# Epidemiological surveys in India and marine natural product drug discovery for malaria

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## Abstract

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This dissertation discusses malaria epidemiology, pathogenesis, and drug discovery projects. Chapter 1 presents background on malaria, its lifecycle, the history of antimalarials and development of resistance, and the Malaria Evolution in South Asia International Center of Excellence for Malaria Research. Chapter 2 details demographic and clinical profiles of *Plasmodium falciparum* and *Plasmodium vivax* patients at a tertiary hospital in the southwestern state of Goa, India. Chapter 3 presents a dynamic cohort study conducted in seven remote villages of the northeastern state of Assam, India and the impact of longitudinal malaria diagnosis and treatment on the remote tribal population. Chapter 4 details the bioactivity of a secondary metabolite of a marine symbiotic bacterium against sexual and asexual *Plasmodium falciparum*. The work presented in this dissertation demonstrates how aspects of different scientific disciplines may be utilized in combination to better understand the biology and community impacts of a major infectious disease and to respond to the urgent need for new antimicrobials.

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## Acknowledgements

At the end of this chapter of my life, I look back on thirteen years of adventure, learning and self-discovery. I am deeply indebted to many unique characters and experiences.

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## Dedication

To my exuberant, courageous, and serious son, Maxwell Xavier Adams:

You are intrinsically worthy and deserving of love.

It is my joy and honor to witness your vitality, growth and trials. It is my immense privilege to shepherd you through the world as you learn and build your life.

I have been working toward this degree since before your conception.

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Bringing your being into existence will forever be my proudest accomplishment. I love you.

## Epigraph

*We shake with joy, we shake with grief.*
*What a time they have, these two,*
*housed as they are in the same body.*
-Mary Oliver

&

*I have walked through many lives,*
*some of them my own,*
*and I am not who I was,*
*though some principle of being*
*abides, from which I struggle*
*not to stray.*
*When I look behind,*
*as I am compelled to look*
*before I can gather strength*
*to proceed on my journey,*
*I see the milestones dwindling*
*toward the horizon*
*and the slow fires trailing*
*from the abandoned camp-sites,*
*over which scavenger angels*
*wheel on heavy wings.*
*Oh, I have made myself a tribe*
*out of my true affections,*
*and my tribe is scattered!*
*How shall the heart be reconciled*
*to its feast of losses?*
*In a rising wind*
*the manic dust of my friends,*
*those who fell along the way,*
*bitterly stings my face.*
*Yet I turn, I turn,*
*exulting somewhat,*
*with my will intact to go*
*wherever I need to go,*
*and every stone on the road*
*precious to me.*
*In my darkest night,*
*when the moon was covered*
*and I roamed through wreckage,*
*a nimbus-clouded voice*
*directed me:*
*“Live in the layers,*
*not on the litter.”*
*Though I lack the art*
*to decipher it,*
*no doubt the next chapter*
*in my book of transformations*
*is already written.*
*I am not done with my changes.*
-Stanley Kunitz

# Chapter 1: Introduction

## *1.1 Malaria overview*

Malaria is a major infectious disease. In 2022, the World Health Organization reported 249 million cases and 608,000 deaths worldwide (1). Human malaria parasites are transmitted by female *Anopheles* species mosquitoes. While there are more than 120 species of *Plasmodium*, only five species (*P. falciparum, P. vivax, P. ovale, P. malariae, and P. knowlesi*) are known to infect humans (2). The most ubiquitous are *P. falciparum* and *P. vivax*.

Malaria is derived from the Spanish term “mal aire” or bad air. Malaria parasites, specifically *P. vivax,* have been infecting humans for an estimated 460,000 years (3, 4). It is believed that *P. falciparum*, arguably more virulent, evolved about 10,000 years ago (5). The cause of malaria disease was only recently determined when the parasites were discovered in 1880 by Dr. Alphonse Laveran and the vector was discovered in India in 1897 by Sir Ronald Ross (6).

Malaria disease is caused by infection by protozoa of the Genus Plasmodium, Phylum Apicomplexa. Apicomplexa are unicellular eukaryotes that are obligate intracellular parasites. The Apicomplexa phylum includes parasitic protozoa responsible for malaria (*Plasmodium),* toxoplasmosis (*Toxoplasma),* cryptosporidiosis (*Cryptosporidium),* babesiosis *(Babesia),* and coccidiosis *(Eimeria*) (7). Gregarines are a group of ancestral Apicomplexa that are closely related to *Plasmodium* and *Toxoplasma* species (8). Some Apicomplexa, including *Plasmodium* and *Toxoplasma,* have apical organelle that are proposed to have been acquired by protist secondary endosymbiosis and are essential for parasite survival (9). Subcellular organelle, such as mitochondria and apicoplasts, are sensitive to some antimicrobials (10).

### *1.2 Lifecycle of Plasmodium falciparum*

The lifecycle of the malaria parasite presents unique challenges due to its complexity and the difficulty of reproducing each phase in laboratory conditions (2, 11). When a female mosquito bites a human, she injects sporozoites into the person, which travel to the liver and incubate in hepatocytes. After about 7-days, an infected hepatocyte releases up to 40,000 merozoites into the peripheral blood of the human. The merozoites invade red blood cells and go through an asexual replication cycle approximately every 48 hours. Mature asexual stage parasites (schizonts and trophozoites) display increased stiffness that allows them to adhere to vasculature and avoid splenic clearance, which causes clinical symptoms.

During each asexual replication cycle, a small subset of parasites diverts into the bone marrow and begins producing male or female progeny or gametocytes. Over 8-10 days, these sexual forms mature from stage I to stage V. Once they reach stage V, gametocytes return to the peripheral blood where they can be taken up by a female mosquito.

Once gametocytes are ingested by a female mosquito, they quickly become gametes. Males differentiate into about 8 microgametes and females into one macrogamete. Fertilization of a macrogamete by a microgamete results in a zygote that goes through meiosis and becomes an ookinete, which penetrates the midgut of the mosquito. The ookinete then forms an oocyst. Inside the oocyst, the parasite asexually replicates until there are several thousand sporozoites. When the oocyst finally ruptures, the sporozoites migrate to the mosquito's salivary glands and wait to be injected into a human.

### *1.3 Antimalarials and resistance*

The Inca of South America used the bark of cinchona trees to treat shivers. The Spanish colonizers brought this remedy to Europe and quinine, a basic alkaloid, was identified as the active ingredient in 1820. The mechanism of action of quinine is unknown, but it works rapidly

against blood stage asexual malaria parasites. To date, quinine is used as a rescue treatment when others fail (12).

In 1934, chloroquine, a 4-aminoquinoline, was synthesized, but was quickly deemed too toxic for humans in its initial evaluation. It was soon determined that the initial toxicity designation had been a mistake. Chloroquine became the frontline malaria drug after World War II and was highly effective worldwide. Then, in 1957, *P. falciparum* resistance to chloroquine arose in Southeast Asia and proceeded to spread worldwide, resulting in millions of deaths annually. Subsequent synthetic antimalarials such as sulfadoxine, pyrimethamine, and mefloquine were brought to market. Yet, resistance developed to each within a decade, despite their diverse mechanisms of action, and quickly spread across regions (13, 14).

The current frontline antimalarials are artemisinin-based combination therapies (ACTs). Dihydroartemisinin (DHA), the active metabolite of artemisinin, is a natural product extracted from sweet wormwood herb used to treat intermittent fever in China as far back as 400 CE. Currently, the artemisinin derivatives are prescribed in combination with one to two other antimalarials to protect their efficacy. Clinical resistance to artemisinin, in the form of delayed parasite clearance or half-life, emerged in the early 2000s, once again in Southeast Asia (15, 16). It is unclear why resistance to antimalarials with different mechanisms of action has arisen in the same location.

The spread of drug resistance to antimalarials has complicated campaigns to control, eliminate and ultimately eradicate malaria and new antimalarials are urgently needed (17, 18). Most approved antimalarials target the asexual blood stage, when parasite populations are greatest, and which causes clinical manifestations in humans. Fewer antimalarials inhibit the replicating liver stage and very few act upon hypnozoites (dormant liver stage) and gametocytes (sexual blood stage) (17).

### *1.4 Malaria Evolution in South Asia International Center of Excellence for Malaria Research*

*The following section is reproduced in entirety from Chakrabarti R,* ***Chery-Karschney L****, White J, Mascarenhas A, Skillman KM, Kanjee U, Babar PH, Patrapuvich R, Mohapatra PK, Patankar S, Smith JD, Anvikar A, Valecha N, Rahi M, Duraisingh MT, Rathod PK. "Diverse Malaria Presentations across National Institutes of Health South Asia International Center for Excellence in Malaria Research Sites in India." Am J Trop Med Hyg. 2022 Oct 11;107(4_Suppl):107-117. doi: 10.4269/ajtmh.21-1344.*

Abstract: The Malaria Evolution in South Asia (MESA) International Center for Excellence in Malaria Research (ICEMR) was established by the US National Institutes of Health (US NIH) as one of 10 malaria research centers in endemic countries. In 10 years of hospital-based and field-based work in India, the MESA-ICEMR has documented the changing epidemiology and transmission of malaria in four different parts of India. Malaria Evolution in South Asia-ICEMR activities, in collaboration with Indian partners, are carried out in the broad thematic areas of malaria case surveillance, vector biology and transmission, antimalarial resistance, pathogenesis, and host response. The program integrates insights from surveillance and field studies with novel basic science studies. This is a two-pronged approach determining the biology behind disease patterns seen in the field and generating new relevant biological questions about malaria to be tested in the field. Malaria Evolution in South Asia-ICEMR activities inform local and international stakeholders on the current status of malaria transmission in select parts of South Asia including updates on regional vectors of transmission of local parasites. The community surveys and new laboratory tools help monitor ongoing efforts to control and eliminate malaria in key regions of South Asia including the state of evolving antimalarial resistance in different parts of India, new host biomarkers of recent infection, and molecular markers of pathogenesis from uncomplicated and severe malaria.

*1.4.1 Introduction*

The Malaria Evolution in South Asia (MESA) International Center of Excellence in Malaria Research (ICEMR) program sponsored by the US National Institutes of Health (NIH) has worked actively in India for a decade (19). The overall goal of this program has been to study variations in malaria parasite evolution through the lens of parasite plasticity, pathogenesis, human genetics, and vector-mediated transmission. The MESA-ICEMR has established field sites in Goa, Maharashtra, Jharkhand, and Assam that stretch across India, from the Southwest to Northeast. The Goa site of the MESA-ICEMR is linked to the Medicine Department of the Goa Medical College and Hospital (GMC), the largest tertiary multispecialty health institution in the state. Goa is India's smallest state, ~120 km North to South and ~80 km East to West. The GMC is centrally situated in Goa and visited by patients from across the state. The Maharashtra site is in Jawaharlal Nehru Medical College and Acharya Vinoba Bhave Rural Hospital (AVBRH) in Wardha district, located at the center of India. Like GMC, the majority of malaria cases at AVBRH are infected with *Plasmodium vivax.* Shalini Hospital, associated with Krishi Gram Vikas Kendra (KGVK), is the site in Ranchi district of Jharkhand. The most remote MESA-ICEMR site is at the Northeastern corner of Assam in Dibrugarh district, within 160 km from the India-Myanmar border. It is hosted by the Regional Medical Research Center-Northeast (RMRC-NE) and has associations with Assam Medical College (AMC). The two Eastern sites KGVK and RMRC-NE see a higher proportion of *Plasmodium falciparum* infections compared with GMC and AVBRH. Within India, Goa is at the low end of the transmission continuum and is classified as a Category 1 state (Elimination Phase) by the National Framework for Malaria Elimination (NFME) (20) in India 2016-2030 and National Strategic Plan (NSP) for Malaria Elimination in India 2017-2022 (21). Maharashtra and Assam have moderate transmission intensity and are Category 2 states (Preelimination Phase), whereas Jharkhand is one of the high-transmission states classified as Category 3 (Intensified

Control Phase). Of these four sites, Goa is at the top of the Healthcare Access and Quality Index (HAX) at 64.8 (59.6-68.8) while Assam is at the other end at 34.0 (30.3-38.1) (22).

Together, the MESA-ICEMR sites are uniquely positioned to capture variables, which contribute to heterogeneity and complexity of malaria transmission in different healthcare accessibility settings in India. The sites regularly report on parasite species distribution, pathogenesis of severe and uncomplicated malaria, changes in malaria transmission, and monitor risk of importing Artemisinin Combination Therapy (ACT) resistance from Southeast Asia. The present perspective reveals the clinical and translational value of MESA-ICEMR conducted research in India, and how such research can contribute to improved malaria control strategies and its implementation. The relative impact of this research on malaria policy-making in India is discussed in an accompanying article titled “International Center of Excellence for Malaria Research for South Asia and Broader Malaria Research in India.”

*1.4.2 Health facility and household-based surveillance*

All four sites under MESA-ICEMR have been conducting health facility-based surveillance. The GMC has been conducting it from 2012 to current date. The AVBRH, KGVK and RMRC-NE conducted surveillance between 2014 and 2017. In addition, RMRC-NE has also conducted longitudinal household-based surveys in Karbi Anglong district in Assam. The RMRC-NE is also the only MESA-ICEMR site that conducts knowledge, attitude, and practice (KAP) surveys to identify the proportion of survey participants who know the main symptoms, treatment, and preventative measures for malaria. This leads to better community engagement, awareness, and receptiveness to existing malaria control measures. In the same region, surveys on indoor residual spraying (IRS) and insecticide-treated bed net (ITN) usage help assess the extent and effectiveness of the current vector-control regimen.

The GMC and RMRC collect data on the total number of febrile patients presenting at these sites who received a parasitological test for malaria. This data informs about the

screening capacity in health facilities, treatment seeking pattern of infected individuals, and sensitivity of surveillance. Our observations reported a slide positivity rate (SPR) at 8.4% for GMC, which went up to 10% at the peak of transmission season during the monsoon (23). Seasonality of infections is a common theme in all the four MESA-ICEMR sites with the number of infections peaking during the monsoon at their lowest during the dry season and a perennial transmission throughout the year. The proportion of infections caused by *P. falciparum* compared with *P. vivax*, however, varies in each region. The ratio of *P. falciparum:P. vivax* infected cases ratio is highest in Assam and lowest in Goa. Having a definitive idea about the seasonal peak of transmission in a region aids in tailoring cost-effective vector-control measures, like intensive spraying, before the start of high-transmission season. It also enables vector-control resources to be deployed effectively at the right time and place.

A common questionnaire used at all the sites collects information about age, gender, occupation, and travel history. We noted a higher proportion of cases in the above 5 years age group compared with 5 years and below age group at all the sites. This is characteristic of a low transmission setting like India. The median age of infection at GMC was 27 years (23). All the sites report a higher proportion of infection in male subjects. These statistics reflect the adult male bias of clinical malaria in hypoendemic regions (24). A detailed travel history helps in estimating the burden of imported cases, particularly in the urban site of Goa, which sees the highest level of incoming migration in all four sites. This is partly caused by Goa's status as a tourist hub in India and partly because of the rapidly expanding construction activities in this state (25), which draws migrant workers from Eastern India (23). In fact, we noted 88.2% of MESA enrolled cases in Goa were born outside Goa and 51.5% of the enrolled cases were construction workers (23). Genome-wide analysis of parasite SNPs from these samples will help in determining the origin of infection (26). This information regarding local versus imported

malaria will be crucial in managing malaria in the urban and periurban transmission settings in Goa that are in the elimination phase (27).

Treatment data collected by the study sites reflect the heterogeneity of *P. falciparum* malaria treatment regimen in India, which involves different ACTs such as artemether-lumefantrine (AL), artesunate-mefloquine (AS-MQ), and artesunate-sulfadoxine-pyrimethamine (AS-SP) along with primaquine (PQ). Northeast states (Assam, Arunachal Pradesh, and Tripura) and Ranchi used AL + PQ, Wardha used AL + PQ, and AS-SP + PQ, Goa used AS-MQ + PQ (before December 2015) and AS-SP + PQ (post December 2015) for *P. falciparum* malaria treatment. All sites used chloroquine (CQ) and PQ for *P. vivax* malaria treatment. These treatment regimens follow the national guidelines for first line of antimalarial use in India (28, 29), which prohibits use of AS-SP in Northeastern India after reports of resistance in that region (30). The RMRC and GMC are also involved in *in vitro* monitoring of artemisinin resistance as detailed in the Antimalarial Resistance section.

The data collected in the MESA-ICEMR sites complement the data collected by National Vector Borne Disease Control Program (NVBDCP) in real time monitoring of the malaria situation and outbreaks in the selected study sites. The MESA-ICEMR data also provide a useful historical record, which can be used to analyze the impact of malaria control measures as well as to improve malaria case management (diagnosis and treatment) in these regions.

*1.4.3 Vector studies*

Vector-related research in MESA-ICEMR involves monitoring the extent and impact of vector-control methods and entomological surveillance. Vector-control measures in India are tailored to transmission intensity settings. This means IRS and long-lasting insecticidal nets (LLINs) are predominantly used in the high-transmission site in Assam and larviciding by biological means is common in the low-transmission setting in Goa (21). We noted ITN usage

and proportion of the surveyed population living in households that have been sprayed at least once in the last 12 months. This in turn, helped us monitor the impact of vector-control measures in our longitudinal household-based survey we conducted in Karbi Anglong, Assam.

*Anopheles stephensi, An. culicifacies, An. fluviatilis, An. dirus (An. baimai),* and *An. minimus* are responsible for malaria transmission at the Indian study sites (31, 32). Of these, *An. stephensi, An. culicifacies* and *An. fluviatilis* have been shown to be responsible for transmission in Goa. We identified *An. subpictus* as the fourth vector for transmission in urban construction sites of Panjim, Candolim, Porvorim, and Margao (33). This puts *An. subpictus* as the second species along with *An. stephensi* to be responsible for malaria in urban regions of Goa. Seasonal distribution of these two species indicates that numbers of both the species peaks during rainy months (May-October). However, *An. subpictus* might be the dominant species responsible for transmission in the dry season (November-April), when *An. stephensi* numbers are low (33). These two vectors, thus, seem to work in tandem in maintaining urban, year-round transmission in Goa. Of these two, we found *An. stephensi* in Goan survey sites to be resistant to the commonly used insecticides; deltamethrin (8%), Malathion (18%), and DDT (48%) (32) while resistance status of *An. subpictus* in Goa still needs to be determined.

In a MESA-ICEMR study, we collected *An. stephensi* larvae from construction sites and later grew them under laboratory conditions in an insectary. These larvae were then propagated to establish a pure colony. We have assessed the ability of these laboratory-raised colonized mosquitoes to support *P. vivax* infection from malaria-infected clinical samples collected at GMC and compared with similar ability of wild mosquitoes. The mosquito infection rates and sporozoite load were higher in wild mosquitoes compared with the colonized mosquitoes. The results may reflect the effect of genetic differentiation associated with long-time mosquito colonization on levels of vector susceptibility to *P. vivax* (34). We were also able to collect valuable data about vector-parasite interactions in this region with these controlled mosquito-

feeding experiments (35). No correlation was noted among *P. vivax* parasitemia/gametocytemia with mosquito infection rates, but weak correlation was seen with parasite development in the mosquitoes. We also found that a higher mosquito infection intensity in wild *An. stephensi* in this region corresponds to a female to male gametocyte sex ratio close to 1 (35). Subsequent mosquito-feeding experiments allowed us to explore the optimal conditions for the routine supply of *P. vivax* sporozoites (36), which could be used to infect hepatic cell cultures in the future. These experiments will expand our understanding of the latent liver-stage *P. vivax* hypnozoites, but also aid in the design of future point-of-care (POC) hypnozoite diagnosis. We found that optimizing the mosquito rearing method and replacing patient plasma with naïve serum led to more than 2-fold increase in mosquito infection and sporozoite levels (36). This finding underscores the importance of monitoring how patient plasma affects mosquito feeding, especially in the regions where there is a high diversity of human host factors. These established mosquito-feeding procedures give a glimpse of the possible vector-parasite interactions happening in this region but are also testament to the growing MESA-ICEMR capacity for vector studies. The MESA insectary in National Institute of Malaria Research (NIMR) Goa is only 5 km away from GMC and this offers the rare opportunity of mosquito-feeding experiments with straight from the arm clinical samples within 45 minutes of blood collection. The optimization of sporozoite production has high potential, not just for generating a ready resource for liver-stage research facilitating the development of antirelapse interventions, but also for other future studies on transmission-blocking immunity and vaccines.

*1.4.4 Antimalarial resistance*

Overall, malaria cases have been declining in India since 1996, however, *P. falciparum* prevalence remains steady (37). Widespread drug resistance to ACTs would alter the trajectory and goal of eliminating malaria in India by 2030. The National Antimalarial Drug Resistance Monitoring System was established in 2009 to continuously monitor sentinel sites throughout

India using a combination of molecular biology and clinical approaches (38). Continued multifaceted approaches for resistance detection are vital to India's efforts to control malaria. Academic-based research investments, like the MESA-ICEMR, play an important role in supplementing resistance surveillance efforts. The MESA-ICEMR laboratories were the first to conduct and combine ring-stage survival assays (RSAs) with genotypic characterization to identify reduced sensitivity to artemisinin in parasites from Northeast and Southwest India (39).

We demonstrated using RSAs that a relatively small patient set (*N=22*) from Northeast India (Assam, Arunachal Pradesh and Tripura) and Goa had parasites with elevated tolerances to dihydroartemisinin (39). In this study, a single isolate out of 22 displayed the A675V nonsynonymous mutation in *Pfkelch13*, which previously had been declared by the World Health Organization (WHO) as a candidate marker for artemisinin resistance (40).

The four confirmed (*in vitro* and *in vivo*) artemisinin resistance *Pfkelch13*mutations (Y493H, R539T, I543T, and C580Y) from Southeast Asia have not been found in India yet. It is unclear which mutations originate from founder populations within India and which are imported from its neighbors. We evaluated the intrinsic capacity of Indian parasites to acquire *in vitro* resistance to novel inhibitors and their rates of mutagenesis are comparable to those from Southeast Asia. Although the capacity to mutate genomes may be similar, South Asia may still be in a good position to minimize the development and spread of artemisinin resistance by vigilant molecular and clinical surveillance. The MESA-ICEMR collection sites spread throughout India have the capacity to identify founder and/or imported resistant populations.

Choice of partner drugs included in ACTs is critical to preserving artemisinin's ability to serve as a frontline antimalarial. Although Northeast India relies on the artemether-lumefantrine combination, most of India is using artesunate-sulfadoxine-pyrimethamine to care for uncomplicated malaria cases (29). A MESA-ICEMR study of 10 culture adapted isolates (2012-2013) from Goa discovered that antifolate resistance genotypes were not predictive of

phenotype. Parasites with two mutations in *Pfdhfr* (59R + 108N) and a single *Pfdhps* mutation (437G), or none, were phenotypically as resistant as triple mutants for both genes. We suspect that folate salvage was the primary modulator of antifolate resistance levels in the Goan parasites. Although rapid parasite clearance is dependent on artemisinins quickly reducing biomass, the day 3 positivity and late-treatment failures point to possible preexisting antifolate resistance genotypes (30, 41-43). The findings make it clear that artemisinin, sooner than later, will have to be partnered with compounds for which there is much less circulating, preexisting resistance in India (44). In the case of antifolate resistance, phenotypic and genotypic surveillance activities will have to be increased to better inform policy-makers. The MESA-ICEMR will continue to monitor resistance to artemisinin and partner drugs at the genotypic and phenotypic levels.

*1.4.5 Pathogenesis and host response*

The MESA-ICEMR team has collected data on around 600 patients hospitalized with severe malaria (SM) and about 2,000 uncomplicated malaria patients, so far. Clinical assessment of the degree of malaria severity was done in all sites. Apart from estimating the burden of SM, we use laboratory-based data from severe and uncomplicated patient samples to explore different facets of the complex pathogenesis involving several intricate processes in the malaria parasite and host.

Steady, year-round access to infected blood from *P. vivax* patients at GMC has allowed the development of tools to overcome experimental shortcomings with the study of *P. vivax*. First, the parasitemia of *P. vivax* infections are usually low within patients and we have improved counting methodologies for low parasitemia infections by light microscopy (45). Investigations into *P. vivax* biology are severely limited by the lack of a continuous *in vitro* culture system. Access to a steady collection of *P. vivax* patient isolates has facilitated significant advances in our ability to short-term culture *P. vivax.* We have discovered that short-

term culturing *P. vivax* in a hematopoietic stem cell medium permits highly efficient maturation of parasites from the ring to schizont stage of the intraerythrocytic developmental cycle (IDC) of the parasite (46). This has allowed us to establish robust *P. vivax* drug and invasion assays using both frozen parasites as well as fresh clinical isolates from GMC. This, in turn, has set the stage for exploring the molecular basis of *P. vivax* invasion, host cell tropism, and pathogenesis. The MESA-ICEMR has shown that gametocytes from cryopreserved patient *P. vivax* isolates are stable for use in mosquito infection (47). This finding, combined with our ability to short-term culture *P. vivax* and the high frequency of gametocytes that we observe in clinical isolates at GMC, will be important resources for future transmission studies.

*P. vivax* invasion of red blood cells is an obligatory step in the parasite life-cycle but there is a marked deficiency in our understanding of the invasion process in *P. vivax*, again primarily due to the lack of a continuous *in vitro* culture system for this parasite. *Plasmodium vivax* is unique due to its strong preference for invading reticulocytes, the youngest red blood cells (48, 49). Using the large number of isolates collected from the GMC, we have shown that the patient isolates exhibit variation in reticulocyte preference (50). We have also developed a flow cytometry-based osmotic lysis assay to demonstrate changes in reticulocyte stability during *P. vivax* infection (51).

The variation in the usage of *P. vivax* invasion ligands, the existence of discrete invasion pathways, and their association with disease severity are poorly understood. The PvDBP/DARC interaction is thought to be essential for *P. vivax* invasion (52) and the lack of DARC on red blood cells of people in Sub-Saharan Africa is likely to have been selected for by *P. vivax* (52, 53). However, there is increasing evidence of *P. vivax* infection in DARC-negative individuals (54-60) that could have profound public health implications for the spread of this parasite. A report of PvDBP duplication (61) in field isolates in Madagascar, Sudan, and Cambodia may present a possible mechanism for invasion of DARC-negative red blood cells. We, therefore,

tested for PvDBP duplication within isolates collected at GMC, but found that they are absent in *P. vivax* isolates from India, suggestive of greater geographical variation in *P. vivax* parasites (62). In future, we plan to test whether there is any protective effect of DARC genotypes by determining the DARC genotypes and *P. vivax* parasite burdens in malaria patients at the GMC.

Our *P. vivax* invasion assay has also allowed us to investigate *P. vivax* invasion ligand gene expression and variations in invasion. In addition to the PvDBP/DARC invasion pathway, the MESA-ICEMR team has also investigated the newly identified reticulocyte-tropic invasion pathway between the *P. vivax* RBP2b invasion ligand and host transferrin receptor (TfR1) (63). We measured invasion inhibition in clinical *P. vivax* isolates using host-targeted small molecules/antibodies (anti-DARC and anti-TfR1) and we observed that while invasion could be inhibited in all strains, there was significant variation in usage of each invasion pathway (64). Furthermore, we observed that combinatorial inhibition using both anti-DARC and anti-TfR1 inhibitors led to inhibition synergy, which may have future implications for *P. vivax* vaccine development (64).

Our work in *P. falciparum* pathogenesis has involved *var* gene (*PfEMP1)* profiling in complicated and uncomplicated malaria patients from Goa (65, 66). We carried out these studies to understand the crucial role of differential expression of *var* genes in pathogenesis of SM through infected RBC (iRBC) sequestration and the role of parasite biomass (65). About 77% of the SM study patients presented more than one severity criterion, as set by the WHO, while 57% showed more than three different severity criteria, indicating multisystem disorder. We demonstrated that SM patients showed higher parasite biomass than uncomplicated malaria patients as indicated by plasma PfHRP-2 levels – the surrogate biomarker assigned for total parasite biomass (65). Quantitative RT-PCR (qRT-PCR) analysis showed that type A *var* transcripts were overrepresented in SM than the type B and C *var* transcripts. Group A *var* variants, specifically DC8 and DC6 PfEMP-1 were previously found to be associated with

pediatric SM (67). This subset of PfEMP-1 includes mediators responsible for “rosetting” and endothelial protein C receptor (EPCR) binding, two distinct erythrocyte adhesion categories known to cause SM (68-70). Higher levels of serum PfHRP-2 positively correlated with elevated transcript levels of EPCR binding (DC8 and DC6) and rosetting (DC5) *var* phenotypes in SM patients. Our data suggests that specific types of PfEMP-1 may promote higher parasite biomass, ultimately contributing to severe pathophysiological outcomes. Machine learning analysis indicated the combination of high parasite biomass (assessed through PfHRP-2 levels) along with DC6 and DC8 encoding *var* transcripts was the strongest indicator of severity and hospitalization in the study subjects from Goa (65). *In vitro* binding studies showed moderate inhibition of EPCR-APC (Activated Protein C) binding using DC8 CIDR$\alpha$ from SM patients, further suggesting that this differential binding might play a role in severe manifestation of the disease in low-transmission settings like India. Molecular analysis of EPCR-APC blockade by PFEMP1 domains further supports this phenomenon (66).

Our subsequent meta-analysis study involving three different cohorts with pediatric malaria in Tanzania and Malawi, and adult malaria in Goa, indicated that parasite biomass and *var* gene expression profiles play independent and complementary roles in development of SM, not only in African children, but also in Indian adults (67). Additionally, even when the *var* gene expression profiles show variation within host and among sites, the *var* gene profile signatures associated with SM were highly similar across three cohorts included in the study. There are differences in disease presentation in African children and Indian adults, which is to be expected owing to differences in transmission intensity in African and Indian regions. Despite these differences, SM in African children and Indian adults is definitively linked to increased transcription of *var* variants predicted to bind EPCR (67). Further, this meta-analysis (67) suggests a strong role of parasite biomass in development of SM and indicates that sequestering parasite populations might increase endothelial cell activation and microvascular

obstruction (71-74). This meta-analysis is consistent with previous studies, where DC8 and group A *var* transcripts were implicated in SM (70, 75).

The interaction/s of malaria parasites with the host that are responsible for clinical outcome vary with parasite biology and host physiology. Naturally acquired immunity (NAI) of the human host to parasites play an important role in malaria pathogenesis. Development of NAI depends on duration and degree of exposure to the parasites (76, 77) and can vary significantly in different endemic areas due to variation in degree of transmission and specificity toward infecting *Plasmodium* species (78). In India, malaria transmission is influenced by many factors such as changing seasons, the existence of multiple *Plasmodium* species and their vectors, and diverse epidemiological profiles that can directly contribute to the development of NAI. Seroreactivity and antibody response against parasite proteins are useful biomarkers of populations-wide NAI, and significantly different levels of these markers had previously been observed at different Indian sites (79). The MESA-ICEMR has optimized the methods to identify seroreactive protein markers using protein arrays in both uncomplicated and complicated patient sera (80). Seroreactivity and antibody response from patients enrolled in Goa were studied with protein microarray chips that display 500 *P. falciparum* and 515 *P. vivax* protein respectively (81). *Plasmodium falciparum* patient Sera showed a broader immune response than *P. vivax* patient Sera. This observation in India is consistent with previous ICEMR protein array data from Kenya (82). Our study identified five *P. falciparum* and two *P. vivax* antigens with differential seroreactivity among inpatients and outpatients. Severe falciparum and vivax malaria patients showed significantly higher antibody response than uncomplicated patients. Interestingly, several conserved proteins like MSP, PHIST, and NOT family proteins were found to be seroreactive only in nonsevere patients in both falciparum as well as vivax infections. Altogether, 248 *P. falciparum* and 73 *P. vivax* seroreactive proteins were identified in the MESA-ICEMR study, which is consistent with similar studies published earlier (82-86). Although the

majority of these putative antigen proteins are predicted to be either exported to the iRBC surface or present on the merozoite surface, some are expected to be nuclear, cytoskeletal, and cytoplasmic. Immunity patterns from the high-transmission endemic areas across age groups differ from the immunity patterns from low-transmission areas. In Africa, adults develop immunity that protects against SM by repeated exposure throughout childhood. Our seroanalysis study provides insights into antibody-dependent immune responses against malaria in low-transmission settings (India). Here, malaria in adults is more likely to evolve into severe disease due to lack of repeated exposures to the parasites during childhood (87). The MESA-ICEMR data provides the first evidence of differential seroreactivity to *P. falciparum* antigens in severe versus non-severe cohorts (80) and complements the efforts undertaken by other ICEMR programs to compare the variation in immunity against malaria (82).

### *1.4.6 Conclusion*

Malaria accounted for 5 million cases in India in 2020 (88). India has reported a 78% decrease in the number of malaria cases from the approximately 200 million cases in 2000 (88). Efforts from government, nongovernment and private sectors have played a significant role in achieving this feat (89, 90). The integrated multidisciplinary research approach of the MESA-ICEMR plays a complementary role in describing malaria in India. Together with national and subnational malaria stakeholders at the MESA-ICEMR study sites, joint efforts are aligned with the goal of malaria elimination in these regions as envisioned by the WHO Global Technical Strategy (GTS) for Malaria 2016-2030 (91), NFME in India 2016-2030 (20), and the NSP 2017-2022 (21). Key MESA-ICEMR contributions include collection and dissemination of health facilities data and survey-based data on malaria in government as well as public/private healthcare facilities. The experience of MESA-ICEMR in real time monitoring of clinical malaria cases through databases like RedCap and ClinEpiDB is important for emerging national digital healthcare surveillance (92). Our household-based surveys provide data on vector-control

coverage and KAP indicators. The surveillance data monitors the transmission metrics in these regions and assesses the impact of interventions (IRS, LLINs, RDTs, and ACTs) on malaria prevalence, incidence, and mortality. This information could help in designing a combination of interventions tailored to local contexts, particularly in the very low-transmission regions in India like Goa. Our entomological surveillance in Assam and in Goa give valuable information about vector species responsible for malaria transmission as well as vector behavior and their insecticide resistance status in these study sites. Our model of determining imported cases through detailed travel history and SNP analysis in Goa could be very useful in similar low-burden Category 1 Indian states in the elimination phase. Adequate case management, involving diagnosis, and treatment is one of the key interventions identified in GTS and NFME. The MESA-ICEMR aims to understand the region-specific molecular variations in host cell invasion and cytoadherence that may precipitate SM at the study sites, along with potential loss of diagnostic tools due to *Pfhrp-2* deletions. We also monitor the current status of ACT sensitivity in the Indian parasites from these sites. Together, these efforts put the MESA-ICEMR team in a competent position for complementing and informing diagnosis and treatment regimens in this region. Moreover, the MESA-ICEMR has built strong research facilities at the study sites, particularly Assam and Goa. This makes us uniquely poised to help deliver on the malaria research and development needs outlined by the WHO (93) and to test the impact of region-specific malaria control measures.

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## Chapter 2: Demographic and clinical profiles of *Plasmodium falciparum* and *Plasmodium vivax* patients at a tertiary care center in southwestern India

*The following chapter is reproduced in entirety from* ***Chery L****, Maki JN, Mascarenhas A, Walke JT, Gawas P, Almeida A, Fernandes M, Vaz M, Ramanan R, Shirodkar D, Bernabeu M, Manoharan SK, Pereira L, Dash R, Sharma A, Shaik RB, Chakrabarti R, Babar P, White J 3rd, Mudeppa DG, Kumar S, Zuo W, Skillman KM, Kanjee U, Lim C, Shaw-Saliba K, Kumar A, Valecha N, Jindal VN, Khandeparkar A, Naik P, Amonkar S, Duraisingh MT, Tuljapurkar S, Smith JD, Dubhashi N, Pinto RG, Silveria M, Gomes E, Rathod PK. "Demographic and clinical profiles of Plasmodium falciparum and Plasmodium vivax patients at a tertiary care centre in southwestern India." Malar J. 2015 Nov 25;15(1):569. doi:10.1186/s12936-016-1619-5.*

Abstract: Malaria remains an important cause of morbidity and mortality in India. Though many comprehensive studies have been carried out in Africa and Southeast Asia to characterize and examine determinants of *P. falciparum* and *P. vivax* malaria pathogenesis, fewer have been conducted in India. A prospective study of malaria-positive individuals was conducted at Goa Medical College and Hospital (GMC) from 2012 to 2015 to identify demographic, diagnostic and clinical indicators associated with *Plasmodium falciparum* and *Plasmodium vivax* infection on univariate analysis. Between 2012 and 2015, 74,571 febrile individuals, 6,287 (8.4%) of whom were malaria positive, presented to GMC. The total number of malaria cases at GMC increased more than two-fold over four years, with both *P. vivax* and *P. falciparum* cases present year-round. Some 1,116 malaria-positive individuals (mean age=27, 91% male), 88.2% of whom were born outside of Goa and 51% of whom were construction workers, were enrolled in the study. Of 1,088 confirmed malaria-positive patients, 77.0% had *P. vivax,* 21.0% had *P. falciparum* and 2.0% had mixed malaria. Patients over 40 years of age and with *P. falciparum* infection were significantly (*p*-values <0.001) more likely to be hospitalized than younger and *P. vivax* patients, respectively. While approximately equal

percentages of hospitalized *P. falciparum* (76.6%) and *P. vivax* (78.9%) cases presented with at least one WHO severity indicator, a greater percentage of *P. falciparum* inpatients presented with at least two (43.9%, *p*-value <0.05) and at least three (29.9%, *p*-value <0.01) severity features. There were six deaths among the 182 hospitalized malaria positive patients, all of whom had *P. falciparum.* During the four-year study period at GMC, the number of malaria cases increased substantially and the greatest burden of severe disease was contributed by *P. falciparum*.

Abbreviations: ACD: Acid citrate dextrose; BP: Blood pressure, BUN: Blood urea nitrogen; DNA: Deoxyribonucleic acid, DMID: Division of Microbiology and Infectious Diseases, GMC: Goa Medical College and Hospital, GOI: Government of India, Hb: Hemoglobin, HCT: Haematocrit, HMSC: Health Ministry Screening Committee, ICEMR: International Center of Excellence for Malaria Research, IMD: India Meteorological Department, IRB: Institutional Review Board, IQR: Interquartile range, MESA: Malaria Evolution in South Asia, NIAID: National Institute of Allergy and Infectious Diseases, NIH: US National Institutes of Health, NVBDCP: National Vector Borne Diseases Control Programme, OR: Odds ratio, RDT: Rapid diagnostic test, SS: Severity score, UW: University of Washington, WHO: World Health Organization.

### *2.1 Background*

Globally, parasites of the *Plasmodium* genus infect more than 200 million people and cause an estimated 438,000 deaths annually [1]. India is the second most populous country in the world with ongoing malaria transmission, with 91% of its more than 1.2 billion population living in areas of malaria risk [1]. The most recent estimates report up to 26 million malaria cases and 55,000 deaths due to malaria annually in India [1], . However, there is ongoing, vigorous debate about these figures, in part due to the vast scale of the country [2-5].

India is co-endemic for *Plasmodium falciparum* and *Plasmodium vivax*, posing challenges for malaria control and elimination planning because the two parasite species may

differ in mosquito vectors, spatial distributions and transmission dynamics and because of the relapsing nature of *P. vivax* infection with a dormant liver stage [6-8]. Overall, approximately 66% of malaria infections in India are caused by *P. falciparum* and 34% are caused by *P. vivax* [1]. However, the proportional distribution varies across India and a wide range of clinical presentations are seen from both predominant species of malaria [6]. In contrast to Africa, malaria transmission in India is more limited, both adolescents and adults are at risk of severe malaria, and a substantial proportion of cases are infected with *P. vivax* rather than the traditionally more virulent *P. falciparum*. Although many expansive and comprehensive studies have been carried out in Africa [9-14] and Southeast Asia [15-20] to examine pathogenesis and mortality determinants in malaria-positive patients, a more limited number of such studies, generally smaller in scope, have been conducted within India [21-29].

India has one of the world's largest and most extensive national surveillance systems to identify malaria incidence [30, 31] and is a highly heterogeneous country with more than 2,000 ethnic groups and 22 official languages spread across 29 states and seven union territories. All of these states have populations in the millions and a more than ten-fold variation in average per capita income [32], which leads to important geographic variations in disease epidemiology and substantial variability in the delivery of malaria diagnosis, care and treatment across the country [33]. It would be costly and operationally difficult to measure all of the diverse malaria settings in India through community-based surveys or reactive case-detection methods [34] and challenging to comprehensively address through national-level strategies, programmes and recommendations [35, 36].

In addition to India's vast countrywide and state-level monitoring of malaria, rigorous hospital-based reports can provide epidemiological data relevant to local control and elimination initiatives as well as clinical data relevant to diagnosis, care and treatment efforts. Clinicians, as well as the wider public health system, may benefit from deep studies of specific patient pools in

order to better understand malaria transmission and pathogenesis in local communities. Such information may also assist in the prioritization of resources for patient care and treatment.

With the aim of conducting methodical studies in a low to mid-endemicity, peri-urban setting, the Malaria Evolution in South Asia (MESA) International Center of Excellence for Malaria Research (ICEMR) established a research site at Goa Medical College and Hospital (GMC) [37-39]. Previously an overseas province of Portugal, Goa is a small, prosperous, southwestern state of India where both *P. falciparum* and *P. vivax* are endemic. GMC is the only government tertiary care center in the state and operates under the auspices of the Government of Goa Public Health Department to provide health care to all, free-of-charge. The relatively advanced diagnostic and clinical capabilities at GMC draw a large, diverse patient pool and allow for deep clinical analysis. As a research site, GMC offers a highly heterogeneous patient population, a constant flow of febrile and malaria-positive cases, and a wide spectrum of clinical presentations of malaria in a relatively affluent, burgeoning peri-urban area. The present study provides a detailed description of the demographic, diagnostic and clinical characteristics of malaria-positive study participants at GMC from 2012 to 2015.

## *2.2 Methods*

### *2.2.1 Institutional ethics approvals*

The present work was part of the US National Institutes of Health-sponsored Program Project [40, 41] entitled Malaria Evolution in South Asia International Center of Excellence for Malaria Research (MESA-ICEMR). The activities of this center were approved by the Government of India (GOI) Health Ministry Screening Committee (HMSC) and the Government of Goa Public Health Department. The human subjects protocol and consent forms for enrollment of *Plasmodium*-positive individuals presenting to Goa Medical College and Hospital (Bambolim, Goa, India) were approved by the institutional review boards (IRB) of the Division of

Microbiology and Infectious Diseases (DMID) at the US National Institute of Allergy and Infectious Diseases (NIAID), GMC, and the University of Washington (UW).

*2.2.2 Study design and enrollment*

All febrile individuals presenting to the outpatient, pediatric, and casualty departments of GMC were tested by hospital staff for *Plasmodium* infection via finger-prick or venous blood draw and examination by microscopy of Giemsa-stained thin blood smear and/or by rapid diagnostic test (RDT), FalciVax, Zephyr Biomedicals). The hospital generally only used one method of testing per individual, usually microscopy. In rare instances, both RDT and microscopy were used. If the RDT or microscopy was positive, the individual was counted as malaria positive. If the RDT and microscopy showed different species of parasite, the individual was counted as mixed infection positive. Hospital-determined malaria positivity and species of infection results are presented for all febrile patients presenting to GMC between January 2012 and December 2015.

Individuals determined to be malaria positive by either microscopy or RDT by the hospital who were between the ages of 12 months and 65 years and not pregnant, were referred to the MESA-ICEMR study team. Enrollment generally occurred during normal working hours and when study staff were not completing enrollment of a previous patient.

Outpatients and individuals admitted to the Medicine, Pediatric, and Intensive Cardiac Care Unit (ICCU) wards were approached for participation in the study. Severely ill individuals requiring use of a ventilator and, therefore, admitted to the Intensive Care Unit (ICU) were not approached for inclusion in this study. Malaria-positive individuals were given oral and written descriptions of the study and were asked to provide written informed consent or, in the case of children between 12 months and 18 years, consent of a parent or guardian or, in the case of children between the ages of eight and 18 years, assent in addition to the consent of a parent or

guardian. Each study participant received a unique numerical code in order to streamline data collection.

Upon enrollment, study participants provided demographic information as well as history of malaria infection, symptoms and travel to study staff. Following venous blood draw into vacutainers (ACD), BD, India), study participants received care and treatment as directed by the attending physician at GMC.

*2.2.3 Sample processing*

Each venous blood sample was transferred to the on-site MESA-ICEMR research laboratory. Research staff immediately prepared three thin and two thick blood smears. *Plasmodium* species and parasitemia were determined by Giemsa-stained thick smear reading by expert microscopists. Research staff then performed an additional RDT (FalciVax, Zephyr Biomedicals, India) and measured hemoglobin (Hb 201, HemoCue, USA) and hematocrit (Iris StatSpin, Beckman Coulter, USA).

In addition to the hospital determination used for initial recruitment, research study staff also made a separate, independent determination of malaria positivity and species of infection based on diagnostic tests conducted in the MESA-ICEMR laboratory. If both RDT and microscopy results were negative in the MESA-ICEMR lab, the patient was classified as an unconfirmed case. If RDT or microscopy was positive, the patient was counted as a confirmed malaria positive case. If the RDT and microscopy showed different species of parasite, the sample was identified as mixed infection positive. Individuals enrolled in the study who were determined to be malaria positive by the hospital, but who were classified as malaria negative by the research study staff were excluded from statistical analysis of demographic, diagnostic and clinical characteristics. Study-determined positivity and species of infection results are presented from April 2012 through December 2015.

*2.2.4 Clinical characteristics and severity scores*

Measured features among all enrolled patients with confirmed malaria infection were: high parasitemia (>4.0% for *P. falciparum*, >1.5% for *P. vivax*); hyperparasitemia (>10% for *P. falciparum*, >2.0% for *P. vivax)*; high fever (>38.1$^{o}$C); severe fever (>38.9$^{o}$C); anemia (hemoglobin (Hb) <9 g/dL in those 12 years and older, Hb <7 g/dL in those one to 11 years); and severe anemia (Hb <7 g/dL in those 12 years and older, Hb <5 g/dL in those one to 11 years). Patients were admitted to GMC based on the clinical judgement of the attending physician and did not have to fulfil any WHO criteria for severe malaria classification.

Enrolled *P. falciparum*- and *P. vivax*-positive patients who were hospitalized were assessed daily by trained GMC clinicians for severity of infection based on WHO criteria [42, 43]. Measured features among enrolled, hospitalized patients associated with severe malaria infection were: cerebral malaria (Glasgow coma score <11 in adults or Blantyre coma score <3 in children and presence of asexual forms in blood); hypoglycaemia (blood glucose <40 mg/dL); metabolic acidosis (plasma bicarbonate <15 mmol/L), renal failure (serum creatinine >3.0 ml/dL and/or blood urea nitrogen (BUN) >17 mmol/L); abnormal bleeding (observable); respiratory distress (breathing rate >20 breaths/min or partial oxygen ($PaO_2$) <75); severe jaundice (total bilirubin >10 mg/dL); circulatory collapse/shock (systolic blood pressure (BP) <80 mmHg with cold extremities); pulmonary oedema (observable); severe anemia (Hb <7 g/dL or haematocrit (HCT) <20% in those 12 years and older, Hb <5 g/dL or HCT <15 % in those one to 11 years); and death. Occasionally, the complete clinical laboratory investigation panel was not ordered [42, 43]. A severity score (SS) was calculated for all hospitalized patients based on the total number of WHO severe malaria criteria met at enrollment and throughout hospitalisation.

*2.2.5 Meteorological data*

Rainfall data for Goa were obtained from the India Meteorological Department (IMD) (Ministry of Earth Sciences, Government of India). Data were collected at the IMD observatory located in the capital city of Goa, Panaji, roughly 5 km from GMC.

*2.2.6 Data and sample management*

Demographic and clinical study data were collected and managed using REDCap electronic data capture tools (Nashville, TN, USA). Diagnostic study data were recorded and stored using LabKey software (Seattle, WA, USA). All samples and associated aliquots were labelled and stored using a customized FreezerPro database (RURO Inc, Frederick, MD, USA).

*2.2.7 Statistical analysis*

Data were initially entered into a customized SQL database (LabKey server) followed by independent verification against the original case report forms. Statistical differences between percent parasitemias between species for all enrolled patients, inpatients and outpatients were determined using an unequal variances t-test using GraphPad Prism 6 software (La Jolla, CA, USA). All other analyses were conducted using R (Vienna, Austria).

For univariate analysis, the primary outcome was the species of malaria infection. Continuous variables were summarized with mean and standard deviation and binary variables were summarized with proportions. Between group univariate comparisons of features for *P. falciparum* and *P. vivax* were analysed with logistic regression. Demographic and diagnostic features were reported for all confirmed *P. falciparum* or *P. vivax*-infected enrolled patients. Clinical laboratory tests and classifications were only reported for confirmed *P. falciparum* or *P. vivax*-infected inpatients and were included in tables only if a threshold of 20% of enrolled inpatients had a particular documented clinical result or classification. Logistic regression was also used to measure differences in hospitalization rates by species of infection between age ranges. Results were represented as odds ratios with 95% confidence intervals (OR (95 %)) as well as *p* values. Differences were considered to be significant at *p* values ≤0.05.

Mixed *P. falciparum* and *P. vivax* infections were excluded from all statistical analyses due to the small sample size.

## *2.3 Results*

### *2.3.1 Febrile and malaria-positive cases at Goa Medical College and Hospital*

A total of 74,571 febrile individuals presented to GMC between January 2012 and December 2015 and all were tested for malaria. Of those, 6,287 (8.4%) were determined to be positive for malaria infection (**Figure 2.1**). Over four years of passive surveillance, the number of malaria-positive cases presenting to GMC steadily and significantly increased, from 889 cases in 2012 to 2,261 cases in 2015 (**Figure 2.2**). While the traditional malaria season in Goa is June to December, *P. falciparum* and *P. vivax* cases were recorded throughout the year at GMC with the peak coinciding with the rainy season (**Figure 2.3**). In 2015, the increase in malaria cases preceded the annual rains and case numbers remained high from April through December. The number of monthly cases observed at GMC during the height of the malaria season (September) was approximately five times greater than at the lowest point during the middle of the dry season (February).

Of the malaria-positive individuals presenting to GMC, the majority (86.8%) were diagnosed by the hospital with *P. vivax* mono-infection. *Plasmodium falciparum* infections accounted for 9.1%, and mixed infections for 4.1% of the total cases over four years (**Figure 2.3**). Month-wise, the number of *P. vivax* infections was always greater than *P. falciparum* and mixed infections. The seasonality of *P. vivax* and *P. falciparum* infections was very similar, with case numbers of both increasing after the start of the rains. An average of 138 *P. vivax*, 16 *P. falciparum*, and seven mixed infection cases presented per week during the traditional malaria high season (June-December), while an average of 57 *P. vivax*, four *P. falciparum* and three mixed infections were seen weekly during the malaria low season (January-May). At the peak of the malaria transmission season as many as 10% of fever cases at GMC were malaria positive (**Figure 2.2**).

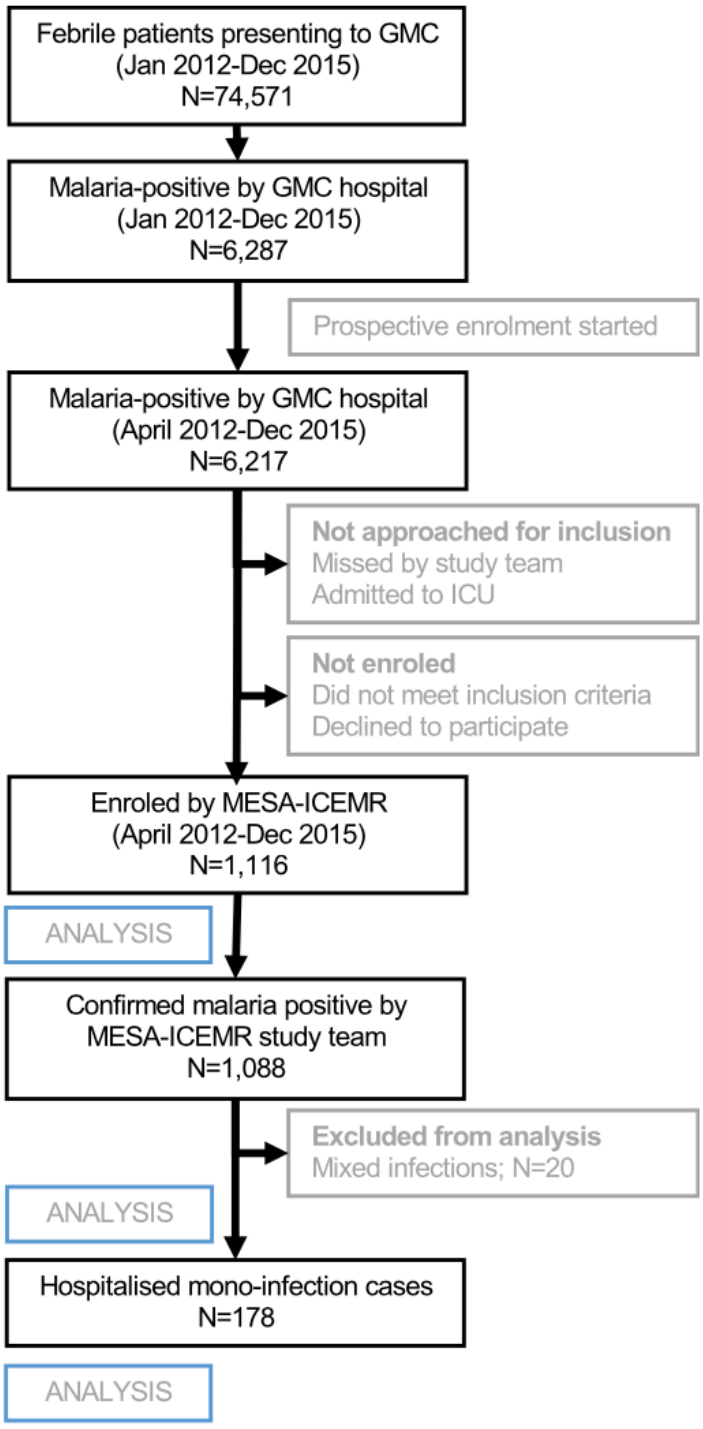


**Figure 2.1 Diagram of study enrollment at Goa Medical College and Hospital.** Of the 74,517 febrile patients who presented to GMC and were tested for malaria, 6,287 were positive by hospital diagnosis. Of the 6,287 cases identified by the hospital between January 2012 and December 2015, 6,217 were referred to the MESA-ICEMR study team between April 2012 and December 2015. Of 6,217, 1,116 study participants were enrolled by the MESA-ICEMR and 1,088 were confirmed by the MESA-ICEMR to be malaria positive. Excluding mixed infections, 178 confirmed malaria cases were hospitalized by GMC.

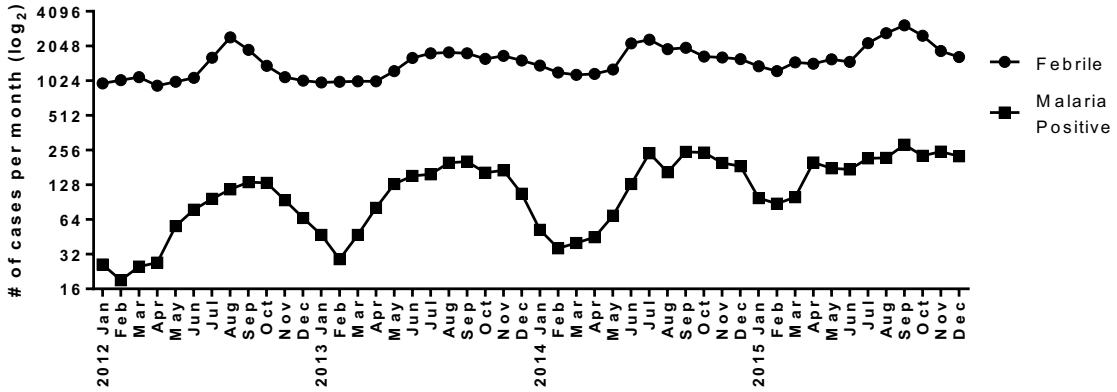


**Figure 2.2 Increasing febrile and malaria positive cases at Goa Medical College and Hospital over four years.** In 2012, 889 malaria cases were identified out of 15,589 febrile cases. In 2013, 1,477 malaria cases were identified out of 17,017 febrile cases. In 2014, 1,660 malaria cases were identified out of 19,454 febrile cases. In 2015, 2,261 malaria cases were identified out of 22,511 febrile cases.

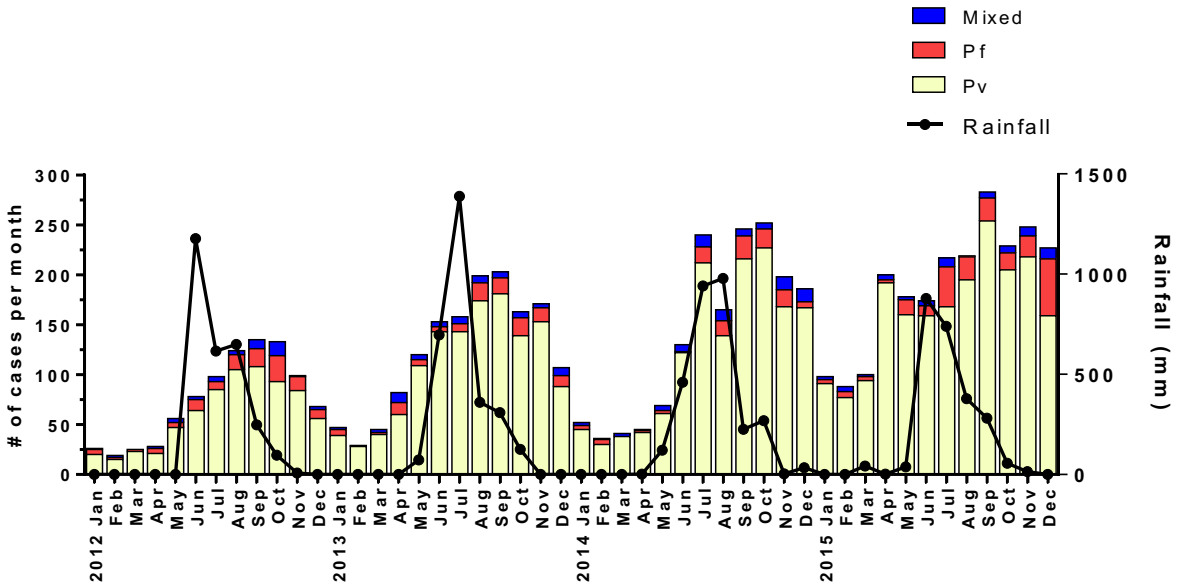


**Figure 2.3 Monthly malaria positive cases by species with rainfall data over four years.** In 2012, 721 *P. vivax*, 120 *P. falciparum* and 48 mixed malaria cases were diagnosed for a total of 889 annual malaria infections at GMC. In 2013, 1,297 *P. vivax*, 116 *P. falciparum* and 64 mixed malaria cases were diagnosed for a total of 1,477 annual malaria infections at GMC. In 2014, 1,467 *P. vivax*, 111 *P. falciparum* and 82 mixed malaria cases were diagnosed for a total of 1,660 annual malaria infections at GMC. In 2015, 1,972 *P. vivax*, 223 *P. falciparum* and 66 mixed malaria cases were diagnosed for a total of 2,261 annual malaria infections at GMC.

*2.3.2 Demographic characteristics of study participants*

A total of 1,116 of 6,217 (18%) febrile individuals identified by GMC as malaria-positive via microscopy or RDT were enrolled in the present study between April 2012 and December 2015 (**Figure 2.1**). Enrollment of this subset of patients was based on the logistical capabilities of the study team. All study participants had a mean age of 27 years (median 24 years; IQR 20-32 years) and were predominantly male (91.0%). The male-to-female ratio of study participants was roughly similar to the gender distribution of febrile cases presenting to GMC (86.7% male and 13.3% female). The mean age of male study participants was 27 years (median 24 years, IQR 20-31), while the mean-age of non-pregnant female study participants was slightly older at 30 years (median 31 years, IQR 19-40). A small minority of malaria-positive cases (51, 4.6%) were children under the age of 16 years, of whom 64.7% were male and 35.3% were female.

Study participants at GMC represented a very heterogeneous Indian population. Approximately one-tenth of study participants (11.8%) were born in Goa and 88.2% were born in 26 other Indian states or outside of India (**Figure 2.4**). The states with the greatest representation of study participants were the eastern states of Bihar with 186 (16.7%) individuals, West Bengal with 179 (16.0%), Uttar Pradesh with 152 (13.6%), and Jharkhand with 111 (9.9%). The majority of study participants (51.5%) self-identified as construction workers while 4.7% self-identified as students, 3.0% as housewives, 1.7% as soldiers or police, 1.5% as office workers, 1.3% as factory workers, and 33.2% selected the option other.

Some 97.5% of study participants were confirmed to be *Plasmodium* positive by the MESA-ICEMR study team, with 77.0% of those cases being *P. vivax*, 21.0% *P. falciparum* and 2.0% mixed-infection positive. The majority of malaria-positive individuals (83.3%) were classified as having uncomplicated malaria and treated on an outpatient basis based on the clinical judgement of the attending physician at GMC. While *P. vivax* infections were much more

common than *P. falciparum*, a higher proportion of hospitalized cases were *P. falciparum* positive (107/182, 58.8%) *versus P. vivax* positive (71/182, 39.0%) and mixed-infection positive (4/182, 2.2%). All analysis presented below includes only patients with mono-infections.

As expected, malaria patients infected with *P. falciparum* only were significantly more likely to have a higher parasitemia than those infected with *P. vivax* mono-infection among both outpatient and inpatient groups (**Table 2.1**) . The mean parasitemia value among *P. vivax* cases was slightly higher for outpatients as compared to inpatients, though the difference was not significant.

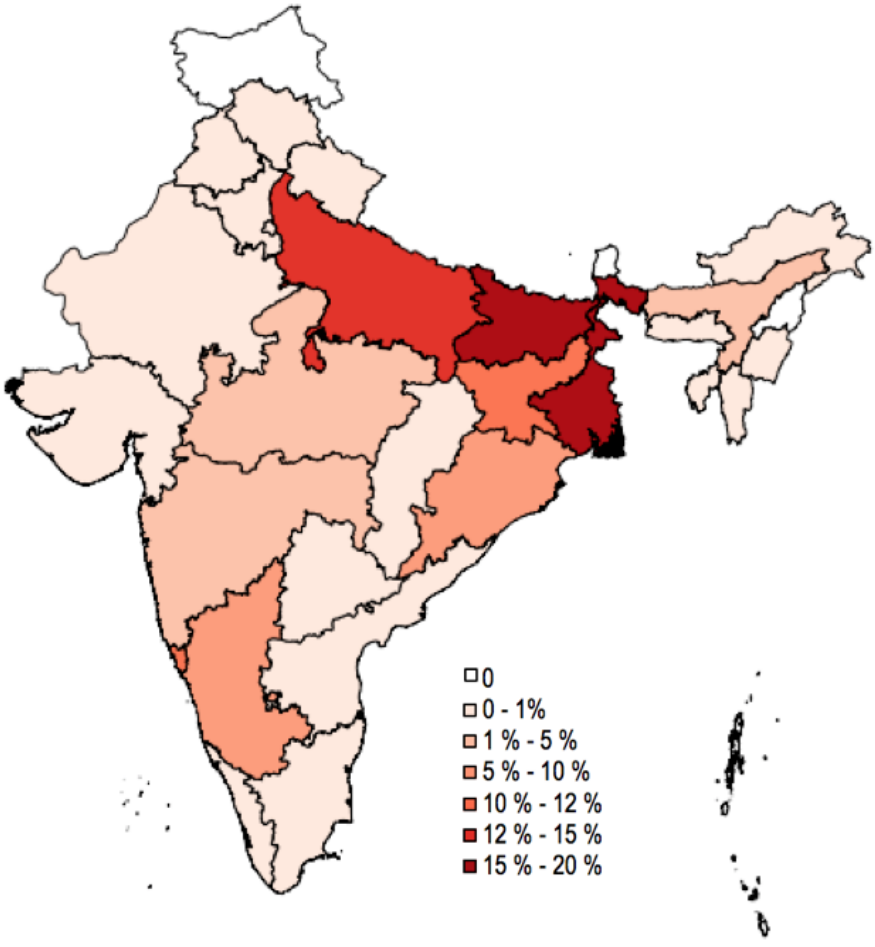


**Figure 2.4 Geographic heterogeneity of Goa Medical College malaria-positive study participants.** Study participants at GMC represented a very heterogeneous Indian population, and most study participants were born outside of Goa. The proportion of patients that were born in each state is shown.

**Table 2.1 Parasitemia profiles by unequal variances t-test of *Plasmodium falciparum* and *Plasmodium vivax* patients**

| | Number of cases[$] | | Per cent parasitemia (Mean ± SD) | | *p*-value |
|---|---|---|---|---|---|
| | ***P. falciparum*** | ***P. vivax*** | ***P. falciparum*** | ***P. vivax*** | |
| Outpatients | 121 | 767 | 1.30% ± 1.94% | 0.56% ± 0.67% | <0.001 *** |
| Inpatients | 107 | 71 | 1.35% ± 3.31% | 0.41% ± 0.65% | 0.005**# |
| All patients | 228 | 838 | 1.32% ± 2.67% | 0.55% ± 0.67% | <0.001 *** |

**: *p value* <0.01, ***: *p value* <0.001, #: Welch correction, $: excludes mixed-infection cases

*2.3.3 Demographic and diagnostic characteristics of malaria patients*

To better understand the *P. falciparum* and *P. vivax* patient populations at GMC, univariate analyses using logistic regression with a primary outcome of malaria parasite species were performed. The demographic and basic diagnostic features associated with species of infection for all malaria-confirmed cases are shown in **Table 2.2**. Occupation as a construction worker was weakly correlated with *P. vivax* infection only (55%), albeit a significant proportion of *P. falciparum* cases (47%) self-identified as construction workers. Higher parasitemia, lower Hb, anemia, severe anemia and hospitalization were all significantly associated with *P. falciparum* infection. Conversely, gender, age, geographic origin, temperature, high fever, and severe fever did not significantly differ between *P. falciparum* and *P. vivax* cases.

**Table 2.2 Univariate logistic regression of demographic and diagnostic features by species of malaria infection**

| **Feature** | ***P. falciparum*[$]** | ***P. vivax*[$]** | **OR (95% CI)** | ***p*-value** |
|---|---|---|---|---|
| Male, n/N (%) | 210/228 (92.1) | 768/838 (91.6) | 1.1 (0.6-1.9) | 0.806 |
| Age, y, Mean ± SD | 27.0 ± 11.2 | 27.4 ± 10.5 | 1.0 (1.0-1.0) | 0.659 |
| Construction workers, n/N (%) | 107/228 (46.9) | 461/838 (55.0) | 0.7 (0.6-1.0) | 0.046 * |

| | | | | |
|---|---|---|---|---|
| Born in Goa, n/N (%) | 30/228 (13.2) | 96/838 (11.5) | 1.2 (0.8-1.9) | 0.337 |
| Born in SW India, n/N (%) | 56/228 (24.6) | 187/838 (22.3) | 1.1 (0.8-1.6) | 0.475 |
| Born in east India, n/N (%) | 134/228 (58.8) | 545/838 (65.0) | 0.8 (0.6-1.1) | 0.103 |
| % Parasitemia, Mean ± SD | 1.3 ± 2.7 | 0.6 ± 0.7 | 1.6 (1.4-1.9) | <0.001 *** |
| Temperature, C, Mean ± SD | 38.1 ± 1.6 | 38.2 ± 1.4 | 1.0 (0.9-1.0) | 0.642 |
| High fever (>38.1$^{o}$C), n/N (%) | 104/228 (45.6) | 380/838 (45.3) | 1.0 (0.8-1.4) | 0.769 |
| Severe fever (>38.9$^{o}$C), n/N (%) | 71/228 (31.1) | 235/838 (28.0) | 1.2 (0.9-1.7) | 0.266 |
| Hemoglobin, g/dL, Mean ± SD | 10.9 ± 2.6 | 11.6 ± 2.1 | 0.9 (0.8-0.9) | <0.001 *** |
| Anemia, n/N (%) | 42/219 (19.2) | 90/837 (10.8) | 2.0 (1.3-2.9) | 0.001 ** |
| Severe anemia, n/N (%) | 15/219 (6.8) | 14/837 (1.7) | 4.3 (2.0-9.2) | <0.001 *** |
| Hospitalization, n/N (%) | 107/228 (46.9) | 71/838 (8.5) | 9.1 (6.4-13.0) | <0.001 *** |

*: *p value* <0.05, **: *p value* <0.01, ***: *p value* <0.001, $: excludes mixed-infection cases

*2.3.4 Clinical characteristics of hospitalized malaria patients*

Overall, 46.9% (107/228) of enrolled *P. falciparum* patients were hospitalized compared with 8.5% (71/838) of enrolled *P. vivax* patients, demonstrating an expected strong skew toward inpatient care and treatment of *P. falciparum* versus *P. vivax* (logistic regression; *p*-value <0.001*)*. Furthermore, there was a significant age-dependent increase in hospitalisations. All malaria-positive patients over 40 years of age infected with either *P. falciparum* or *P. vivax* were significantly more likely to be hospitalized than those between the ages of 1-20 and 21-40 with the same species of infection (logistic regression; *P. vivax p*-value <0.001, *P. falciparum p*-value=0.033).

Among inpatients with malaria-confirmed infections at GMC, those with *P. falciparum* were more severely ill according to the WHO severe malaria criteria (**Table 2.3**) [42, 43]. Approximately equal percentages of hospitalized *P. falciparum* (76.6%) and *P. vivax* (78.9%) cases presented with at least one severity indicator. Of these criteria, respiratory distress,

jaundice and renal failure were the most common symptoms present among both *P. falciparum* (51, 40.7, 42%) and *P. vivax* (42.9, 32.1, 19.7%). However, a significantly greater percentage of *P. falciparum* inpatients presented with at least two (43.9%, *p*-value <0.05) and at least three (29.9%, *p*-value <0.01) severity features compared with *P. vivax* inpatients, indicating that multi-organ involvement was much more common in hospitalized *P. falciparum* cases. There were six deaths among the 182 enrolled malaria positive inpatients at GMC (6/182, 3.3%), all of which were *P. falciparum* cases (6/107, 5.6%).

**Table 2.3 Univariate logistic regression of clinical features by species of malaria infection among hospitalized patients**

| **Feature** | ***P. falciparum*[$]** | ***P. vivax*[$]** | **OR (95% CI)** | ***p*-value** |
|---|---|---|---|---|
| Glasgow coma score, Mean ± SD | 13.8 ± 2.5 | 14.5 ± 1.5 | 0.8 (0.7-1.0) | 0.040 * |
| Coma, n/N (%) | 10/107 (9.3) | 2/71 (2.8) | 3.7 (0.8- 17.9) | 0.099 |
| Blood urea nitrogen, mg %, Mean ± SD | 75.7 ± 82.0 | 38.3 ± 24.7 | 1.0 (1.0-1.0) | 0.003 ** |
| Serum creatinine, mg/dL, Mean ± SD | 2.2 ± 3.2 | 1.1 ± 0.5 | 1.4 (1.1-2.0) | 0.027 * |
| Renal failure, n/N (%) | 42/100 (42.0) | 12/61 (19.7) | 3.0 (1.4-6.3) | 0.004 ** |
| Respiration rate (breaths/m), Mean ± SD | 23.2 ± 6.7 | 21.8 ± 8.7 | 1.0 (1.0-1.1) | 0.395 |
| Respiratory distress, n/N (%) | 50/98 (51.0) | 27/63 (42.9) | 1.5 (0.8-2.8) | 0.219 |
| Total bilirubin, mg/dL, Mean ± SD | 5.9 ± 8.6 | 3.5 ± 4.1 | 1.0 (1.0-1.0) | 0.812 |
| Jaundice, n/N (%) | 35/86 (40.7) | 18/56 (32.1) | 1.5 (0.8-3.1) | 0.225 |
| Severe jaundice, n/N (%) | 15/86 (17.4) | 5/56 (8.9) | 2.3 (0.8-6.7) | 0.136 |
| Systolic BP, mm Hg, Mean ± SD | 99.0 ± 13.0 | 95.8 ± 13.4 | 1.0 (1.0-1.0) | 0.107 |
| Shock, n/N (%) | 3/105 (2.9) | 5/70 (7.1) | 0.4 (0.9-1.8) | 0.217 |
| Pulmonary oedema, n/N (%) | 9/106 (8.5) | 5/72 (6.9) | 1.5 (0.8-3.1) | 0.225 |
| Abnormal bleeding, n/N (%) | 7/107 (6.5) | 1/71 (1.4) | 5.0 (0.6-43.4) | 0.136 |
| % Parasitemia, Mean ± SD | 1.4 ± 3.3 | 0.4 ± 0.7 | 1.5 (1.0-2.1) | 0.024 * |
| Temperature, C, Mean ± SD | 37.7 ± 1.5 | 99.5 ± 1.4 | 1.0 (0.9-1.2) | 0.481 |
| High fever (>38.1$^{o}$C), n/N (%) | 35/107 (32.7) | 22/71 (31.0) | 1.2 (0.6-2.3) | 0.599 |

| | | | | |
|---|---|---|---|---|
| Severe fever (>38.9ºC), n/N (%) | 19/107 (17.8) | 13/71 (18.3) | 1.0 (0.5-2.3) | 0.918 |
| Hemoglobin, g/dL, Mean ± SD | 10.1 ± 2.7 | 10.6 ± 2.5 | 0.9 (0.8-1.0) | 0.159 |
| Anemia, n/N (%) | 33/107 (30.8) | 16/71 (22.5) | 1.6 (0.8-3.3) | 0.157 |
| Severe anemia, n/N (%) | 12/107 (11.2) | 2/71 (2.8) | 4.6 (1.0-21.9) | 0.049 * |
| Days in hospital, Mean ± SD | 6.0 ± 5.5 | 4.6 ± 2.0 | 1.1 (1.0-1.2) | 0.044 * |
| Long hospital stay (>8 days), n/n (%) | 18/107 (16.8) | 3/71 (4.2) | 4.7 (1.3-17.2) | 0.016 * |
| Severity, Mean ± SD | 2.0 ± 2.02 | 1.2 ± 1.14 | 1.3 (1.1-1.67) | 0.005 ** |
| Severe malaria (SS>0), n/N (%) | 82/107 (76.6) | 56/71 (78.9) | 1.1 (0.5-2.2) | 0.818 |
| Severe malaria (SS>1), n/N (%) | 47/107 (43.9) | 19/71 (26.8) | 2.2 (1.2-4.3) | 0.014 * |
| Severe malaria (SS>2), n/N (%) | 32/107 (29.9) | 9/71 (12.7) | 3.0 (1.3-7.0) | 0.007 ** |

*: *p value* <0.05, **: *p value* <0.01, $: excludes mixed-infection cases, SS: # of WHO severe malaria criteria met

In addition to increasing clinical severity (*p*-value <0.01), lower Glasgow coma score, higher blood urea nitrogen, higher serum creatinine, renal failure, per cent parasitemia, severe anemia, more days in the hospital and hospitalization for more than eight days were all significantly correlated (*p*-value <0.05) with *P. falciparum* infection. Shock or circulatory collapse was the only feature that showed a greater frequency among hospitalized *P. vivax* cases as compared to *P. falciparum*, albeit numbers were low in both species of infections. Though hypoglycemia and acidosis were not routinely measured, one case of hypoglycemia was observed in a *P. falciparum* patient (1/45, 2.2%), while it was not seen among any of the tested *P. vivax* patients (0/13, 0%). Metabolic acidosis, when tested for, was observed more frequently in *P. falciparum* cases (12/33, 36.4%) as compared to *P. vivax* (2/19, 10.5%).

### *2.4 Discussion*

The present four-year study was undertaken to better understand malaria disease burden in a peri-urban setting in India. The study points to a complex malaria situation in Goa with both *P. vivax* and *P. falciparum* cases year-round, a recent, substantial increase in the

number of malaria-positive patients annually, a greater burden of disease from *P. falciparum*, and a highly diverse population of Indian patients from 26 of 29 states.

Between 2012 and 2015, GMC saw 6,277 malaria-positive cases, with the number of *P. falciparum* and *P. vivax* patients increasing more than two-fold, from 889 in 2012 to 2,261 in 2015. In contrast, between 2012 and 2015, the Indian National Vector Disease Control Programme (NVBDCP) reported 4,854 cases of malaria in the state of Goa, 443 of which were caused by *P. falciparum,* and showed a more than two-fold decline in cases between 2012 (1714 cases) and 2015 (786 cases) [44]. Whether this discrepancy in malaria cases is due to an increase in the number of patients from other states, higher local transmission, or some other cause remains to be determined. At the same time, these figures suggest that a large proportion of the total malaria cases in the state of Goa may be presenting to GMC and, thus, GMC may provide an approximation of malaria transmission and disease burden in the local community.

Despite the expected increase in number of malaria cases during and after the monsoon season, both *P. vivax* and *P. falciparum* patients were seen year-round at the GMC. While the year-round presence of *P. vivax* cases may be explained by the ability of *P. vivax* parasites to lie dormant as hypnozoites in liver stages with small numbers re-emerging during the dry season, this would not be the case for *P. falciparum* [8, 45]. Although it remains to be established, a more perennial transmission cycle of both *P. vivax* and *P. falciparum* may be aided by the recent discovery of a previously unsuspected vector in this region, *Anopheles subpictus* [46]. This vector has been shown to peak in numbers and transmission capacity both after the monsoons when the traditional urban malaria vector *Anopheles stephensi* peaks, but also in the dry season when *An. stephensi* numbers are low.

Although *P. vivax* infections were much more common at GMC, *P. falciparum* infections were associated with greater severity. Although approximately equal proportions of hospitalized patients with *P. falciparum* and *P. vivax* had at least one WHO severe malaria feature, patients with two or more severity criteria were more common to *P. falciparum* cases. The six deaths

that occurred among study participants were all *P. falciparum* patients. In recent years, there have been a number of studies pointing to the importance of severe *P. vivax* in Asia, with some suggesting that *P. vivax* may be as or nearly as virulent as *P. falciparum* [47-52]. In the present study, though *P. vivax* caused some severe illness, multi-organ involvement was not typical. *Plasmodium falciparum* infections showed greater severity with substantial multi-organ involvement.

There was a strikingly greater proportion of adult males (86.7%) presenting to GMC with *P. falciparum* and *P. vivax* malaria than adult females. The same gender bias was smaller for children enrolled in the study, with 64.7% male children and 35.3% female children.. Such findings are consistent with previous reports of age-dependent sex-bias in clinical malarial disease in hypo-endemic regions [53] and with the gender distribution of malaria-positive patients at other MESA-ICEMR government tertiary hospital sites in India (unpublished data). It is also important to consider the profile of individuals who commonly seek care at government hospitals, which may be a contributing factor.

Both *P. falciparum* and *P. vivax* patients over 40 years of age were significantly more likely to be hospitalized by GMC than those between the ages of one and 20 years and 21 and 40 years, which points to a possible increase in the susceptibility of older populations to severe malaria in lower transmission settings. Future analysis will explore age-related symptomology and outcomes as well as parasite and gametocyte carriage to better understand if malaria virulence is increased in older populations in Goa. Other studies have shown that presenting syndromes in severe malaria depend on age and that age is an independent risk factor for a fatal outcome of malaria [16, 54, 55].

Construction sites in India are commonly suggested as potential transmission hot-spots, especially of severe *P. falciparum*, in low prevalence, relatively prosperous areas such as Goa [6, 56]. Though construction workers, who live and work at these sites and traditionally hail from the east and northeast states of India, accounted for roughly half of the malaria-positive study

participants at GMC, they were nearly equally likely to be infected with *P. vivax* as *P. falciparum*. These findings may have implications for the conventional understanding of risk factors as well as the basis of targeted control measures at and around construction sites in the low transmission state of Goa. In the future, investigation to determine the possible effect of pre-existing *P. falciparum* and *P. vivax* immunity, compared across age, gender, origin and occupation, on transmission in Goa will be carried out.

There are a number of limitations to the present study. Due to logistical limitations and the high number of cases, only 18% of the hospital-identified malaria positive cases that presented to GMC over the study period were enrolled. To date, there is not a complete, widely agreed-upon definition of what clinical features constitute severe *P. vivax* disease, as compared to severe *P. falciparum* disease, globally or on the Indian subcontinent [52, 57]. As such, the WHO definition of severe malaria for *P. falciparum* infection was used for both species, as is generally the case, which may bias or weaken associations seen among *P. vivax* cases. The present study does not include data for splenomegaly, spleen rupture or thrombocytopaenia, which have been reported to be common among severe *P. vivax* cases and are not reflected in the WHO criteria [22, 24, 52]. Lactate, hypoglycaemia, and metabolic acidosis, all of which are important indicators of severe malaria, were not routinely measured. Malaria patients admitted to the GMC who required use of a ventilator, presumably due to respiratory distress [29, 47], which also has been commonly reported in severe *P. vivax,* were not included in the present study. This may impact the disease burden being reported among *P. vivax* and/or *P. falciparum* cases. It was also not always possible to accurately determine whether a patient had co-infections and/or had been treated with anti-malarials prior to presentation at GMC, a tertiary care center. Co-infections and/or prior treatment may have impacted the measurement of clinical severity and/or course of malaria infection [58]. Future studies will seek to present the dynamic course of infection among severe malaria patients at GMC.

## *2.5 Conclusion*

The present study highlights the demographic, diagnostic and clinical profiles of *P. falciparum* and *P. vivax* patients at the only government tertiary care facility in a peri-urban setting in southwestern India. The number of malaria cases presenting to GMC increased more than two-fold from 2012 to 2015, which may be of interest in the context of elimination efforts. The data from GMC show a greater burden of disease contributed by *P. falciparum* than *P. vivax* and suggest a potential age-dependent increase in susceptibility that will be further investigated.

## *2.6 Ethics approval and funding*

The human subjects protocol and consent forms for enrollment of *Plasmodium* positive individuals presenting to Goa Medical College and Hospital were approved by the institutional review boards of the Division of Microbiology and Infectious Diseases at the US National Institute of Allergy and Infectious Diseases (DMID 11-0074), GMC (no number assigned), and the University of Washington (42271).

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## Chapter 3: Impact of malaria diagnosis and treatment on a remote tribal cohort in Assam, Northeastern India

*This chapter contains ongoing analysis at the time of this dissertation's submission. The work is outlined below. The anticipated author list is:* ***Karschney L****, Sarma DK*, Gillespie K, Chakrabarti R, Maki JN, Pal-Bhomick I, Bora K, Bhattacharyya DR, Narain K, Mahanta J, Kublin JG, Rathod PK, Janes HE, Mohapatra PK. (*authors contributed equally)*

Abstract: India is a vast country with an estimated population of 1.4 billion and accounts for 66% of the malaria cases in the WHO Southeast Asia region. The northeastern region of India is of particular interest due to higher prevalence rates, the predominance of *P. falciparum*, and its proximity and ecological similarity to the Greater Mekong Subregion.

A remote seven-village cluster in the Karbi Anglong district of Assam, India was selected for a community-wide dynamic cohort study from May 2015 to June 2017. Three surveys were conducted annually, during which each consenting resident was clinically assessed, tested for malaria by microscopy and rapid diagnostic test, and given a short questionnaire. Throughout the rest of the year, residents experiencing symptoms were provided with malaria testing. Identified malaria cases were referred for treatment per the Indian National Drug Policy.

During the first survey (May-Sept 2015), 152 of 2331 individuals from 455 households (6.5%) were malaria positive by microscopy or RDT; 143 (6.1%) with *P. falciparum*, 6 (0.3%) with *P. vivax* and 3 (0.1%) with mixed infection. By the fifth survey, the overall prevalence had decreased by 95% in roughly two years to 0.33%. This represents a 96% reduction in *Pf* cases, an 83% reduction in *Pv* cases and a 100% reduction in mixed cases.

The demographics of the seven villages were similar, though differences in prevalence of malaria were observed. The highest prevalence was in the village of Bhaktegaon (88/483; 18.2%). Among the total population, more malaria cases were asymptomatic (120/2787; 4.3%) than symptomatic (106/2787; 3.8%). It was noted that while most (2665/2777; 95.9%)

individuals reported using a bed net, few (319/2661; 12.0%) reported treated bed net usage. Despite a malaria prevalence of 6.5% during the first survey, 98.4% of study participants reported no prior malaria infection.

Though the Government of India has committed to eliminating malaria by 2030, the low usage of treated bed nets (12%) and prevalence of symptomatic malaria (3.8%) indicate that routine prevention, diagnostic and treatment options may not be adequate or accessible to this population. The prevalence of asymptomatic malaria (4.3%) and percentage of individuals reporting no known prior infection (98.4%) indicates a reservoir of malaria that may not be effectively addressed by surveillance or elimination efforts relying solely on identification and treatment of symptomatic cases.

Abbreviations: ASHA: Accredited Social Health Activist, DMID: Division of Microbiology and Infectious Diseases, GOI: Government of India, GMS: Greater Mekong Subregion, Hb: Hemoglobin, HMSC: Health Ministry Screening Committee, ICEMR: International Center of Excellence for Malaria Research, IRB: Institutional Review Board, MESA: Malaria Evolution in South Asia, NIAID: National Institute of Allergy and Infectious Diseases, NIH: US National Institutes of Health, NVBDCP: National Vector Borne Diseases Control Programme, OR: Odds ratio, RDT: Rapid diagnostic test, Regional Medical Research Center-NE Region (RMRC-NE), UW: University of Washington, WHO: World Health Organization.

### *3.1 Introduction*

Despite ongoing control, elimination and eradication efforts, malaria remains a major infectious disease worldwide. In 2022, the WHO reported 249 million cases and 608,000 deaths globally (1). Between 2000 and 2020, India reported a 78% decrease in the number of malaria cases, from approximately 200 million cases in 2000 to approximately 5 million cases in 2020 (2). According to the National Vector Borne Disease Control Programme of India (NVBDCP),

malaria cases in the northeastern state of Assam dropped from 83,939 in 2008 to 3,816 in 2018 (3).

The northeast region of India is of particular interest due to higher prevalence rates, the predominance of *P. falciparum*, and its proximity and ecological similarity to the Greater Mekong Subregion (3-6). Northeast India offers the only land route for vectors and parasites to enter India from Southeast Asia through Myanmar (7). Over many decades, malaria parasites have developed drug resistance to a variety of antimalarials with diverse mechanisms of action in Southeast Asia (8). These include chloroquine (CQ), sulfadoxine-pyrimethamine (SP), mefloquine and, most recently artemisinin derivatives (8).

Assam, one of eight states in northeastern India, is located in the tribal belt and is a biodiversity hotspot (3). The climate is humid and the terrain is mostly mountainous and hilly. The primary vectors of malaria parasites are *An. baimai* and *An. minimus* and the predominate species of parasite are *Plasmodium falciparum* and *Plasmodium vivax*, with *Plasmodium falciparum* contributing the majority of malaria infections in Assam (3, 9). In 2013, Artemisinin-Lumefantrine (AL) was introduced as the frontline antimalarial against *Pf* in NE India, including Assam, due to widespread resistance to both CQ and SP (10-12).

The present study was designed to determine whether human hemoglobin variants, specifically HbAE and HbAS, reduced susceptibility to and/or altered the clinical course of *Plasmodium* infection (13-18). We were additionally interested in determining the prevalence of asymptomatic and symptomatic malaria infections by species over time and space in a remote, community-based cohort (19). Secondarily, we sought to identify any instances of delayed parasite clearance in the population, which could indicate reduced efficacy of artemisinin-based combination therapies (ACTs) (7, 8). Finally, as an unintended consequence of providing access to malaria diagnosis and treatment to the cohort of study participants, the present study

observed the impact of a comprehensive test and treat strategy on malaria prevalence in an isolated community (20, 21).

## *3.2 Methods*

### *3.2.1 Ethics approval*

Approvals for this study were obtained from the University of Washington Institutional Review Board (STUDY00001192), the Government of India Health Ministry Screening Committee (TDR/689/2017-ECD-II) and the Indian Council of Medical Research-Regional Medical Research Center Northeast (ICMR RMRC-NE) institutional ethics committee.

### *3.2.2 Study site selection*

We designed a dynamic cohort study to include all permanent residents of seven contiguous villages in the malaria endemic district of Karbi Anglong, Assam state, India (**Figure 3.1**). Six villages inhabited by native Karbi people and one village (Japijuri) inhabited by tea estate workers (22) who migrated from Central India about 150 to 200 years ago were included in the seven-village cluster. Karbi people are known to have a high prevalence of HbAE and individuals from Central India have a high prevalence of HbAS (14).

The study villages (Bachekrang, Bhaktegaon, Dolamara Market, Jamindar, Japijuri, Rangjangphang and Sartheterang) sit south of the Kohora range of the Kaziranga National Park at an elevation of about 100 meters and are surrounded by hilly, forested areas. Farming was the main occupation of the villagers including shifting cultivations on the hills, locally known as ‘Jhum’. Others worked in the nearby tea gardens or in the forests. The study area contained a private health clinic and the nearest government health facility, the Dolamara mini Primary Health Center (mPHC) was located about 10 km away (19).

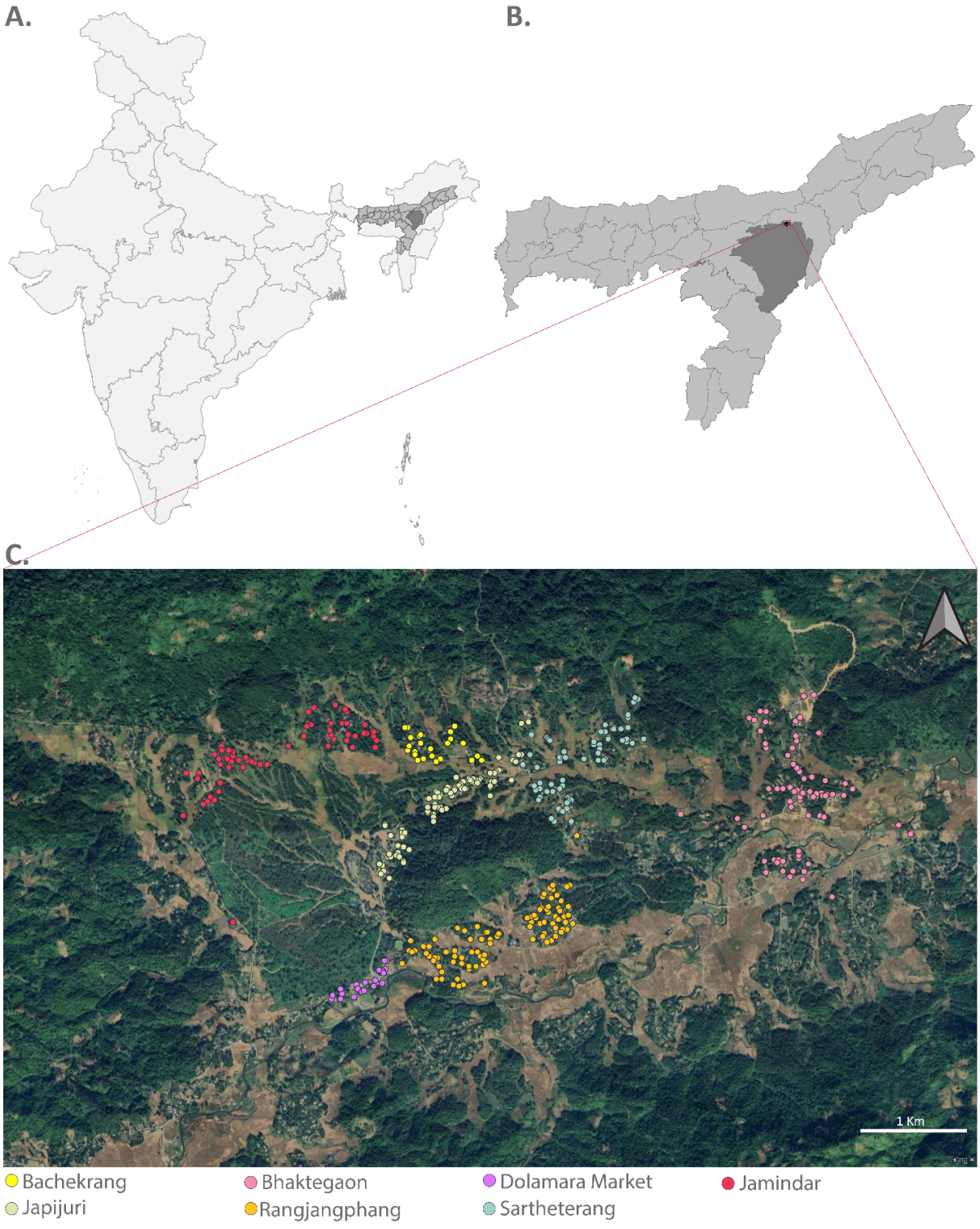


**Figure 3.1 Household locations included in the Karbi Anglong cohort study.** The A.) Indian state of Assam contains B.) the Karbi Anglong district where C.) the seven villages in this survey are located.

*3.2.3 Study site preparation, cohort enrollment and follow-up*

A field laboratory was established in the nearby town of Bokakhat. Before commencing, study investigators met all the head men (Gaon Burha) of the villages to inform them of the project, seek their permission, and explain the study methodology. A team of six trained individuals recruited from the study villages covered the entire area on foot to identify and enumerate all households. A household was defined as any single permanent or semi-permanent entity that served as a primary residence for a person or group of peoples from the same family. All households were assigned unique identifying IDs, written prominently on the front doors, along with the number of permanent residents in the houses. A permanent resident of a household was defined as a person who was staying in the household for at least the last one month.

Study investigators explained the study procedures to heads of the households and sought informed consent from every household member. Individuals providing informed consent were enrolled in the study and co-ordinates of the study household were noted with a handheld GPS unit. Portable weather stations were set up in Sartheterang and Japijuri to locally monitor meteorological parameters of rainfall, temperature and humidity.

All enrolled participants were asked to participate in three surveys annually: one during the pre-monsoon (March-April), one during the monsoon (July-August) and one during the post-monsoon season (October-November). Village-based Accredited Social Health Activist (ASHA) (23) services were sought to monitor febrile cases for malaria infection throughout the year (non-survey period) with Rapid Diagnostic Tests (RDT, FalciVax, Zephyr Biomedicals). ASHAs are empowered by the National Health Mission (NHM) to ‘provide a minimum package of curative care as appropriate and feasible for that level and make timely referrals’.

Each study participant was requested to donate a fingerprick blood sample, which was subsequently used for malaria diagnosis through RDT and smears (thick and thin). Blood samples were also spotted on Whatman FTA™ Elute Micro cards (Merck) and stored for future analysis. Participants were subjected to a brief physical examination that included body temperature recording and a short questionnaire including demographic information, medical and travel history, and malaria-related knowledge, attitudes and practices (KAP).

Any participant who was found to be malaria positive by RDT was treated by the ASHA on-site with the appropriate antimalarial regimen per the National Drug Policy (11, 24). Severe cases were referred to the nearest government facility with transport aided by the study team. The thin and thick smears were examined for malaria parasites in the MESA-ICEMR field laboratory at Bokakhat the following day. If any RDT negative participant was found to be malaria-positive by smear, they were contacted immediately, and the appropriate antimalarial regimen was provided through the ASHAs.

Each malaria positive study participant was followed up by the study team on Day 2 (D2), Day 3 (D3), Day 5 (D5) and Day 7 (D7) post detection. A fingerprick blood sample was drawn on each follow up visit for thick and thin smears as well as FTA™ card. Follow-up visits also included a brief physical examination, recording of temperature, and a short questionnaire about any malaria-related symptoms. Study participants who were still parasite positive on Day 7 were referred to the nearest government health facility for rescue treatment according to the National Guidelines (11, 24). FTA cards collected during all study visits, including those from follow-up visits, were transported to the main MESA-ICEMR laboratory at RMRC, Dibrugarh for further analysis using qPCR. Any case of death, birth and migration in or out of each village or refusal to continue in the study cohort was recorded in real-time.

The study site, comprised of all seven villages, was divided into six sectors based on population for supervisory purposes. Each sector was overseen by one supervisor along with

one ASHA member, both permanent residents of that area. The supervisors were responsible for tracking fever cases during non-survey periods, assisting ASHA members to carry out malaria diagnosis, completing the non-survey visit questionnaires, and transferring all questionnaires and samples from the field to the field laboratory in Bokakhat. All data forms were reviewed on site and matched to samples and blood smears. The supervisors ensured that at least three attempts were made to monitor participants before a scheduled visit was considered missed. When possible, information from friends or neighbors of missing families or participants was collected to determine possible reasons for absence.

### *3.2.4 Vector studies*

CDC miniature light traps were set up in four households in Bhaktegaon and Sartheterang between June 2015 and January 2017. The light traps were operated from dusk to dawn in four households (two fixed and two randomly selected) at weekly intervals. Morphological typing of the collected mosquitoes was done at the species level. Thereafter, each mosquito's head and thorax were detached from the abdomen under sterile conditions and each head and thorax were placed in a 1.5ml micro-centrifuge tube with 1X PBS. Genomic DNA was extracted from each individual head and thorax portion using the QIAamp DNA micro kit (QIAGEN, CA, USA). DNA samples were eluted in 20µL of DNase/RNase free water and stored in -20 °C for subsequent analysis.

## *3.3 Results*

A total of 2,787 unique study participants enrolled in the dynamic cohort from seven villages over the course of five surveys. Enrollment varied by survey, with an average of 2,294 individuals participating per survey. The number of study participants in individual villages varied between the five surveys as some missed survey visits while other participants joined (**Table 3.1**). Five surveys were conducted between May 2015 and June 2017 (**Figure 3.2**), with the visits in each village generally overlapping with one another.

**Table 3.1 Enrollment of study participants in each survey, by village**

| Village | First | Second | Third | Fourth | Fifth |
|---|---|---|---|---|---|
| Bachekrang (n = 179) | 151 | 156 | 160 | 146 | 140 |
| Bhaktegaon (n = 483) | 415 | 392 | 399 | 395 | 368 |
| Dolamora Market (n = 147) | 93 | 95 | 107 | 106 | 82 |
| Jamindar (n = 421) | 366 | 371 | 373 | 375 | 322 |
| Japijuri (n = 476) | 402 | 394 | 404 | 396 | 353 |
| Rangjanphang (n = 673) | 564 | 545 | 566 | 569 | 482 |
| Sartheterang (n = 408) | 340 | 356 | 360 | 371 | 357 |
| Total (n = 2787) | 2331 | 2309 | 2369 | 2358 | 2104 |

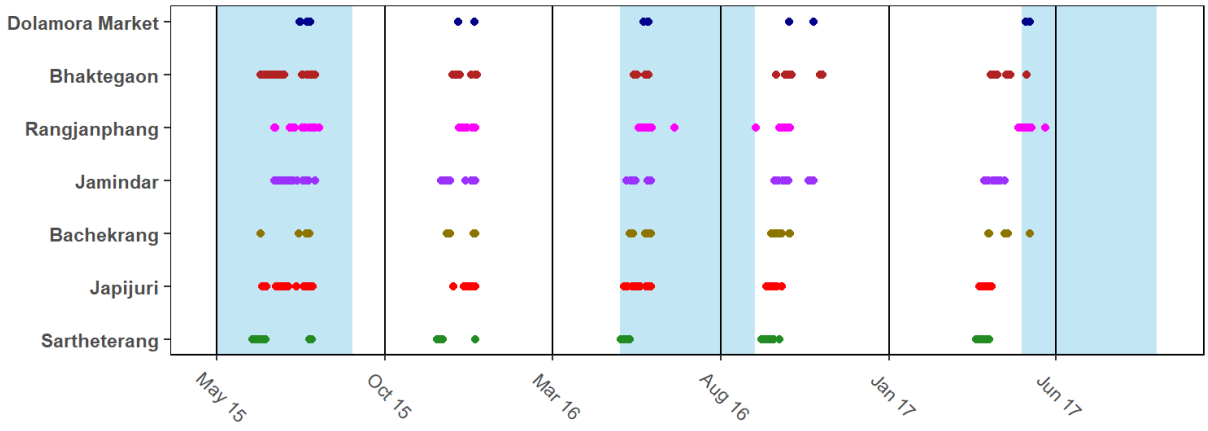


**Figure 3.2 Timing of longitudinal surveys across all villages.** Actual timing of surveys within a period between villages. Each point represents the date at which a participant from a village was surveyed during a survey period. In general, the clusters of visits for each village overlapped with those of other villages within a window. Blue stripes represent monsoon season in the study region.

The prevalence of malaria in the study population was calculated based on rapid diagnostic test (RDT) and/or smear positive malaria results. A total of 215 individuals, out of

2,787, tested positive for malaria during the study period, accounting for an overall malaria prevalence of 7.7%. The number of malaria infections was slightly higher (226) as nine study participants had multiple infections over the two-years. During the first survey (**Figure 3.3**), some malaria-positive study participants (13/152; 8.5%) were lost to follow-up due to the logistical challenges presented by the study location and the high prevalence.

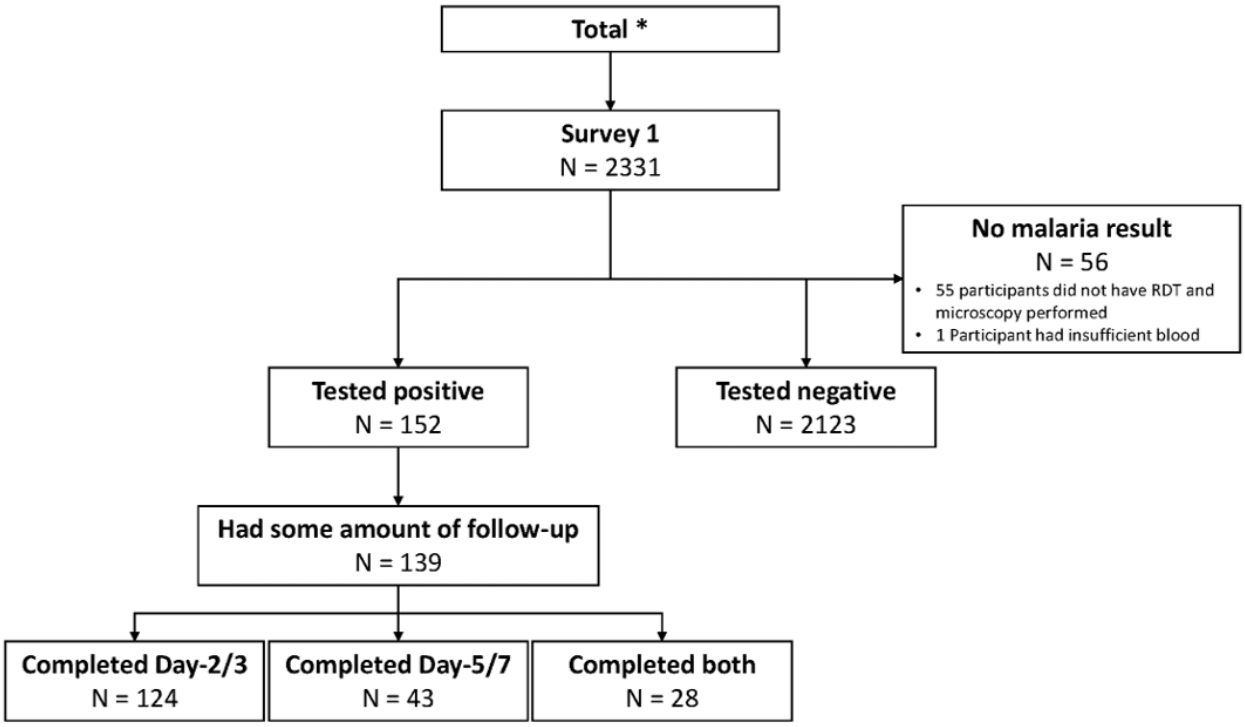


**Figure 3.3 Consort diagram of first survey enrollment**

Over the course of the two-year study, malaria prevalence in the seven villages decreased by 95% (**Figure 3.4**). During the first survey (May-Sept 2015), 152 of 2331 individuals from 455 households (6.5%) were malaria positive by microscopy or RDT. Almost all positive cases were due to *P. falciparum*. 143 (6.1%) were positive with *P. falciparum*, 6 (0.3%) with *P. vivax* and 3 (0.1%) with mixed infection. By the fifth survey, the prevalence of malaria

was 0.33% (7/2104), which accounted for a 96% reduction in *Pf*, an 83% reduction in *Pv*, and a 100% reduction in mixed infections over two years.

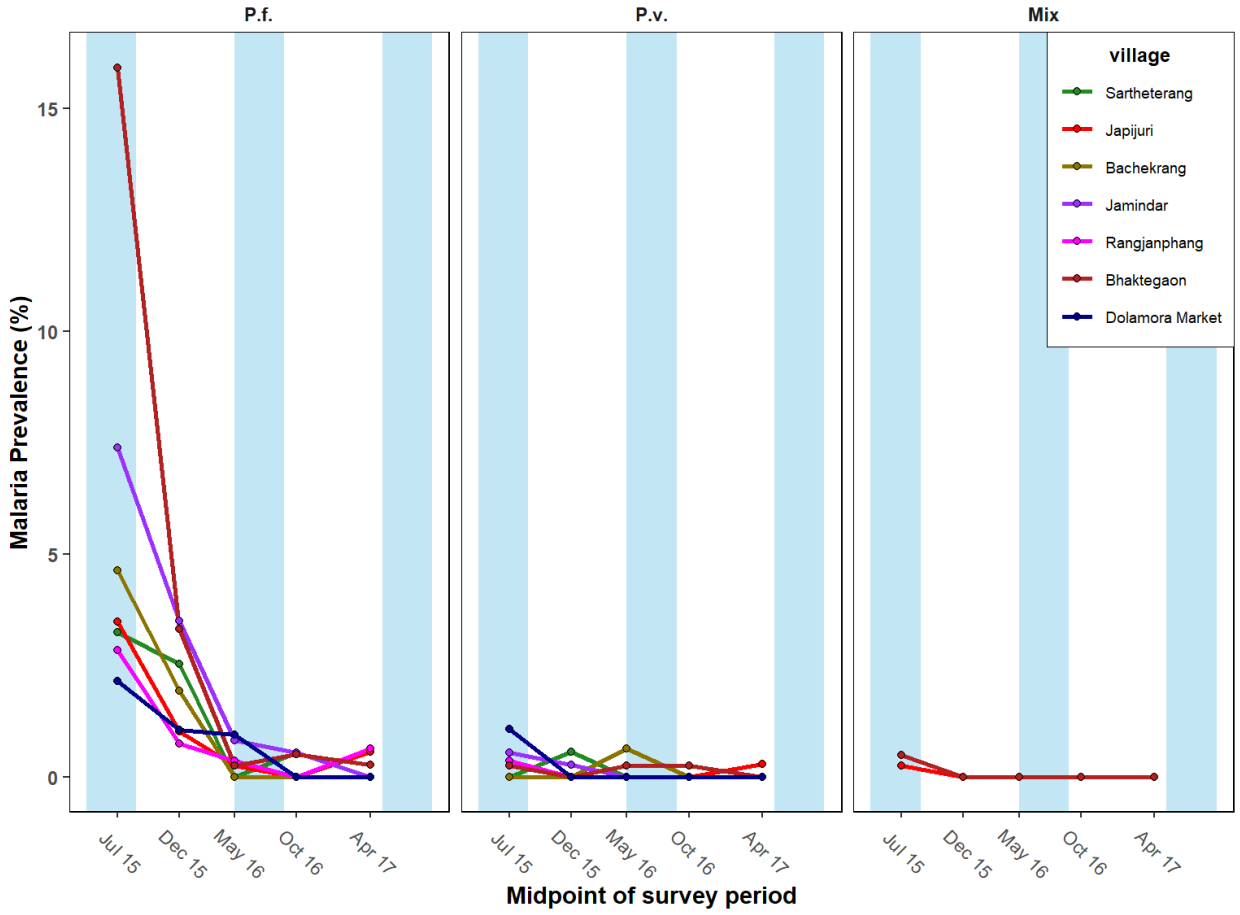


**Figure 3.4 Prevalence of different species of *Plasmodium* infection, by village and survey period.** The different species each get a panel and the prevalence for each village is drawn by survey period. Village prevalence is calculated by dividing the number of species-specific malarial infections during the survey period in the village by the number of visits during the period from the same village. The light blue rectangles represent the annual rainy season (May - September) in the Assam region.

Demographic data collected from the 2,787 study participants reflected a near-equal gender distribution (1,390 male vs 1,397 female) with 371 participants under age 5, 729 between 5 and 15 years of age, and 1,687 above age 15 (**Table 3.2**). Most (2,665/2,777; 95.9%) individuals reported using a bed net. However, many fewer (319/2,661; 12.0%) reported using an insecticide-treated bed net. Most (2,733/2,777; 98.4%) individuals reported no known

prior malaria infection with only 1.5% (42/2,777) reporting a prior malaria infection and 0.1% (2/2,777) uncertain. There was no statistical difference observed between symptomatic and asymptomatic malaria-positive study participants.

**Table 3.2 Cohort demographics by symptomatic and asymptomatic case load in all villages**

| Variable | Level | n | Symptoms n (%) | No symptoms n (%) | Overall n (%) |
|---|---|---|---|---|---|
| | Total enrolled | 2787 | 104 (3.7%) | 111 (3.9%) | 215 (7.7%) |
| Gender | Male | 1390 | 55 (3.9%) | 68 (4.8%) | 123 (8.8%) |
| | Female | 1397 | 49 (3.5%) | 43 (3.0%) | 92 (6.5%) |
| Age | <5 | 371 | 4 (1.0%) | 5 (1.3%) | 9 (2.4%) |
| | 5-15 | 729 | 40 (5.4%) | 32 (4.3%) | 72 (9.8%) |
| | >=15 | 1687 | 60 (3.5%) | 74 (4.3%) | 134 (7.9%) |
| Occupation | Housewife | 369 | 14 (3.7%) | 11 (2.9%) | 25 (6.7%) |
| | Student | 937 | 50 (5.3%) | 43 (4.5%) | 93 (9.9%) |
| | Unemployed | 540 | 11 (2.0%) | 18 (3.3%) | 29 (5.3%) |
| | Unknown | 15 | 0 (0%) | 0 (0%) | 0 (0%) |
| | Working class | 924 | 29 (3.1%) | 39 (4.2%) | 68 (7.3%) |
| | Missing | 2 | 0 (0%) | 0 (0%) | 0 (0%) |
| Education | Too young | 370 | 4 (1.0%) | 4 (1.0%) | 8 (2.1%) |
| | Illiterate | 693 | 27 (3.8%) | 42 (6.0%) | 69 (9.9%) |
| | Some school | 1721 | 73 (4.2%) | 65 (3.7%) | 138 (8.0%) |
| | Missing | 3 | 0 (0%) | 0 (0%) | 0 (0%) |
| Bed net use | Always | 2589 | 90 (3.4%) | 94 (3.6%) | 184 (7.1%) |
| | Sometimes | 76 | 4 (5.2%) | 10 (13.1%) | 14 (18.4%) |
| | Never | 105 | 10 (9.5%) | 7 (6.6%) | 17 (16.1%) |
| | Missing | 17 | 0 (0%) | 0 (0%) | 0 (0%) |
| Treated bed net | Yes | 319 | 10 (3.1%) | 9 (2.8%) | 19 (5.9%) |
| | No | 2336 | 84 (3.5%) | 95 (4.0%) | 179 (7.6%) |
| | Uncertain | 6 | 0 (0%) | 0 (0%) | 0 (0%) |
| | Missing | 126 | 10 (7.9%) | 7 (5.5%) | 17 (13.4%) |
| History of malaria | Yes | 42 | 10 (23.8%) | 6 (14.2%) | 16 (38.1%) |
| | No | 2733 | 92 (3.3%) | 104 (3.8%) | 196 (7.1%) |
| | Uncertain | 2 | 0 (0%) | 0 (0%) | 0 (0%) |
| | Missing | 10 | 2 (20%) | 1 (10%) | 3 (30%) |

The prevalence of asymptomatic malaria (120/2787; 4.3%) was higher than the prevalence of symptomatic malaria (106/2787; 3.8%) (**Table 3.3**). The highest prevalence was

in the village of Bhaktegaon (88/483; 18.2%). The largest number of study participants (673) resided in Rangjangphang whereas Bachekrang had the lowest study population (179). Each village had an approximately equal distribution of symptomatic and asymptomatic cases, with the exception of Bachekrang, which had only asymptomatic cases (11/11 cases).

**Table 3.3 Prevalence of symptomatic and asymptomatic malaria positive cases by species and village**

| Categories | *P. falciparum* asymptomatic | *P. falciparum* symptomatic | *P. vivax* asymptomatic | *P. vivax* symptomatic | Mixed asymptomatic | Mixed symptomatic | Total *P. falciparum* malaria | Total *P. vivax* malaria | Total mixed malaria |
|---|---|---|---|---|---|---|---|---|---|
| | n (%) | n (%) | n (%) | n (%) | n (%) | n (%) | n | n | n |
| **a) By species** | 114 (54.2%) | 96 (45.7%) | 5 (38.4%) | 8 (61.5%) | 1 (33.3%) | 2 (66.6%) | 210 | 13 | 3 |
| **b) By village** | | | | | | | | | |
| Bachekrang (n=179) | 10 (100%) | 0 (0%) | 1 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 10 | 1 | 0 |
| Bhaktegaon (n=483) | 47 (56.6%) | 36 (43.3%) | 0 (0%) | 3 (100%) | 0 (0%) | 2 (0%) | 83 | 3 | 2 |
| Dolamara Market (n=147) | 2 (50%) | 2 (50%) | 0 (0%) | 1 (100%) | 0 (0%) | 0 (0%) | 4 | 1 | 0 |
| Jamindar (n=421) | 24 (53.3%) | 21 (46.6%) | 3 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 45 | 3 | 0 |
| Japijuri (n=476) | 10 (47.6%) | 11 (52.3%) | 0 (0%) | 1 (100%) | 1 (100%) | 0 (0%) | 21 | 1 | 1 |
| Rangjangphang (n=673) | 10 (40%) | 15 (60%) | 0 (0%) | 2 (100%) | 0 (0%) | 0 (0%) | 25 | 2 | 0 |
| Sartheterang (n=408) | 11 (50%) | 11 (50%) | 1 (50%) | 1 (50%) | 0 (0%) | 0 (0%) | 22 | 2 | 0 |

## *3.4 Discussion*

Over the course of about two years, the prevalence of malaria in the study population decreased from 6.5% to 0.33%. Such a substantial decrease in a relatively short period of time indicates that providing testing to all and treating positive cases through ASHAs was very successful in reducing malaria in the study population. Such a strategy could be considered to drive down malaria prevalence in high-endemicity regions.

The prevalence of malaria during the first survey (6.5%), which lasted from May 2015 through September 2015, was higher than the prevalence reported in Karbi Anglong district data collected from the Government of India (GOI) (unpublished). From May 2015 through September 2015, the GOI reported that it tested 59,768 individuals for malaria in Karbi Anglong and that a total of 2,450 individuals (4.1%) were positive for *Plasmodium falciparum* (2,315/59,768; 3.9%) or *Plasmodium vivax* (135/59,768; 0.2%). The difference in prevalence

may reflect testing of the entire population versus testing of fever cases only, as the GOI prevalence more closely matched the symptomatic prevalence observed in the cohort (3.8%).

It is notable that only 1.5% of the cohort reported a known prior infection with malaria. This finding signifies that there may be a lack of access to health education, diagnosis and/or treatment available to the study population. Similarly, though 95.9% of participants reported sleeping under a bednet, only 12% reported using an insecticide treated bednet. Distribution of long-lasting insecticidal nets (LLINs) are a staple of malaria control and elimination measures (25, 26). Possible reasons for the lack of LLIN use could be related to a lack of access or distribution as well as concerns about LLIN's possible effect on silkworms, which were being cultivated in many study households.

Overall, the prevalence of asymptomatic malaria (4.3%) was higher than the prevalence of symptomatic malaria (3.8%). This indicates that a reservoir of malaria existed in the study population that may not be effectively addressed through existing GOI surveillance and testing efforts that rely on the identification and treatment of symptomatic cases only (27, 28). Without identification and treatment of asymptomatic cases, changes in ecological factors (vectors, climate, reduced antimalarial sensitivity of parasites) could result in unexpected increases in prevalence and malaria pathogenesis (29-32).

The present study was conducted in a remote part of northeastern India, which presented myriad challenges to the study team. Lack of phone reception, frequent flooding and washout of roads, hilly terrain, subsistence farming away from the household during daytime hours, traditional dwelling structures that are raised many meters from the ground, transportation time between the villages and the field lab in Bokakhat, and separatist activity all presented challenges on the ground. Though follow-up of malaria cases was planned on D2/3 and D5/7, completion by the study team, particularly during the first survey when the prevalence was higher than expected based on preliminary data, was spotty and incomplete. As such, it

was not possible to reliably assess the prevalence of delayed parasite clearance after 7 days. Future studies in this or similar regions may wish to account for such challenges when planning and staffing.

A follow-up study was scheduled to begin in early 2020 but was not completed due to disruptions caused by the global covid pandemic. That planned work was to again assess the malaria prevalence in the population after a three-year break in comprehensive diagnosis and treatment access and to monitor parasite clearance rates over 42 days to assess the effectiveness of ACTs in the Karbi Anglong district.

### *3.5 Conclusion*

Though the Government of India has committed to eliminating malaria by 2030 (25), the low usage of treated bed nets (12%) and prevalence of symptomatic malaria (3.8%) indicate that routine prevention, diagnostic and treatment options may not be adequate or accessible to this population. The prevalence of asymptomatic malaria (4.3%) and percentage of individuals reporting no known prior infection (98.4%) indicates a reservoir of malaria that may not be effectively addressed by surveillance or elimination efforts relying on identification and treatment of symptomatic cases alone.

### *3.6 Ethics approval and funding*

The human subjects protocol and consent forms for enrollment in the selected villages in Karbi Anglong District, Assam, India were approved by the institutional review boards of the Division of Microbiology and Infectious Diseases at the US National Institute of Allergy and Infectious Diseases (DMID 14-0109), Regional Medical Research Center, NE Region (Indian Council of Medical Research) (no number assigned), and the University of Washington (STUDY00001377).

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## Chapter 4: Tartrolon E, a secondary metabolite of a marine symbiotic bacterium, is a potent inhibitor of asexual and sexual *Plasmodium falciparum*

*The following chapter is reproduced in entirety from* ***Chery-Karschney L****, Patrapuvich R, Mudeppa DG, Kokkonda S, Chakrabarti R, Sriwichai P, O'Connor RM, Rathod PK, White J. "Tartrolon E, a secondary metabolite of a marine symbiotic bacterium, is a potent inhibitor of asexual and sexual Plasmodium falciparum." Antimicrob Agents Chemother. 2024 Feb 7;68(2):e0068423. doi: 10.1128/aac.00684-23.*

Abstract: Due to the spread of resistance to front-line artemisinin derivatives worldwide, there is a need for new antimalarials. Tartrolon E (TrtE), a secondary metabolite of a symbiotic bacterium of marine bivalve mollusks, is a promising antimalarial because it inhibits the growth of sexual and asexual blood stage *Plasmodium falciparum* at sub-nanomolar levels. The potency of TrtE warrants further investigation into its mechanism of action, cytotoxicity, and the ease with which parasites may evolve resistance to it.

### *4.1 Introduction*

Malaria is a major infectious disease caused by *Plasmodium spp.* parasites. Approximately 247 million cases and 619,000 deaths were attributed to malaria in 2021, mainly in sub-Saharan Africa (1). Of the five species that infect humans, *Plasmodium falciparum* is responsible for the vast majority of malaria-caused morbidity and mortality. The development and spread of resistance to derivatives of the front-line antimalarial artemisinin has intensified the need for discovery of novel inhibitors that target unique pathways (2, 3).

Natural products make up more than half of the FDA-approved drugs over the last forty years and have long been an important source of and inspiration for antimicrobials due to their structural diversity (4, 5). Some of the most efficacious and widely-used antibiotics are polyketides, a large, diverse class of natural products that includes the tetracyclines (6) and the macrolides azithromycin and erythromycin (7), which all possess antimalarial properties. Two of

the most successful antimalarials in history are the natural products quinine and artemisinin, which have complex mechanisms of action and require multifactorial processes for acquisition of resistance (8). Looking forward, the ocean is an underexplored frontier for natural product discovery and marine organisms have been shown to produce a wide variety of unique chemical scaffolds with biomedical potential (9).

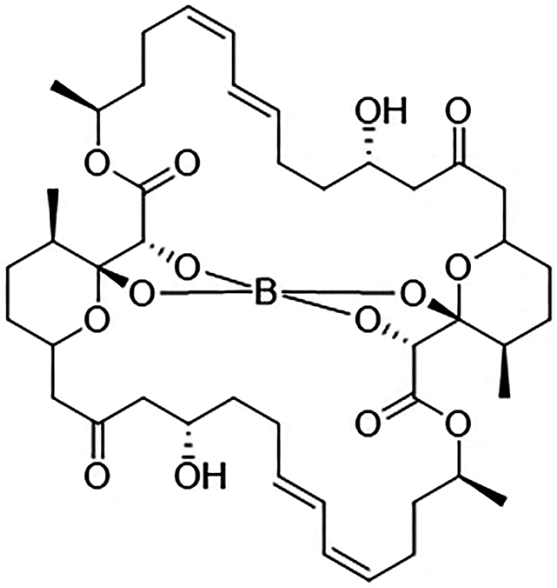


**Figure 4.1** Tartrolon E. TrtE ($C_{44}H_{64}BO_{14}$; molecular weight = 827.79) was isolated from a culture of the *T. turnerae* bacterium and purified as previously described (11) (Purification Method 2). The identity of TrtE was confirmed by high resolution mass spectrometry electrospray ionization (HRMS-ESI) as well as $^{1}H$ and $^{13}C$ nuclear magnetic resonance (NMR) (**Appendix Figures A4.1-A4.3).**

Tartrolon E (TrtE) (**Figure 4.1**) is a secondary metabolite macrolide polyketide with a central complexed tetraborate. It was isolated from *Teredinibacter turnerae*, an intracellular endosymbiotic gammaproteobacteria of marine wood-boring bivalve mollusks of the family *Teredinidae* (shipworms) (10). Recently, we showed potent inhibition of a diverse range of apicomplexan parasites including *Plasmodium*, *Toxoplasma*, and *Cryptosporidium* by TrtE (11). This was consistent with the hypothesis that *T. turnerae* may produce TrtE to protect mollusks

against gregarines, the most ancestral organism of the apicomplexan phylum (12). Here, we describe the bioactivity of TrtE on asexual and sexual blood stages of *Plasmodium falciparum.*

*4.2 Results*

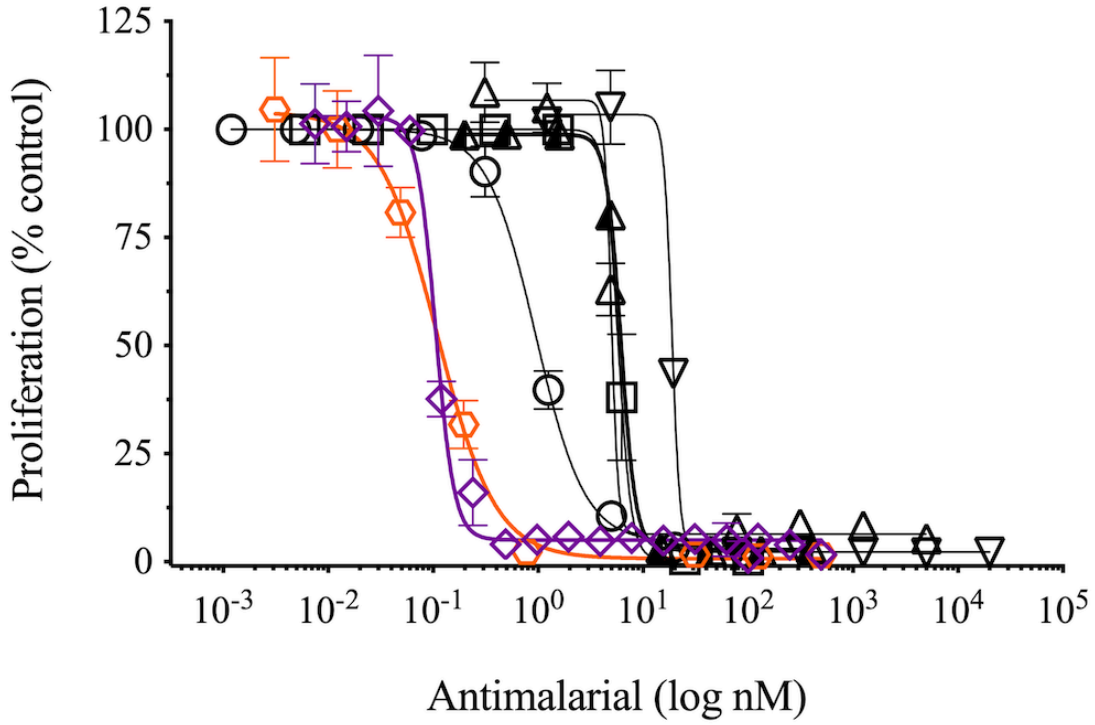


**Figure 4.2 Inhibition by TrtE vs. established and novel antimalarials.** Standard, 72-hour inhibitor-response assays (14) were utilized to determine the 3D7 $EC_{50}$s for Tartrolon E (purple diamond, 105pm), dihydroartemisinin (orange hexagon, 129 pM), atovaquone (circle, 1 nM), DSM265 (triangle, 5 nM), artemisinin (half-filled triangle, 6 nM), chloroquine (square, 6 nM), pyrimethamine (upside down triangle, 19 nM).

Historically, the best antimalarials are highly potent, tolerable and efficacious across multiple steps of the parasite life cycle (13). In parasite proliferation assays (14), TrtE exhibited sub-nanomolar potency against the asexual blood stages of *P. falciparum (Pf)* (3D7 $EC_{50}$ = 105 pm), responsible for the clinical manifestations of malaria disease. *In vitro* inhibition of parasite growth by TrtE was approximately equivalent to that of dihydroartemisinin (DHA; 3D7 $EC_{50}$ = 129 pm), the active metabolite of the front-line artemisinin. (**Figure 4.2**).

**Table 4.1 *Pf* clone-specific sensitivities to TrtE.** TrtE displays high potency against a broad set of parasite genetic backgrounds. R, *P. falciparum* resistant as determined by $EC_{50}$ or, for ART, ring-stage survival assay (RSA); S, sensitive; and U, undetermined. CQ (chloroquine), QN (quinine), MQ (mefloquine), CG (cycloguanil), PYR (pyrimethamine), SDX (sulfadoxine), and ATO (atovaquone). Parasite clones were obtained from BEI Resources except for GMC1, which was collected in Goa, India by the MESA-ICEMR. Cambodian clones were reported as resistant to artemisinin by RSA (BEI, Didier Menard).

| | | EC50, nM (95% CI) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cell Line | Origin | TrtE | DHA | CQ | QN | MQ | CG | PYR | SDX | ART | ATO | DSM1 |
| IPC 5202 | Battambang, Cambodia | 0.81 (0.76-0.86) | 0.94 (0.72-1.03) | R | R | R | R | R | R | R | S | S |
| IPC 3445 | Palin, Cambodia | 0.73 (0.69-0.79) | 0.92 (0.91-1.10) | R | R | R | R | R | R | R | S | S |
| Dd2 | SE Asia | 0.28 (0.27-0.32) | 0.14 (0.12-0.16) | R | R | R | R | R | R | S | S | S |
| GMC1 | Goa, India | 0.26 (0.23-0.30) | 0.21 (0.19-0.24) | R | U | S | R | R | R | S | S | S |
| TM90C2A | Thailand | 0.16 (0.14-0.17) | 0.091 (0.086-0.097) | R | U | R | R | R | U | S | S | S |
| 7G8 | Brazil | 0.18 (0.16-0.19) | 0.095 (0.083-0.110) | R | R | S | R | R | U | S | S | S |
| V1/S | Vietnam | 0.31 (0.29-0.33) | 0.084 (0.072-0.090) | R | R | S | R | R | U | S | S | S |
| HB3 | Honduras | 0.13 (0.12-0.14) | 0.16 (0.13-0.20) | S | S | S | S | R | S | S | S | S |
| NF54 | Netherlands | 0.20 (0.17-0.25) | 0.15 (0.14-17) | S | S | S | S | S | R | S | S | S |
| 3D7 | Netherlands | 0.16 (0.14-0.18) | 0.11 (0.10-0.12) | S | S | S | S | S | R | S | S | S |
| D6 | Sierra Leonne | 0.14 (0.13-0.15) | 0.17 (0.14-0.20) | S | S | S | S | S | S | S | S | S |
| D10 w/ yDHODH | Papua New Guinea | 0.19 (0.17-0.21) | 0.12 (0.11-0.13) | S | R | S | U | U | U | S | R | R |

A diverse panel of *P. falciparum* clones, collected from four continents with varying genetic backgrounds and well-characterized susceptibilities to novel and existing antimalarials, was treated with TrtE and with DHA in standard growth inhibition assays. All clones were inhibited by TrtE at sub-nanomolar concentrations, including two clones from Western Cambodia that were collected in a province with clinical artemisinin resistance and that display *in vitro* resistance to artemisinin in ring-stage survival assays (BEI; Didier Menard) (**Table 4.1**). The potency of TrtE is comparable to DHA across all parasite lines tested, with both compounds displaying slightly lower proliferation inhibition to the Cambodian cell lines. Based on these data, preliminary indications are that the mechanism of action utilized by TrtE is orthogonal to those of all commonly used antimalarials except DHA. Additional studies are needed to determine whether there is any similarity in mechanism between TrtE and DHA.

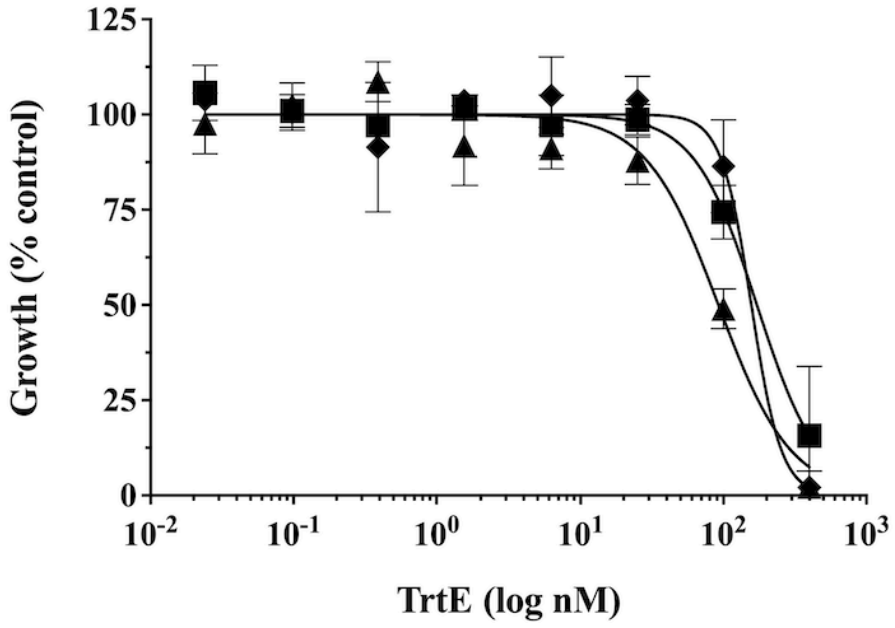


**Figure 4.3 TrtE activity against gametocytes.** Enriched, late-stage gametocytes were treated for three days with TrtE and then viability-tested with alamarBlue (Invitrogen) (15, 16). Three independent experiments (squares, triangles, diamonds) determined the average $EC_{50}$ to be 140.1 ± 42.2 nM.

Gametocytes of *Pf* NF54, the competent gametocyte producing clone, were treated with TrtE as previously described (15, 16). In three independent experiments, TrtE inhibited the progression of *Pf* NF54 late stage gametocytes (stage III to stage V) with an average $EC_{50}$ of 140.1 nM, which is superior to the gametocytocidal antimalarials pyronaridine (4.26 μM) and primaquine (>40 μM) (17) (**Figure 4.3**). To validate the *in vitro* gametocytocidal activity of TrtE, mosquito infectivity assays were conducted. In four independent experiments, treatment with 100 nM TrtE, the approximate gametocidal $EC_{50}$, significantly inhibited mosquito infection by *P. falciparum* ($p$ = 0.0155) (**Figure 4.4**). Oocyst development in mosquitoes represents a significant bottleneck in the lifecycle of *Plasmodium spp.* where transmission can be efficiently interrupted (18).

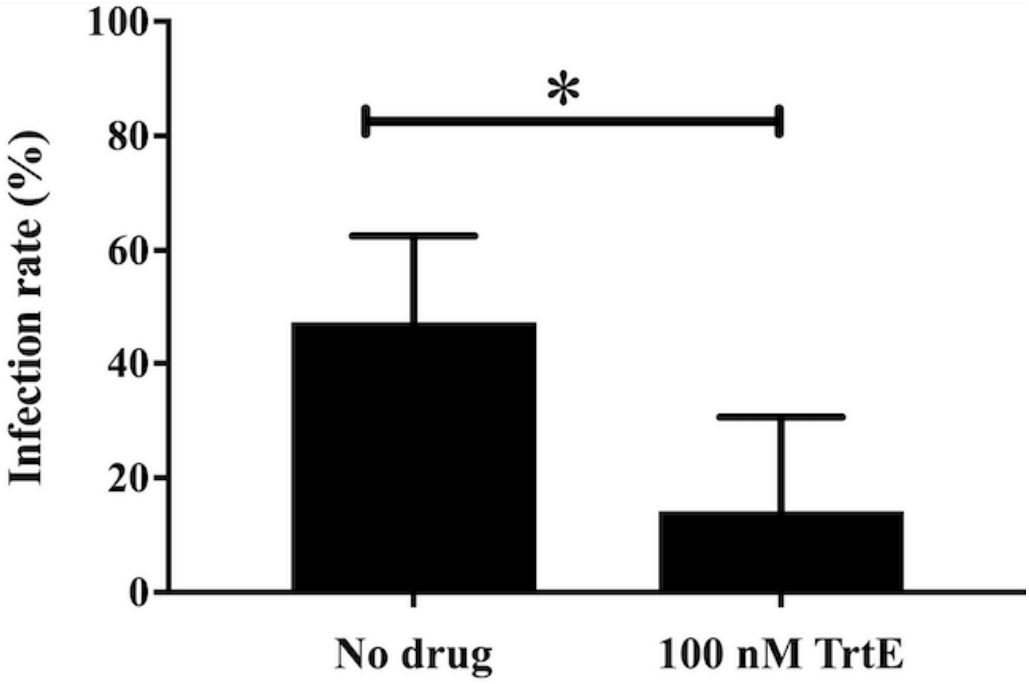


**Figure 4.4 TrtE inhibition of oocyst development in mosquitoes.** Late-stage *Pf* NF54 gametocytes were incubated with 100nM TrtE, the approximate gametocidal $EC_{50}$, or left untreated. Following incubation and development to stage V, the cultures were fed to female *Anopheles dirus* mosquitoes using standard membrane feeding techniques. After nine-days, the mosquitoes were dissected and oocysts stained and counted. The infection rate was measured as the ratio of mosquitoes with one or more oocysts to the number of dissected mosquitoes. *, $P < 0.05$

In preliminary experiments to evaluate cytotoxicity to mammalian cells, the murine leukemia L1210 cell line and the human liver cell line Hep G2 were grown under identical conditions alongside *Pf* 3D7. These were treated with TrtE, the chemotherapeutic methotrexate, and DSM265, an inhibitor of parasite dihydroorotate dehydrogenase (19). *In vitro* cytotoxicity assays demonstrated that TrtE inhibits L1210 growth at sub-micromolar concentrations, though the compound was 2000-fold more toxic to parasites (**Table 4.2**). Hep G2 was approximately three-fold more sensitive to TrtE compared to L1210 cells (Hep G2 $EC_{50}$ 0.07 nM vs L1210 $EC_{50}$ 0.23 nM). Previous toxicity selectivity indices (1302-2633) were comparable (11). A wide range of cellular effects of TrtE is consistent with other macrolides. In the future, selectivity may be improved with chemical modification.

**Table 4.2 Cellular sensitivity of TrtE**. The selectivity index ($EC_{50}$ of HepG2 or L1210 / $EC_{50}$ 3D7) of TrtE was compared to the antimalarial DSM265 (Phase IIa clinical trial) and the FDA-approved anti-arthritic, anti-cancer drug methotrexate.

| | $EC_{50}$, µM (95% CI) | | | |
|---|---|---|---|---|
| **Compound** | **3D7** | **HepG2** | **L1210** | **Selectivity** |
| **Tartrolon E** | 0.00016 (0.00014-0.00018) | 0.071 (0.063-0.081) | 0.23 (0.21-0.25) | 444, 1438 |
| **DSM265** | 0.005 (0.0038-0.006) | 9.9 (8.2-12.0) | >20 | 1980, >4,000 |
| **Methotrexate** | 0.047 (0.041-0.054) | >1 | 0.012 (0.011-0.013) | 0.26, 21.3 |

## *4.3 Conclusion*

TrtE may be considered for further development because of its high potency across multiple stages of the malaria parasite life cycle. Preliminary *in vitro* single-step selection experiments indicate that pressure from TrtE presents high hurdles to resistance for asexual parasites (data not shown). TrtE is a complex molecule that requires significant effort to extract and would be challenging to synthesize, however further investigation may disclose a minimum scaffold responsible for activity. In addition to further defining windows of parasite-host cytotoxicity, high priorities include characterization of the kinetics of TrtE-mediated killing and identification of the cellular target(s) of TrtE using forward genetic approaches.

## *4.4 Ethics approval and funding*

Animal use ethics approval for the mosquito components of this study was obtained from the Mahidol University Faculty of Tropical Medicine Animal Care and Use Committee (FTM-ACUC 029/2020). This study was supported by grants from the NIH/National Institute of Allergy and Infectious Diseases (U19 AI089688 to PKR) and NIH/National Center for Complementary and Integrated Health (R21 AT009174 to RMO).

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# Appendix

## *A. Supplementary Information for Chapter 4*

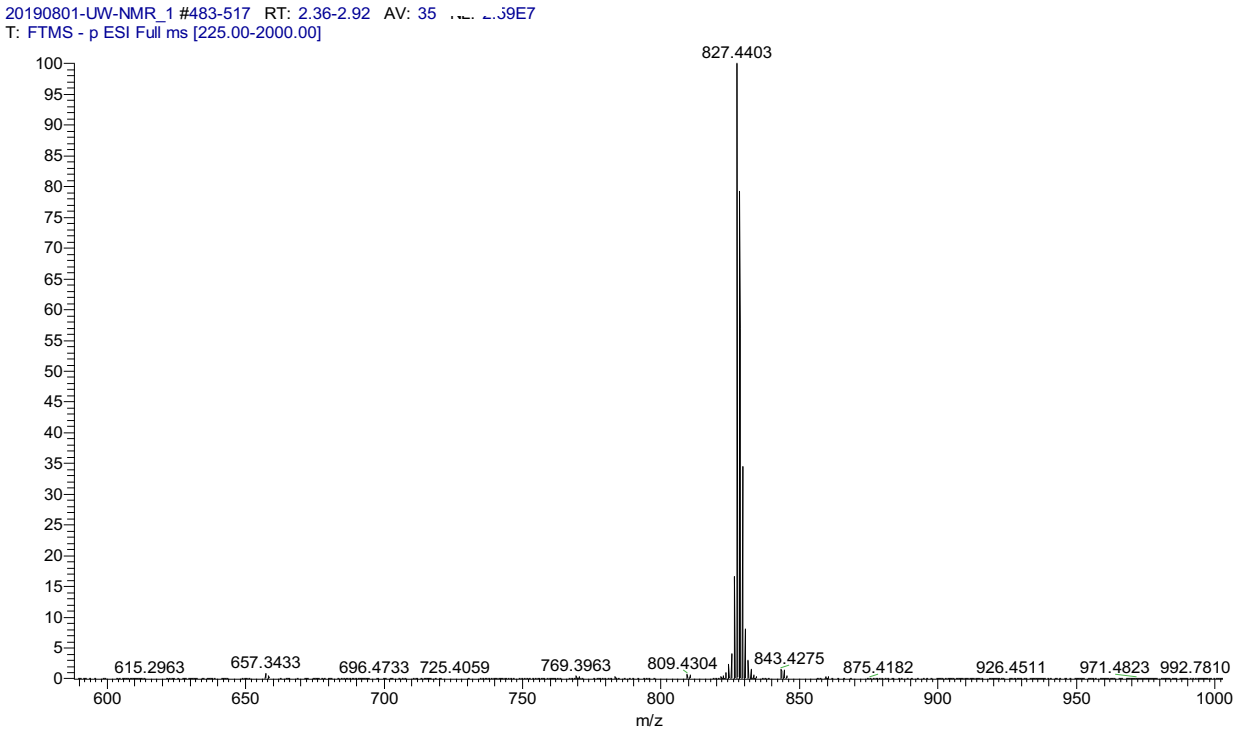


**Figure A4.1 High Resolution Mass Spectrometry Electron Spray Ionization (HRMS-ESI).** HRMS (ESI) was recorded on a Thermo LTQ-Orbitrap XL, resolution setting 60,000, negative ionization mode. HRMS ($ESI^-$) m/z calculated for $C_{44}H_{64}O_{14}B$ $[M - H]^-$ : 827.4391 found: 827.4403

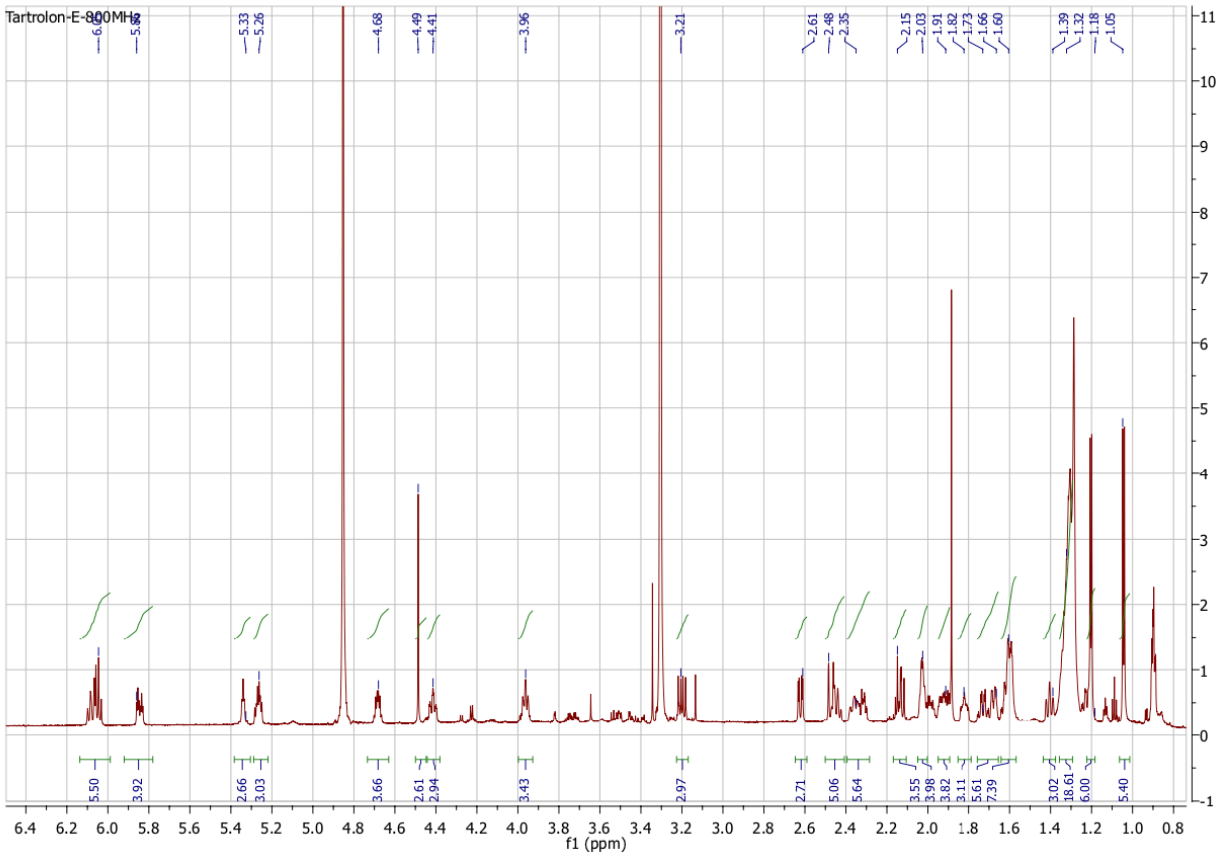


**Figure A4.2 Proton Nuclear Magnetic Resonance (NMR) Spectroscopy**. $^{1}$H NMR (800 MHz, MeOD) δ: 6.12 – 6.01 (m, 4H), 5.84 (dt, *J* = 15.2, 4.7 Hz, 2H), 5.34 (t, *J* = 4.6 Hz, 2H), 5.30 – 5.23 (m, 2H), 4.68 (dt, *J* = 16.3, 5.8 Hz, 2H), 4.48 (s, 2H), 4.46 – 4.38 (m, 2H), 3.97 (dt, *J* = 16.7, 6.6 Hz, 2H), 3.20 (dd, *J* = 18.3, 10.2 Hz, 2H), 2.62 (dd, *J* = 14.1, 4 Hz, 2H), 2.47 – 2.41 (m, 3H), 2.39 – 2.28 (m, 3H), 2.14 (dt, *J* = 14.1, 9.7 Hz, 2H), 2.05 – 2.0 (m, 2H), 1.96 - 1.87 (m, 2H), 1.85 – 1.79 (m, 2H), 1.76 – 1.71 (m, 2H), 1.69 – 1.65 (m, 3H), 1.64 – 1.57 (m, 4H), 1.43 – 1.38 (m, 2H), 1.37 - 1.25 (m, 9H), 1.20 (d, *J* = 6.2 Hz, 6H), 1.04 (d, *J* = 6.6Hz, 6H).

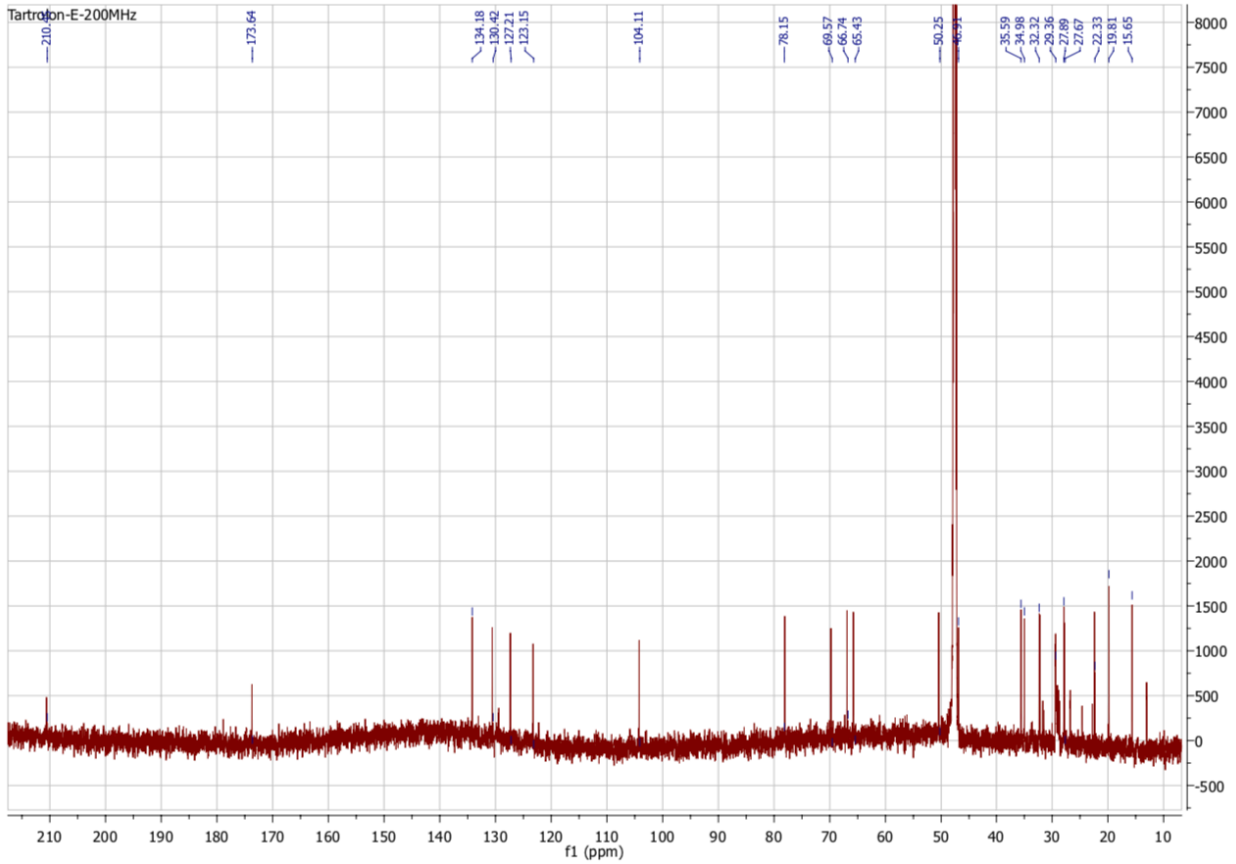


**Figure A4.3 Carbon Nuclear Magnetic Resonance (NMR) Spectroscopy.** $^{13}C$ NMR (201 MHz, MeOD) δ: 210.45, 173.64, 134.18, 130.42, 127.21, 123.15, 104.11, 78.15, 69.57, 66.74, 65.43, 50.25, 46.91, 35.59, 34.98, 32.32, 29.36, 27.89, 27.67, 22.33, 19.81, 15.65.