longitudinal cohort of pregnant women in Mali, malaria was common and increased the risk of stillbirth nearly 4-fold among primigravidae despite widespread use of IPTp-SP (18).

Perinatal death

Perinatal death refers to death of a baby between the 28th week of pregnancy to 7 days after birth, encompassing the outcomes of stillbirth and early neonatal death. Rates of perinatal mortality are overall higher in malaria-endemic countries with an estimated perinatal mortality rate of 34.7 per 1000 births (61). However, actual perinatal mortality rates are believed to be even higher than reported since a large percentage of perinatal deaths are unreported in malaria-endemic countries (62).

Maternal malaria increased the risk of perinatal death and LBW (risk ratio = 12.4), in a study from the Democratic Republic of the Congo (known as Zaire at the time of the study) (63). On the Thai-Myanmar border, *falciparum* and *vivax* MiP increase the risk of mortality by 2.55-fold and 1.98-fold, respectively (64). Among pregnant women in rural Malawi, the risk of neonatal death increased as birth weight decreased (65). The risk of LBW is known to be increased by placental malaria infection, thereby highlighting a link between malaria and perinatal mortality (18). Further, in a study with pregnant women in Kenya, impaired uteroplacental blood flow has been found to be predictive of perinatal death (14). Malaria infection in the third trimester was associated with abnormal uterine artery flow velocity waveforms, suggesting impaired uteroplacental blood flow may be related to the pathology of infected maternal erythrocytes in the placenta.

Successful prevention of *falciparum* infections reduces the risk for perinatal mortality by 27% among primigravidae (66). When it was still effective as an antimalarial, chloroquine prophylaxis was shown to protect against perinatal death (risk ratio = 0.38) (63). While more comprehensive data on perinatal mortality are necessary to accurately determine the impact of preventative measures, new interventions are clearly necessary to reduce the burden of malaria sequelae during pregnancy.

Risk factors for fatal outcomes of neonates and infants

Intrauterine growth restriction (IUGR), PTB, and LBW are all risk factors for death at delivery or in early infancy (Table 1). IUGR, the condition in which a fetus does not grow to normal weight during pregnancy, is a major cause for LBW. High density parasitemia in placental smears has been associated with IUGR in Benin (67). Malaria both in early and late pregnancy is associated with IUGR. Women with early pregnancy malaria have 2.2 times the risk of IUGR measured on multiple ANC visits compared to women without early infection (95% CI: 1.1, 4.2) (68).

Malaria infection also increases the risk of PTB (hazard ratio = 2.41, p = 0.003) (18), the other major contributor to LBW in malaria exposed populations. PTB is related to stillbirth, with around three-quarters of stillbirths born preterm globally (5). MiP is estimated to be responsible for 36% of PTB and 70% of IUGR in areas with stable malaria transmission in Africa (58).

LBW, defined as a birthweight less than 2,500 grams, is a major risk factor for infant mortality. In malaria-endemic regions, an estimated 19% of LBW births are due to malaria, and an estimated 6% of overall infant deaths are due to malaria-induced LBW (69). MiP increases the risk of both LBW and prematurity, and some studies find that the risk is greater when the infection is acquired in early pregnancy (32, 70, 71). Frequency of infection is also a relevant factor as the risk of LBW increases as the number of malaria episodes during pregnancy increases [one episode: prevalence ratio (PR) 1.62 (95% CI 1.07–2.46); two episodes: PR 2.41 (95% CI 1.39–4.18)] (70). A study with pregnant women in Gambia found a four-fold risk of delivering LBW babies if mothers had parasitized placentae (72). Another cohort in Papua New Guinea found that histopathologically diagnosed chronic placental malaria is associated with both LBW and PTB (73).

Preventative measures are effective against adverse pregnancy outcomes. Three or more doses of IPTp-SP was associated with a 66% reduced risk of LBW (74). Further, a regimen of more than three doses IPTp-SP was associated with an improved birth weight compared to fewer doses of treatment. However, treatment with IPTp in the third trimester did not prevent IUGR, stressing the need to protect women from malaria from early in pregnancy (75). Furthermore, consistent antenatal care is of importance as PTB has been associated with <5 antenatal care visits (aOR 2.2, 95% CI 1.3-3.7) (60). Altogether, the evidence suggests measures focused directly on malaria prevention in conjunction with broader health care access are critical to address risk factors for MiP.

Limitations

There are contradicting data in the literature regarding which adverse birth outcomes are significantly related to malaria. This could be due to the lack of consensus on what markers of malaria should serve as surrogates of adverse birth outcomes. A 2020 study found that detection of parasites through placental histopathology was associated with an increased risk of adverse birth outcomes, while parasite detection by microscopy was not (76). The variation in sensitivity of malaria detection methods may contribute to discrepancies in reports of malaria impact on adverse birth outcomes.

Further, there is a lack of first trimester cohort studies in Africa, with most studies of first trimester pregnancies being conducted in Southeast Asia. This is at least partially attributable to the later gestational age at first ANC visit in most African sites, as well as a lack of established data collection sites in the region. Recent efforts to address this include a pregnancy registry established in Mali to provide baseline information on maternal and fetal outcomes in preparation for vaccine trials in pregnant women (44).

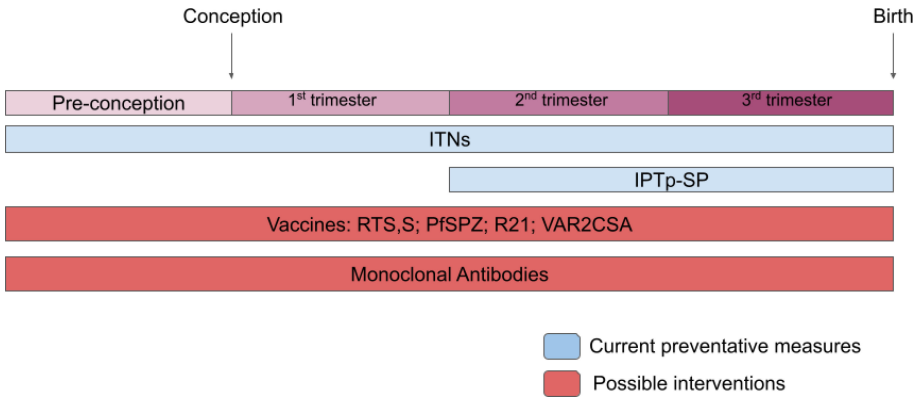

FIGURE 2
Current and future interventions for MiP. Summary of interventions for MiP from before conception to time of birth. Blue regions represent established interventions and red regions represent possible interventions for the future.

Finally, increased ANC monitoring and care (IPTp; bed-nets; clinic visits) during any vaccine or interventional trial in pregnancy will likely reduce the burden of parasitemia and therefore improve pregnancy outcomes. This benefit of study participation will thus reduce the endpoints needed to assess malaria vaccine efficacy, highlighting the need to estimate this effect *a priori* to ensure sufficient sample size and power for statistical analysis of the clinical benefit of the vaccine. On the other hand, fewer adverse pregnancy outcomes in the control group due to increased care and malaria prevention might facilitate identification of any concerning safety signals specifically related to the vaccine.

Future directions

There have been significant strides in reducing the burden of MiP through the distribution of ITNs, and implementation of IPTp/SP (Figure 2). However, with continued rates of maternal mortality, stillbirth, and other malaria-related sequelae, MiP persists as a perennial problem requiring stronger solutions. In light of growing resistance to current therapeutics, a more sustainable and effective approach to preventing pregnancy malaria is warranted (77). The development of a vaccine targeting MiP has possibly the greatest potential to prevent enduring adverse birth outcomes. By intensifying research on adverse pregnancy outcomes caused by MiP, we can best equip ourselves to confirm the safety and efficacy of vaccines against MiP and accelerate their ultimate deployment to end these preventable health consequences.

Author contributions

AB and JD wrote the original draft of the manuscript. PD reviewed and edited the manuscript. All authors read and approved the submitted version of the manuscript.

Funding

This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Acknowledgments

The authors thank J. Patrick Gorres for editing the manuscript.

Conflict of interest

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

The authors JD and PD declared that they were editorial board members of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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frontiers | Frontiers in Public Health

TYPE Original Research
PUBLISHED 20 October 2023
DOI 10.3389/fpubh.2023.1258594

OPEN ACCESS

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RECEIVED 14 July 2023
ACCEPTED 02 October 2023
PUBLISHED 20 October 2023

CITATION
Addis D and Gebeyehu Wondmeneh T (2023) Assessment of malaria prevention knowledge, attitude, and practice and associated factors among households living in rural malaria-endemic areas in the Afar Pastoral Region of Ethiopia.
Front. Public Health 11:1258594.
doi: 10.3389/fpubh.2023.1258594

Assessment of malaria prevention knowledge, attitude, and practice and associated factors among households living in rural malaria-endemic areas in the Afar Pastoral Region of Ethiopia

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Background: Malaria morbidity has reduced significantly in most regions of Ethiopia, but it is still a serious issue in the northeast, particularly in the Afar region.

Objective: The study aimed to evaluate household heads' knowledge, attitudes, and practices toward malaria prevention and its associated factors in rural Ada'ar woreda district in the Afar region.

Methods: A community-based cross-sectional study was conducted among 422 households living in Ada'ar woreda district. A systematic sampling technique was used to select households. A pre-tested, structured questionnaire was used to interview randomly selected adult household heads. Frequency and percentage were computed. Logistic regression was used to determine the association between independent and dependent variables. Statistical significance was considered to be a p-value <0.05.

Results: Nearly two-thirds (64.2%) of household heads had good knowledge of malaria prevention, and 46.9% had a positive attitude toward it. About 17.3 and 56.9% of study participants had good malaria prevention practices and good healthcare seeking behaviors, respectively. Illiterate (AOR = 2.62, 95% CI: 1.49–4.63) and low-income (AOR = 2.6, 95% CI: 1.2–5.6) participants were more likely to have poor knowledge of malaria prevention (malaria signs and symptoms, malaria transmissions, and malaria prevention methods). Married participants (AOR = 2.52, 95% CI: 1.02–6.29) and illiterates (AOR = 2.83, 95% CI: 1.69–4.73) had negative attitudes toward malaria prevention. Household heads with poor knowledge of malaria prevention had 85% higher rates of practicing poor malaria prevention methods (regular bed nets used; AOR = 1.85, 95% CI: 1.2–2.8). Young adults (18–25 years) were more likely to have poor healthcare seeking behaviors (AOR = 3.5, 95% CI: 1.73–7.1), while pastoralists had a lower likelihood (AOR = 0.46, 95% CI: 0.28–0.8).

Conclusion: Knowledge, attitude, and practices toward malaria prevention remain a problem in malaria-endemic rural areas of the Afar region of Ethiopia. There is a need for the implementation of interventions that will focus on increasing knowledge of malaria prevention and encouraging positive attitudes toward it, as well as promoting regular bed net usage and healthcare seeking behaviors.

KEYWORDS

knowledge, attitude, practice, malaria, household, endemic, Afar, Ethiopia

Background

Malaria is a vector-borne disease endemic in most of sub-Saharan Africa, where the most common parasite to infect humans is *Plasmodium falciparum* (1). It is a disease with complicated patterns of transmission that is linked to significant geographical and temporal variation (2). Malaria poses a threat to millions of people living in tropical and subtropical countries (3). Throughout the world, malaria causes 300–500 million cases and up to three million fatalities annually; of these, Africa alone bears more than 90% of the burden, and more than 80% of malaria deaths take place there, while <15% occur in Asia and Eastern Europe (4). Globally, malaria cases increased by 5.8% and deaths by 11% in 2020 compared to 2019. Two-thirds of the additional deaths in 2020 compared to 2019 were attributable to the disruption in malaria prevention and control efforts brought on by the COVID-19 pandemic (5). East Africa is an important region to focused on in the global fight against malaria because it accounted for 25% of all cases worldwide (6). In Djibouti, the prevalence of malaria was 70.8% (7). In Ethiopia, the pooled prevalence of malaria among adults was 13.6% (8). In the Afar region, the prevalence of malaria among febrile under-five children was 64% (9). Seasonal malaria transmission persists when the maximum monthly rainfall is <600 mm and the temperature is markedly above 15°C or below 40°C (10). The use of medication and bed nets is an efficient malaria prevention strategy that can be implemented in malaria-endemic areas (11). The government of Ethiopia distributes the bed nets through an ongoing effort campaign every three years (12). Malaria infection depends on people's knowledge and awareness of the disease (13). Understanding the biology of malaria transmission at the individual level is an essential aspect of the strategies used to stop the spread of malaria parasites (14). In sub-Saharan Africa, schoolchildren's knowledge of malaria's causes and transmission ranged from 19.2 to 85%, and 51.2% of them had a positive attitude. Schoolchildren practice low to moderate levels of malaria prevention, ranging from 32.4 to 67.9% (15). In Senegal, nearly a third of adolescents had good knowledge of malaria (34.4%) and good practice for using bed nets (32.8%), whereas 59.0% had a positive attitude and 73.8% had good care-seeking practice toward malaria (16). Adults in Nigeria had a high level of comprehensive knowledge (90%) and practiced malaria prevention (80%). Seeking hospital care was a good practice (68.5%), while attitudes about antimalarial treatment were poor (56.7%) (17). In Cameroon, 88% of pregnant women and mothers had a good level of knowledge, 99% had a good attitude toward ITNs, and 57% of respondents used ITNs to prevent malaria (18). Women of reproductive age in Burkina Faso had 56.1% accurate knowledge of the preventive measures, causes, and symptoms of malaria. About 97.4% of women said they slept under a mosquito net, and most of the women (80%) reported sleeping within an insecticide-treated net (19). In South Africa, 63% of household heads were able to identify at least three symptoms of malaria. Participants' attitudes toward indoor residual spraying (IRS) were favorable for 76% of respondents. Only 2% of the participants used bed nets (20). In Mozambique, 90.0% of households' heads had knowledge of malaria prevention. About 81.7% of respondents slept under an ITN at night (21). In Tanzania, 47.3 and 13.8% of household heads had moderate and high-level knowledge of malaria prevention, respectively. The majority of the households (83.9%) had bed nets hanging on the sleeping spaces, while 95% of household heads agreed that it was beneficial to sleep beneath a bed net (22). In a study of Southern Ethiopia, household heads who had good knowledge, a positive attitude, and good practices toward malaria prevention were 50.4%, 55.1%, and 67.7%, respectively (23). Approximately two-thirds of participants in the Amara region of Ethiopia had good knowledge (63.1%) and a positive attitude (62.6%) toward malaria, whereas only half had good practice (50.8%) with regard to malaria prevention and control measures (24). In a study of the Oromia region in Ethiopia, about 85% of household heads had good knowledge of malaria prevention, 87.8% had a positive attitude toward malaria prevention, and 51.3% regularly practiced malaria prevention (25). Self-medication was practiced by some people to prevent malaria (26). The majority of Afar people (71%) preferred to receive their medical care from modern health facilities, 16% used traditional practices, and 3% used self-medication during their symptoms of malaria. The use of traditional medicine may increase as people get older, whereas the use of modern medication rose as people's educational levels increased (27). Education and knowledge are used to combat malaria (28). Poor knowledge resulted in poor management of malaria (29). A person's knowledge about malaria prevention was affected by male gender, rural residence, low income level, and illiterate educational level (16, 18, 19, 22–24). Male gender, rural residence, low educational level, low income (16, 24, 30), and Islamic religion (23) were associated with lower rates of use of malaria prevention measures, whereas good knowledge boosted the use of malaria prevention practices (31, 32). A person's attitude toward malaria prevention was affected by a low wealth quintile and a low educational level (16).

Even though malaria is endemic and has a seasonal outbreak in the pastoral region of Afar, there is no data on knowledge, attitudes, or practices about malaria prevention in the region. Therefore, the current study investigated malaria-related knowledge, attitudes toward the disease, adoption of prevention practices, and care-seeking practices among household heads living in rural areas with persistent malaria transmission in the Afar region. The study also explored factors associated with household heads knowledge, attitudes, and practices toward malaria.

The aim of the study was to generate data that will aid in the development of social and behavioral change interventions targeted specifically at household levels in order to improve households' awareness and adherence to malaria control and preventative measures in the Afar region.

Methods

Study area and design

A community-based cross-sectional study design was conducted in Ada'ar woreda, located in Zone 1 (also known as Awsi Rasu), Afar Region, northeast Ethiopia, from February 30th

Abbreviations: SPSS, social science statistical package; ITN, insecticide-treated net; COVID-19, Coronavirus disease 2019; COR, crude odd ratio; AOR, adjusted odd ratio; CI, confidence interval; Fig, figure; ETB, Ethiopian Birr; TV, television.

to May 10th, 2023, to assess knowledge, attitude, and practice of malaria prevention and control. There were five zones in the Afar region, with Zone 1 (Awsi Rasu Zone) being one of them. Geographically, the region is located between 9°N−12°N latitude and 40°E−42°E longitude at the northern tip of the Great East African Rift Valley and at 432 m altitude. It is a pastoral region in north-eastern Ethiopia, 576 km from Addis Ababa. The regional weather condition is sunny, and recurrent natural disasters like drought and flooding are common in the region. Poverty remains high and multidimensional in the region. The population's mean index of health care deprivation was 64.6%, which indicates that the majority of the population did not consult any medical practitioner within a year. The absence or scarcity of health centers and practitioners and the inability of households to access health services due to financial and other constraints were contributing factors (33). According to the Ada'ar woreda Health Bureau report, there were 13 kebeles in the woreda with a total estimated population of 69,111. The study was conducted in six rural kebeles out of the 13 kebeles (Table 1).

There are three health centers, 12 health posts, and three private clinics in the woreda. The map of the study zone in the Afar region is depicted in Figure 1.

TABLE 1 The total population and total households for the selected kebeles.

Selected kebeles	Total populations	Total households
Ada'ar	7,054	841
Siylu na Woki	5,517	968
Burka	5,677	902
Ledi	4,331	618
Woranso na Hormati	6,283	1,102
Hado	4,355	764

Sample size determination

To determine the sample size of this study, we put the prevalence (p) at 50% because of the lack of existing research on the knowledge, attitudes, and practices of malaria prevention and control in the Afar region. With a confidence interval (CI) of 95%, precision (d) of 5%, and q of 50%, the sample size became 384. With the addition of a 10% non-response rate, the final sample size became 422.

Source and study population

The source populations were all households in Ada'ar woreda, whereas the study populations were all households in the selected kebeles.

Inclusion and exclusion criteria for study participants: study participants should live permanently in the selected districts, and interviewees should be at least 18 years old (head of household), regardless of gender. Participants who are seriously ill or mentally ill, unable to communicate, or refuse to give consent were excluded from the study.

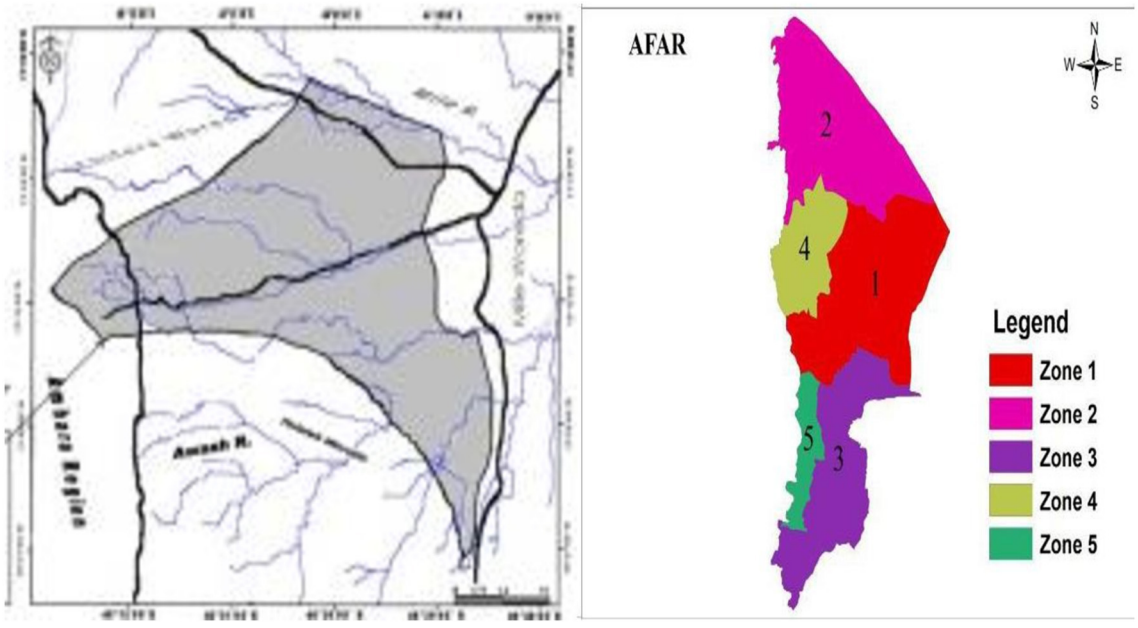

FIGURE 1
Map of the study zone.

Sampling method and procedure

For this study, Ada'ar woreda in Zone 1 (Awsi Rasu Zone) of the Afar region was selected purposefully because of the representativeness of the population dimension and the densely populated nature, which enabled the recruitment of an adequate sample. To ensure representativeness, the six kebeles were first selected by simple random sampling methods using lottery methods. Then the sample size is allocated proportionally to each of the six kebeles. The households in each kebele were selected using the following technique: initially, a list of households (a sampling frame) was developed by assigning a number to each household. Next, the sample interval (the number of households divided by the sample size) was calculated for each kebele, and a random start number was picked. Finally, from this first random number, households were systematically selected using the sampling interval until the calculated sample size was met. Interviews were conducted with the head of the selected household regardless of sex, and in the absence of the head of the household, a responsible person over 18 years old who had been appointed by the family was interviewed. When there are two or more heads in one household, the lottery method is used to select interview participants.

Data collection methods and tools

The study questionnaires were adapted from various similar prior studies and reviewed. The questionnaire was first designed in English, and then translated into Afar, the local language in the study area. Closed questions on the following topics were included in the questionnaire: (i) socio-demographic characteristics of participants; (ii) knowledge of malaria transmission, symptoms, and signs of malaria and how to prevent malaria; (iii) practices for prevention; (iv) attitudes toward malaria; (v) practices for seeking care for malaria; and (vi) information channels. Five percent of the questionnaires were pre-tested among respondents in Chifra, a location not in the study area, to ensure word consistency, and some words were modified in light of the findings of the pilot study. Data was collected by prior-experienced data collectors. During the training of data collectors, the study protocol, the questionnaire, and the consent procedure were covered. Monitoring visits were made during the data collection phase to examine the accuracy of the data and determine whether the study's informed consent standards were being followed. In the event that the head of the household was not present, a responsible adult who is at least 18 years old was selected to take part in the interview.

Operational definition and measurement of outcome variables

In this study, Kebele is the smallest administrative unit contained within a woreda in Ethiopia (34).

Malaria: Malaria diagnosis includes a patient's clinical assessment (for non-malaria endemic areas, fever, headache, chillness, fatigue, and vomiting; for malaria-endemic areas, only fever is suspected), microscopic examination of blood slides, and rapid diagnostic test (RDT) in accordance with the level of the health facility. Microscopic diagnosis remains the standard of diagnosis in health centers and hospitals of different levels, whereas multi-species rapid diagnostic tests (RDTs) are the main diagnostic tool at the health post level. Artemisinin-based combination therapies (ACTs) are the first-line drug for the treatment of uncomplicated *P. falciparum* malaria. Chloroquine is used for the treatment of *Plasmodium vivax* (35).

The primary outcomes investigated in this study were knowledge and attitudes about malaria, use of malaria prevention and control practices, and practices for seeking medical care when feeling ill. Participants' knowledge of malaria was assessed using three items: correctly identifying the mode of transmission, recognizing the sign and symptoms of malaria, and identifying methods to prevent malaria. The participant's scores on the three knowledge questions were added up to produce a knowledge score. Participants received a score of one if they mentioned a mosquito bite as the method of malaria transmission; otherwise, they received a score of zero. One point was given if at least three of the five basic symptoms of malaria—fever, headache, chills, fatigue and vomiting—were correctly identified. Participants who correctly identified a bed net as a method of preventing malaria were given a score of one, and those who correctly identified any of the other prevention methods—mosquito coils, wearing long clothes, applying insecticide spray, weeding, or disposing of sewage—were given a half-point. The overall score's median was used as the cutoff point for classifying knowledge of malaria into two levels. Participants' levels of malaria knowledge were rated as poor (below or equal to the median) or high (above the median). Six items pertaining to the population at risk for malaria, prevention, and treatment were used to measure attitudes about the disease. According to Likert's method of scoring, there were five possible responses: strongly agree (score 5), agree (score 4), neutral (score 3), disagree (score 2), and strongly disagree (score 1). For one item (malaria can be cured without medical care), the scale was reversed. An attitude score was estimated by adding up each participant's 'score across the six variables. Participants were regarded as having a positive attitude if their total score was at or above the median; otherwise, they were considered to have a negative attitude. Participants who own a bed net and use it more than three times per week or every night are classified as practicing good prevention, while those who don't own a bed net or who do but don't use it more than three times per week are categorized as practicing poor prevention. We examined whether households actually required care within 24 h of the onset of symptoms in order to assess their behavior for seeking care for recent malaria. Households were deemed to have good care-seeking behavior if they visited a healthcare facility within 24 h of needing care; otherwise, they were deemed to have poor care-seeking behavior (16, 36, 37).

Data management and analysis

Epidata version 3.1 was used to enter the data, which was subsequently exported to SPSS software version 26. For categorical variables, frequency and percentage were computed. Bivariate and multivariate logistic regressions were used to assess factors associated with household heads' knowledge, attitudes,

TABLE 2 Socio-demographic characteristics of participants.

Variables	Categories	Frequency	Percentages (%)
Age (in year)	18–25	85	20.1
	26–35	168	39.8
	36–45	102	24.2
	≥46	67	15.9
Sex	Males	236	55.9
	Females	186	44.1
Marital status	Married	264	62.6
	Single	134	31.8
	Divorced	24	5.7
Religious	Muslim	347	82.2
	Christian	75	17.8
Occupation	Pastoralist	315	74.6
	Agro pastoral	107	25.4
Educational level	Illiterate	201	47.6
	Primary school	115	27.3
	Secondary school	106	25.1
Average household income (ETB)	<2,000	58	13.7
	2,000–4,000	78	18.5
	4,001–6,000	109	25.8
	6,001–8,000	100	23.7
	>8,000	77	18.2

ETB, Ethiopian birr.

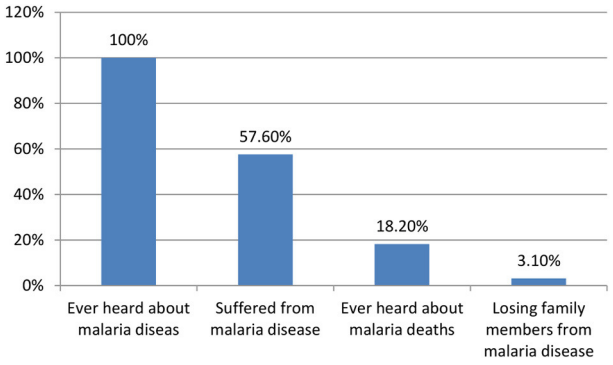

FIGURE 2
Participant's information regarding malaria.

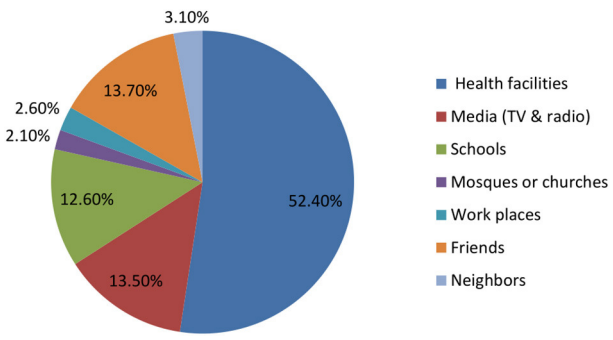

FIGURE 3
The source of information about malaria.

and practices of malaria prevention. The multivariable regression models included variables that had a p-value of ≤0.25 in the bivariate analysis. Statistical significance was considered when the p-value <0.05.

Results

Socio-demographic characteristics of participants

The response rate for this study was 100%. Most of the respondents (39.8%) were in the age range of 26–35 years old. Male respondents were 55.9%. Married respondents constituted about 62.6%. The Muslim religion accounted for 82.2%. Most participants were pastoralists (74.6%). Illiterate respondents were 47.6%. About 25.8% of respondents had an average monthly income of 400–1,500 ETB (Table 2).

Participants' information about malaria

All participants heard about malaria; 57.6% suffered from malaria disease, and 18.2% heard about malaria deaths. About 3.1% of participants lost family members from malaria (Figure 2).

Source of information about malaria

Health facilities were the source of malaria information for more than half (52.4%) of the study's participants. Nearly comparable percentages of study participants reported that the sources of information about malaria were the media (TV and radio; 13.5%), friends (13.7%), and schools (12.6%; Figure 3).

Participants' knowledge of malaria transmission, signs and symptoms, and prevention methods

The majority of study participants (74.9%) reported that the transmission of malaria occurs through mosquito bites. Most respondents identified body pain (35.8%), fever (31.5%), and fatigue (27.5%) as malarial symptoms. Using mosquito bed nets was reported as a means of malaria prevention by the majority of respondents (43.1%). Nearly two-thirds (64.2%) of study participants had good knowledge of malaria (Table 3).

TABLE 3 Knowledge of malaria transmission, signs and symptoms, and prevention methods among participants.

Variables	Categories	Frequency	Percentage (%)
How is malaria transmitted?	Mosquito bite	316	74.9
	Contacting with malaria patients	61	14.5
	Using contaminated water	23	5.5
	I don't know	22	5.2
Malaria signs and symptoms	Fever	133	31.5
	Headache	97	23
	Vomiting	85	20.1
	Chilies	54	12.6
	Fatigue	116	27.5
	Body pain	151	35.8
	Feeling of thirsty	62	14.7
Malaria prevention	Using mosquito bed net	182	43.1
	Insecticide spray	94	22.3
	Using mosquito repellents lotion	31	7.3
	Using ventilators	66	15.6
	Avoiding stagnant water	49	11.6
Level of knowledge	Good	271	64.2
	Poor	151	35.8

TABLE 4 Attitude of participants toward malaria prevention.

Statements	Likert scales				
	Strongly disagree	Disagree	Neutral	Agree	Strongly agree
	No (%)	No (%)	No (%)	No (%)	No (%)
Everybody can have malaria	49 (11.6)	74 (17.5)	78 (18.5)	118 (28)	103 (24.4)
Malaria can be cured without medical treatment	86 (20.4)	94 (22.3)	61 (14.5)	95 (22.5)	86 (20.4)
It is important to be tested before taking malaria treatment	70 (16.6)	64 (15.2)	80 (19)	113 (26.8)	95 (22.5)
Malaria can be prevented	77 (18.2)	74 (17.5)	58 (13.7)	127 (30.1)	86 (20.4)
It is necessary to finish malaria treatment	76 (18)	88 (20.9)	48 (11.4)	95 (22.5)	115 (27.3)
Malaria is deadly	66 (15.6)	84 (19.9)	47 (11.1)	80 (19)	145 (34.4)
Attitude score	Positive		198 (46.9%)		
	Negative		224 (53.1%)		

Attitude of participants toward malaria prevention

Nearly half of participants (52.4%, $n = 221$) believed that everybody can have malaria, and almost identical numbers also believed that malaria is deadly (53.3%, $n = 225$). Similarly, the same number of participants perceived that it is important to be tested before taking malaria treatment (49.3%, $n = 208$) and that it is necessary to finish malaria treatment (49.8%, $n = 210$). Half of the respondents (50.5%, $n = 213$) also stated that malaria can be prevented. Regarding the overall score of attitude, 46.9% of respondents exhibited a positive attitude (Table 4).

Practices of participants for malaria prevention

About 41.9% of participants reported owning a bed net; of those, 7.3% reported using it every night, and 10% reported using it more than three days a week. Nearly one-fifth (21.1%) and 6.6% of study participants used insecticide spray and repellent lotion, respectively. About 13.5% of study participants considered ventilators for malaria prevention, and 16.8% never used any malaria prevention methods. Overall, 17.3% of participants had good malaria prevention practices (Table 5).

TABLE 5 Practices of participants for malaria prevention.

Variables	Categories	Frequency	Percentages (%)
Participants owned bed nets		177	41.9
Respondents' use of mosquito bed nets	Every night	31	7.3
	Over three days a week	42	10
	Three or less days per week	104	24.6
Insecticide spray		89	21.1
Using mosquito repellent lotion		28	6.6
Using ventilators		57	13.5
Never use any things		71	16.8
Family members slept under a bed net	All family members	73	17.3
	Fathers and mothers	31	7.3
	Children and mothers	43	10.2
	Mothers	15	3.6
	Children	15	3.6
Overall level of practices for malaria prevention	Good level of practice	73	17.3
	Poor level of practice	349	82.7

Healthcare seeking behavior

More than half of respondents (56.9%) reported visiting health facilities within 24 h after having malaria symptoms, while a quarter of respondents (25.1%) reported visiting health facilities more than 24 h after experiencing malaria symptoms. Eighteen percent of study participants never visited health facilities when they encountered malaria symptoms. Those study participants who attended a health facility within 24 h (56.9%) after experiencing malaria symptoms were regarded as having good care-seeking behavior for preventing malaria (Figure 4).

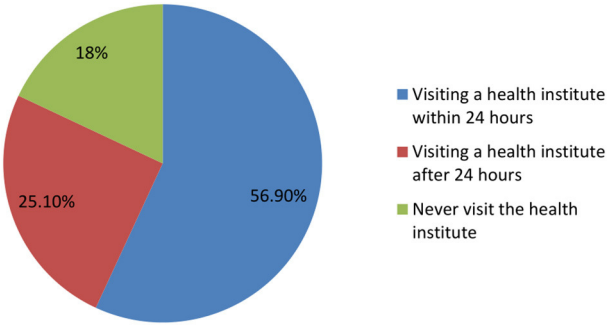

FIGURE 4
Care seeking practices of the study participants.

Factors associated with malaria prevention knowledge

In the binary logistic regression analysis, the age range of 18–25 years, illiteracy, and an average household income of <2,000 ETB were significantly associated with poor knowledge of malaria prevention. In the multivariable analysis, participants with illiteracy (AOR = 2.62, 95% CI: 1.49 – 4.63) and low average monthly income (<2,000 ETB; AOR = 2.6, 95% CI: 1.2–5.6) were more likely to have poor knowledge of malaria prevention than those with secondary school and average monthly income >8,000 ETB, respectively (Table 6).

Factors associated with attitudes toward malaria prevention

In the binary logistic regression, illiterates and low household average monthly income were significantly associated with a higher negative attitude toward malaria prevention. In the multivariable logistic regression analysis, married participants (AOR = 2.52, 95% CI: 1.02–6.29) and illiterate participants (AOR = 2.83, 95% CI: 1.69–4.73) were more likely to have negative attitudes toward malaria prevention than study participants who were divorced and secondary school, respectively (Table 7).

Factors associated with malaria prevention practices

No statistically significant variables were identified in the binary logistic regression; however, in the multivariable logistic regression, only one variable (poor knowledge) had a statistically significant association with poor malaria prevention practices. Study participants with poor knowledge were more likely to have poor malaria prevention practices than those with good knowledge (AOR = 1.85; 95% CI: 1.2–2.8; Table 8).

TABLE 6 Factors associated with malaria prevention knowledge.

Variables	Categories	Knowledge		COR	AOR
		Good	Poor		
		No (%)	No (%)		
Age (in year)	18–25	48 (11.4)	37 (8.8)	2.27 (1.13–4.56)*	1.92 (0.91– 4.0)
	26–35	103 (24.4)	65 (15.4)	1.86 (0.99–3.5)	1.82 (0.94–3.54)
	36–45	70 (16.6)	32 (7.6)	1.35 (0.67–2.68)	1.3 (0.64–2.71)
	≥46	50 (11.8)	17 (4)	1	1
Sex	Males	160 (37.9)	76 (18)	1.42 (0.95–2.1)	0.74 (0.48–1.13)
	Females	111 (26.3)	75 (17.8)	1	1
Educational status	Illiterates	108 (25.6)	93 (22)	3.11 (1.8–5.3)*	2.62 (1.49–4.63)*
	Primary	80 (19)	35 (8.3)	1.58 (0.86–2.9)	1.4 (0.8–2.61)
	Secondary	83 (19.7)	23 (5.5)	1	1
Average household monthly income (ETB)	<2,000	22 (5.2)	36 (8.5)	3.6 (1.77–7.4)*	2.6 (1.2–5.6)*
	2,000–4,000	61 (14.5)	17 (4)	0.62 (0.3–1.27)	0.53 (0.25–1.1)
	4,001–6,000	69 (16.4)	40 (9.5)	1.28 (0.69–2.4)	1.3 (0.67–2.5)
	6,001–8,000	66 (15.6)	34 (8.1)	1.14 (0.6–2.15)	1.26 (0.65–2.46)
	>8,000	53 (12.6)	24 (5.7)	1	1

ETB, Ethiopian birr.
*Statistical significant.

TABLE 7 Factors associated with attitudes toward malaria prevention.

Variables	Categories	Attitude		COR	AOR
		Positive	Negative		
		No (%)	No (%)		
Age (in years)	18–25	34 (8.1)	51 (12.1)	1.64 (0.86–3.1)	1.27 (0.64–2.55)
	26–35	71 (16.8)	97 (23)	1.45 (0.846–2.64)	1.44 (0.79–2.62)
	36–45	58 (13.7)	44 (10.4)	0.83 (0.45–1.54)	0.766 (0.4–1.47)
	≥ 46	35 (8.3)	32 (7.6)	1	1
Marital status	Married	118 (28)	146 (34.6)	2.1 (0.87–4.9)	2.52 (1.02–6.29)*
	Single	65 (15.4)	69 (16.4)	1.77 (0.72–4.32)	1.9 (0.75–4.96)
	Divorced	15 (3.6)	9 (2.1)	1	1
Household occupation	Pastoralist	153 (36.3)	162 (38.4)	0.77 (0.49–1.2)	0.91 (0.56–1.47)
	Agro pastoralist	45 (10.7)	62 (14.7)	1	1
Educational status	Illiterates	71 (16.8)	130 (30.8)	3.02 (1.86–4.9)*	2.83 (1.69–4.73)*
	Primary	61 (14.5)	54 (12.8)	1.46 (0.85–2.5)	1.47 (0.84–2.55)
	Secondary	66 (15.6)	40 (9.5)	1	1
Household average monthly income (ETB)	<2,000	18 (4.3)	40 (9.5)	2.5 (1.24–5.2)*	1.85 (0.86–3.99)
	2,000–4,000	35 (8.3)	43 (10.2)	1.4 (0.74–2.63)	1.31 (0.66–2.59)
	4,001–6,000	53 (12.6)	56 (13.3)	1.2 (0.67–2.2)	1.1 (0.6–2.1)
	6,001–8,000	51 (12.1)	49 (11.6)	1.09 (0.6–1.98)	1.2 (0–62–2.17)
	>8,000	41 (9.7)	36 (8.5)	1	1

ETB, Ethiopian birr.
*Statistical significant.

TABLE 8 Factors associated with malaria prevention practices.

Variables	Categories	Prevention practices		COR	AOR
		Good	Poor		
		N (%)	*N* (%)		
Household occupation	Pastoralists	61 (14.5)	254 (60.2)	0.5 (0.27–1.02)	0.56 (0.28–1.1)
	Agro pastoralist	12 (2.8)	95 (22.5)	1	1
Educational level	Illiterates	25 (5.9)	176 (41.7)	1.74 (0.921–3.3)	1.43 (0.74–2.8)
	Primary	27 (6.4)	88 (20.9)	0.81 (0.42–1.53)	0.76 (0.4–1.46)
	Secondary	21 (5)	85 (20.1)	1	1
Knowledge	Poor	19 (4.5)	132 (31.3)	1.73 (0.98–3.04)	1.85 (1.2–2.8)*
	Good	54 (12.8)	217 (51.4)	1	1
Attitude	Negative	34 (8.1)	190 (45)	1.37 (0.83–2.27)	1.16 (0.68– 1.96)
	Positive	39 (9.2)	159 (37.7)	1	1

*Statistical significant.

TABLE 9 Factors associated with care-seeking practices to prevent malaria.

Variables	Categories	Care seeking practices		COR	AOR
		Good	Poor		
		No (%)	No (%)		
Age	18–25	29 (6.9)	56 (13.3)	3.46 (1.8–6.8)*	3.5 (1.73–7.1)*
	26–35	105 (24.9)	63 (14.9)	1.08 (0.6–1.94)	1.2 (0.63–2.2)
	36–45	63 (14.9)	39 (9.2)	1.12 (0.59–2.1)	1.3 (0.66–2.5)
	≥46	43 (10.2)	24 (5.7)	1	1
Marital status	Married	156 (37)	108 (25.6)	0.58 (0.25–1.36)	0.53 (0.21–1.3)
	Single	73 (17.3)	61 (14.5)	0.71 (0.3–1.69)	0.55 (0.2–1.36)
	Divorced	11 (2.6)	13 (3.1)	1	1
Occupation	Pastoralists	194 (46)	121 (28.7)	0.47 (0.3–0.73)*	0.46 (0.28–0.8)*
	Agro pastoral	46 (10.9)	61 (14.5)	1	1
Educational status	Illiterates	95 (22.5)	106 (25.1)	1.92 (1.18–3.1)	1.27 (0.75–2.15)
	Primary	78 (18.5)	37 (8.8)	0.82 (0.47–1.42)	0.71 (0.4–1.27)
	Secondary	67 (15.9)	39 (9.2)	1	1
Knowledge	Poor	74 (17.5)	77 (18.2)	1.65 (1.1–2.5)*	1.43 (0.92–2.2)
	Good	166 (39.3)	105 (24.9)	1	1
Attitude	Negative	116 (27.5)	108 (25.6)	1.56 (1.06–2.3)*	1.33 (0.86–2.01)
	Positive	124 (29.4)	74 (17.5)	1	1

*Statistical significant.

Factors associated with healthcare-seeking practices for malaria prevention

In the binary logistic regression, participants with ages ranging from 18 to 25 years, poor knowledge, and a negative attitude were more likely to have poor healthcare seeking practices, but pastoralists were less likely to have poor healthcare seeking behavior. In the multivariable logistic regression analysis, participants with ages ranging from 18 to 25 years old (AOR = 3.5, 95% CI: 1.73–7.1) were nearly three times more likely to have poor healthcare seeking behavior than those with ages >45. Pastoralist participants (AOR = 0.46; 95% CI: 0.28–0.8) were 54% less likely to have poor healthcare seeking practices than agro-pastoral participants (Table 9).

Discussion

Understanding malaria transmission and preventative measures at the individual and household level, which depend

on populations' knowledge of the disease (13), attitude toward the disease, and implementation of prevention activities (11), are essential strategies to stop the spread of malaria in malaria-endemic areas (14). There must be a reduction in the number of malaria cases and deaths at the global (1–5), regional (6, 7), national (8, 9), and district (9) levels. Therefore, this study assessed malaria prevention knowledge, attitude, and practice among households living in malaria-endemic areas.

In this study, 64.2% of household heads had good knowledge of malaria prevention. This magnitude is lower than the previous studies in sub-Saharan Africa (15), Nigeria (17), Cameroon (18), Mozambique (21), and Ethiopia (25). The discrepancy could be due to educational status, i.e., the presence of a high rate of illiteracy in the study region (38), and the COVID-19 pandemic may also have disrupted malaria prevention efforts by making it more difficult to provide health education about the symptoms, transmission, and prevention of malaria (5), and the study areas, which are rural pastoral areas. However, compared to earlier studies conducted in Senegal (16), Burkina Faso (19), South Africa (20), Tanzania (22), and southern Ethiopia (23), the magnitude of the current study's knowledge of malaria prevention was higher. This variation could be due to different study participants, such as in age group and gender, as well as different outcome measures or definitions and different study periods. Another reason could be that long-term residence in a malaria-endemic region exposed study participants to the disease's symptoms and transmissions there. The current findings on knowledge of malaria prevention were consistent with a study conducted in the Amhara region of Ethiopia (24).

In the current finding, 46.9% of study participants exhibited a positive attitude. This magnitude is lower than previous studies conducted in African nations (13, 15–18, 20). The reasons for this variation could be due to different study participant characteristics, such as the fact that some participants were adults in some studies (17, 20, 22), adolescents or children in others (15, 16), and only women in another study (18), as well as the pastoralist life styles and cultures of the current study participants. The other reasons may be variations in outcome measurement, the disruption of malaria prevention and control efforts as a result of COVID-19 (5), and differences in study setting. Furthermore, the current study's magnitude of attitude toward malaria prevention was lower than studies conducted in different parts of Ethiopia (23–25). The reason for this discrepancy may also be that the use of different tools to measure attitude and the effect of COVID-19 on health services led to the neglect of malaria prevention efforts (5).

The present study showed that 17.3% of participants had good malaria prevention practices (bed net use). This result is significantly lower than previous studies conducted in the Africa nations (15–19, 21, 22), as well as studies in Ethiopia (23–25). The difference may be the measurement of malaria prevention practices; in the current study, only bed nets were used to measure malaria prevention practices, but other malaria prevention activities may have been used in other studies. The adoption of traditional methods (27) and self-medication (26) may be other factors contributing to this disparity. Despite the fact that using bed nets to combat malaria is an efficient method in places where the disease is endemic (11), only a few households among the study participants were using bed nets, which may be a reason for the high malaria prevalence in the region (9). The other reason for these poor malaria prevention practices may also be the COVID-19 pandemic, which disrupted malaria preventive services (5), leading to a challenge in distributing bed nets to households. Lack of access to healthcare services brought on by a scarcity of healthcare facilities and medical professionals could also impede households from engaging in malaria prevention (33). On the other hand, the magnitude of bed net use practices among the current study participants was higher than in a previous study in South Africa (20). The perceived favorability of indoor residual spray (76%) and other malaria preventive methods over bed netting among South African study participants may be the cause of this variation.

In this study, 56.9% of study participants had good healthcare seeking behaviors, which is lower than the previous studies conducted in Senegal (16) and Nigeria (17). Moreover, the percentage of study participants with good healthcare seeking behaviors was lower than an earlier study in the Afar region that stated that most Afar people (71%) preferred to receive their medical care from modern health facilities (27). The participants' favorable attitudes regarding the use of self-medication (26) and traditional medication practices may be the cause of the difference. The low healthcare seeking practices in the present study led to a decrease in the use of medication for malaria prevention strategies implemented in malaria-endemic areas (11). Due to these reasons and the study area's favorable environmental conditions (due to little rainfall) (2, 10), malaria transmission might have increased there (9). Therefore, the uptake of healthcare services should increase, and in turn, the use of antimalarial drugs should improve. Eighteen percent of study participants in this study never visited health facilities when they encountered malaria symptoms, which is comparable with the previous study conducted in the Afar region of Ethiopia, in which a total of 19% of participants (3% used self-medication and 16% used traditional practices) did not use health care facilities (27). The absence of health facilities and financial constraints may also be contributing factors for the 18% of the study participants who never went to a health facility when they experienced malaria symptoms (33).

In the current study, low-income and illiterate household heads were more likely to have poor malaria prevention knowledge than secondary school and high-income participants, respectively. This evidence is in line with the previous findings (16, 18, 19, 22–24). Government programs that can improve economic and educational opportunities should be established to advance malaria prevention knowledge in the Afar region. Poor malaria prevention knowledge was significantly associated with poor malaria prevention practices (poor bed net use). This evidence is consistent with the previous findings that poor knowledge resulted in poor malaria management (29). Household heads should be given community health education about malaria to get adequate knowledge of malaria prevention that aids in combating malaria (29).

Participants who were married or illiterate had a statistically significant association with a negative attitude toward malaria prevention. This finding is in line with a previous study (16). The participants' attitudes that are more supportive of traditional medicine (27) and self-medication (26) may be the cause of this negative attitude toward malaria prevention. Household heads in the age range of 18–25 years old were more likely to have poor healthcare seeking behaviors than older age participants (≥46

years). The implementation of age-specific malaria healthcare-seeking intervention strategies is necessary, with a particular emphasis on young adults. Pastoralist participants were also less likely to have poor healthcare-seeking behaviors.

The study's limitation is its cross-sectional nature, which reflects data obtained at a certain time point and may alter in subsequent periods, which can result in some bias, including recall bias. A cause-and-effect relationship was also not examined. A longitudinal study design should thus be conducted to examine the causality of the proposed relationship. It used the head of the household as a stand-in for the knowledge, attitude, and practices regarding malaria prevention that all household members possess. It would have been preferable to adopt a sample strategy that included a wider range of adults from the study community, but due to a lack of financing, this was not achievable. As a result, the findings could not accurately reflect the viewpoints of the entire community. Due to the fact that some of the data included in the study was self-reported by the participants, the estimates may have been overestimated or underestimated. For instance, the participants may have overstated the social desirability of bed net use because it was self-reported. Furthermore, using closed-ended questions may limit the range and depth of responses, omit relevant contextual information that isn't covered by the options, introduce researcher bias or other questionnaire-related factors, and reduce respondents' engagement and interest. The questionnaire wasn't also developed to evaluate the quality of bed nets, such as bed net insecticide and the presence of holes. However, this study is the first in the region where malaria transmission is high, and it is used as an input to establish intervention strategies to improve malaria prevention knowledge and increase uptake of bed nets. Health promotion-based interventional study designs (pre-test and post-test) should be carried out in the future to improve malaria prevention knowledge and attitude. This will help increase the uptake of bed net usage.

Conclusion

Although the majority of participants had good knowledge of malaria prevention, there are still a significant number of participants with poor knowledge of malaria prevention. Nearly half of participants had good healthcare-seeking behavior and a positive attitude toward malaria prevention. Only a small percentage of individuals used bed nets regularly to avoid malaria. Economic reform and community health education were required to address issues with low-income and illiterate household heads' poor knowledge and married and illiterate participants' negative attitudes. Young adults' poor healthcare-seeking behavior should also improve in order to promote malaria prevention.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was obtained from a Research and Ethics Review Committee of the Health Science College, Samara University, Ethiopia. All methods were performed in accordance with the relevant guidelines and regulations. Informed consent was obtained from all the study participants. No one was harmed as a result of participating in this study. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

DA: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing—original draft, Writing—review and editing. TG: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing—original draft, Writing—review and editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

The authors like to thank all study participants and other individuals involving in any process of this study.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Tropical Diseases

TYPE Original Research
PUBLISHED 29 January 2024
DOI 10.3389/fitd.2024.1287025

OPEN ACCESS

EDITED BY
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RECEIVED 01 September 2023
ACCEPTED 08 January 2024
PUBLISHED 29 January 2024

CITATION
Hugo LE, van Huyssteen K, Oloniniyi O, Donnelly L, Conn A, Collins KA, Mitchell H, McCarthy JS and Macdonald J (2024) Rapid low-resource detection of Plasmodium *falciparum* in infected *Anopheles* mosquitoes. *Front. Trop. Dis* 5:1287025.
doi: 10.3389/fitd.2024.1287025

Rapid low-resource detection of Plasmodium *falciparum* in infected *Anopheles* mosquitoes

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Vector surveillance of Plasmodium *falciparum* is critical for monitoring and reducing one of the most severe forms of malaria, which causes high morbidity and mortality in children under five and pregnant women. Here we developed a rapid and highly sensitive test for the detection of *P. falciparum (Pf)*-infected mosquitoes (Rapid *Pf* test), with high suitability for low-resource vector surveillance implementation. The Rapid *Pf* test had similar analytical sensitivity to laboratory-based tests, detecting down to 4 copies/µL of a 18S rRNA DNA standard. In addition, the Rapid *Pf* test could be completed in less than 30 minutes, and only required a liquid sample preparation reagent, pestle, tube, and 39°C heating block for operation, indicating amenability for low-resource implementation. Diagnostic testing was performed using *Anopheles stephensi* mosquitoes, either uninfected, or fed with *P. falciparum* gametocyte cultures. These *P. falciparum* fed mosquitoes were determined to have 79% infection prevalence based on parallel microscopy and qPCR testing on a subset of 19 mosquitoes. However, our Rapid *Pf* test determined a 90% positive test rate when testing individual infected mosquitoes (n=30), and did not detect 40 uninfected mosquitoes regardless of blood-fed status (n=40), suggesting the true prevalence of infection in the mosquitoes may have been higher than calculated by qPCR and microscopy. The Rapid *Pf* test was demonstrated to detect infection in individual mosquitoes (both fresh and frozen/thawed), as well as pools of 1 infected mosquito mixed with 19 known uninfected mosquitoes, and individual mosquitoes left in traps for up to 8 days. After testing on infected and uninfected mosquitoes (n=148) the Rapid *Pf* test was conservatively estimated to achieve 100% diagnostic sensitivity (95% confidence interval, CI: 91%-100%) and 97% diagnostic specificity (CI: 92%-99%) compared to the estimated prevalence from combined microscopy and qPCR results. These results indicate the Rapid *Pf* test could provide a highly effective tool for weekly surveillance of infected mosquitoes, to assist with *P. falciparum* monitoring and intervention studies.

KEYWORDS

isothermal (DNA) amplification, rapid test, malaria - mosquito vectors, rapid sample preparation, nucleic acid lateral flow, *Plasmodium* falciparum

Introduction

Malaria is a mosquito-borne disease that causes significant morbidity and mortality, especially in sub-Saharan Africa (1, 2). Global malaria incidence decreased by 27% between 2000-2015, the result of measures funded by initiatives including Roll Back Malaria, 2000, the Global Fund, 2002, and the United States Presidents Initiative against Malaria, 2005 (3). Concerningly, global malaria incidence increased from 2020-2021, due in part to the disruption of malaria prevention and control strategies by the COVID-19 pandemic (2, 4). In 2021, there were an estimated 247 million cases of malaria, 619,000 deaths and with approximately 95% of these cases occurring in Africa (2).

The causative agents of malaria are protozoan parasites of the genus Plasmodium, which are transmitted by female *Anopheles* mosquitoes. Of the five species of protozoan parasites that cause human malaria, *P. falciparum* causes the most severe form of the disease with high morbidity and mortality, especially in children under five and pregnant women. Mosquitoes ingest gametocytes while blood feeding on an infected person. The sexual stage of the parasite life cycle is completed in the mosquito as the gametocytes develop through a series of intermediate stages into oocysts embedded in the walls of the mosquito stomach, or midgut. Sporozoites emerge 10-12 days later (5). Sporozoites migrate to the salivary gland of the mosquito and are transmitted to new host when the mosquito takes its next blood meal. Given the crucial role mosquitoes play in the transmission of malaria, vector control remains an important method in the quest to eradicate malaria (6). Indoor residual spraying (IRS), insecticide treated nets (ITNs), and larval source management are critical entomological tools to achieve this goal (7).

Monitoring the prevalence of Plasmodium infection in mosquito populations is a critical part of the management of malaria. Detection of Plasmodium prevalence in mosquitoes can be applied as a xenomonitoring tool for detecting the presence of human reservoirs of the parasite. Sensitive and non-invasive detection of reservoirs is becoming increasingly important as nations approach malaria elimination.

There are several methods for detection of Plasmodium parasites in mosquitoes. The traditional method and gold standard for detection is dissection and microscopic examination of mosquito midguts for the presence of oocysts (indicating infection of the mosquito) and salivary glands for the presence of sporozoites (indicating the potential for onward transmission of the parasite) (8). However, these methods are time consuming and require expert technical assistance. Circumsporozoite protein enzyme-linked immunosorbent assay (CS-ELISA) is another method which is used to detect circumsporozoite antigen, but there are issues with detection when parasites are in low abundance (9). A number of PCR assays have been developed for the detection of Plasmodium in mosquitoes. In particular, the nested PCR assay designed by Snounou et al. (10) is considered the gold standard among PCR assays for its sensitivity, however post PCR processing of samples is required and the assay requires hours to complete. Newer quantitative PCR (qPCR) assays provide sensitive detection and the ability to differentiate between the separate species of Plasmodium without any requirement for post PCR processing (11–14). These assays, however, require the use of expensive thermocyclers and are not suitable for use in resource poor settings. Several rapid diagnostic tests (RDTs) based on immuno-chromatography assays have been developed for the detection of human infection with Plasmodium, including tests targeting *P. falciparum* histidine rich protein 2 (HRP2) and Plasmodium lactate dehydrogenase (pLDH) (15–17). However, these tests have not been applied to test infections in mosquitoes and in some cases antigens may not be expressed during the mosquito life stages. A dipstick enzyme immunoassay based test was developed to detect sporozoite protein antigens from *P. falciparum* and two *P. vivax* strain infections in mosquitoes (18, 19). This was developed commercially as the VectorTest (formerly Vectest™) Malaria Antigen Panel (VecTOR Test Systems, Thousand Oaks, CA, USA). Rigorous testing demonstrated VecTest to have equivalent sensitivity with marginally lower sensitivity than CS-ELISA (20). However, sensitivity was substantially lower when compared to PCR (21). Novel diagnostic assays include isothermal nucleic acid-based tests that offer the sensitivity of PCR combined with portability and applicability to low-resource settings. These include tests based on Loop mediated isothermal amplification (LAMP) and Recombinase Polymerase Amplification (RPA) (22). These technologies have not been applied for the detection of Plasmodium in mosquitoes or applied in a limited capacity.

Here we report the development of a test for the detection of *P. falciparum* (Rapid *Pf* test) in mosquitoes that is suitable for low-resource implementation and offers equivalent sensitivity to PCR. The test uses a novel 10-minute sample preparation method that requires only tube, pestle, and a liquid reagent (23–28). Sensitive 10-minute isothermal amplification of the Plasmodium 18S rRNA gene is performed using recombinase polymerase amplification (RPA) followed by lateral flow detection, as described for other viruses and bacteria (23–34), thus requiring only a single temperature heating block for operation. In this study, we report analytical sensitivity of the test, and demonstrate detection of *P. falciparum* in experimentally infected *Anopheles stephensi* mosquitoes. Mosquito testing was performed both individually and in pools, using freshly-infected and frozen mosquitoes, as well as mosquitoes left in traps for up to 8 days in a simulated field environment.

Materials and methods

Mosquitoes

An. stephensi (Sind-Kasur strain) were obtained from the University of Nijmegen, The Netherlands, and maintained in colony at the QIMR Berghofer Medical Research Institute insectary at 27°C ± 1, 70-80% relative humidity and 12:12 hour day:night light cycle. Adult mosquitoes were fed on 8% sucrose with

para-aminobenzoic acid (PABA) (14). Female mosquitoes were collected at 3-4 days old, starved for 15 hours, and 100 mosquitoes were placed into paper cups with gauze lids. For *P. falciparum*-exposed mosquitoes, a mosquito blood-meal (0.22% mature gametocytemia) was prepared by mixing 650 µL of Plasmodium gametocyte infected red blood cells obtained from a *P. falciparum* (NF54 strain) gametocyte culture with 650 µL of AB blood serum. Mosquitoes were allowed to feed on the blood-meal maintained at 37°C via an artificial membrane feeding apparatus for 30 minutes. After feeding, mosquitoes that were not engorged were discarded and 110 remaining mosquitoes were incubated in an environmental growth chamber (Panasonic MLR-352H-PE, Panasonic, Japan) set at 28 ± 1°C, 75% relative humidity and 12:12 hr light cycle for 10 days.

After incubation, (1) *39* P. *falciparum*-exposed mosquitoes were dissected to observe oocysts in midguts, and of these, 19 were frozen and then tested by qPCR to determine the prevalence of Plasmodium infection; (2) Forty-eight *P. falciparum*-exposed mosquitoes were used to trial the RPA test: (i) 20 were tested immediately; (ii) 10 were frozen at –20°C for 7 days and then thawed before testing; (iii) 9 were used for testing pools of mosquitoes, and (iv) 9 were left in traps for 8 days in a chamber that simulated a tropical environment, as described in the next paragraph.

We tested the performance of the Rapid *Pf* test in a real-world scenario in which mosquitoes were tested only after a holding period in a mosquito trap under field conditions. We placed mosquito samples at 10 d after blood-feeding into gauze bags and placed the bags into a BG Sentinel mosquito trap (Biogents AG, Regensburg, Germany) positioned inside a second environmental growth chamber that was set to simulate tropical ambient conditions, including a daily cycling temperature profile varying between 23.5-31°C, 70% relative humidity and 12:12 hr day: night lighting.

RPA oligonucleotides

De-Cifer *P. falciparum* RPA primers and probe (BioCifer Pty Ltd, Brisbane, QLD) were used in the Rapid *Pf* test, which target the 18S rRNA gene. A 157bp DNA sequence from *P. falciparum* 18S rRNA gene (accession number KJ170099.1) cloned into the pBIC-A plasmid, was used as the 18S rRNA DNA standard. The concentration (18S gene copies per ml) of the resuspended plasmid was determined using the dsDNA HS Assay Kit (Life Technologies, Singapore).

Microscopy

Mosquitoes were dissected 10 d after feeding, cold-anaesthetized and dissected to remove midguts into PBS. The midguts were then stained using 0.5% mercurochrome in PBS and examined to determine the presence and quantity of oocysts using a brightfield compound microscope.

Quantitative PCR assay

Quantitative PCR was performed as described by Wang et al. (14) with some modifications. DNA was extracted from dissected midguts using the QIAGEN DNeasy blood and tissue DNA extraction kit (QIAGEN, Hilden, Germany). Samples were homogenized in 50 µl of QIAGEN ATL buffer using a micropestle and stored at -20°C. Samples were thawed, 130 µl of QIAGEN ATL buffer and 20 µL Proteinase K was added to each tube and samples were incubated at 56°C overnight. DNA was then extracted from samples according to the manufacturer's instructions. Quantitative PCR was performed using the QIAGEN QuantiNova Probe qPCR kit. qPCR reactions consisted of 7.5 µl of QIAGEN QuantiNova 2x qPCR master mix, 1.5 µl of 1:1 dilution of DNA and QuantiNova yellow sample dilution buffer, 0.1 µM of each primer and 0.1 µM of a CY5-conjugated Taqman probe targeting a conserved region of the *P. falciparum* 18S gene (35) in a total volume of 15 µl. The qPCR assay was performed on a Corbett Rotorgene 6000 thermocycler (Corbett Research, Sydney, Australia) under the following conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s.

Rapid *P. falciparum* test

Sample preparation

P. falciparum fed individual whole mosquitoes were crushed with a disposable blue polypropylene tube pestle (Sigma-Aldrich, Castle Hill, NSW, AU), in 50 µL of TNA-Cifer Reagent (BioCifer Pty Ltd, Brisbane, QLD), and mosquito pools (1 P. *falciparum*-exposed mosquito with 19 uninfected mosquitoes, or 20 uninfected mosquitoes) were crushed in 200 µL TNA-Cifer Reagent. Samples were incubated for 10 minutes at room temperature, and then 10 µL was added to 40 µL of RNAse and DNAse-free water and 1 µL immediately used for isothermal amplification.

Isothermal amplification

Sample (1 µL) was mixed with 8 µL recombinase polymerase amplification (RPA) mixture, followed by addition of 1 µL 140 mM Magnesium acetate to start the reactions, and incubation at 39°C for 10 mins. The final concentration of reactants in each tube was: 420 nM Forward and Reverse primers, 120 nM probe, 1x Rehydration buffer & pellet mixture, and 14 mM Magnesium acetate.

Lateral flow detection

Immediately following incubation, 2 µL of the amplicons were transferred to the sample pad of a HybriDetect lateral flow strip (Milenia Biotec GmbH, Gießen, Germany), which detects DNA dual labelled with Biotin/fluorescein. Strips had been pre-prepared by the addition of 8 µL blocking buffer (0.4% casein, 0.1% Tween in PBS, pH 9) to the sample pads of the strips (32). Strips were subsequently placed into tubes with the sample pads immersed in 100 µL of borate buffer (100 mM H_3BO_3, 100 mM $Na_2B_4O_7$, 1% BSA, 0.05% Tween 20, pH 8.8), and left to wick along the strips for 5 minutes before appearance of test bands was observed by eye and imaged.

Data analysis

Strips were imaged using the MultiDoc-It™ Digital Imaging System (Upland, CA, USA) or a flatbed scanner (Hewlett-Packard) and analyzed using ImageJ software (National Institutes of Health, MD, USA). Greyscale-converted images were used to determine band-intensity, by measuring the mean grey value (limit to threshold), using a fixed area measurement, and subtracting from the maximum threshold value. For each test band, an average of the neighboring white space of all LF strips in that experiment was subtracted from the band intensity to normalize the results. To define a sample as positive the test band values were standardized by subtracting a cutoff value (three standard deviations above the average of the negative control test band intensities). A standardized value of greater than 0 was a positive result.

Comparative analysis of tests was performed using a diagnostic sensitivity and specificity calculator with exact Clopper-Pearson 95% confidence intervals (36).

Results

A recombinase polymerase amplification-lateral flow test for *P. falciparum*

To develop a rapid and sensitive test for *P. falciparum*, we chose recombinase polymerase amplification (RPA) followed by lateral flow detection (LFD) (29–34), which enables sensitive and rapid amplification, but requires only a simple heating block for operation. Analytical sensitivity of our *Pf* RPA-LFD test (Figure 1) using a quantified DNA standard indicated strips producing clearly visible test lines at all concentrations tested (Figure 1A). Analytical sensitivity analysis, as determined both by eye (Figure 1A) and by image analysis of pixel density (Figures 1B-D), indicated a limit of detection of 4 copies/µL (the lowest concentration evaluated).

Estimated infection prevalence in mosquitoes fed on *P. falciparum* gametocyte culture

To evaluate our developed test for detection of infected mosquitoes, we first had to determine the infection prevalence of *P. falciparum* in a batch of *P. falciparum*-exposed *An. stephensi* mosquitoes, which had fed on *P. falciparum* gametocyte cultures and harvested 10 days post blood-meal. Microscopy was performed on 39 *An. stephensi* mosquitoes fed on *P. falciparum* gametocyte cultures. A total of 24 mosquitoes were observed to have oocysts in their midgut (oocysts range 1 – 19, mean 4), indicating a microscopy positive prevalence rate of 62% (24/39; Table 1). While microscopy is considered the gold standard of detection of Plasmodium in mosquitoes, there is the possibility of failing to identify oocysts that have not stained well, or are not clearly visible, especially when present at low numbers. Therefore, DNA was

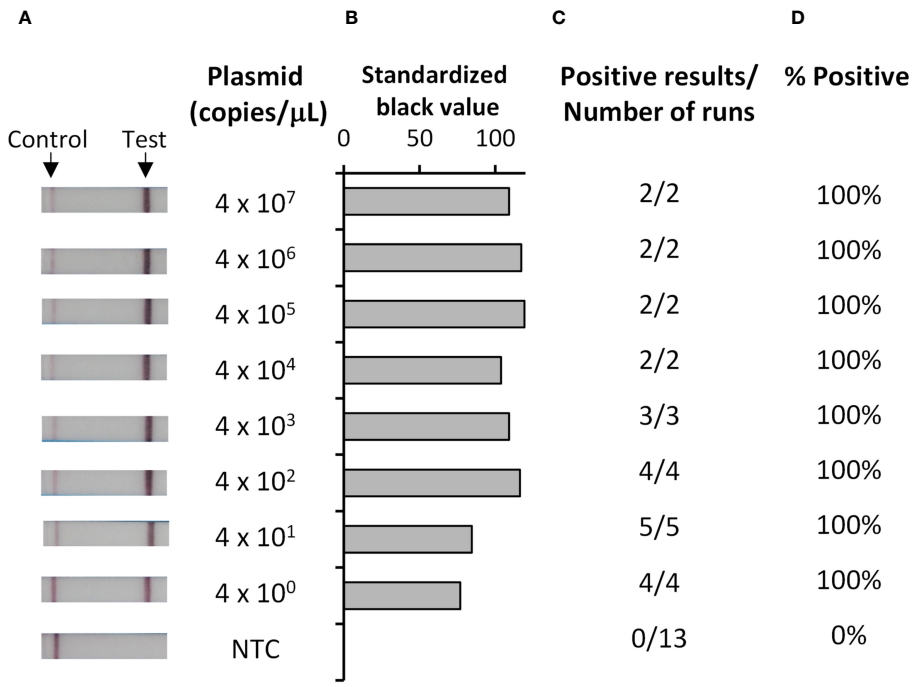

FIGURE 1
Analytical sensitivity of the *Pf* RPA-LFD test was 4 copies/µL. Ten-fold dilutions of plasmid containing a portion of the *P. falciparum* 18S rDNA target gene, or a water no template control (NTC), were tested with the *Pf* RPA-LFD test. Representative photographs of resultant lateral flow strips **(A)** show position of control and test lines alongside plasmid concentration (copies/µL) from which standardized black value **(B)** was determined by ImageJ analysis of test line. The number of times a positive result was obtained per total number of replicate experiments **(C)** was used to determine percentage positive tests **(D)**.

extracted from the midguts of 19 of the mosquitoes already examined by microscopy and evaluated using qPCR, resulting in 14 mosquitoes testing positive by qPCR (Table 2) and indicating a qPCR-positive prevalence rate of 73% (14/19). Two additional microscopy negative mosquitoes were detected as positive by qPCR, and one qPCR negative result contained the largest oocyst number by microscopy, indicating this sample was also positive. By combining results, we determined four mosquitoes were negative by both tests, suggesting an estimated "true" infection prevalence of 79% (15 positives out of 19 mosquitoes; Table 2).

Low-resource sample preparation enables a Rapid *Pf* test for detecting infected mosquitoes

To construct our low-resource Rapid *Pf* test for detection of infected mosquitoes, our *Pf* RPA-LFD was combined with a novel TNA-Cifer Reagent (BioCifer Pty Ltd, Brisbane, QLD), which enables low-resource sample preparation for molecular testing (23–28). Thirty *P. falciparum*-exposed mosquitoes, from the same batch assessed previously by qPCR and microscopy, were tested (20 tested fresh, and 10 frozen and subsequently thawed for testing; Figure 2). Of the *P. falciparum*-exposed mosquitoes, 27 were positive using the Rapid *Pf* test, indicating a 90% prevalence of *P. falciparum* in the blood-fed mosquitoes (Table 1). We also tested 40 known uninfected mosquitoes (20 fed on uninfected blood cultures, and 20 not blood-fed; Supplementary Figure S1), which produced a clear negative result for all uninfected mosquitoes tested, demonstrating 100% diagnostic specificity (95% Confidence interval, CI: 91% - 100%; n=40). These results indicated that the crushed blood-fed mosquito background did not affect test specificity, and that any observable band, regardless of intensity, was indicative of a mosquito that had been fed on *P. falciparum*. A comparison of microscopy, qPCR and our Rapid *Pf* test for determination of infection prevalence in the mosquitoes is shown in Table 1. Comparing our Rapid *Pf* test results to the "gold standard" combined microscopy and qPCR determined infection rate of 79%, our Rapid *Pf* test would be determined have a diagnostic sensitivity of 100% (95% confidence interval, CI: 86-100%) and 93% specificity (CI:82%-99%). However, as our testing showed improved sensitivity compared to the estimated prevalence from microscopy and qPCR, and did not have false positives when detecting known uninfected mosquitoes, our test is likely more accurate compared to the observed microscopy and qPCR results, with 100% diagnostic sensitivity (CI: 87% to 100%) and 100% diagnostic specificity (CI: 92% to 100%).

Detection of *P. falciparum* in mosquito pools and mosquitoes left in traps

Parasite detection in wild caught mosquitoes is usually performed from pools of mosquitoes, due to the large number collected for examination in the field. We therefore tested if our Rapid *Pf* test could detect parasites when a single *P. falciparum*-exposed mosquito was pooled with 19 known uninfected mosquitoes. Because of the larger sample mass, pools were homogenized in 200 µL TNA-Cifer Reagent. Eight of 9 pools were determined to be positive (Figure 3, top panel), indicating an 89% positive rate, which was consistent with the individual mosquito testing estimates. All three pools containing only uninfected mosquitoes were negative, confirming the specificity of the Rapid *Pf* test. We also sought to determine if the rapid *P. falciparum* test could detect infected mosquitoes left in traps for up to a week, to simulate a weekly testing regime. Individual *P. falciparum*-exposed mosquitoes were left in traps for 8 days in a chamber that simulated a tropical environment. Six of 9 individual *P. falciparum*-exposed mosquitoes tested using the rapid *P. falciparum* test were positive after being held in these conditions (Figure 3, bottom panel). This 67% positive rate is lower than the positive rate obtained from individual and fresh pool testing, but within the range of the standard error, indicating the rapid *P. falciparum* test could be informative for weekly trap testing regimes. A summary of results from all mosquito trials and the resultant positive rate is provided in Table 3. Using the results from all Rapid *Pf* trials combined, and comparing our test results to the observed combined microscopy and qPCR determined infection rate of 79%, our test is conservatively calculated to have a diagnostic sensitivity of 100% (CI: 91-100%) and 97% specificity (CI:92%-99%).

TABLE 1 Performance of Microscopy, qPCR and Rapid *Pf* test for estimation of infection prevalence.

Test method	*An. stephensi* mosquito testing result[a]				Infection prevalence determined by each test
	Mosquito exposure status	Total number tested	Positive result	Negative result	
Microscopy	*P. falciparum*-exposed	39	24	15	62%
qPCR	*P. falciparum*-exposed	19	14	5	74%
Microscopy + qPCR	*P. falciparum*-exposed	19	15	4	79%
Rapid *Pf* test	*P. falciparum*-exposed	30	27	3	90%
	Uninfected	*40*	*0*	*40*	0%

(a) Test results obtained when testing *An. stephensi* mosquitoes fed on *P. falciparum*-gametocyte cultures (*P. falciparum*-exposed) or known uninfected mosquitoes (Uninfected).

TABLE 2 Estimation of Infection prevalence for *P. falciparum*-exposed *An. stephensi*, determined by microscopy and qPCR on a subset of 19 mosquitoes.

		Microscopy		Total
		Positive	Negative	
qPCR	Positive	12	2	14
	Negative	1	4	5
	Total	13	6	19

Discussion

Elimination of malaria is an expressly stated goal in both the WHO Millennium Development Goals (MDG) and the current Sustainable Development Goals (SDG; Target 3.3) (37). To achieve these goals, improved vector surveillance and monitoring/evaluation of interventions aimed at vector control is critical. We sought to improve vector surveillance capabilities by developing a rapid test for detecting mosquitoes infected with *P. falciparum*, which causes the most severe form of malaria, causing high

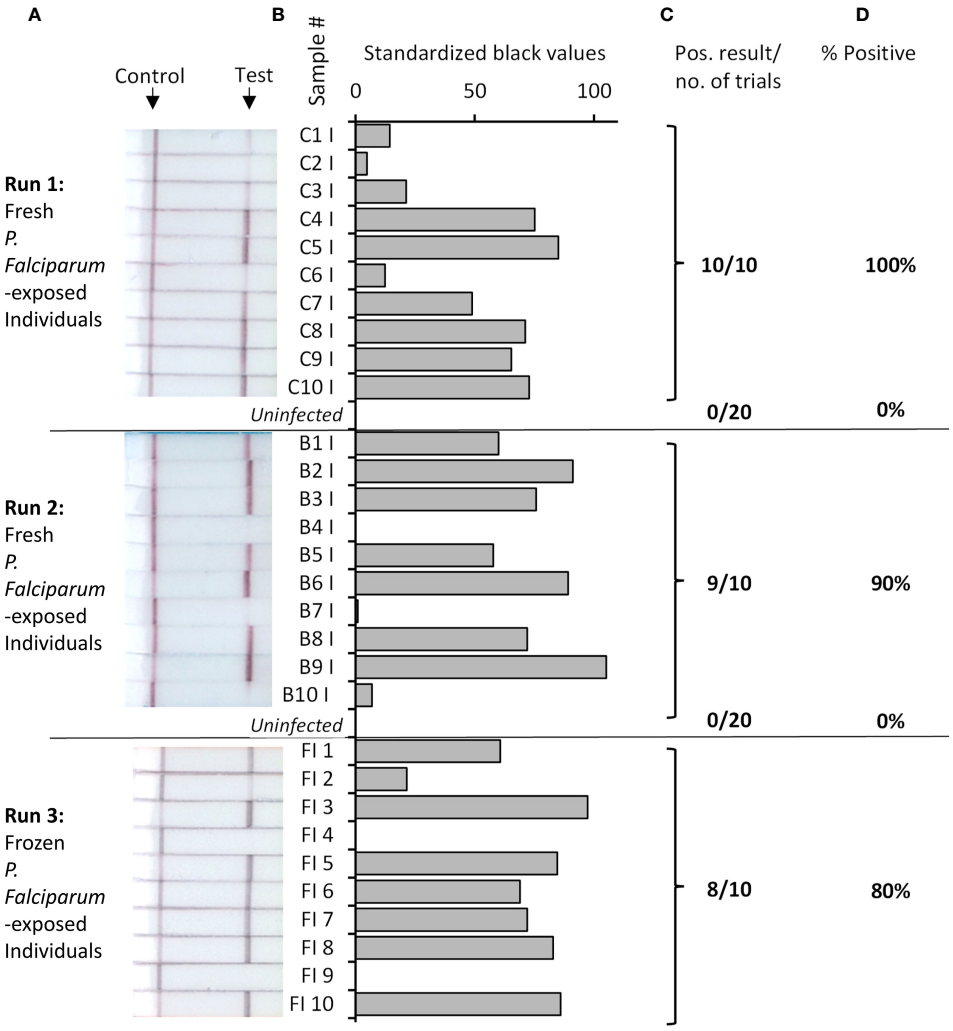

FIGURE 2
Rapid *Pf* test applied to detection of *P. falciparum*-exposed or uninfected *An. stephensi* mosquitoes. Fresh or frozen *P. falciparum*-exposed mosquitoes were tested in batches as indicated, alongside uninfected mosquitoes (pictures of all uninfected mosquitoes strips provided in Supplementary Figure S1). Photographs of resultant lateral flow strips **(A)** show position of control and test lines alongside mosquito sample groups from which standardized black value **(B)** was determined by ImageJ analysis of test line. The number of times a positive result was obtained per total number of samples tested **(C)** was used to determine percentage positive **(D)**.

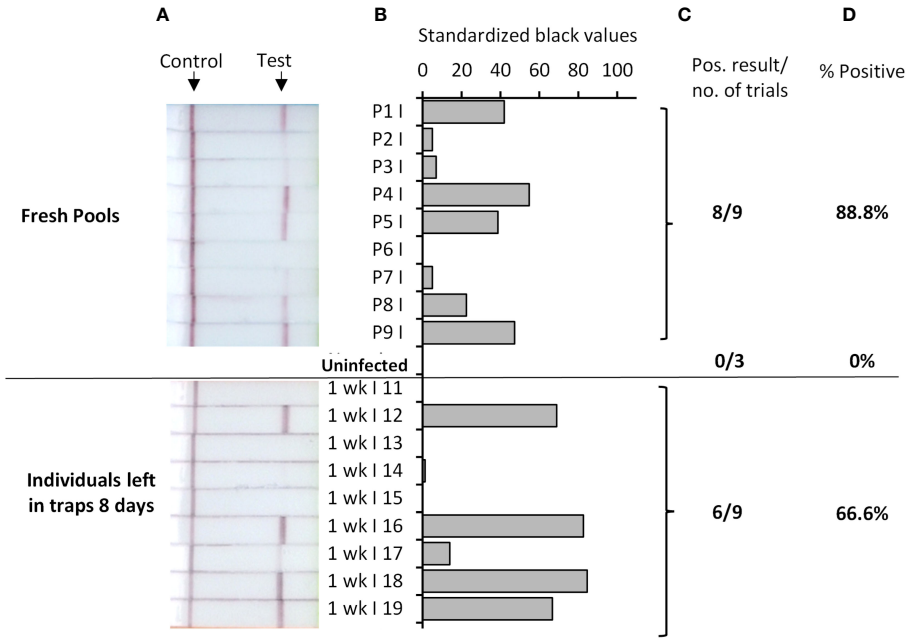

FIGURE 3
Rapid *Pf* test applied to detection of *P. falciparum*-exposed *An. stephensi* mosquitoes in pools (top), or when left in traps for up to 8 days (bottom). Top panel shows test results for *P. falciparum*-exposed pools that each contained one *An. stephensi* mosquito fed from *P. falciparum* gametocyte blood cultures, mixed with 19 known uninfected mosquitoes (or 20 known uninfected mosquitoes; Uninfected pools). Bottom panel shows results from testing individual *P. falciparum*-exposed *An. stephensi* mosquitoes (fed with *P. falciparum* infected blood cultures), that were subsequently left in environmental chambers simulating a tropical environment for 8 days, prior to testing. Photographs of resultant lateral flow strips **(A)** show position of control and test lines alongside mosquito sample groups from which standardized black value **(B)** was determined by ImageJ analysis of test line. The number of times a positive result was obtained per total number of samples tested **(C)** was used to determine percentage positive **(D)**.

morbidity and mortality in children under five and pregnant women. Here we report a rapid *P. falciparum* test (Rapid *Pf* test) with specifications equivalent to laboratory-based tests, but with potential to be implemented in low-resource settings and be effective under the rigors of standard vector screening protocols, such as testing of mosquitoes collected in traps and left for up to one week, or processing large numbers of mosquito pools.

Our Rapid *Pf* test is a molecular test, yet suitable for implementation in low-resource settings. The test employs a novel TNA-Cifer Reagent that prepares the mosquito sample for molecular testing in as little as 10 minutes, using only a liquid reagent, tube, and pestle. This reagent has previously been demonstrated useful for detection of viruses (23–28), and here we report, for the first time, the utility of the reagent for detection of a

TABLE 3 Positive test rate of the Rapid *Pf* test when applied to the different trials performed in this study.

Mosquito trial	*An. stephensi* mosquito testing result[a]				Positive test rate
	Mosquito exposure status	Total number mosquitoes or pools tested	Positive result	Negative result	
Individual	*P. falciparum*-exposed	30	27	3	90%
	Uninfected	40	0	40	0
Pools	*P. falciparum*-exposed pools (#E + #U)[b]	9	8 (8 E + 152 U)	1 (1 E+ 19 U)	89%
	Uninfected pools (#E + #U)[b]	3 (0 E + 60 U)	0	3 (0 E + 60 U)	0
8 days old	*P. falciparum*-exposed	9	6	3	67%
All mosquito trials combined	*P. falciparum*-exposed	48	41	7	85%
	Uninfected[c]	100	0	100	0

(a) Test results obtained when testing *An. stephensi* mosquitoes fed on *P. falciparum*-infected blood cultures (*P. falciparum*-exposed, E) or known uninfected mosquitos (Uninfected, U); noting qPCR and microscopy estimated prevalence of *P. falciparum* in this batch of infected mosquitoes was 79% (see Table 2). (b) *P. falciparum*-exposed pools contained 1 *P. falciparum*-exposed (E) mosquito and 19 uninfected (U) mosquitoes; uninfected pools contained 20 uninfected mosquitoes; (c) total number of uninfected includes the sum of all individual uninfected mosquitoes tested in uninfected pools.

parasitic infection. After sample preparation, a simple dilution step reduces inhibitory amounts of reagent and mosquito debris, allowing an isothermal amplification protocol that requires incubation for only 10 minutes using a simple 39° C heating block. Detection is performed using a lateral flow strip, such that the appearance of a test band indicates presence of *P. falciparum*. The simplicity of the test is exemplified when considering what equipment is not required for testing, including standard DNA extraction and testing equipment: centrifuges, silica membrane spin-columns, magnetic beads, and thermocycling machines. In addition, the entire test, from sample to result, can be performed in less than 30 minutes.

Testing of detection parameters in this study demonstrated excellent analytical sensitivity, down to 4 copies/µL of a *P. falciparum* 18S rRNA DNA standard. Diagnostic testing of *P. falciparum*-exposed or known uninfected individual mosquitoes indicated the Rapid *Pf* test detected more positives than would have been estimated by microscopy and qPCR, resulting in 100% diagnostic sensitivity (CI: 86%-100%), but lowering the diagnostic specificity (93%; CI: 82-99%). Further pool testing, however, continued to demonstrate excellent diagnostic specificity when testing known uninfected mosquitoes. Combining all testing results together (n=148), the diagnostic sensitivity was conservatively calculated to be 100% (CI: 91-100%), and diagnostic specificity was 97% (CI: 92%-99%). Testing mosquito pools demonstrated the Rapid *Pf* test could detect a single *P. falciparum*-exposed mosquito when mixed with 19 uninfected mosquitoes, giving the same diagnostic sensitivity and specificity of testing compared to individual mosquito testing. In addition, we demonstrated the Rapid *Pf* test could detect mosquitoes left in tropical humidity conditions for up to 8 days with similar positive rates to microscopy, qPCR, and fresh or frozen/thawed Rapid *Pf* test results. These results indicate our Rapid *Pf* test is highly suitable for low-resource implementation, and could enable effective weekly surveillance of *P. falciparum* prevalence in hot-spot areas.

The diagnostic specificity and sensitivity of our Rapid *Pf* test is class-leading for rapid assays detecting Plasmodium in mosquitoes. The only commercial rapid assay specifically designed for detection of Plasmodium in mosquitoes is the Vectortest Malaria Sporozoite Antigen Assay. A large multicenter trial found the assay had an overall diagnostic sensitivity of 92% and specificity of 98.1% when compared against the circumsporozoite (CS) ELISA assay (19). However, sensitivities dropped to below 18% when compared against PCR as the gold standard (21). Our experiments were designed to overcome inherent difficulties in determining the "true" positives for the purpose of determined the diagnostic sensitivity and specificity of our assay. We calculated "true" prevalence from a combination of positive determined by microscopy (which can underestimate prevalence) and qPCR (which may detect residual non-oocyst DNA and can be affected by intracellular inhibitors). Like qPCR and other molecular assays, our Rapid *Pf* test may also detect *P. falciparum* residual DNA from mosquito feeding but previous research indicates this is unlikely (14). However, our observation that a single midgut containing a large number of oocysts was qPCR negative was unexpected, which could be due to a sample processing error or PCR inhibition from mercurochrome. Regardless, testing with our Rapid *Pf* test showed higher positive rates compared to both microscopy and qPCR combined, and did not show false-positive results when testing uninfected mosquitoes, regardless of blood-fed status. These results highlight the difficulty in determining diagnostic sensitivity and specificity when a new test shows improved results compared to the gold standard, which could result in potentially true positives being labelled as false positives.

To the best of our knowledge, our Rapid *Pf* test is the first assay to apply RPA and LF to the detection of Plasmodium infection in mosquitoes. As an indirect means of comparison, the diagnostic specificity of our Rapid *Pf* test was equivalent to an 18S *P. falciparum* RPA and LF assay developed for clinical application (38). Specificity to *P. falciparum* was 100% among genomic DNA samples obtained from a range of prokaryotic and eukaryotic organisms and sensitivity was 100 fg of gDNA. Similarly, an 18S RPA-LF assay against Plasmodium *knowlesi* achieved 100% specificity and sensitivity of 10 parasites per µl (39). As with other RDTs targeting Plasmodium 18S, the Rapid *Pf* test will detect all *P. falciparum* life stages present in mosquitoes and will not be limited to the detection of sporozoites. Without specific quantification of sporozoites, it is not possible to directly calculate the entomological inoculation rate for specific epidemiological studies (9). However, targeting *P. falciparum* 18S as a ubiquitous marker of infection provides increased sensitivity (39). Detection of 18S provides a sensitive assay for the detection of parasite reservoirs and a high-throughput means of determining infectivity. Strong agreement between 18S and CS ELISA detections from the same mosquitoes provides a rationale for testing 18S as a high throughput proxy measure for transmission (40). Oocyst infection intensities from our experimentally infected mosquitos were lower than that observed from a study of *Plasmodium* infection intensities in wild-caught mosquitoes (41). The arithmetic mean number of oocysts for our infected mosquitoes was 4.72 oocysts per midgut, whereas the mean number of oocytes that developed in wild-collected mosquitoes ranged from 10.3 to 14.7. Thus, while additional studies testing more *Anopheles* spp., including mosquitoes naturally infected with *P. falciparum*, are required to confirm test diagnostic sensitivity, our results suggest that our test will also perform well with field-caught infected mosquitoes.

Limitations of this study include the inability to use the same panel of mosquitoes for both PCR and rapid testing, as to demonstrate the simplicity of the TNA-Cifer Reagent sample preparation, an entire mosquito was used; testing if TNA-Cifer Reagent prepared samples could be used for parallel PCR testing would also be valuable, as this could assist with standard diagnostic sensitivity testing. Testing more replicates and pools for mosquitoes stored for 1 week, as well as frozen mosquitoes, would also be valuable; while our study showed some drop in the number of positives, however, these differences were not statistically significant due to the sample size tested, and were still equivalent to the infection rate of our mosquito population when estimated by PCR and microscopy. In addition, analytical specificity should be confirmed by testing mosquitoes infected with other *Plasmodium*

species and other pathogens. Further replicate studies (n=>20 at each DNA concentration) should be performed to confirm the true limit of detection as well as repeatability and reproducibility. In addition, a simple lateral flow strip reader would assist with low-resource interpretation of results. We also note that a low-resource test should also remain low cost, noting that cost is often country-dependent, and calculation of cost is outside the scope of this publication.

In conclusion, in this study we report a Rapid *Pf* test with similar analytical sensitivity to laboratory-based molecular testing; detecting down to 4 copies/µL of a 18S rRNA DNA standard. The Rapid *Pf* test was superior to microscopy and equivalent to qPCR, yet the entire Rapid *Pf* test could be completed in less than 30 minutes, and only required a liquid sample preparation reagent, pestle, tube, and 39°C heating block for operation, indicating suitability for low-resource implementation. Testing demonstrated excellent diagnostics sensitivity (100%, CI: 91-100%) and specificity (97%, CI: 92-99%) (n=148). The test successfully detected infection in individual mosquitoes both fresh and frozen/thawed, as well as pools of 1 *P. falciparum*-exposed mosquito mixed with 19 known uninfected mosquitoes. This, combined with detection of individual mosquitoes left in traps for up to 8 days, indicates our Rapid *Pf* test could provide a highly effective tool for weekly surveillance of *P. falciparum* levels in infected mosquitoes, to assist with elimination of malaria. Having demonstrated highly sensitive and robust detection of total *P. falciparum* life stages in mosquitoes, further developments will include detection of sporozoites to determine sporozoite rates.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

LH: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. Kv: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. OO: Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft. LD: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. AC: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. KC: Investigation, Resources, Writing – review & editing. HM: Investigation, Resources, Writing – review & editing. JaM: Resources, Supervision, Writing – review & editing. JoM: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This project was funded by the Bill and Melinda Gates Foundation [OPP1140133]. Under the grant conditions of the Foundation, a Creative Commons Attribution 4.0 Generic License has already been assigned to the author Accepted Manuscript version that might arise from this submission. The project was also funded by the National Foundation of Medical Research Innovation and the NSW Department of Primary Industries. The Bill and Melinda Gates Foundation, the National Foundation of Medical Research Innovation, and the NSW Department of Primary Industries had no involvement in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

Acknowledgments

The authors are grateful to Matthew Adams, Greg Robinson, and Bridget Barber (Clinical Tropical Medicine Laboratory, QIMR-Berghofer Medical Research Institute) for assistance with mosquito infection and dissections, and guidance with *P. falciparum* qPCR. The authors are also grateful to Claire Wang (Queensland Paediatric Infectious Diseases (QPID) Research Group, University of Queensland) for Guidance with *Plasmodium* qPCR and provision of qPCR standards. The authors are also grateful to Nina Pollak (Centre for Bioinnovation, University of the Sunshine Coast) for assistance with supervision of students.

Conflict of interest

JoM is a co-founder, shareholder, and director of BioCifer Pty. Ltd., who has licensed the technology. Information in this paper has been included in an International Patent Application PCT/AU2022/051506 PCT filing date 14th Dec 2022; Applicants BioCifer Pty Ltd, University of the Sunshine Coast, DMTC Limited; Title: Sample preparation reagents; Inventor: JoM.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fitd.2024.1287025/full#supplementary-material

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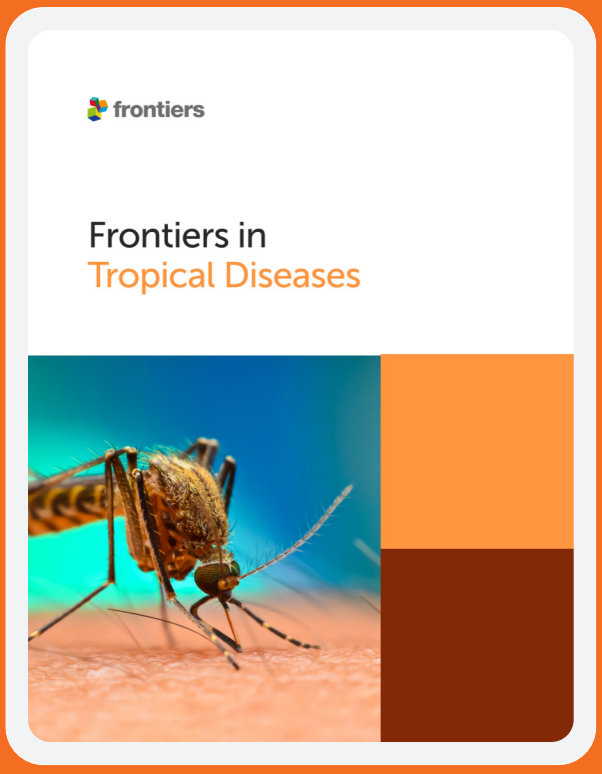

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