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Epidemiology of Malaria and Other Diseases of Public Health Importance and Implications for Interventions in High Transmission Settings in Sub-Saharan Africa

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ABSTRACT

Epidemiology of malaria and other diseases of public health importance and implications for interventions in high transmission settings in sub-Saharan Africa

by

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November 11, 2020

Infectious diseases remain a major cause of disability of death in low-resource settings. Malaria alone was responsible for an estimated 405,000 deaths globally in 2018, with the 94% of these deaths occurring in sub-Saharan Africa. In Mozambique and Democratic Republic of the Congo (DRC), communicable diseases, including malaria, lower respiratory infections, and neonatal disorders are among the top causes of disability and death. Understanding malaria and co-endemic diseases in these two countries can aid the planning, evaluation, and targeting of public health interventions. Additionally, studying the efficacy of the drugs used to treat malaria will preserve the ability for malaria cases to be treated successfully.

The three studies in this dissertation describe the epidemiology of malaria and co-endemic diseases of public health importance in Mozambique and evaluate the efficacy of medicines used to treat malaria in DRC. The first study will describe the spatial epidemiology of malaria in two high-burden districts in northern Mozambique to explore the utility of exploration of local spatial heterogeneity in high-transmission settings. The second study will investigate patterns in antibody responses to several infectious pathogens of public health importance in Mozambique, providing an opportunity to understand common predictors of infectious diseases endemic in this region. The third study will examine the efficacy of three artemisinin-based combination therapies used to treat uncomplicated malaria and molecular markers of antimalarial resistance in five sites in DRC.

Collectively, the three studies in this dissertation describe factors that have implications for intervention planning and disease surveillance in areas with high malaria and other tropical disease burden and limited health resources. Careful consideration of transmission setting can support more efficient and higher quality data collection and may allow for intervention design tailored to the local realities that can target multiple diseases of public health importance.

Epidemiology of malaria and other diseases of public health importance and implications for interventions in high transmission settings in sub-Saharan Africa

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I applied for this degree program while responding to the 2014 West Africa Ebola Virus Disease epidemic and am completing it during the COVID-19 pandemic. These bookends underscore critical role of epidemiologists in this world and the mentors that guide us through the education and real-world experience needed to protect the public from health threats. I thank the mentors who encouraged me to pursue this degree, guided me through this degree, and taught me how to be a mentor for future scholars.

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Author's Statement Page

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Chapter 1: Literature review and statement of purpose

Introduction

Malaria

Malaria is a disease caused by the parasite belonging to the genus *Plasmodium.* Five species are known infect humans: *P. falciparum* (the most common and associated with the highest mortality), *P. vivax, P. ovale,* and P. *malariae,* and more recently *P. knowlesi* [1,2]. The epidemiology of *P. falciparum* in central and southern Africa is well documented[3,4], and there is evidence that *P. vivax, P. ovale,* and *P. malariae* are also circulating[5–7].

Malaria is transmitted through the female *Anopheles* mosquito, of which there are 30 species are implicated in malaria transmission[8]. Malaria transmission is affected by mosquito breeding and feeding behavior and survival, which are affected by climatic conditions such as rainfall, altitude, and temperature. While *Anopheles* behavior is diverse, most species tend to be active at dusk[2]. In central and southern Africa, *Anopheles arabiensis* and *funestus* have been found to be common malaria vectors[9,10].

Symptoms of uncomplicated malaria are non-specific; the main symptom is fever and can also include chills, sweats, headaches, muscle pain, nausea, and vomiting[11]. Clinical symptoms of severe malaria, most commonly caused by *P. falciparum,* include impaired consciousness, respiratory distress, convulsions, coma, shock, pulmonary edema, bleeding, and jaundice[12].

The gold standard for the diagnosis of malaria in a clinical setting has historically been blood film microscopy where blood is taken from an individual who has suspected malaria, spread on a blood smear stained with Giemsa and read by a microscopist trained to diagnose and distinguish parasite species[2]. Malaria rapid diagnostic tests (RDTs) have increased the accessibility of malaria diagnostics to people without access to a healthcare structure with microscopy among its services. The RDT requires a few drops of capillary blood that are mixed with a lysing agent in a plastic test strip, which detects the target antigen in the blood, providing easily interpretable results within about 20 minutes. The RDT is widely used at the peripheral levels of the healthcare system, including at the community level. There are several

types of malaria RDTs, many of which are single species for *P. falciparum* and some of which are multispecies[13]. Other diagnostic tools for malaria outside the point of care setting include molecular methods, which are highly sensitive and can detect low density infection as well as be used to identify malaria species[14]. Evidence of recent or past exposure to malaria can be detected using serological tests, for which blood samples are taken and tested for a quantifiable reaction to species-specific antigens[15]. Serological studies can be helpful in a household survey setting, where the goal is to learn about how the population has been affected by malaria rather than how much of the population has active malaria infection[16].

To treat uncomplicated malaria, the World Health Organization (WHO) recommends the use of artemisinin-based combination therapy (ACT), a type of medication containing an artemisinin derivative, which works quickly to reduce initial parasitemia, and a partner drug that works to clear remaining parasitemia and prevent the emergence of artemisinin resistance. There are six ACTs recommended by WHO for the treatment of uncomplicated malaria[17,18].

Additional infectious diseases of public health importance

Infectious diseases remain a major cause of disability of death in low-resource settings. Malaria alone was responsible for an estimated 405,000 deaths globally in 2018, with the 94% of these deaths occurring in sub-Saharan Africa[4]. WHO estimates that 96 million people become ill and 40,000 people die from Dengue infection each year[19]. Local transmission and periodic outbreaks of Chikungunya and Rift Valley Fever are reported throughout sub-Saharan Africa[20,21]. Lymphatic filariasis, a parasitic infection also transmitted by mosquitoes that can cause elephantiasis, infects an estimated 51 million people worldwide[22].

Despite tremendous gains in implementing childhood vaccination programs throughout sub-Saharan Africa, there is evidence that high coverage of basic childhood vaccinations has not reached the entire continent, resulting avoidable morbidity and mortality, notably among children[23–25]. Diarrhea also remains

a significant cause of disease and death, also disproportionately affecting young children. Rotavirus, Cryptosporidium spp., and Shigella spp., *Escherichia coli*, and salmonella have been found to be significant causes of severe diarrheal illness and death in low-income regions[26,27].

Finally, neglected tropical diseases (NTDs), a group of illnesses representing bacterial, parasitic, viral, and fungal etiologies associated with poverty, are significant causes of disability with debilitating physical, economic, and social impact on the afflicted individuals and communities[28].

Mozambique & Democratic Republic of the Congo (DRC)

Communicable, maternal, neonatal and nutritional diseases have remained the most common cause of disability and death in Mozambique. Under five mortality was an estimated 73.8 per 1,000 live births as of 2017. In DRC, malaria, lower respiratory infections, and neonatal disorders cause the most deaths and disability, and under five mortality was an estimated 81.9 per 1,000 live births in 2017. Mozambique and DRC are among the countries with the highest rates of child mortality in the world[29].

Mozambique and DRC are among six countries worldwide that accounted for over half of all malaria cases in 2018. DRC accounts for 11% and Mozambique accounts for 4% of malaria deaths globally[4]. Both countries are part of a new WHO initiative called “High Burden High Impact” that seeks to reduce the impact of malaria in the highest burden countries. In Mozambique, malaria is responsible for 42% of deaths among children under five years old. All 29.7 million persons living in Mozambique are at risk for malaria, but the burden of disease is highest in the center and northern of the country, particularly the provinces of Nampula and Zambézia[30,31]. In DRC, malaria is responsible for 19% of deaths among children under five years old, and 100% of the population of 81.3 million persons is at risk for malaria, and the highest burden provinces include Bas-Congo, Orientale, and Kasai Oriental[3].

Review of the Literature

Spatial epidemiology of malaria

The method of transmission and therefore its predictors are associated with location, increasing the likelihood of a spatially nonrandom distribution of the malaria in endemic areas. Spatially nonrandom

predictors include *Anopheles* breeding sites and their proximity to humans, housing materials and type, occupation as well as environmental variables including rainfall and altitude[32–36]. There are several methods available to detect and quantify spatial autocorrelation and used to describe malaria epidemiology with differing assumptions, methods, and nuances. Understanding the spatial epidemiology of malaria using methods that are practical and interpretable will assist public health practitioners to design interventions targeting the most at-risk areas and people.

The most common method used to identify clusters of infectious diseases, including malaria in a given area is the use of Kulldorff's spatial scan statistic, or SatScan. This method uses geographic point data to detect whether the outcome of interest is distributed randomly over space and detects clusters and assigns statistical significance to them. The general null hypothesis is that the outcome of interest is uniformly distributed across space. The SatScan statistic can use one of two models: a Poisson-based model, for count data and a Bernoulli model for case/non-case data[37]. To detect clusters, an area is scanned gradually with a predefined circular window size for which the radius of which can be up to 50% of the area of interest, or by a pre-defined radius size. For malaria, researchers have chosen varying pre-defined sizes, many correlating with the farthest distance traveled by the *Anopheles* mosquito[38]. At each window, the number of observed and expected cases is calculated. Maximum likelihood estimation is used to define clusters, and Monte Carlo hypothesis testing is used to determine the statistical significance of each cluster[39]. Spatial scan statistics have been used widely in the context of malaria with the goal of identifying areas to target for malaria control efforts in high and low transmission settings with data collected in surveys and through routine surveillance[40–44].

B.D. Ripley's K(r) function is another method used to assess spatial heterogeneity in epidemiology. The null hypothesis of the K function is a random labeling assumption, that the assignment of cases and controls are distributed randomly given unit location[45]. *K(r)* estimates the frequency of an event relative to the distance of other events in the area of interest[46]. Deviation from complete spatial randomness is determined by examining a plot of the K function by distance r and comparing it to a random Poisson

point process, or πr^2. If there was a tendency for events to cluster in an area, the K function would be higher than the πr^2 curve, indicating that there would be more events than expected under the assumption of complete spatial randomness. If there was a tendency for dispersion at a certain location, the plot of the K function line would be lower than the πr^2 curve. Though less frequently than spatial scan statistics, the K function has also been used to assess spatial distribution of malaria to spatially target or assess malaria interventions in high and low transmission settings in Africa and Asia[47–51].

Global and local methods to answer the question "how similar are neighbors to one another?" include Moran's I and the Local Indicator of Spatial Autocorrelation (LISA). These methods of detection of spatial autocorrelation can provide information to further investigate other similarities between defined geographic areas or neighborhoods that are similar to or different from one another[52]. While this method more difficult to interpret using spatial point data, it has been used to identify hot spots and cold spots of malaria[44,53,54]. Other methods of assessing spatial clustering of malaria have been used, including several Bayesian geostatistical modeling techniques[55–58].

The use of spatial methods in malaria epidemiology

In lower transmission settings, researchers have used spatial methods to identify spatially dependent drivers of persistent infection in a community[41]. Findings from these settings often do help identify straightforward predictors of malaria positivity such as distance from a river or other type of vector breeding site that may be targeted with a vector control activity such as indoor residual spraying targeting those households[35,42]. In medium to high transmission settings, results of spatial analyses have varied, but have generally been on a larger scale, examining either an urban area or using spatial data to look at spatial distribution of malaria nationwide. One study in Mozambique found spatial heterogeneity of malaria in the study area that did not change based on season, and higher transmission persisted in areas close to a swampy area[59]. A study in Kampala, Uganda had similar results when examining an urban cohort of children where risk of malaria infection was associated with distance from a swamp[43]. Studies in

Malawi and Mali used household survey data to predict nationwide malaria prevalence, describing spatial heterogeneity at a large scale; authors suggested that their findings could be used to guide strategies to stratify interventions based on malaria burden[56,58].

While there have been several analyses of malaria spatial data throughout Africa, evidence regarding spatial distribution of malaria in high-transmission settings in southern Africa, including Mozambique and even more so, from the highest transmission regions, is limited[57,59–62]. Additionally, the foremost methodology for understanding malaria burden has been through periodic nationwide household surveys that measure parasitemia often at a regional level[63]. The sampling methodology of most of these national surveys do not allow for the assessment of spatial heterogeneity beyond the region or province, which limits the ability to reliably use methods to examine hotspots or explore the feasibility of targeting interventions at a health zone, village, or even neighborhood level, especially in lesser-populated rural areas. The implementation of more focused household surveys that target a region or district level and collect spatial point data may provide opportunities to apply spatial analysis techniques that examine spatial point processes.

The use of serological tools to examine exposure to several tropical infectious diseases

Specimens collected in household surveys have generally targeted one disease to understand prevalence along with questions about predictors of those specific diseases[64]. Examples include malaria indicator surveys that measure malaria prevalence with microscopy or RDT[63] and AIDS indicator surveys measuring HIV prevalence[65]. Neglected tropical disease surveillance is typically based on the collection of blood spots, eye swabs, urine, or stool samples in periodic surveys[66]. These surveys and specimen collection can be resource intensive and require multiple specimens to be collected from the same populations[67].

Availability of multiplexing technology for surveillance allow for more efficient data collection, minimize the number of biological specimens taken from participants in surveys and research studies, and

allow public health practitioners to look beyond the disease-specific descriptive epidemiology and interventions[67,68]. Additionally, within a disease, different serological markers may be used to deepen the epidemiologic understanding of one disease. For example for malaria, antibody response for different antigens will vary in duration and by number of infections[69].

Luminex-bead antibody assays allow for the analysis of antibody responses to several antigens at once from a single dried blood spot collected on filter paper with consistently high sensitivity and specificity[68,70]. Sample analysis using this technology has been integrated into household surveys and have provided valuable information about antibody prevalence of diseases of public health importance in several countries[70–73].

While multiplex assays have been used for sample analysis, increasing efficiency in data collection and sample processing, often the statistical analysis is limited to just one pathogen or group of pathogens such as vaccine preventable diseases, malaria, or NTDs to describe the distribution of seropositivity or assess the effectiveness of an intervention[5,72,74–77]. Literature including information about several diseases has generally been limited to seropositivity prevalence estimates[70–73] and few have integrated survey data with those results to examine common risk or protective factors[78]. Integrating survey with biomarker data provides an opportunity to not only implement integrated disease surveillance and could provide evidence about situations and behaviors that can inform the effectiveness of integrated disease control programs.

Antimalarial resistance

WHO recommends the implementation of therapeutic efficacy studies (TES) at least every two years in malaria endemic countries to quickly identify reduced sensitivity to artemisinin-based combination therapies used for the treatment of uncomplicated malaria[79]. Early identification of waning efficacy of a drug may inform national malaria control program policy for malaria treatment.

During TES, patients are enrolled and followed for a defined period, during which they are monitored for early treatment failures, late recurrence, or adequate clinical and parasitological response (ACPR)[80]. The

primary results of a TES include uncorrected and PCR-corrected cumulative efficacy and per-protocol efficacy. In malaria endemic settings in which TES are implemented, patients enrolled in the study may become infected with a new parasite over the study period. To generate PCR-corrected estimates, distinguishing new infections from recrudescent infections that indicate possible failure of the drug is critical, especially in high transmission settings where individuals are likely to be reinfected. There are molecular correction techniques available, and it is important to account for areas of high malaria transmission where it is also likely for individuals to have multiple malaria infections at once[81]. There is evidence that classic molecular correction techniques, such as comparisons of the genes for the merozoite surface proteins (*msp*) 1, 2, and glutamate-rich protein (glurp)[80], may be subject to amplification bias and suppressing fragments in the case of multiple infections, increasing the likelihood to misclassify recurrent parasitemia as reinfections, leading to overestimations of efficacy[82,83].

Another method used for molecular correction includes the use of seven neutral microsatellite markers, for which fragment lengths of neutral loci are measured and compared between day 0 and day of recurrent parasitemia[84]. A Bayesian algorithm for comparison of the two samples that accounts for the baseline prevalence of fragment lengths was developed that assigns each late recurrence a probability of recrudescence, which can be used to calculate the final PCR-corrected efficacy estimates[85]. There is evidence that this approach provides unbiased classification in all transmission settings, and a simulation study has shown that this may especially useful in areas of higher transmission to prevent misclassification bias[82].

In addition to monitoring ACT efficacy, therapeutic efficacy studies may monitor molecular markers of antimalarial resistance among *Plasmodium falciparum* parasites. Specific polymorphisms in the propeller domain of the *pfkelch13* (*pfk13)* gene[86] have been associated with artemisinin resistance, a finding described in Southeast Asia[87] and recently in Rwanda, the first sub-Saharan African county with published evidence of *pfk13* mutation association with susceptibility to artemisinin[88]. Decreased susceptibility to lumefantrine and amodiaquine has been associated with polymorphisms in the gene

pfmdr1 and decreased susceptibility to amodiaquine has been associated with polymorphisms in the gene *pfcrt*[89].

Recent TES completed in DRC have demonstrated that the three ACTs used to treat uncomplicated malaria in the public and private sector, artemether lumefantrine (AL), artesunate-amodiaquine (ASAQ), and dihydroartemisinin piperaquine (DP) are efficacious in DRC, with per-protocol PCR-corrected efficacies of over 90% in studies conducted between 2015–2017[4,90,91].

The results of an updated TES in DRC is needed to provide data to the DRC NMCP to make decisions about treatment for uncomplicated malaria as recommended by WHO. As a country representing a large portion of malaria cases on the continent, threats to ACT efficacy in DRC are especially important. Furthermore, while the Bayesian statistical algorithm has been validated for higher transmission settings, it has not been validated on the highest transmission settings found in areas like DRC. Further evidence that may be used to prevent misclassification bias in molecular correction can ensure that accurate estimates of therapeutic efficacy are used by decision-makers.

Statement of purpose

The purpose of this dissertation is to describe the epidemiology of malaria and co-endemic diseases of public health importance in Mozambique and evaluate the efficacy of three ACTs in DRC, which represent a malaria intervention critical for the reduction of disease in high transmission settings in sub-Saharan Africa.

In 2014, a survey was conducted in the Nampula province of Mozambique to assess the impact of a universal long-lasting insecticide treated net (LLIN) campaign completed the year prior. Antibody responses to 39 antigens representing malaria, diarrheal, respiratory, neglected tropical diseases, and vaccine preventable diseases were measured using a multiplex bead assay, and a malaria RDT was performed on all consenting survey participants. The first two studies will analyze data collected during this survey. The third study will analyze data collected in five sites in DRC for therapeutic efficacy monitoring.

The first study will describe the spatial epidemiology of malaria in two high-burden districts in northern Mozambique to explore the utility of exploration of local spatial heterogeneity in high-transmission settings in targeting interventions. Understanding the spatial epidemiology of malaria using methods that are practical and interpretable will contribute to WHO strategy and of stratifying and tailoring interventions according to malaria burden[92] in addition to addressing the evidence of meaningful variation of malaria endemicity at local levels[93]. Furthermore, applying spatial analysis techniques to areas of high transmission settings will fill a gap in the literature of describing spatial variation beyond a regional or national level, and provide baseline information to progress overtime, with the ultimate goal of spatially targeting persistent reservoirs of transmission.

The objective of the second paper is to investigate patterns in comorbidities for infectious diseases in Nampula Mozambique. The understanding of multiple exposures present in the population of interest can allow for more comprehensive understanding of the important causes of morbidity and mortality and to monitor the success of programs such as mass drug administration for trachoma or routine immunization programs[71,94,95]. Having the ability to examine evidence of exposure to multiple pathogens in tandem could provide an understanding of the epidemiology of endemic diseases, which may support the clinical management of illness for which there is no easy diagnosis at the peripheral levels of the health system and be used to improve upon integrated disease prevention and treatment programs. This study will fill the gaps in the literature of going beyond describing prevalence of seropositivity to multiple diseases or focusing on one or a group of pathogens, and examine shared patterns, risk factors, and protective factors between 40 diseases of public health importance in Mozambique.

The goal the third paper is to describe the results of a study examining the therapeutic efficacy of three ACTs used for the treatment of uncomplicated Plasmodium falciparum malaria in five sites in DRC and present the prevalence of molecular markers of resistance to artemisinin derivatives and partner drugs. Current information about the efficacy of ACTs in DRC will support decisions about the national malaria treatment guidelines in DRC. Ensuring that the ACTs used in a high-burden country such as DRC will

prevent excess morbidity and mortality due to ineffective drugs. Additionally, understanding the prevalence of molecular markers of resistance in a large country may provide early warning of any drug resistance issues for the continent.

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Chapter 2: An exploratory spatial analysis of malaria in two high-burden districts in Mozambique

Target Journal: Malaria Journal

Abstract

Background

Mozambique has one of the highest malaria burdens in the world. Nampula province of Mozambique had an estimated 66% malaria prevalence as measured by RDT in 2015, second only to Zambézia, which had an estimated prevalence of 68%. As a “high burden high impact” country defined by the World Health Organization, there an emphasis on using tools such as spatial analysis methods to tailor interventions at more granular level to eliminate persistent reservoirs of transmission.

Methods

A household survey was conducted in 2014 in two districts in Mozambique’s Nampula Province: Mecubúri and Nacala-a-Velha. A two-staged cluster sampling technique was used to select houses and a questionnaire was administered to all consenting individuals present at the visit and included questions regarding malaria knowledge and preventive behaviors, demographic, and socioeconomic indicators. A *P. falciparum* malaria rapid diagnostic test was also administered as a measure of active or recent malaria infection. To investigate spatial heterogeneity of acute malaria infection, three methods were used, the Moran’s I statistic, B.D. Ripley’s K function, and spatial scan statistics.

Results

The Moran’s I test of global spatial autocorrelation provided evidence of clustering of households with similar proportions of household members at short distances. Another test of global spatial clustering, the K statistic demonstrated no evidence of difference in spatial autocorrelation between households with malaria cases versus households without cases. Local clustering analysis found eight statistically significant malaria clusters. The SatScan analysis malaria RDT positivity among children under five years old revealed evidence of five cold spots, one of which was statistically significant and two hotspots, none of which were statistically significant.

Conclusions

While there was some evidence of clustering of cases or non-cases in a few instances, overall, the spatial distribution of malaria infection across the study area was homogeneous. The results of this analysis show that stratification at levels lower than a district level in regions of high malaria burden may not be warranted.

Keywords: Malaria, P. falciparum, spatial analysis, Mozambique, household survey

Background

Mozambique has one of the highest malaria burdens in the world, representing four percent of the world's cases and four percent of malaria deaths in 2018(1). Deaths attributed to malaria represent 42 percent of all deaths among children under five years old, and 27 percent of deaths of people of all ages(2). Malaria burden in Mozambique is heterogenous, with regional parasitemia estimates measured by rapid diagnostic test (RDT) ranging from two percent in the capital of Maputo to 66 and 68 percent in the northern provinces of Nampula and Zambézia, respectively(3).

The strategy for malaria control, led by the national malaria control program (NMCP) in Mozambique includes providing access to vector control interventions such as insecticide-treated nets and Indoor Residual Spraying , and ensuring that malaria cases are confirmed using a parasitological test and treating positive tests with an appropriate antimalarial at the facility and community levels. Targeting preventive and care seeking behaviors with social behavior change (SBC) activities and strengthening surveillance and program management at all levels of the health system are also included(2).

The World Health Organization (WHO)'s Global Technical Strategy for Malaria 2016-2030(4) calls for the reduction of malaria incidence by 90% by 2030. This ambitious goal will require the use of data, including data collected in household surveys to track progress in addition to understanding the epidemiology of malaria at increasingly smaller spatial scales to precisely target interventions. The WHO strategy includes a special focus on driving down burden in highly endemic areas such as northern Mozambique(5).

The method of malaria transmission is associated with location, increasing the likelihood of a spatially nonrandom distribution of the malaria. Spatially nonrandom predictors include *Anopheles* breeding sites and their proximity to houses, housing materials and type, occupation as well as environmental variables including rainfall and altitude(6–10). There are several methods available to detect and quantify spatial autocorrelation and used to describe malaria epidemiology with differing assumptions, methods, and data availability.

Much of the information, including spatial data about malaria burden and its predictors come from household survey data, which are not usually powered to make precise estimates of spatial clustering that regional count data representative of an area of interest such as census or surveillance data may have the power to do(11,12). Methods to assess spatial point patterns with survey data include assessment of global spatial autocorrelation with methods such as Moran's I and the K function to determine the tendency of a variable of interest to cluster spatially, and local cluster identification techniques such as the local indicator of spatial autocorrelation and spatial scan statistics(11). These methods provide different outputs that may be triangulated to make inferences about disease patterns and spatially nonrandom drivers of those patterns in the absence of population-wide spatial data.

Spatial methods have been widely used in the context of malaria in low to moderate transmission areas that are candidates for malaria elimination to identify reservoirs of infection that are driving most of the transmission in an otherwise low transmission area(9,10,13–16), but the use of these methods in high transmission areas to identify areas with higher or lower than average prevalence has been limited or used to describe or model malaria distribution at a national-scale(17–20).

Understanding the spatial epidemiology of malaria in high transmission areas using methods that are practical and interpretable will contribute to WHO strategy of stratifying and tailoring interventions according to malaria burden at a more granular level(14). The goal of this analysis is to utilize methods practical for programmatic use to describe local spatial patterns of malaria in a high transmission setting to identify opportunities to target control activities at a local level.

Methods

Data collection

A household survey was conducted in 2014 in two districts in Mozambique's Nampula Province: Mecubúri and Nacala-a-Velha. Detailed methods of the data collection processes are described elsewhere(21). Briefly, A two-staged cluster sampling method was used to randomly select 20 clusters in each district and 16 houses within each cluster in 2013 prior to a mass bed net distribution campaign. The

2014 survey targeted the households that were included in the prior year's survey. Every individual present in the household was invited to participate in a questionnaire that included indicators of household and individual socioeconomic and behavioral factors for which there is evidence of association with malaria. All respondents were asked to be administered a *P. falciparum* HRP2-specific RDT (SD Bioline, Yongin, Republic of Korea). Those who tested positive were treated according to the national case management guidelines(22). The longitude and latitude of each household was recorded by the survey enumerator using an electronic data collection tool. RDT result was used as a measure of current or recent parasitemia and as the main outcome variable in the analysis. Techniques to identify clusters and patterns of clustering were used to describe the spatial distribution in the study population. Only the 2014 results are included in the present analysis.

Moran's I

The Moran's I statistic measures spatial autocorrelation of a spatial variable with variation in that variable by neighboring areas. Global Moran's I can be used to evaluate evidence of influence of space on a variable of interest(23). A positive value for Moran's I indicates evidence of spatial clustering, or a tendency for observations to have values similar to their neighbors. A negative value indicates that observations tend to have values dissimilar to their neighbors. Values of 0 indicate no evidence of spatial autocorrelation. The null hypothesis under the Moran's I test of spatial association is that that values of the variable of interest are randomly distributed. In its basic form the Moran's I statistic calculation is below where *i* and *j* are two locations or observations; z_i is the difference between the observation and its mean. Spatial weights are denoted by $w_{i,j}$ for features *i* and *j*, *S* is the sum of all spatial weights, and *n* is the total number of observations. Statistical significance is evaluated for the index through a z-score and a p-value. If there is evidence of clustering in an area, *I* will be positive, and if dispersed, negative, if close to zero, then there is evidence of random distribution of that spatial attribute(11).

$$I = \frac{n}{S_0} \frac{\sum_{i=1}^{n} \sum_{j=1}^{n} w_{ij} z_i z_j}{\sum_{i=1}^{n} z_i^2}$$

Global Moran's I analysis was used to evaluate clustering based on proportion of household RDT positivity in the study area. Households for which there are no RDT results were not included in the analysis. Moran's I statistics for proportion of household members testing positive by RDT were calculated at 10 distances classes from the latitude and longitude of each house up to two kilometers. Statistical significance was defined as $p<0.05$.

The Local Indicator of Spatial Autocorrelation (LISA), based on the Moran's I statistic, is a measure of spatial autocorrelation that allows for analysis at the observation-level. The global Moran's I statistic is simply the sum of the LISA values for all observations. A single LISA statistic provides information about the extent to which the value of one observation is similar to its neighbors(23).

To investigate local clusters, weights were calculated based in Euclidian distance between households. Local Moran's I statistics were calculated for each household. Statistically significant ($p<0.05$) clusters of households were defined as high proportion of RDT positivity with high neighbors, low near low neighbors, low near high neighbors, and high near low neighbors.

K Function

B.D. Ripley's *K(r)* function was used to determine the spatial dependence of RDT status in the study area(24). The null hypothesis of the K function is a random labeling assumption, that the assignment of cases and controls are distributed randomly given unit location(25). *K(r)* estimates the frequency of an event relative to the distance of other events in the area of interest and is described below; λ is the intensity of events in the study area(24).

$$K(r) = \frac{E[n\ events\ within\ r\ of\ a\ randomly\ chosen\ event]}{\lambda}$$

Deviation from complete spatial randomness is determined by examining a plot of the K function by distance r and comparing it to a random Poisson point process, or πr^2. If there was a tendency for events to cluster in an area, the K function would be higher than the πr^2 curve, indicating that there would be

more events than expected under the assumption of complete spatial randomness. If there was a tendency for dispersion at a certain location, the plot of the K function line would be lower than the πr^2 curve. The random labeling assumption can be tested by examining the difference in K functions between two groups, in this case, RDT positive cases, and RDT negatives, or controls. If the difference in K functions is zero, one may conclude that there is no meaningful difference in distribution of cases and controls in the study area, or that there is no relationship between malaria RDT positivity and space. Significance testing of the K function can be calculated through a Monte Carlo test, comparing the observed point labels to independent N random permutations of the outcomes(26).

Households with at least one positive RDT were defined as case households, and households with no RDT positives were defined as controls. The difference in the K function estimates of cases and controls was plotted and compared with zero to test the hypothesis that cases and controls are randomly distributed by distance *r*. Isotropic edge correction was used to estimate *K(r)* to account for observations near the border of the study area(11). Monte Carlo simulations (n=999) were used to construct bands around the expected value under the assumption of complete spatial randomness(26).

Spatial scan statistic

A common method used to identify clusters of malaria or other infectious diseases in a given area is the use of Kulldorff's spatial scan statistic(27–34). This method uses geographic point data to detect whether the outcome of interest is distributed randomly over space and detects clusters and assigns statistical significance to them. To detect clusters, an area is scanned gradually with a predefined circular window size, representing potential clusters. At each scanning window, the number of observed and expected cases is calculated and tested using a likelihood ratio test, with expected number of cases representing random distribution of cases and controls across households. Monte Carlo hypothesis testing generating a permutations of observed labeling of cases across the study area is used to determine the statistical significance of each cluster(35).

For this analysis, a scanning window of 10% of the population was used with the Bernoulli model to compare cases to non-cases at each household. To calculate statistical significance, 999 Monte Carlo replications were used. A case was defined as a child under five years old in the dataset testing positive by RDT, a negative was a child under five years old with a negative RDT. A significance level of P=.05 was used to determine statistical significance(36).

SaTScan™ software (version 9.6) was used for the spatial scan cluster analysis. R version 3.5.2 (R Foundation for Statistical Computing, Vienna Austria) was used for the remainder of analyses. R packages ggplot2(37) and spatstat(38) were used to generate figures and perform the K function analysis(38).

Results

Description of study sample

A total of 365 households and 1282 individuals were included in the analysis, 184 households and 704 individuals in Mecubúri and 181 households and 578 individuals in Nacala-a-Velha. There was an average of 3.5 persons reported in each household. Nineteen percent of the study sample was composed of children under five years old.

Among persons included in the analysis, 782 (61%) tested positive for malaria by RDT, 498/704 (71%) in Mecubúri, and 284/578 (49%) in Nacala-a-Velha. Among children under five, 209 (78%) tested positive for malaria by RDT, 129/146 (88%) in Mecubúri and 90/123 (65%) in Nacala-a-Velha. The mean proportion of household members testing positive by RDT in the study sample was 53% (standard deviation 39%).

Figure 1.1: Location of households included in sample and malaria rapid diagnostic test (RDT) results in Mecúburi and Nacala-a-Velha, Nampula Province, Mozambique

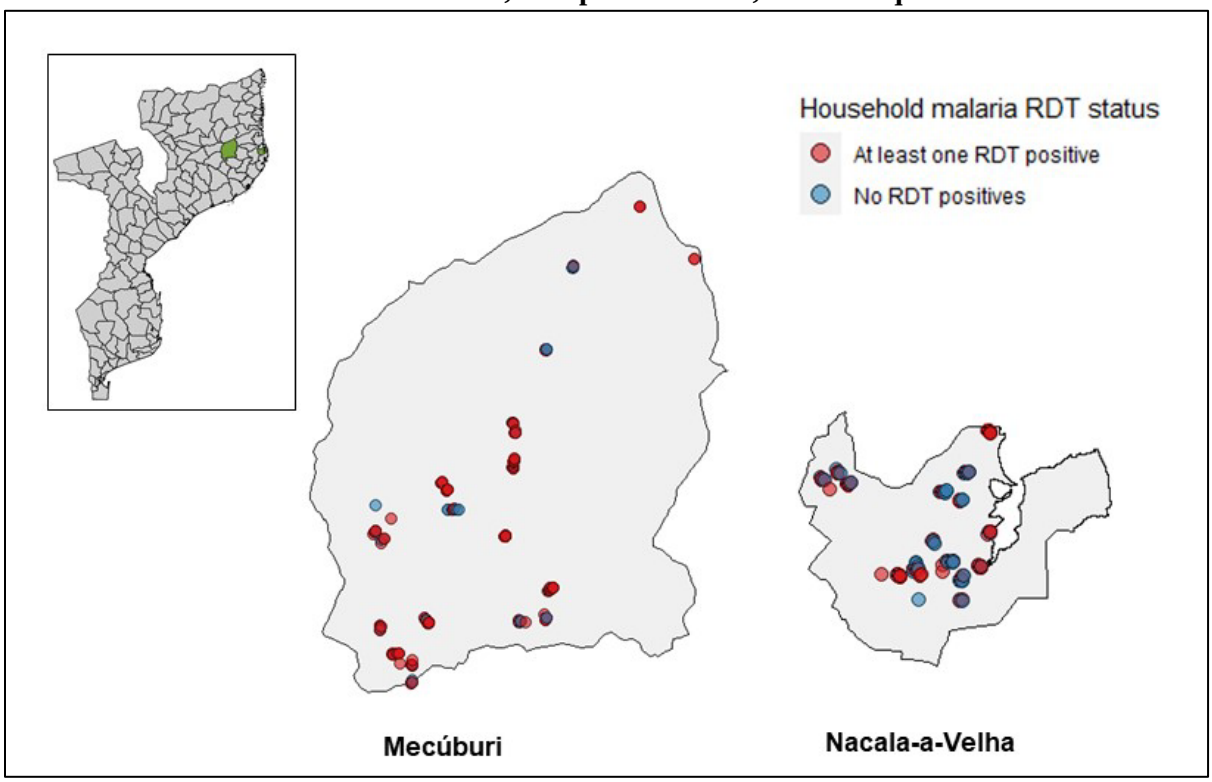


Table 1.1: Summary of study sample of malaria RDT positivity in two districts in Nampula Province, Mozambique, 2014

		All ages		Under five years old	
	Households	Individuals[a]	Positive RDT (%)	Individuals[a]	Positive RDT (%)
Mecubúri	184	704	498 (71)	146	129 (88)
Nacala-a-Velha	181	578	284 (49)	123	80 (65)
Total	365	1282	782 (61)	269	209 (78)

[a]excluding individuals with no RDT result

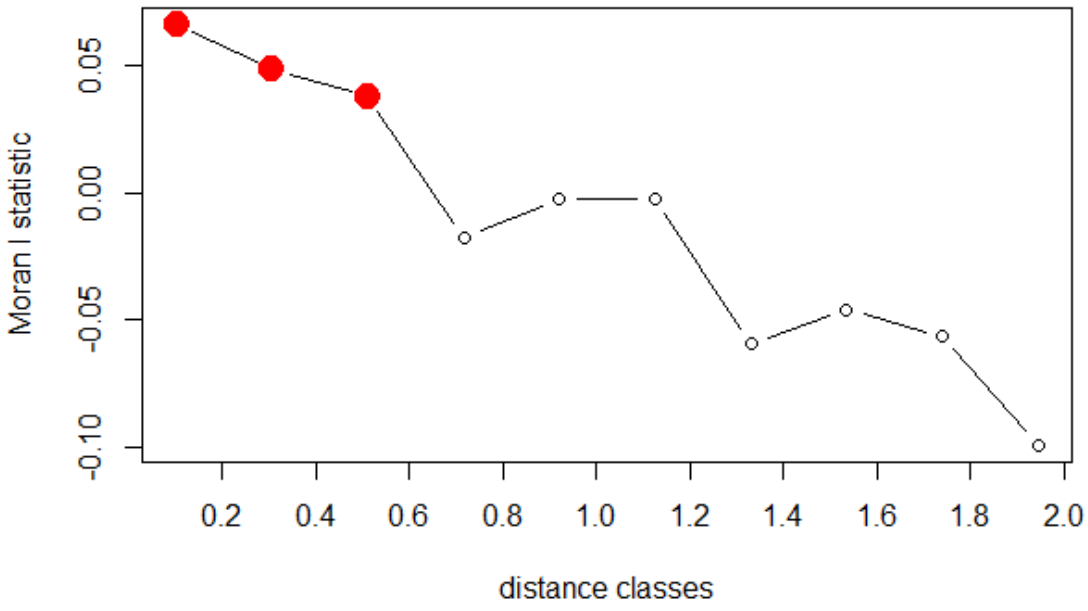
Moran's I & Local Moran's I

All households for which RDT results were available (n=365) were included in the calculation of the Moran's I statistic. Moran's I was calculated over 10 distance classes from 0 to 2 kilometers. The distance classes of 0.1 km (coefficient: 0.06, $p<0.01$), 0.3 km (coefficient: 0.048, $p<0.01$). and 0.5km (coefficient: 0.048, $p=0.01$) yielded statistically significant clustering with values of above 0, indicating a pattern of

households to be similar to their neighbors with respect to proportion of RDT-positive persons in their households when the neighbors are less than one half of a kilometer apart (Figure 2).

The local analysis yielded eight instances (two percent of households) of local spatial autocorrelation; three (0.8%) where a household had a relatively high prevalence relative to its neighbors, three (0.8%) where a household had a relatively low prevalence relative to its neighbors, and two (0.5%) where a household and their neighbors all had lower than average prevalence.

Figure 1.2: Moran's I statistic for global spatial autocorrelation of proportion of household members testing positive for malaria by rapid diagnostic test over 10 distance classes in Nampula Province, Mozambique, 2014

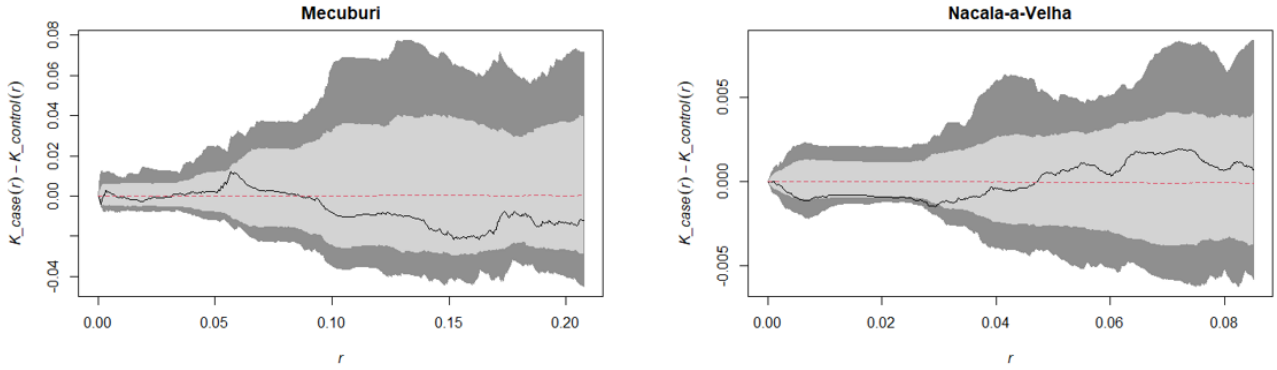


K Function

In Mecubúri, there were 148 (80%) case households with at least one person testing positive, and 36 (20%) control households where nobody tested positive. There was no evidence of a difference in K functions of the cases versus control households at any distance (p= 0.68).

In Nacala-a-Velha, there were 115 (64%) case households with at least one person testing positive, and 65 (35%) control households, with no person testing positive. There was no evidence of a difference in K functions of the cases versus control households at any distance (p=0.75).

Figure 1.3: Difference in K Functions between households with at least one positive P. falciparum malaria rapid diagnostic test and households with only negative P. falciparum rapid diagnostic tests in two districts in Nampula Province, Mozambique



Spatial Scan statistics

The spatial scan analysis revealed two clusters of RDT negative households in Mecubúri with including two households each, one of which was statistically significant (p=.00045). In Mecubúri, 2.9% of households were identified within one of the identified cold spots. In Nacala-a-Vela, five clusters were identified: two hotspots and three cold spots, none of which were statistically significant. The hotspots included five and six households, and the cold spots included ten, nine, and two households. In Nacala-a-Vela, 9.7% of households were within a hotspot and 18.6% of households located within a cold spot.

Figure 1.4: Results of hot and cold spot analysis using spatial scan statistics in Nacala-a-Velha and Mecubúri, Nampula, Mozambique.
Dots indicate households included in the survey sample for which RDT results were available for children under five years old. Circles indicate hot or cold spots of RDT positivity. Blue denotes cold spots, and red denotes hotspots.

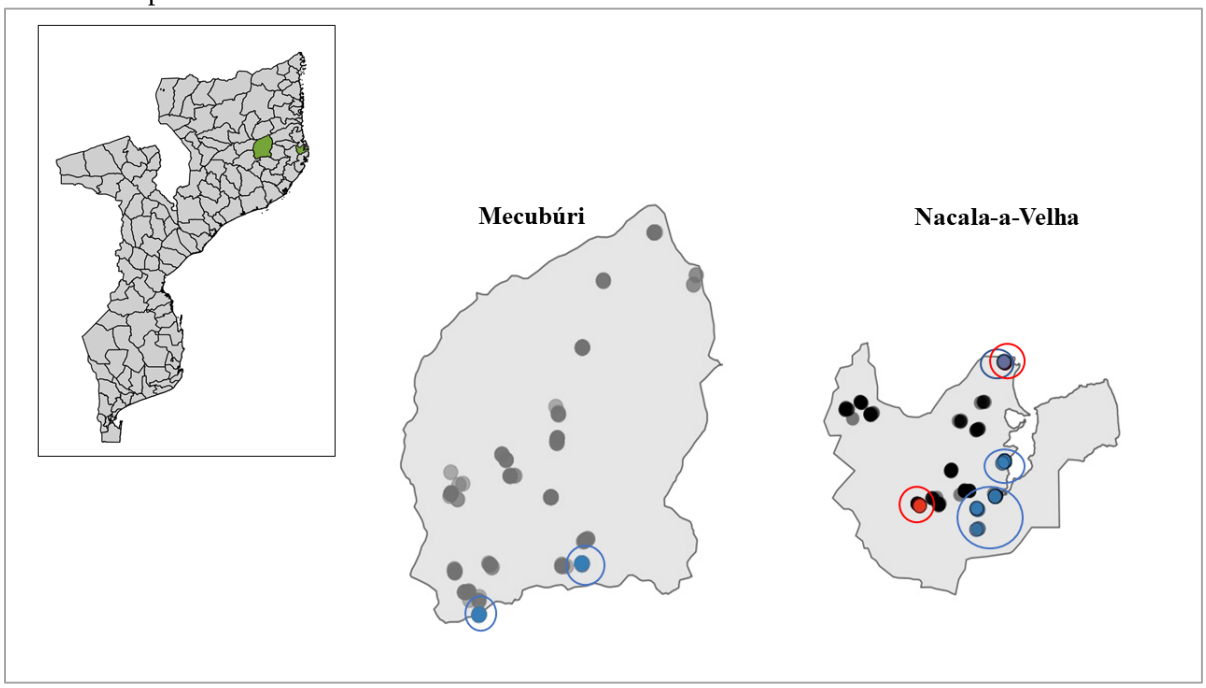


Table 1.2: Hotspots and coldspots of P. falciparum rapid diagnostic test identified using spatial scan statistics in two districts of Nampula province, Mozambique, 2014

	Cluster	Number of households (individuals)	# RDT positive (%)	Observed/expected	P-value
Mecubúri	Coldspot 1	2 (7)	1(14)	0.16	.00045[a]
	Coldspot 2	2(3)	1(33)	0.38	0.9165
Nacala-a-	Coldspot 3	8 (8)	1 (13)	0.19	0.1703
Velha	Coldspot 4	10 (10)	2 (20)	0.31	0.2500
	Coldspot 5	2 (3)	0 (0)	0	0.8975
	Hotspot 1	6 (6)	6 (100)	1.54	0.9755
	Hotspot 2	4 (5)	5 (100)	1.54	0.9959

[a]Statistically significant

Discussion

The Moran's I test of global spatial autocorrelation provided evidence of clustering of households with similar proportions of household members at short distances. Another test of global spatial clustering, the

K statistic demonstrated no evidence of difference in spatial autocorrelation between households with malaria cases versus households without cases.

Local clustering analysis yielded a handful of statistically significant instances of local spatial autocorrelation. The SatScan analysis malaria RDT positivity among children under five years old revealed evidence of five cold spots, one of which was statistically significant and two hotspots, none of which were statistically significant.

This analysis shows that malaria prevalence in this district as measured by RDT, which is evidence of active or recent infection(39) is extremely high and homogenous among young children at the district-level, indicating widespread high transmission intensity(40). The difference in malaria prevalence between the two districts shows that even in a very high-burden region, it might be worth exploring the between-district heterogeneity and predictive factors of these differences including ecological such as differences in rainfall or altitude, environmental such as access to care or other preventive or curative services, and cultural or normative differences that may affect preventive or care seeking behaviors.

This analysis included the use of three methods that required the use of three different ways of measuring the outcome variable. The Moran's I and LISA statistics used proportion of RDT positivity at the household level; The K function used a binary outcome at the household level (at least one positive case in a household); The spatial scan statistic used individual-level data for each location. The common thread across the methods was the use of RDT positivity as a measure of current or recent infection. The variability of these three measurements allows for triangulation of the spatial distribution of malaria infection in the study area, and all yielded consistent findings of spatial homogeneity of malaria infection in this sample.

Intervention stratification should be informed by data regarding the epidemiologic setting and spatial distribution of malaria and its predictors. At very low transmission settings, there could be reason to tailor interventions to the very local level, especially when implementing resource-demanding programs such as

reactive case detection and treatment(41). While a handful of anomalous groupings of households were identified in this analysis, there was no obvious clustering pattern identified using any of the methods in this analysis. This confirms that in high-burden settings, the focus of ensuring strong coverage of malaria control measures including universal ITN coverage, access to case management, IPTp and behavioral interventions to drive down transmission should be the priority. Strong surveillance to track progress and identify areas that remain drivers of transmission as incidence declines is also critical. Any tailoring of interventions in the context of limited resources such as targeting indoor residual spraying may be considered at the district level.

Limitations

While helpful in an exploratory analysis, the survey and sampling methodologies were not originally designed for precise spatial analyses, therefore the number and distribution of observations may not have been sufficient to make meaningful conclusions about the spatial clustering patterns. Additionally, this analysis did not include information about covariates, especially spatially heterogeneous predictors that may drive hotspots. Finally, while monospecies RDTs have been deemed appropriate for use in Mozambique(42), there are still limitations to their use to determine the prevalence of malaria in a given area, given their limits of detection in the case of low parasite density which can be a common occurrence in areas of high endemicity(43). Additionally, the use of a *P. falciparum*-only test does not capture additional malaria species that may be circulating.

Conclusions

Further exploration of the spatial distribution between districts may help inform strategy for intervention stratification in high transmission settings. In a high transmission setting, it may be more useful to examine temporal variation in the spatial distribution of malaria by conducting longitudinal studies. Understanding the areas that may be serving as reservoirs of transmission in a period of relatively lower transmission may be useful in spatial targeting of interventions in high-transmission areas(14). Finally, spatially representative sampling and collection of more spatially dependent covariates, or the use of routine surveillance data would add to the precision and validity of future spatial analyses(44).

This exploratory analysis of spatial distribution within two high-burden districts of Mozambique presented evidence of within-district spatial homogeneity of malaria. When considering tailoring malaria interventions to the epidemiology of a certain area, malaria control programs may consider tailoring to the district level at the lowest in high burden settings.

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Declarations

Ethical approval and consent to participate

The Mozambique National Bioethics committee approved the study and written consent was obtained from adult participants for themselves and on behalf of their children before participating in the survey and agreeing to be given an RDT. CDC investigators provided technical assistance and were not considered to be engaged in the research (CDC research determination number 2014-268).

Availability of data and material

Data analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they do not have any competing interests.

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Chapter 3: Investigation of patterns of antibody responses to infectious diseases using multiplex serology in Nampula, Mozambique

Running title: Patterns of antibody response to infectious diseases Mozambique

Target Journal: The American Journal of Tropical Medicine and Hygiene

Key words: Serology, Mozambique, Multiplex, Neglected tropical diseases

Abstract word count: 250

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Abstract

Communicable, maternal, neonatal and nutritional diseases have remained the most common cause of disability and death in Mozambique and surveillance for infectious diseases has typically been pathogen specific. The availability of multiplex platforms for the assessment of antibody responses to antigens of interest allows for the collection of information about several antigens of interest in a single specimen. In 2014, a household survey was undertaken in Nampula Province of Mozambique. A questionnaire was administered to selected households and dried blood spots were collected for all consenting household members. Multiplex analysis was used to measure antibody responses to 39 antigens representing infectious diseases of public health importance. The survey data were used to calculate adjusted log-MFI values for each antigen. A comparison matrix to compare adjusted log-MFI values was developed using the Pearson correlation coefficient for each pairwise comparison. Data representing 1,156 participants were included in the analysis. Of 702 pairwise comparisons of adjusted log-MFI values, 684 (97%) were statistically significant, 460 (67%) of which had positive correlations, and 224 (33%) were negatively correlated. Age, sex, and district were the most common predictors of log-MFI. Multiplex assays coupled with cross-sectional survey data regarding practices, socioeconomic indicators, and access and use of healthcare services can provide insights about common predictors that may be associated with exposure to multiple pathogens. By understanding the epidemiology of several pathogens and their common predictors, public health practitioners can target common risks of exposure to diseases making more efficient and effective public health interventions in resource-limited settings.

Introduction

Communicable, maternal, neonatal and nutritional diseases have remained the most common cause of disability and death in Mozambique. Under 5 mortality was an estimated 73.8 per 1,000 live births as of 2017, making Mozambique among the countries with the highest rates of child mortality in the world[1]. Malaria is a substantial contributor to morbidity and mortality in Mozambique and is responsible for 29 percent of all deaths and 42 percent of deaths among children under 5 years old, with the highest burden in the provinces of Zambézia and Nampula[2]. In 2017 in Mozambique, malaria was the third most common cause of death in all ages following HIV/AIDS and neonatal disorders[3]. While nationwide administrative childhood vaccination coverage reported to be high, there is evidence that only 65.8% of children received all eight basic vaccinations in 2015[4,5]. Neglected tropical diseases remain a significant cause of disability throughout the country, including lymphatic filariasis, which shares a common vector with malaria[6,7]. In a 2015 nationwide survey, 11% of children under 5 reported having diarrhea in previous 2 weeks[5]; the most frequent causes of diarrhea in rural Mozambique include Rotavirus, Cryptosporidium sp., Shigella sp., *E. coli*, and adenovirus[8].

In 2010, the World Health Organization (WHO) recommended the universal use of diagnostic tests for before treatment to avoid indiscriminate use of antimalarials and minimize resistance, reduce costs associated with antimalarials, and improve treatment of persons with non-malaria fevers[9]. The accessibility of point of care diagnostic tools for malaria clinical management allows for understanding its epidemiology at granular levels of the health system through routine surveillance data in electronic surveillance systems[10]. Surveillance for vaccine preventable diseases, respiratory infections, and diarrheal diseases commonly relies on case-based surveillance or sentinel surveillance systems with strong laboratory capacity [8,11,12]. Neglected tropical disease surveillance is typically based on the collection of blood spots, eye swabs, urine, or stool samples in periodic surveys[13].

The availability of multiplex platforms for the assessment of antibody responses to antigens of interest allows for the collection of information about several pathogens of interest in a single assay of eluate

from a dried blood spot, the collection of which is simple compared to other types of samples. This capability can allow for the understanding of multiple exposures present in the population of interest, allowing for more comprehensive understanding of the important causes of morbidity and mortality and to monitor the success of programs such as mass drug administration for trachoma or routine immunization programs[14–16]. Having the ability to examine evidence of exposure to multiple pathogens in tandem could provide an understanding of the epidemiology of endemic diseases, which may support the clinical management of illness for which there is no easy diagnosis at the peripheral levels of the health system and be used to improve upon integrated disease prevention and treatment programs. Moreover, understanding the common predictors of exposure to different infectious pathogens may inform public health programming that targets behaviors or circumstances that make some more vulnerable to multiple illnesses than others.

In 2014, a survey was conducted in the Nampula province of Mozambique to assess the impact of a universal long-lasting insecticide treated net (LLIN) campaign completed the year prior. Antibody responses to 39 antigens representing malaria, diarrheal, respiratory, neglected tropical diseases, and vaccine preventable diseases were measured using a multiplex bead assay. This objective of this analysis is to investigate patterns in comorbidities for infectious diseases in Nampula Mozambique, a highly endemic for malaria and other tropical diseases.

Methods

Description of study area

Nampula is a coastal province in the northeast of Mozambique and has one of the highest poverty rates in the country, measured at 64.9% in 2014/2015[17]. Nampula's economy is largely based on agriculture and the province is a large producer of cotton[18]. Among persons between 15 and 49 years old, the mean years of education is an estimated 4.4 years among males and 3.39 years among females[19].

In 2014, the under 5 mortality per 1,000 live births in Nampula province was estimated as 86.1, and the mean diarrhea prevalence among children under 5 years old was an estimated 31 per 1,000. Prevalence of

stunting among children under 5 was an estimated 0.43%. The coverage of the first dose of the diphtheria-pertussis-tetanus vaccine was 93.2% and third dose was 84.9%. The estimated prevalence of *P. falciparum* infection in 2014 in Nampula was 48% among all ages. Nampula, along with Zambézia province have the highest prevalence of malaria among 11 provinces in Mozambique, and Nampula has the highest prevalence of schistosomiasis. The prevalence of HIV among persons between 15 and 49 years old was an estimated 6.2% in 2015[5,19,20].

Survey

A household survey was conducted October-November in 2013 and 2014 in 2 districts in Mozambique's Nampula Province: Mecubúri and Nacala-a-Velha. A 2-staged cluster sampling method was used to randomly select 20 clusters in each district, and 16 houses within each cluster were chosen in the first survey. In the second survey, the same households were visited with no replacement households. Every individual present in the household at the time of data collection was invited to participate in a questionnaire that included indicators of household and individual socioeconomic and behavioral factors for which there is evidence of association with malaria. Up to 6 10 mcL spots of capillary blood were collected on filter paper (TropBio, Cellabs, Sydney, Australia) in addition to administration of a *P. falciparum* HRP-2 specific rapid diagnostic test (SD Bioline, Yongin, Republic of Korea). If the RDT was positive, participants were treated for malaria according to the national guidelines for malaria treatment[21]. The present analysis only includes data from the second survey completed in 2014.

Laboratory analysis

Laboratory analysis was carried out in the malaria laboratory at the Centers for Disease Control and Prevention (CDC) in Atlanta, GA, USA. Details of the laboratory analysis are previously described[22]. Briefly, dried blood spots were diluted to a final dilution of 1:400 of serum and immunoglobulin G (IgG) antibody response to 39 antigens plus one control antigen were analyzed using a multiplex bead platform[23]. Antigens were coupled to Seromap (Luminex Corp., Austin, TX), or BioPlex COOH (BioRad,

Hercules, CA) beads. Consistency was ensured by including a buffer-only blank and 6 control sera and by duplicating runs. The 2 median fluorescence intensity (MFI) values minus the buffer-only blank value were used to calculate the final average MFI minus background value (MFI-bg). Antigen responses were repeated for samples for which there were discordant results between the 2 runs as defined by a coefficient of variation of more than 15%[14]. Antigen descriptions and coupling descriptions are described in Supplemental Table 2.1.

Table 2.1: Antigens and corresponding infectious agents measured in Mozambique household survey, 2014

Antigen	Infectious Agent
Malaria	
Pvivax	*Plasmodium vivax*
Pfalcip	*Plasmodium falciparum*
Pmalar	*Plasmodium malariae*
Povale	*Plasmodium ovale*
PfCSP	*Plasmodium falciparum*
Pflsa1	*Plasmodium falciparum*
Non-malaria mosquito-borne	
CHIKV	Chikungunya
Wb123	Lymphatic filariasis
Bm14	Lymphatic filariasis
Bm33	Lymphatic filariasis
VLP3	Dengue
RVFV	Rift Valley fever
Vaccine Preventable	
MeaslesQ	Measles
Rubella	Rubella
Dip	Diphtheria
Tet	Tetanus
Diarrheal	
Norwalk	Norovirus
Sydney	Norovirus
StCloud	Norovirus
VSP3	Giardia
VSP5	Giardia
SEA	Schistosomiasis
ETEC	*Escherichia coli*
Cholera	Cholera
SalB	Salmonella

SalD	Salmonella
Campyp18	Campylobacter
Campyp39	Campylobacter
Other neglected tropical diseases	
pgp3	Trachoma
CT694	Trachoma
YAWSrp17	Yaws
YawsTmpA	Yaws
T24H	Cysticercosis
SAG2	Toxoplasma
AscHb	Ascaris
NIE	Strongyloides
Cp17	Cryptosporidium
Cp23	Cryptosporidium
CpP2	Cryptosporidium

Statistical Analysis

Only participants for whom results from the multiplex analysis were generated were included in statistical analyses. Density plots were generated for log-MFI values for all ages and by age category[24]. To generate adjusted log-MFI values, linear regression models built using log-MFI values for each pathogen as the outcome variable were built using forward and backward stepwise selection using the Akaike Information Criterion[25]. The full model included items collected during the household survey including district, cluster, mosquito net received during 2013 campaign, socioeconomic status category, malaria knowledge index score, household size, bed net ownership, sex, relationship to the head of household, malaria RDT result, age category, whether the sleeping space had a bed net, household distance from a health facility, household income type, occupation of the head of household, highest level of schooling for the head of household, number of sleeping spaces, and sufficient number of mosquito nets to cover sleeping spaces.

The adjusted log-MFI using each pathogen's regression model for each individual in the sample was calculated. A comparison matrix to compare corrected log-MFI values was developed using the Pearson correlation coefficient for each pairwise comparison. Statistical significance was defined at $\alpha=0.01$. Statistical analyses were carried out using R (R Foundation for Statistical Computing, Vienna Austria).

Ethical Considerations

The Mozambique National Bioethics Committee approved the study. Written consent was provided by adult participants and on behalf of child participants. CDC staff provided technical assistance in data collection and analysis and were considered to be non-engaged in this research by the Office of the Associate Director for Science, Center for Global Health.

Results

Data representing 1,156 participants were included in the analysis. A total of 721 participants were excluded due to missing data.

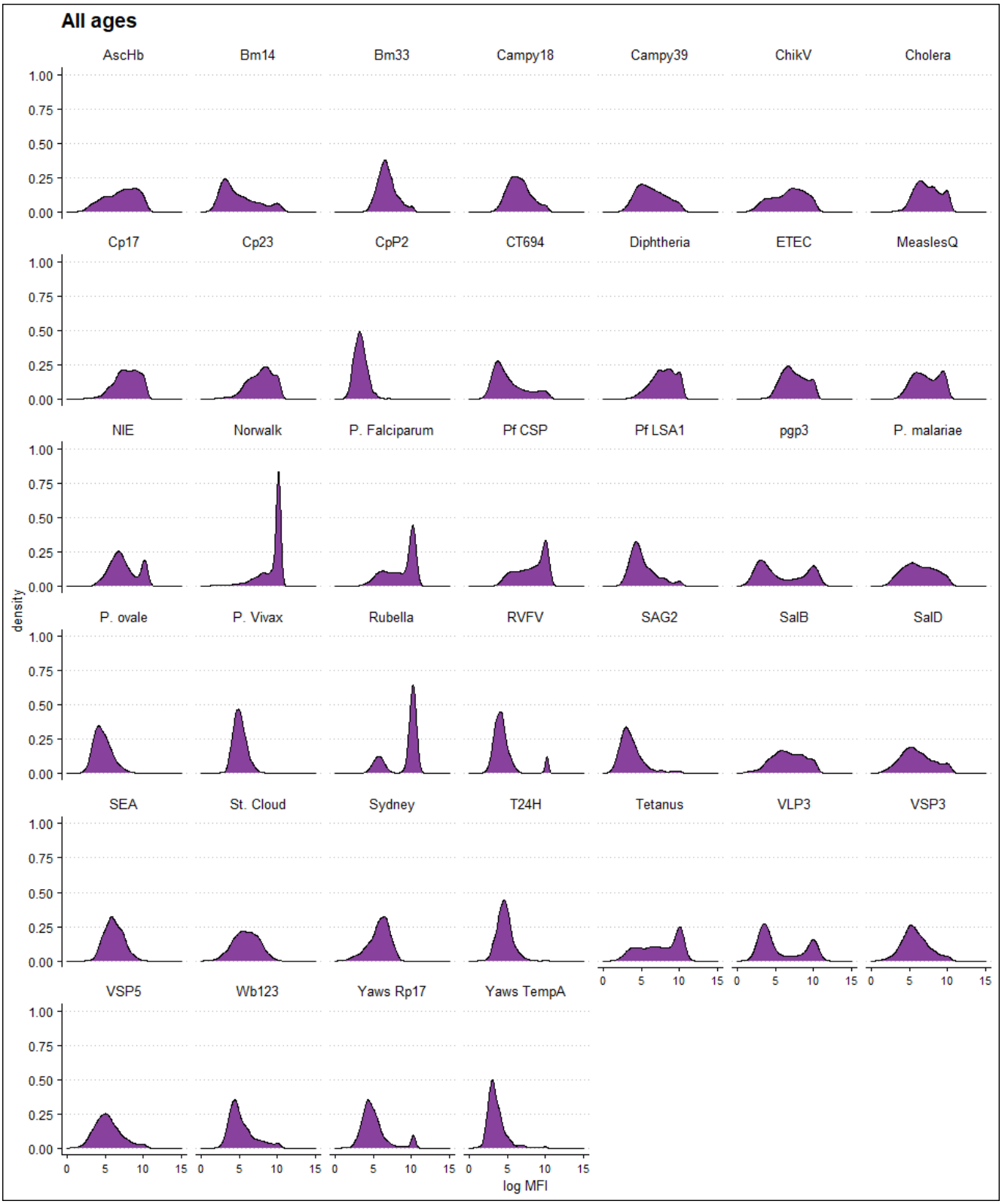
Table 2.2: Characteristics of sample of household survey conducted in 2 districts of Nampula Mozambique, 2014

Characteristic	Mecubúri N = 619	Nacala-a-Velha N = 537
Age (in years)		
<5	126 (20%)	117 (22%)
5-10	137 (22%)	114 (21%)
10-14	56 (9.0%)	44 (8.2%)
14-20	54 (8.7%)	32 (6.0%)
20-30	87 (14%)	70 (13%)
30-40	76 (12%)	53 (9.9%)
40-90	83 (13%)	107 (20%)
Sex		
Female	339 (55%)	289 (54%)
Male	280 (45%)	248 (46%)
Relationship to head of household		
Head	115 (19%)	122 (23%)
Spouse	119 (19%)	95 (18%)
Child	286 (46%)	232 (43%)
Other	99 (16%)	88 (16%)

Malaria knowledge index score	0.24 (0.02, 0.43)	0.28 (0.13, 0.46)
Sleeping space has an ITN	296 (48%)	394 (73%)
Net received during campaign	330 (53%)	462 (86%)
Household size	5 (4, 7)	5 (4, 7)
Household socioeconomic status index		
1 - lowest	135 (22%)	94 (18%)
2	112 (18%)	61 (11%)
3	132 (21%)	99 (18%)
4	90 (15%)	137 (26%)
5 - highest	150 (24%)	146 (27%)
Distance from closest health facility		
Less than 30 min on foot	161 (26%)	127 (24%)
1 to 2 hours on foot	159 (26%)	138 (26%)
30 min to 1 hour on foot	45 (7.3%)	50 (9.3%)
More than 2 hours on foot	254 (41%)	222 (41%)
Household income		
Salaried	27 (4.4%)	78 (15%)
None	408 (66%)	255 (47%)
Do not know	4 (0.6%)	49 (9.1%)
Occasional	180 (29%)	155 (29%)
Head of household occupation		
Farmer	544 (88%)	354 (66%)
Manual laborer	34 (5.5%)	96 (18%)
Other	5 (0.8%)	27 (5.0%)
Fisherman	0 (0%)	7 (1.3%)
Vendor	36 (5.8%)	53 (9.9%)
Head of household school		
Primary 1	207 (33%)	251 (47%)
Primary 2	73 (12%)	43 (8.0%)
Middle	16 (2.6%)	21 (3.9%)
Secondary	40 (6.5%)	21 (3.9%)
None	276 (45%)	194 (36%)
Do not know	7 (1.1%)	7 (1.3%)
Number of sleeping spaces in household		
1	73 (12%)	42 (7.8%)
2	187 (30%)	200 (37%)

3	249 (40%)	194 (36%)
4	67 (11%)	70 (13%)
5	20 (3.2%)	20 (3.7%)
6	21 (3.4%)	11 (2.0%)
7	2 (0.3%)	0 (0%)
Pf RDT result		
Negative	188 (30%)	272 (51%)
Positive	431 (70%)	265 (49%)
At least one ITN per sleeping space	174 (28%)	323 (60%)

Figure 2.1: Density plots of Log MFI values by antigen, Nampula Mozambique household survey, 2014



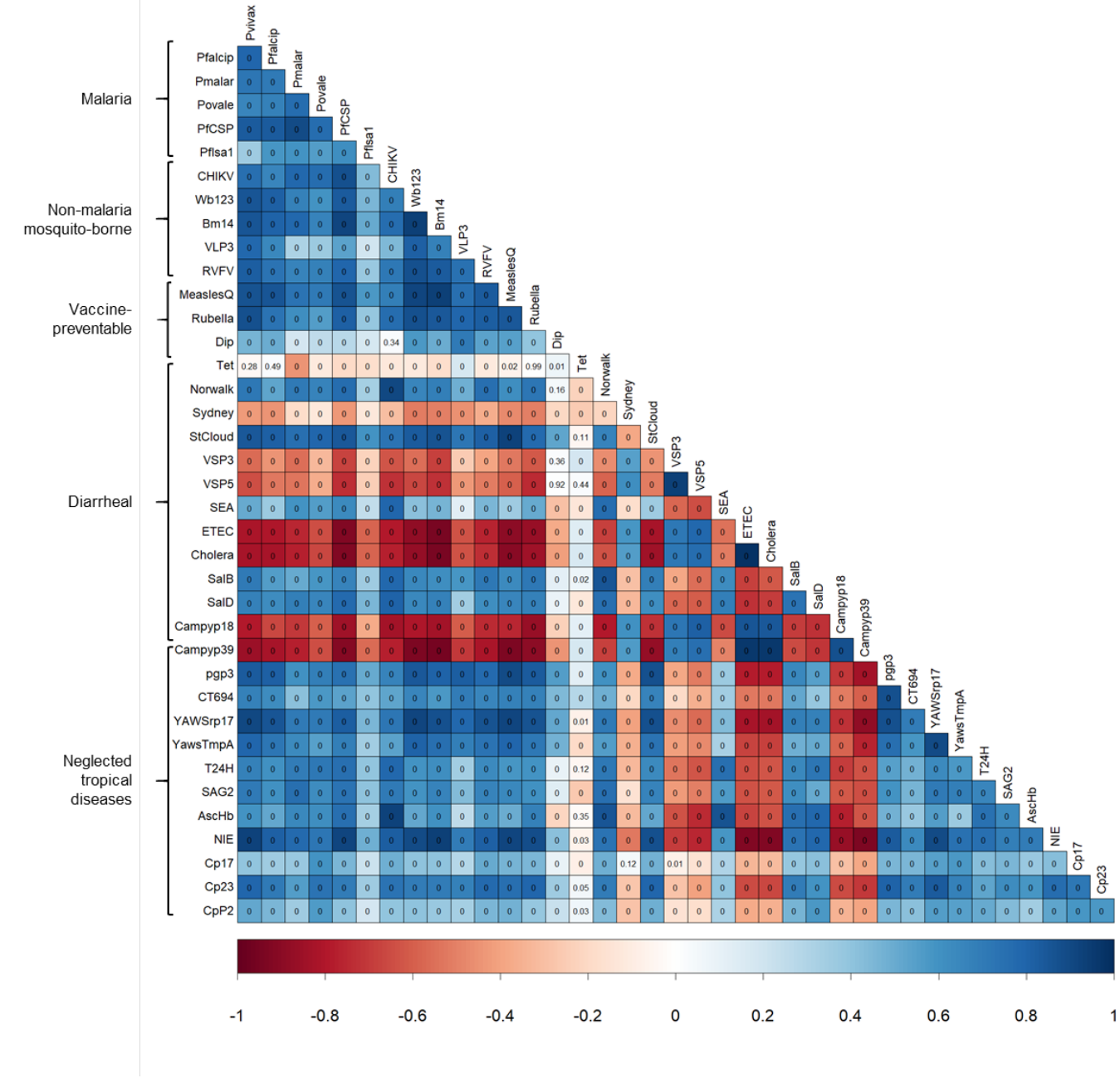
Associations

Of 702 pairwise comparisons of adjusted log-MFI values, 684 (97%) were statistically significant, 460 (67%) of which had positive correlations, and 224 (33%) were negatively correlated. All adjusted log-MFI values for antigens for mosquito-borne illnesses were positively correlated with one another. Similarly, comparisons among all mosquito-borne illnesses, including all malaria markers were positive.

Among vaccine preventable diseases, measles was positively associated with rubella and diphtheria as were rubella and diphtheria. Measles was negatively associated with tetanus. There was no evidence of statistically significant association between tetanus and rubella and diphtheria.

Of the 3 norovirus serotypes, Norwalk and Sydney were statistically significantly negatively associated with each other, as were Sydney and St. Cloud. Norwalk and St. Cloud were positively associated with one another. The 2 types of *Giardia intestinalis* antigens (VSP3 & VSP5) were positively associated, as were the 2 types of Salmonella sp. lipopolysaccharides (LPS B & LPS D) and 2 *Campylobacte jejuni* antigens (p18 & p39). There were no other apparent positive or negative trends among the diarrheal diseases as a group. Nearly all pairwise comparisons between neglected tropical diseases were positive.

Figure 2.2: Matrix of Pearson correlation coefficients and p-values of adjusted log-MFI for 40 antigens measured using a multiplex bead assay, Nampula, Mozambique, 2014



Blue denotes positive correlation
Red denotes negative correlation
Shade denotes strength of correlation
Numbers in boxes denote p-values, 0 denotes p-value of <0.01

Predictors

Age was the strongest predictor of seropositivity and included in the majority (97%) of the models and increased age was more likely to be associated with increased log-MFI than decreased.

Sex was included in over half of the models (54%), and district, relationship to head of household, socioeconomic status, and distance from health facility were included in between 38 and 44% of the models. Presence of a net on the sleeping space was a predictor included in the fewest models (18%).

Higher scores on the malaria knowledge index was associated with decreased odds of seropositivity for 4 non-malaria antigens and 3 malaria antigens. Receipt of an LLIN during the recent universal coverage campaign was associated with decreased log-MFI values for 7 non-malaria antigens and 3 malaria antigens (*P. ovale*).

Table 2.3: Variables included in linear regression models regressing covariates on log-MFI for 39 antigens, Nampula, Mozambique, 2014

Variable	Number of models included (%)	Risk factor (%)	Protective (%)
Age in years (ref: <5)			
5-10	38 (97)	30 (79)	8 (21)
10-14		29 (76)	9 (24)
14-20		29 (76)	9 (24)
20-30		30 (79)	8 (21)
30-40		30 (79)	8 (21)
40-90		30 (79)	8 (21)
Relationship to head of household (ref: head)			
spouse	16 (41)	6 (38)	10 (62)
child		8 (50)	8 (50)
other		9 (56)	7 (44)
Household socioeconomic status index (ref: 1)			
2	17 (44)	10 (59)	7 (41)
3		10 (59)	7 (41)
4		12 (71)	5 (29)
5		9 (53)	8 (47)
Distance from closest health facility (ref: less than 30 min on foot)			
30 min to 1 hour on foot	15 (38)	5 (33)	10 (66)
1 to 2 hours on foot		6 (40)	9 (60)
More than 2 hours on foot		6 (40)	9 (60)
Household income (ref: salaried)			
do not know	12 (31)	5 (42)	7 (58)
none		4 (33)	8 (66)
occasional		7 (58)	5 (42)
Head of household occupation (ref: farmer)			
manual laborer	15 (38)	4 (27)	11 (73)
fisherman		6 (40)	9 (60)
vendor		3 (20)	12 (80)
other		0 (0)	15 (100)

Head of household school (ref: primary 1)			
primary 2		8 (80)	2 (20)
middle		2 (20)	8 (80)
secondary	10 (26)	5 (50)	5 (50)
do not know		8 (80)	2 (20)
None		5 (50)	5 (50)
Number of sleeping spaces in household	10 (26)	3 (30)	7 (70)
Result of *Pf* RDT	15 (38)	12 (80)	3 (20)
At least one ITN per sleeping space	20 (51)	17 (85)	3 (15)
Village	21 (54)	NA	NA
District Nacala-a-Velha	16 (41)	3 (19)	13 (81)
Sex male	21 (54)	1 (5)	20 (95)
Malaria knowledge index score	12 (31)	5 (42)	7 (58)
Sleeping space has a net	7 (18)	3 (43)	4 (57)
Net use index	9 (23)	7 (78)	2 (82)
Received an ITN during campaign	13 (33)	3 (23)	10 (77)
Household size	11 (28)	7 (64)	4 (36)

Discussion

This analysis examined the tendency for immune status to 39 antigens representing pathogens endemic to the Nampula province of Mozambique to share common predictors defined by individual and household level variables collected during a household survey. Age was the strongest predictor of serostatus, followed by sex and district.

Many associations between pathogens were expected based on common route of transmission, particularly the mosquito-borne diseases such as malaria, lymphatic filariasis and dengue. *P. vivax* did not follow this pattern but was likely because there were very few individuals in the sample who had higher MFI-values indicating seropositivity for *P. vivax*, reducing the ability to identify strong predictors.

Among the vaccine preventable diseases, measles, rubella, and diphtheria shared common predictors, which may be an indication of comprehensive EPI coverage among those with any EPI coverage (i.e. those with evidence of antibody response to one are more likely to have evidence of antibody response to all 3). The negative or null association of these 3 antigens with tetanus may be an indication of weaker coverage of tetanus boosters coverage, which are scheduled for first and second grades of school. Additionally, male sex was associated with decreased log-MFI for tetanus, which may also be an indication of effective booster coverage among women of reproductive age, confirming previous findings from Mozambique and other countries[26].

The negative associations among the norovirus serotypes may be explained by outbreaks of different serotypes occurring at different periods, as age was a predictor for all 3 serotypes. For example, older age was associated with increased log-MFI values for the Norwalk and St. Cloud serotypes, but with decreased log-MFI values for the Sydney serotype. It is possible there was a more recent outbreak among young children for the Sydney serotype to which adults were less likely to be exposed[27]. This pattern is congruent with evidence that predominant strains of norovirus emerge, replacing the previous predominant strain with lack of evidence of cross-immunity between strains[28].

Most antigens representing neglected tropical diseases were co-linear, which is logical given that many share routes of transmission, many associated with water, sanitation and hygiene access and practices, which were not measured directly in the survey questions, but other predictors such as SES may have served as a proxy[29].

Age was the most common predictor of antibody response and was included in the majority of the prediction models. Age can be a predictor of disease exposure[27], access to or use of prevention or healthcare[8], or waning immunity[26] and varies by pathogen and antigen used to measure exposure. While SES had predictive power in the prediction models, there was no clear pattern of higher or lower SES being associated with higher adjusted MFI-values in any grouping of pathogens. Notably, higher scores on the malaria knowledge index and LLIN use as protective factors for several non-malaria antigens may signal that knowledge and practice of malaria transmission and prevention activities may be a proxy for practicing general health prevention behaviors.

Overall, the majority of pairwise associations of log-MFI values were positive, indicating that higher log-MFI values for most antigens tested for in this sample share predictors and the direction of the association. With the exception of the vaccine preventable diseases, for which immune response is more likely to be an indication of interaction with the healthcare system and preventive behavior, there is an indication that individuals in this sample are likely to have several cumulative disease exposures. While

only exposure status and not disease status can be known, the impact of multiple infectious disease exposure can have serious health and economic impacts on the individual and community levels[30].

Limitations

There are several limitations to this analysis. The household survey administered during which the samples were collected was intended to measure malaria and associated behaviors, and many variables that would likely be strong predictors of non-mosquito borne diseases such as vaccine status, water source, food handling practices, and intervention coverage such as MDA administration were not included in the survey. Second, while seroprevalence studies can be used to gain information about pathogen exposure, they cannot provide information about whether these exposures happened at the same time, or even if exposure was associated with illness, limiting the ability to make inferences about co-occurrence of active infections. While the use of log-MFI values, rather than assigning a binary cutoff for seropositive and seronegative reduces misclassification bias due to challenges in determining appropriate cutoff values, log-MFI is not as easily interpretable, and the range of values vary by antigen.

Conclusions

Multiplex assays coupled with cross-sectional survey data regarding practices, socioeconomic indicators, and access and use of healthcare services can provide insights about common predictors that may be associated with exposure to multiple pathogens. By understanding the epidemiology of several pathogens at once, public health practitioners can target the most common predictors of exposure to diseases in an area making more efficient and effective public health interventions in resource-limited settings.

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Declarations

Availability of data

All data generated or analysed during this study are available from the corresponding author upon reasonable request.

Disclaimer

The findings and conclusions in this paper are those of the authors and do not necessarily represent the views of the U.S. Centers for Disease Control and Prevention.

Conflict of Interest

All co-authors have no conflicts of interest to declare.

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Chapter 4: Decreased efficacy of artemisinin-based combination therapies in Democratic Republic of the Congo and investigation of molecular markers of antimalarial resistance

Running title: Decreased antimalarial efficacy in DRC

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Abstract

Background

Routine assessment of artemisinin-based combination therapies (ACTs) efficacy is critical for the early detection of antimalarial resistance. We evaluated the efficacy of ACTs recommended for treatment of uncomplicated malaria in five sites in Democratic Republic of the Congo (DRC): artemether-lumefantrine (AL), artesunate-amodiaquine (ASAQ), and dihydroartemisinin-piperaquine (DP).

Methods

Children aged 6-59 months with confirmed *P. falciparum* malaria were treated with one of three ACTs and monitored. The primary endpoints were uncorrected and PCR-corrected 28-day (AL & ASAQ) or 42-day (DP) cumulative efficacy. Molecular markers of resistance were investigated.

Results

Uncorrected efficacy estimates ranged from 63% to 88% for AL, 73% to 100% for ASAQ, and 56% to 91% for DP. PCR-corrected efficacy estimates ranged from 86% to 98% for AL, 91% to 100% for ASAQ and 84% to 100% for DP. No *pfk13* mutations previously found to be associated with ACT resistance were observed. Statistically significant associations were found between certain *pfmdr1* and *pfcrt* genotypes and treatment outcome.

Conclusions

There is evidence of efficacy below the 90% cutoff recommended by WHO to consider a change in first-line treatment recommendations of two ACTs in one site each. Confirmation of these findings in future therapeutic efficacy monitoring in DRC is warranted.

Abstract word count: 199

Key words: Antimalarial, *P. falciparum*, efficacy, resistance, malaria, DRC

Introduction

Democratic Republic of the Congo (DRC) accounts for an estimated 12% of the malaria morbidity and 11% of malaria mortality globally, with 25 million reported malaria cases and 46,000 deaths annually[1]. In 2005, artemisinin-based combination therapies (ACTs) were introduced in DRC for the treatment of uncomplicated malaria as recommended by the World Health Organization (WHO) to prevent or delay resistance to artemisinin derivatives and partner drugs[2]. Two ACTs, artemether-lumefantrine (AL) and artesunate-amodiaquine (ASAQ), are used as first-line treatments, and dihydroartemisinin-piperaquine (DP) is considered as an alternative first-line treatment for uncomplicated *Plasmodium falciparum* malaria in DRC. All three ACTs circulate freely in the private sector[3].

WHO recommends the implementation of therapeutic efficacy studies at least every two years in malaria endemic countries to quickly identify reduced sensitivity to ACTs[4]. Early identification of waning efficacy of a drug may inform DRC national malaria control program (NMCP) policy for malaria treatment. Recent studies have demonstrated that AL, ASAQ, and DP are efficacious in DRC, with per-protocol PCR-corrected efficacies of over 90% in studies conducted between 2015–2017[5,6].

In addition to monitoring ACT efficacy, therapeutic efficacy studies may monitor molecular markers of antimalarial resistance among *Plasmodium falciparum* parasites. Specific polymorphisms in the propeller domain of the *pfkelch13* (*pfk13)* gene[7] have been associated with artemisinin resistance, a finding described in Southeast Asia[8] and polymorphisms identified in one country in sub-Saharan Africa[9]. Decreased susceptibility to lumefantrine and amodiaquine has been associated with polymorphisms in the gene *pfmdr1*[10] and decreased susceptibility to amodiaquine has been associated with polymorphisms in the gene *pfcrt* .

This report will describe the results of a study examining the therapeutic efficacy of three ACTs used for the treatment of uncomplicated *Plasmodium falciparum* malaria in five sites in DRC. Prevalence of molecular markers of resistance to artemisinin derivatives and partner drugs will also be presented.

Methods

The standard WHO protocol for *in vivo* therapeutic efficacy studies[4] was followed to assess the efficacy of AL, ASAQ, and DP in five sentinel sites representing different epidemiologic zones of DRC. Study recruitment took place from March 2017 to January 2018.

Study sites and population

Three sites in the equatorial zone of DRC were included: Kabondo, in Kisangani in the northern Tshopo province, where malaria prevalence measured by rapid diagnostic test (RDT) among children 6–59 months old was 52.2% in the 2017–2018 Multiple Indicator Cluster Survey[11]; Mikalayi, in the Kasai Central province, where malaria prevalence measured by RDT among children 6-59 months old was 45.5%; and Kimpese, in the Kongo Central province next to the border with Angola, where malaria prevalence measured by RDT among children 6–59 months old was 40.0%. The fourth site, Rutshuru, located in the mountainous zone next to the border with Rwanda in the North Kivu province of eastern DRC, where malaria prevalence measured by RDT among children 6-59 months old was 11.4%. The fifth site, Kapolowe, is in the Haut Katanga province in the southern part of the country, next to the border with Zambia, and is in the tropical zone, where malaria prevalence measured by RDT among children 6-59 months old was 42.7%. The national malaria prevalence measured by RDT among children 6-59 months old was 38.5%[11].

Figure 3.1: Location of antimalarial therapeutic efficacy monitoring sites, Democratic Republic of the Congo, 2017

Children aged 6–59 months old with uncomplicated *Plasmodium falciparum* malaria infection were recruited at participating health centers. A sample size of 88 children per arm per site was targeted and calculated assuming 5% drug failure, 95% confidence level, 5% precision and the assumption of 20% loss to follow-up.

Study Procedures

Criteria for inclusion included *Plasmodium falciparum* infection measured by microscopy with parasite density between 2,000–200,000 trophozoites per µl, axillary temperature of 37.5°C or higher, ability to take oral medication, ability to adhere to the study follow-up procedures, declared consent from a parent or guardian, absence of signs of severe illness, malnutrition, or other illness associated with fever, and

absence of past allergic reaction to the study medication. Children with severe anemia (hemoglobin <5g/dl or hematocrit <15%), weighing less than 5kg, taking regular medication contraindicated with the study medication, or presenting with signs of severe illness were excluded from the study.

Microscopic blood examination was performed by trained microscopists using thick and thin smears on the same slide to determine parasite species. Two slides were collected for each patient, one for screening and one for quantification of parasitemia. The slide for screening was stained with 10% Giemsa for 10 minutes and the second with 6% Giemsa for 30 minutes. Quality control of the study slides was carried out at two levels: first, at each study site by two trained and experienced microscopists and a third in case of discrepancy. Second, at the end of the study, 10% of slides from each site were also read by the national malaria reference laboratory located at the Institut National de Recherche Biomédicale.

Study participants were randomly assigned one of three ACTs: ASAQ (Winthrop; Sanofi Aventis, Paris, France), AL (Coartem, Novartis, Basel, Switzerland), or DP (Eurartesim, Alfasigma, Bologna, Italy). Weight-based dosage was determined using the WHO malaria treatment guidelines[2]. Medications were procured by the DRC NMCP and its partners, notably WHO, and underwent quality control at the laboratory of the Faculty of Pharmaceutical Sciences of the University of Kinshasa (DRC). Medication was administered on days 0, 1, and 2 under supervision of study staff. AL intake was also accompanied by the intake of milk and biscuits. All children enrolled in the study were administered paracetamol for fever management in the first 48 hours after seeking care at the health facility. All doses, including the evening dose of AL, were observed by a study team member and were observed for 30 minutes after each dose was administered. Any child who vomited during this 30-minute window received the same dose of medication and was observed for an additional 30 minutes. In case of vomiting a second time, the child was removed from the study and given a rescue treatment[12].

There were nine total pre-planned visits in the 42 days of follow-up (day 0, 1, 2, 3, 7, 14, 21, 28, 35, 42). However, parents and guardians were instructed to return to the study site for any reason, including sickness or adverse event related to the study medication. A clinical evaluation was performed at all

follow-up visits. On day 0, medical history and demographic information were recorded in addition to screening for malnutrition by measuring body weight by Salter or baby scale, brachial circumference, and checking for the presence of nutritional edema. Hemoglobin was measured on days 0, 14, 28, and 42 by sampling capillary blood using Hemocue® (Angelholm, Sweden). Parasitological examination was done by a trained microscopist on all follow-up visits. Capillary blood was collected on Whatman (GE Healthcare, Chicago, IL) filter paper for PCR-genotyping to differentiate reinfections from recrudescent infections and characterization of molecular markers of antimalarial resistance on days 0, 7, 14, 21, 28, 35, 42, and unplanned visits. Rescue treatment was administered in case of recurrent parasitemia or severe illness in accordance with the national case management guidelines[12].

Supervision was organized in each site by the monitor and instructors at the start of the study, once during recruitment, and at the closure of the study site. A supervision tool was used during each supervision visit and feedback was provided to study staff if any inconsistency in enrollment or follow-up procedures were noted.

All study participants were assigned a classification at the end of follow-up. Early treatment failures were defined as parasitemia higher on day 2 than on day 0, parasitemia on day 3 with fever, parasitemia on day 3 of ≥ 25% of the count on day 0, or danger signs or severe malaria in the presence of parasitemia on days 1–3. Recurrences were defined as recurrent parasitemia on days 4 through 28 for AL and ASAQ, and on days 4 through 42 for DP. Adequate clinical and parasitological response (ACPR) was defined as absence of recurrence on either day 28 (AL and ASAQ) or 42 (DP). Children who were lost to follow-up or met exclusion criteria during the study were excluded from the analysis.

Adverse events were recorded by using standard forms and shared with the DRC National Pharmacovigilance Center (CNPV-RDC). Serious adverse events were communicated by the principal investigator to the CNPV-RDC and the drug manufacturer within 24 hours. Adverse events were defined as any unfavorable sign, symptom, syndrome or unexpected illness appearing or worsening with the use of the study medication. Serious adverse events were defined as any medical occurrence with use of the

study medication that resulted in death or was life threatening, required hospitalization, or resulted in significant or persistent disability.

Molecular analysis

Molecular analyses were performed at the U.S. Centers for Disease Control and Prevention (CDC) Malaria Laboratory in Atlanta, GA. Genomic DNA extraction from dried blood spots collected on day of enrollment and day of recurrence was performed using the QIAamp blood minikit (Qiagen Inc., Hilden, Germany) following the manufacturer's instructions. PET-PCR was used to confirm *Plasmodium* infection and species[13].

The analysis of seven neutral microsatellites was used to distinguish reinfections from recrudescence among study participants classified with recurrent infection. Fragment lengths from day 0 and day of recurrence samples were measured after amplification of seven neutral microsatellite loci over six chromosomes by non-nested or semi-nested PCR using previously described methods[14,15].

Sanger sequencing was used to investigate *pfk13, pfcrt,* and *pfmdr1* single nucleotide polymorphisms (SNPs) on day 0 and day of recurrence samples for all classified recurences. Polymorphisms within codons 389–649 of the propeller domain region of *pfk13*[16], codons 86, 184, 1034, 1042, and 1246 of *pfmdr1,* and within codons 72-76 of *pfcrt* were analyzed. SNPs were identified using the Geneious software package (Biomatters, Inc. San Francisco, CA).

Data management and statistical analysis

Data were entered into a secure study database with independent double entry. Descriptive statistics of study participants were performed in addition to descriptions of any adverse events. Uncorrected and PCR-corrected efficacy estimates were calculated using per-protocol and Kaplan-Meier analyses[4]. Uncorrected per-protocol efficacy was calculated per arm and per site, including all recurrences and ACPR. Those lost to follow-up or excluded from the study were not included in the uncorrected or PCR-

corrected per-protocol estimates. Participants lost to follow-up or excluded were included until last day of follow-up in the Kaplan-Meier analysis.

To perform the PCR-corrected per-protocol and Kaplan-Meier analyses, microsatellite data were used to assign each recurrent parasitemia a posterior probability of recrudescence using a previously used Bayesian algorithm further validated for this study[17]. Samples for which amplification was not possible were assigned the average posterior probability of recrudescence from all amplified samples. For the PCR-corrected per-protocol analysis, the total number of recrudescences was defined as the sum of probability of recrudescence in all recurrent infections per arm and site. The number of reinfections equaled the total number of recurrences minus the sum of the posterior probability of recrudescence and was excluded from the PCR-corrected per-protocol calculation. For the PCR-corrected Kaplan-Meier analysis, posterior sampling was used to generate Kaplan-Meier estimates and 95% confidence intervals from the posterior probability of recrudescence. A sensitivity analysis was done to assess the use of a cutoff approach to distinguish recrudescence vs. reinfection, defining a recrudescence as above or equal to a predetermined number of matches over the seven loci vs. the use of the sum of probabilities of recrudescence to tally the number of recrudescences as done to derive the efficacy results in this study.

The combination of SNPs at *pfmdr1* codons 86, 184, and 1246 were used to define *pfmdr1* haplotypes, and the combination of SNPs at *pfcrt* codons 72-76 were used to define *pfcrt* haplotypes. For mixed infections, all possible haplotypes (i.e., wild type and mutant) were counted for *Pfmdr1* and included in the analysis. For *Pfcrt*, the wildtype (CVMNK) and most likely mutant haplotypes were reported for mixed infections. To investigate differences in *pfmdr1* between cleared and uncleared parasites, haplotypes of day 0 samples of reinfections (cleared infections) were compared to the haplotypes of day of recurrence samples among recrudescences and reinfections (uncleared infections) using Fisher's exact test. Reinfection for day of recurrence were included as "uncleared" infections due to the lack of susceptibility of parasites to post-treatment prophylaxis[18]. For tabulation, samples with a posterior probability of recrudescence of ≥50% were defined as a recrudescence and those with a posterior

probability of <50% were considered reinfections. Multiplicity of infection (MOI) was calculated per site in samples from those who experienced recurrent infection as the maximum number of SNPs detected among the seven neutral microsatellite markers.

Microsoft Excel and R version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria) were used to perform statistical analyses.

Ethical considerations

The DRC Ethics Committee of the School of Public Health of the University of Kinshasa provided ethical clearance for the study in DRC. The study protocol was registered in an approved public register (ClinicalTrials.gov) whose registration number is NCT02940756. Informed consent was available in French and translated into local languages Lingala, Kikongo, Swahili, and Tshiluba. All patient information was kept confidential and was known only to the research team. Staff from the Centers for Disease Control and Prevention (CDC) provided technical assistance[19]; the protocol was approved as a non-research program evaluation by the Office of the Associate Director for Science, Center for Global Health at CDC.

Results

A total of 1,356 children were enrolled in the study over the three drug arms and five sites (range per arm: 75 to 96), with 1,271 (93.7%; range 83.1 to 97.9%) reaching a study endpoint. A total of 85 children were withdrawn or lost to follow-up over all arms. Day 3 slide positivity rates were null in all arms except for the Kabondo ASAQ arm, where the day 3 slide positivity was 1% (1/81), and the Kabondo DP arm, where the day 3 slide positivity rate was also 1% (1/74).

Description of study participants

Table 3.1: Baseline characteristics and enrollment of patients participating in the DRC therapeutic efficacy study, 2017

		Characteristics at day of study enrollment				Enrollment and follow-up		
Site	Drug	Age, years (mean, sd)	Female, n (%)	Weight (kg) (mean, sd)	Parasitemia, geometric mean, parasites/µL (range)	Lost to follow up, n (%)	Excluded, n (%)	Reached study endpoint, n (%)
Kabondo	AL (n=89)	2.7 (1.3)	37 (41.6)	12.3 (3.2)	19944 (2046, 179760)	6 (6.7)	0 (0)	83 (93.3)
	ASAQ (n=84)	2.8 (1.1)	32 (38.1)	12.7 (2.7)	27450 (2272, 195564)	3 (3.6)	0 (0)	81 (96.4)
	DP (n=89)	2.6 (1.2)	45 (50.6)	11.9 (2.9)	22436 (4078, 168915)	15 (16.9)	0 (0)	74 (83.1)
Kapolowe	AL (n=96)	2.4 (1.3)	52 (54.2)	11.9 (3.1)	39426 (2039, 192554)	2 (2.1)	1 (1.0)	93 (96.9)
	ASAQ (n=94)	2.6 (1.3)	51 (54.3)	12 (3.1)	36842 (2032, 207761)	2 (2.1)	3 (3.2)	89 (94.7)
	DP (n=96)	2.4 (1.3)	43 (44.8)	11.9 (3.4)	33949 (2095, 198059)	3 (3.1)	1 (1.0)	92 (95.8)
Kimpese	AL (n=75)	3.3 (1.3)	38 (50.7)	14 (4.4)	17625 (2008, 199446)	1 (1.3)	0 (0)	74 (98.7)
	ASAQ (n=90)	3.3 (1.3)	44 (48.9)	13.7 (3.5)	21520 (2015, 199538)	8 (8.9)	0 (0)	82 (91.1)
	DP (n=85)	3.1 (1.2)	28 (32.9)	12.9 (3.2)	22189 (2080, 199095)	4 (4.7)	0 (0)	81 (95.3)
Mikalayi	AL (n=94)	2.0 (1)	43 (45.7)	10.7 (2.8)	25437 (2016, 163200)	2 (2.1)	0 (0)	92 (97.9)
	ASAQ (n=92)	2.1 (1.4)	45 (48.9)	10.5 (3.1)	23619 (1050, 179428)	2 (2.2)	6 (6.6)	84 (91.3)
	DP (n=94)	2.2 (1.3)	52 (55.3)	11.6 (8.7)	24995 (1454, 188800)	4 (4.3)	2 (2.1)	88 (93.6)
Rutshuru	AL (n=93)	2.5 (1.4)	44 (47.3)	11.1 (3.2)	27273 (2088, 195246)	1 (1.1)	3 (3.2)	89 (95.7)
	ASAQ (n=91)	2.5 (1.2)	39 (42.9)	11.3 (2.9)	32539 (2000, 301900)	1 (1.1)	8 (8.8)	82 (90.1)
	DP (n=94)	2.5 (1.2)	56 (59.6)	11.4 (3.3)	32613 (2679, 198579)	0 (0)	7 (7.4)	87 (92.6)

DRC: Democratic Republic of the Congo
sd: standard deviation
AL: artemether lumefantrine
ASAQ: artesunate amodiaquine
DP: dihydroartemisinin piperaquine

Efficacy

There were 268 recurrent infections, including no early treatment failures observed in any arm. The mean MOI among all day 0 and day of recurrence samples among recurrent infections was 2.0 (standard deviation, 0.83, range 1, 5) (Supplemental Figure 1).

Uncorrected 28-day cumulative efficacy for AL ranged from 63% (95% CI 55, 74) in Mikalayi to 88% (95% CI 82, 95) in Kabondo. PCR-corrected 28-day cumulative efficacy for AL ranged from 86% (95% CI 79, 93) in Mikalayi to 98% (95% CI 95, 100) in Kabondo.

Uncorrected 28-day cumulative efficacy for ASAQ ranged from 73% (95% CI 64, 83) in Rutshuru to 100% (95% CI 100, 100) in Kabondo. PCR-corrected 28-day cumulative efficacy for ASAQ ranged from 91% (95% CI 85, 98) in Rutshuru, to 100% (95% CI 100, 100) in Kabondo and Kapolowe.

Uncorrected 28-day cumulative efficacy for DP ranged from 75% (95% CI 67, 85) in Rutshuru to 99% (95% CI 97, 100) in Kimpese. PCR-corrected 28-day cumulative efficacy for DP ranged from 97% (95% CI 93, 100) in Rutshuru to 100% (95% CI 100, 100) in Kabondo. Uncorrected 42-day cumulative efficacy for DP ranged from 56% (95% CI 47, 67) in Mikalayi to 91% (95% CI 85, 98) in Kabondo. PCR-corrected 42-day cumulative efficacy for DP ranged from 84% (95% CI 75, 93) in Mikalayi to 100% (95% CI 99, 100) in Kabondo.

The sensitivity analysis examining the use of a cutoff approach based on number of matches at each loci yielded highly variable failure rates for each drug dependent on the cutoff used, influenced by the relatively high number of intermediate values of posterior probability of recrudescence for each instance of recurrent parasitemia (Supplemental Figures 3.2 & 3.3).

Table 3.2: Classification of patients with the outcome of late recurrence, DRC therapeutic efficacy study, 2017

	Kabondo			Kapolowe			Kimpese			Mikalayi			Rutshuru		
	AL (n=83)	ASAQ (n=81)	DP (n=74)	AL (n=93)	ASAQ (n=89)	DP (n=92)	AL (n=74)	ASAQ (n=82)	DP (n=81)	AL (n=92)	ASAQ (n=84)	DP (n=88)	AL (n=89)	ASAQ (n=82)	DP (n=87)
Early treatment failure	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Late recurrence	10	0	7	23	0	33	11	6	8	34	20	39	18	23	36
Recrudescence[1]	1	0	0	4	0	6	2	0	0	14	3	12	3	6	3
Day 7-13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Day 14-21	1	0	0	1	0	1	1	0	0	3	2	1	2	1	1
Day 22-28	0	0	0	3	0	1	1	0	0	11	1	1	1	5	1
Day 29-35	-	-	0	-	-	2	-	-	0	-	-	6	-	-	1
Day 36-42	-	-	0	-	-	2	-	-	0	-	-	4	-	-	0
Reinfection	9	0	7	19	0	27	9	6	8	20	17	27	15	17	34
Day 7-13	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Day 14-21	1	0	1	10	0	1	7	1	1	7	8	3	4	12	9
Day 22-28	8	0	0	9	0	7	2	4	7	13	9	10	11	5	11
Day 29-35	-	-	4	-	-	11	-	-	0	-	-	8	-	-	9
Day 36-42	-	-	2	-	-	8	-	-	0	-	-	6	-	-	5
ACPR	73	81	67	70	89	59	63	74	73	58	64	49	71	59	51

[1]Recrudescence defined as a late recurrence with a posterior probability of recrudescence ≥ 0.5

DRC: Democratic Republic of the Congo

AL: artemether lumefantrine

ASAQ: artesunate amodiaquine

DP: dihydroartemisinin piperaquine

ACPR: adequate clinical and parasitological response

Table 3.3: Therapeutic efficacy of three artemisinin-based combination therapies at five monitoring sites, Democratic Republic of the Congo, 2017

		Kaplan-Meier				Per-protocol			
		Uncorrected % (95% CI)		PCR-corrected % (95% CI)[a]		Uncorrected % (95% CI)		PCR-corrected % (95% CI)[a]	
Site	Drug	28 days	42 days	28 days	42 days	28 days	42 days	28 days	42 days
Kabondo	AL	88 (82, 95)		98 (95, 100)		88 (79, 94)		98 (92, 100)	
	ASAQ	100 (100–100)		100 (100, 100)		100 (96, 100)		100 (96, 100)	
	DP	99 (96, 100)	91 (85, 98)	100 (100, 100)	100 (99, 100)	99 (93, 100)	91 (81, 96)	100 (95, 100)	100 (94, 100)
Kapolowe	AL	76 (68, 85)		94 (89, 99)		75 (65, 84)		93 (84, 97)	
	ASAQ	100 (100, 100)		100 (100, 100)		100 (96, 100)		100 (96, 100)	
	DP	89 (83, 96)	64 (56, 75)	98 (94, 100)	93 (87, 99)	89 (81, 95)	64 (53, 74)	96 (90, 99)	91 (82, 97)
Kimpese	AL	85 (78, 94)		96 (92, 100)		85 (75, 92)		96 (88, 99)	
	ASAQ	93 (88, 99)		99 (98, 100)		93 (84, 97)		100 (95, 100)	
	DP	99 (97, 100)	90 (84, 97)	100 (100, 100)	100 (99, 100)	99 (93, 100)	90 (81, 96)	100 (96, 100)	100 (95, 100)
Mikalayi	AL	63 (55, 74)		**86 (79, 93)**		63 (52, 73)		**82 (71, 90)**	
	ASAQ	77 (69, 86)		96 (91, 99)		76 (66, 85)		95 (86, 99)	
	DP	83 (76.91)	56 (47, 67)	97 (94, 100)	**84 (75, 93)**	83 (73, 90)	56 (45, 66)	**88 (89, 94)**	**80 (68, 89)**
Rutshuru	AL	80 (72, 89)		96 (92, 100)		80 (70, 88)		96 (88, 99)	
	ASAQ	73 (64, 83)		91 (85, 98)		72 (61, 81)		91 (81, 97)	
	DP	75 (67, 85)	59 (50, 70)	97 (93, 100)	95 (90, 100)	75 (65, 84)	59 (48, 69)	99 (92, 100)	93 (83, 98)

Bold indicates point estimate of PCR-corrected efficacy <90%

[a]Number of treatment failures calculated as the sum of posterior probabilities of recrudescence

CI: confidence interval

AL: artemether-lumefantrine

ASAQ: artesunate-amodiaquine

DP: dihydroartemisinin-piperaquine

Safety

Among the 1,356 children enrolled in the study, five serious adverse events were reported including two deaths (one in the Rutshuru DP arm and one in the Mikalayi ASAQ arm). Three instances of respiratory distress were reported among participants in the Rutshuru DP arm. After clinical assessment, it was concluded that it was unlikely that these events were associated with the study drug or concomitant medication.

Molecular markers of antimalarial resistance

There were 577 dried blood spots available for analysis of molecular markers of antimalarial resistance. Samples taken on day 0 from participants with recrudescent infections (n=54) were excluded from the analysis to avoid double counting a parasite.

pfk13

A total of 251/263 day 0 reinfections were successfully sequenced for *pfk13* (95%) (cleared initial infection), and 235/260 (90%) samples were successfully sequenced from day of recurrence samples (uncleared infections).

Most samples (96%) included in the analysis were wild type for *pfk13*. Synonymous mutations (P417P, C469C, R471R, S477S, T478T, G496G, Y511Y, R539R, S576S) were found in 17 (3%) samples, and a non-synonymous mutation, S477Y, was observed in one day of recurrence sample in the DP arm.

pfmdr1

A total of 466 dried blood spots from those in the AL and ASAQ arms were included for analysis. A total of 204/206 (99%) day 0 reinfections were successfully sequenced (cleared initial infection), and 258/260 (99%) samples were successfully sequenced from day of recurrence samples (uncleared infections).

More than half of the samples analyzed had the N86 *pfmdr1* SNP (263/462, 57%). Eleven percent (52/462) had mixed N/Y, and 32% (147/462) had the 86Y SNP. More than half (268/462, 58%) carried

the Y184 *pfmdr1* SNP, with 102 (22%) with mixed Y/F, and 92 (20%) with the 184F *pfmdr1* SNP. Most samples (379/462, 82%) carried the D1246 pf*mdr1* SNP, 35 (8%) had mixed D/Y, and 48 (10%) carried the 1246Y SNP. The most common *pfmdr1* haplotypes were NYD (N86, Y184, D1246) (50%), NFD (N86, 184F, D1246) (31%), and YYD (86Y, Y184, 1246Y) (28%; percentages total > 100% due to mixed infections). In the AL arm, there was a statistically significant increased risk of failure in samples carrying the N86 versus the 86Y *pfmdr1* SNP (p=.007, Fisher's exact test). Also in the AL arms, there was a statistically significant increased risk of treatment failure among samples with the NYD compared to the YFD haplotype (p= 0.003, Fisher's exact test). No statistically significant associations were found between treatment outcome and *pfmdr1* SNP or haplotypes in any other treatment arm.

Pfcrt

Investigation of *pfcrt* SNPs was performed in samples from the ASAQ arms only. There were 85 samples included in the analysis. A total of 38 samples from day 0 of those who would experience a reinfection were successfully sequenced (100%), and 47 samples from day of recurrence were successfully sequenced (100%).

At codon positions 72 and 73, all samples were wild type (C and V respectively). Most samples were found to have the 74I (88%), 75E, (87%), and 76T (88%) *pfcrt* SNPs, and the most common haplotype among samples analyzed was CVIET (C72, V73, 74I, 75E, 76T) (88%). There was a statistically significant increased risk of treatment failure among those with the 75E versus the N75 SNP (p= 0.042, Fisher's exact test), and having the 76T versus the K76 SNP (p= 0.013, Fisher's exact test). There was also a statistically significant increased risk of treatment failure among samples with the CVIET versus the CVMNK haplotype (p= 0.013, Fisher's exact test) (Supplemental Tables 3.1-3.3).

Table 3.4: Molecular markers of resistance, all drug arms, Democratic Republic of the Congo therapeutic efficacy study, 2017

	All samples	Reinfection Day 0	Recrudescence + Reinfection Day of recurrence
pfk13			
Successfully sequenced	486/523 (93%)	251/263 (95%)	235/260 (90%)
Wild type	468 (96%)	244 (97%)	224 (95%)
Synonymous[a]	17 (3%)	7 (3%)	10 (4%)
Non-synonymous[b]	1 (0.2%)	0 (0%)	1 (0.4%)
pfmdr1[c]			
Successfully sequenced	462/466 (99.1%)	204/206 (99%)	258/260
N86	263 (57%)	109 (53%)	154 (60%)
86N/Y	52 (11%)	26 (13%)	26 (10%)
86Y	147 (32%)	69 (34%)	78 (30%)
Y184	268 (58%)	119 (58%)	149 (58%)
184Y/F	102 (22%)	45 (22%)	57 (22%)
184F	92 (20%)	40 (20%)	52 (20%)
D1246	379 (82%)	166 (81%)	213 (83%)
1246D/Y	35 (8%)	13 (6%)	22 (9%)
1246Y	48 (10%)	25 (12%)	23 (9%)
NYD	232 (50%)	89 (44%)	143 (55%)
NFD	141 (31%)	53 (26%)	88 (34%)
NFY	20 (4%)	7 (3%)	13 (5%)
NYY	31 (7%)	10 (5%)	21 (8%)
YFD	69 (15%)	27 (13%)	42 (16%)
YFY	0 (0%)	0 (0%)	0 (0%)
YYD	130 (28%)	48 (24%)	82 (32%)
YYY	20 (4%)	20 (10%)	0 (0%)
pfcrt[d, e]			
Successfully sequenced	85/85 (100%)	38/38 (100%)	47/47 (100%)
M74	8 (9%)	6 (16%)	2 (4%)
74M/I	2 (2%)	2 (5%)	0 (0%)

74I	75 (88%)	30 (79%)	45 (96%)
N75	10 (12%)	8 (21%)	2 (4%)
75N/E	1 (1%)	0 (0%)	1 (2%)
75E	74 (87%)	30 (79%)	44 (94%)
K76	9 (11%)	8 (21%)	1 (2%)
76K/T	1 (1%)	0 (0%)	1 (2%)
76T	75 (88%)	30 (79%)	45 (96%)
CVMNK	9 (11%)	8 (21%)	1 (2%)
CVIET	75 (88%)	30 (79%)	45 (96%)
CVMNT	1 (1%)	0 (0%)	1 (2%)
CVINK	3 (6%)	2 (6%)	1 (2%)

[a]Synonymous mutations include P417P, C469C, R471R, S477S, T478T, G496G, Y511Y, R539R, S576S,
[b]Non-synonymous mutation, S477Y
[c]*pfmdr1* haplotype constructed according to amino acids at positions 86, 184, and 1246; mixed infections included in numerator for each haplotype
[d]*pfcrt* haplotype constructed according to amino acids at positions 72, 73, 74, 75, and 76; mixed infections included in numerator for each haplotype
[e]all samples were wildtype for positions 72 (C) and 73 (D)

Discussion

This therapeutic efficacy study assessed three ACTs and molecular markers of antimalarial resistance in five sites representing different epidemiologic zones of DRC. Uncorrected efficacy estimates ranged from 63% to 88% for AL, 73% to 100% for ASAQ (at 28 days), and 56% to 91% for DP (at 42 days). PCR-corrected efficacy estimates ranged from 86% to 98% for AL, 91% to 100% for ASAQ and 84% to 100% for DP at the aforementioned endpoints. No *pfk13* mutations previously found to be associated with ACT resistance were observed. There were statistically significant associations between certain *pfmdr1* and *pfcrt* genotypes and haplotypes and treatment outcome in the AL and ASAQ arms, respectively.

There was a significant number of recurrent parasitemia in all arms in the present study, yielding low uncorrected efficacy estimates for all drugs (but not in each site). The high number of reinfections, particularly in the AL arm, provide evidence of limited post-treatment prophylaxis resulting in a large proportion of children being reinfected within 4 weeks of an initial infection. Repeated malaria infection among young children can result in severe health consequences[20–22].

There were observed efficacies below 90% in two of 15 arms in the study: in the Mikalayi AL and DP arms. Past studies in DRC have not shown evidence of suboptimal efficacy of these ACTs. In all estimates except for the 42-day per-protocol estimate for the Mikalayi DP arm, the confidence intervals spanned above 90% PCR-corrected efficacy. However, in seven additional arms where the point estimate observed was 90% or above, confidence intervals spanned below the 90% threshold (Kapolowe AL and DP arms, Kimpese AL arm, Rutshuru AL, ASAQ, and DP arms, and Mikalayi ASAQ arm). The precision in PCR-corrected estimates can be lost in areas of high-transmission with high rates of reinfection such as DRC, where up to 34 participants were excluded from the PCR-corrected estimates due to reinfection. This reduction in sample size limits the ability to make conclusions with certainty about ACT efficacy. Additionally, in a study conducted in Angola in 2019, one site, Lunda Sul, which is located across the border not far from Mikalayi showed evidence of AL efficacy <90%[23], raising concerns about the use of this drug for the region. .

The proportionally large number of intermediate values of posterior probability of recrudescence derived from the Bayesian algorithm for interpretation of microsatellite markers molecular correction demonstrates the complexity of the parasite population in this study. Using a molecular correction method that accounts for uncertainty of classifications of recurrent parasitemia, rather than a cutoff approach assigning a number of loci matches to define a recrudescence, is particularly useful in these settings[24].

The lack of observed *pfk13* mutations is consistent with the finding that there were no early treatment failures and a slide positivity rate of almost null in all arms, suggesting that artemisinin derivatives are effective at initial reduction of parasitemia in all arms and all sites in DRC. However, the molecular findings suggest that susceptibility to lumefantrine and amodiaquine may be decreasing in DRC. The findings of this study are consistent with previous evidence of higher risk of treatment failure among those carrying the *pfmdr1* N86 SNP versus the 86Y SNP in the AL arm[10], providing molecular evidence of reduced susceptibility to lumefantrine in the parasite population in DRC. This outcome is consistent with the reduced efficacy of AL found in Mikalayi. In the ASAQ arm, we observed a high risk of recurrent parasitemia among samples with the 76T SNP (and CVIET haplotype) compared with K76 (and CVMNK haplotype), findings congruent with the reduced ASAQ efficacy observed in Rutshuru and previous studies from other countries [10,25,26].

Limitations

Samples collected on day 0 for participants classified as ACPR were not available for molecular analysis. Therefore, a proxy, day 0 samples from those who cleared their initial infection but would later experience a reinfection, was used to evaluate associations between treatment outcome and presence of SNPs. This may have introduced classification bias into statistical tests performed as there may have been systematic differences between this group of participants who would go on to be reinfected and those who were not.

Recommendations and conclusions

Therapeutic efficacy monitoring of three drugs in five sites demonstrate evidence of inadequate efficacy of AL and DP in one site each in addition to molecular findings of SNPs and haplotypes associated in

prior studies with reduced susceptibility to lumefantrine and amodiaquine. Further investigation of these findings to rule out other reasons for treatment failure, including measurement of drug levels to investigate potential issues of drug absorption, additional validation of molecular genotyping techniques, and increased sample size to improve precision of efficacy estimates in this setting with high rates of reinfection are warranted. Decreased efficacy of ACTs in DRC, which has the second highest number of *Plasmodium falciparum* malaria infections in the world, could have a strong negative impact on the fight against malaria in sub-Saharan Africa.

Declarations

Funding information

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Availability of data

All data generated or analysed during this study are available from the corresponding author upon reasonable request.

Disclaimer

The findings and conclusions in this paper are those of the authors and do not necessarily represent the views of the U.S. Centers for Disease Control and Prevention.

Conflict of Interest

All co-authors have no conflicts of interest to declare.

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Chapter 5: Dissertation Summary and Future Directions in Research

Summary

The three papers in this dissertation described different factors impacting the epidemiology of diseases of public health importance in Democratic Republic of the Congo (DRC) and Mozambique, two countries in sub-Saharan Africa that are deeply affected by malaria and other tropical diseases. The first paper described the spatial distribution of malaria in Nampula Province, Mozambique. The analyses found evidence that active or recent malaria infection as defined by a positive malaria rapid diagnostic test is highly prevalent and spatially homogenous. This evidence does not support spatial targeting of interventions in high transmission areas below the district level.

In the second paper, patterns of comorbidities for infectious diseases were explored in Nampula, Mozambique. The results of the analysis showed several instances of shared predictors of antibody responses between many infectious diseases of public health importance, with age, sex, and district as the most common predictors. By understanding the epidemiology of several pathogens at once, public health practitioners can target the most common predictors of exposure to diseases in an area making more efficient and effective public health interventions in resource-limited settings such as Mozambique.

The third paper described the results of an antimalarial therapeutic efficacy study investigating three artemisinin-based combination therapies (ACTs) recommended for the treatment of uncomplicated malaria in DRC. The study found evidence of efficacy below the 90% cutoff recommended by WHO to consider a change in first-line treatment recommendations of two ACTs in one site each. If confirmed in a subsequent efficacy study, inadequate efficacy of ACTs in a high-transmission country such as DRC can have substantial impacts on malaria morbidity and mortality. Additionally, the need to re-treat more than 10% of malaria cases due to ineffective malaria treatment will have grave financial impacts on the health system in a country that treats over 18 million cases of malaria per year.

Directions for future research

Both in the results of the analyses and limitations, all three papers demonstrated that high quality and complete data are needed to plan, track, and evaluate interventions targeting malaria and other tropical diseases. Paper 1 showed that that there are spatial analysis methods available to describe spatial epidemiology at a very local level. However, household surveys are not always designed with these types of analyses in mind. As countries continue to drive down malaria transmission in high burden areas, more granular data collection will inform interventions that will target reservoirs of transmission. Paper 2 demonstrated that multiplex platforms can efficiently provide information about prevalence of antibody responses for several pathogens. Socioeconomic, behavioral, and health access data can complement biomarkers and allow for further inference about interventions that may target multiple diseases responsible for morbidity and mortality at once. Indicators with implications for all diseases to be analyzed in a multiplex platform such as coverage of interventions such as vaccines or mass drug administration should be considered in future household surveys. Paper 3 demonstrated that clinical and molecular data can be analyzed together to paint a complete picture of drug efficacy that can impact effective malaria case management, one of the most impactful interventions needed to decrease disease burden in high-transmission settings. Consideration of transmission setting and results of previous efficacy studies should be included in future study planning and may have implications for sample size, molecular correction techniques, and additions of other assays such as drug levels.

Collectively, the three studies in this dissertation describe factors that have implications for intervention planning and disease surveillance in areas with high malaria and other tropical disease burden. Careful consideration of transmission setting can support more efficient and higher quality data collection and may allow for intervention design tailored to the local realities that can target multiple diseases of public health importance.

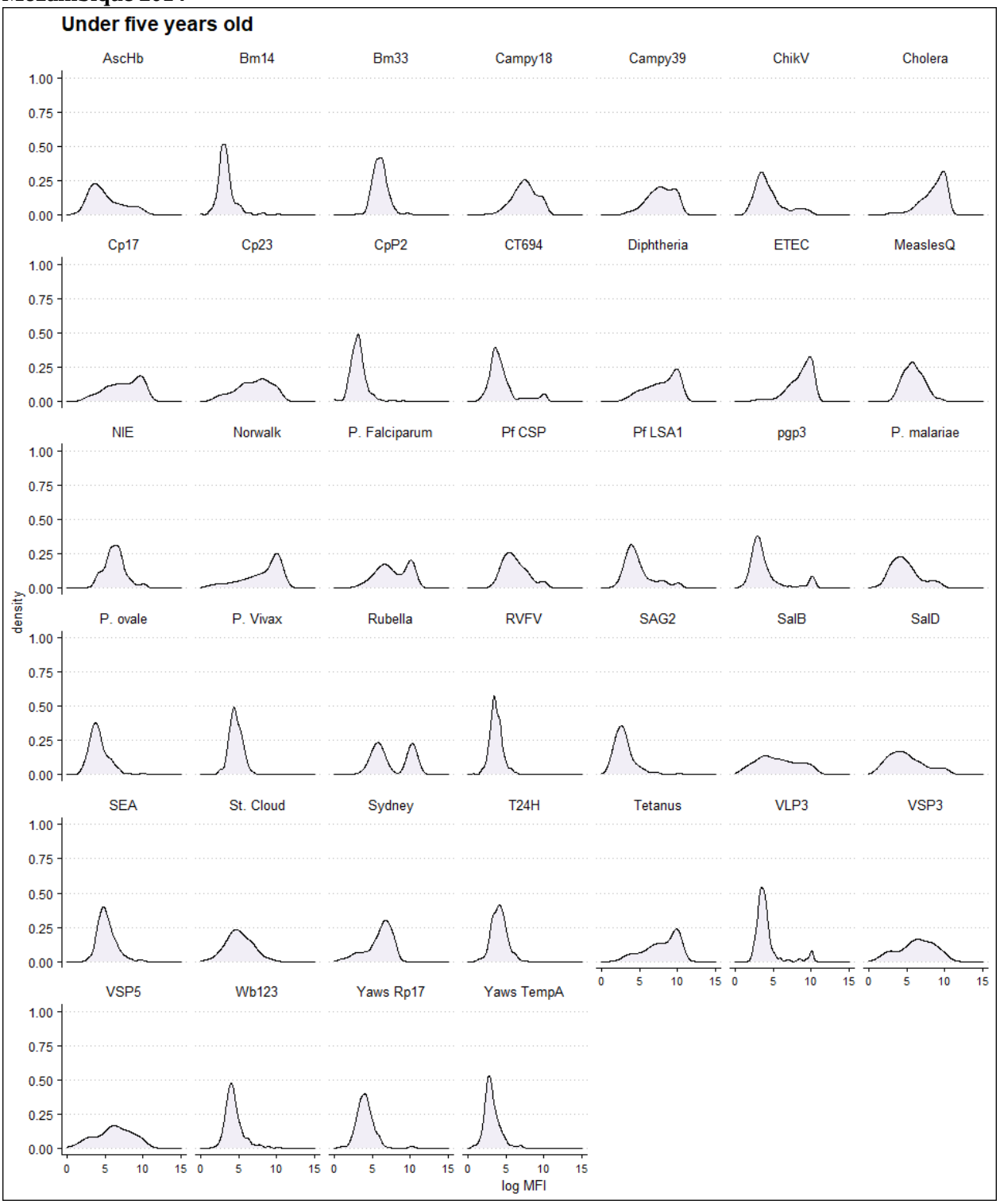
Appendix: Supplemental Tables & Figures

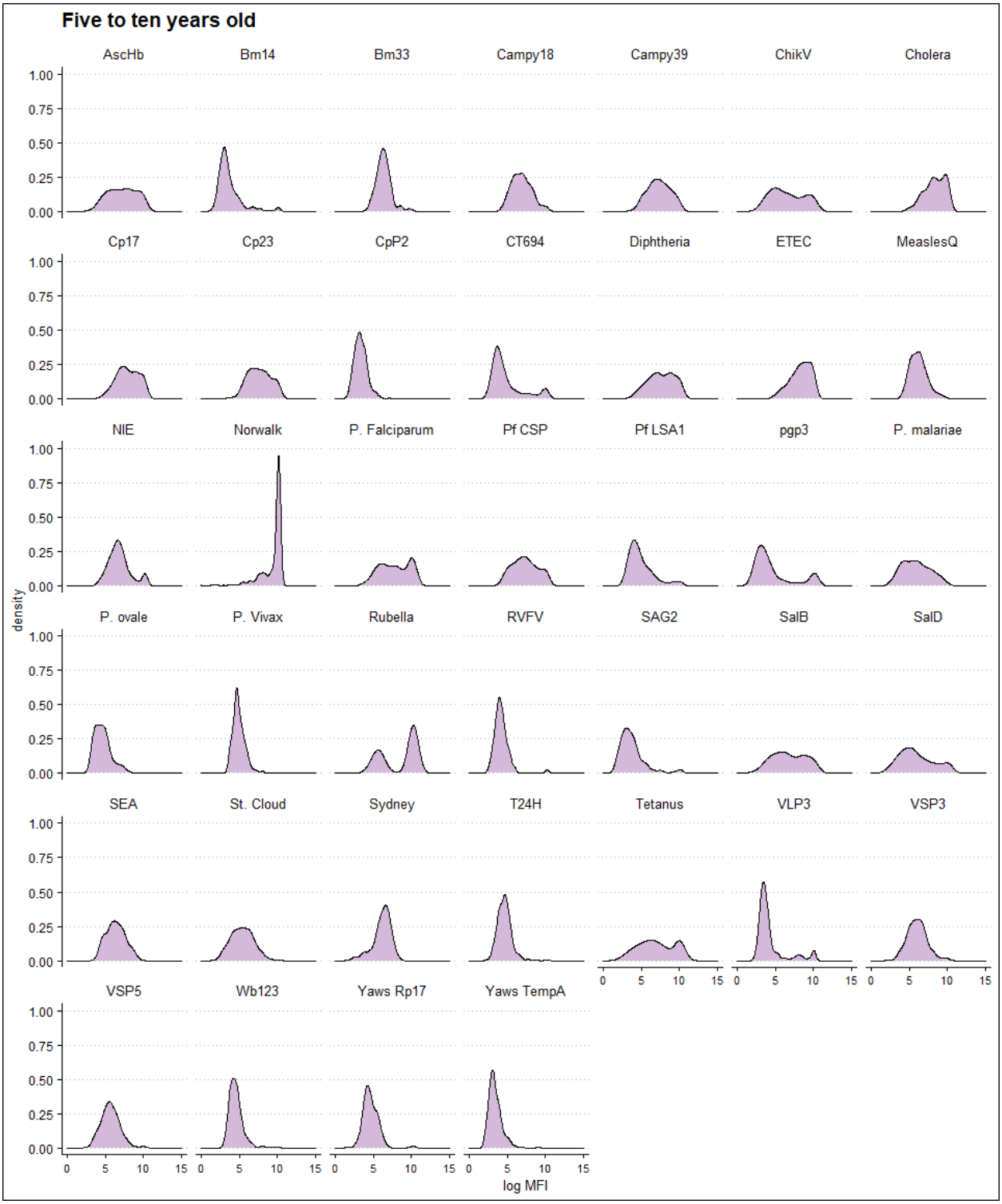
Supplemental Table 2.1: Protein amounts & buffer conditions used for the analysis of 39 antigens plus one control

Antigen	mg Protein/ml beads	Buffer	pH	Fusion	Source	Reference
RVFV N Protein	17	MES/NaCl	5	GST	S. Nichol, CDC, USA	unpublished
Norovirus strain Norwalk	30	PBS	7.2	None	Jan Vijne, CDC, USA	unpublished
Norovirus strain Sydney	30	PBS	7.2	None	Jan Vijne, CDC, USA	unpublished
Norovirus strain St. Cloud	30	PBS	7.2	None	Jan Vijne, CDC, USA	unpublished
Dengue VLP3	30	PBS	7.2	None	J. Chang, CDC Ft. Collins, USA	Poirier et al., 2016, Bull WHO, 94:817-825
CHIKV E protein (wt and mutant)	7.5 each	PBS	7.2	None	CTK Biotech, San Diego, CA	Poirier et al., 2016, Bull WHO, 94:817-825
P. vivax MSP1/19	20	MES/NaCl	5	GST		Plucinski et al., 2018, PLOS NTDs, e0006278
P. falciparum MSP1/19	30	MES/NaCl	5	GST		Plucinski et al., 2018, PLOS NTDs, e0006278
P. malariae MSP1/19	30	MES/NaCl	5	GST		Plucinski et al., 2018, PLOS NTDs, e0006278
P. ovale MSP1/19	30	MES/NaCl	5	GST		Plucinski et al., 2018, PLOS NTDs, e0006278
csp (P. falciparum)	30	MES/NaCl	5	None		Plucinski et al., 2018, PLOS NTDs, e0006278
Wb123	120	PBS	7.2	GST	T. Nutman, NIH, USA	Plucinski et al., 2018, PLOS NTDs, e0006278
Bm14	120	PBS	7.2	GST		Plucinski et al., 2018, PLOS NTDs, e0006278
Bm33NS	20	MES/NaCl + 2 M urea	6	GST/HIS		Plucinski et al., 2018, PLOS NTDs, e0006278
Tetanus	12.5	MES/NaCl	5	None	Mass. Biological Laboratories, Boston, MA	Scobie et al., 2016, CVI, 23:546-554
MeaslesQ	6	MES/NaCl	5	None	Meridian Life Sciences, Memphis, TN	Njenga et al., 2020, AJTMH, 102:164-176
Rubella	30	MES/NaCl	5	None	Meridian Life Sciences, Memphis, TN	Feldstein et al., 2020, PLOS Med, 17:e1003071
Diphtheria	60	MES/NaCl	5	None	List Biological Laboratories, Campbell, CA	Njenga et al., 2020, AJTMH, 102:164-176
Cysticercosis T24H	120	MES/NaCl	5	GST		Priest et al., 2016, PLOS NTDs, e0004699
Toxoplasma SAG2	12.5	MES/NaCl	5	GST		Priest et al., 2015, Epidemiol Infect, 143:618-630
Giardia VSP3s	30	MES/NaCl	5	GST		Priest et al., 2010, CVI, 17:1695-1707
Giardia VSP5	30	MES/NaCl	5	GST		Priest et al., 2010, CVI, 17:1695-1707
Salmonella LPS B CHAPS	10	MES/NaCl + CHAPS	5	None	Sigma Chemical Co., St. Louis, MO	Aiemjoy et al., 2020, PLOS NTDs, 14:e0008647
Salmonella LPS D CHAPS	10	MES/NaCl + CHAPS	5	None	Sigma Chemical Co., St. Louis, MO	Aiemjoy et al., 2020, PLOS NTDs, 14:e0008647
S. mansoni SEA	120	PBS	7.2	None	E. Secor, CDC, USA	Won et al.,2017, AJTMH, 96:1460-1467
Strongyloides NIE	20	PBS + 2M Urea	7.2	GST		Plucinski et al., 2018, PLOS NTDs, e0006278
Ascaris Hb	120	PBS	7.2	None	P. Geldhof, Ghent Univ., Belgium	Njenga et al., 2020, AJTMH, 102:164-176

Cp23	12.5	MES/NaCl	5	GST		Priest and Moss, 2020, Meth. Mol. Biol. 2052: 61-85
Cp17	6.8	MES/NaCl	5	GST		Priest and Moss, 2020, Meth. Mol. Biol. 2052: 61-85
CpP2(100) peptide	30	MES/NaCl	5	None		Benitez et al., 2011, Vaccine, 29:9239-9245
ETEC labile TX Beta subunit	30	MES/NaCl	5	None	Sigma Chemical Co., St. Louis, MO	Arnold et al., 2017, PLOS NTDs, 11:e0005616
Campy p18	25	MES/NaCl	5	GST		Zambrano et al., 2017, AJTMH, 97:876-887
Campy p39	25	MES/NaCl	5	GST		Zambrano et al., 2017, AJTMH, 97:876-887
Trachoma pgp3	120	PBS	7.2	GST	D. Martin, CDC, USA	Goodhew et al., 2012, PLOS NTDs, 6:e1873
Trachoma CT694	30	PBS	7.2	GST	D. Martin, CDC, USA	Goodhew et al., 2012, PLOS NTDs, 6:e1873
Yaws rp17	15	MES/NaCl	5	GST	D. Martin, CDC, USA	Cooley et al., 2016, J Clin Microbiol, 54:1321-1325
Yaws TmpA	15	PBS	7.2	GST	D. Martin, CDC, USA	Cooley et al., 2016, J Clin Microbiol, 54:1321-1325
GST (Control)	15	MES/NaCl	5	GST		Priest and Moss, 2020, Meth. Mol. Biol. 2052: 61-85

Supplemental Figures 2.1 – 2.7: Density plots by age group of log-MFI values by antigen, Nampula, Mozambique 2014

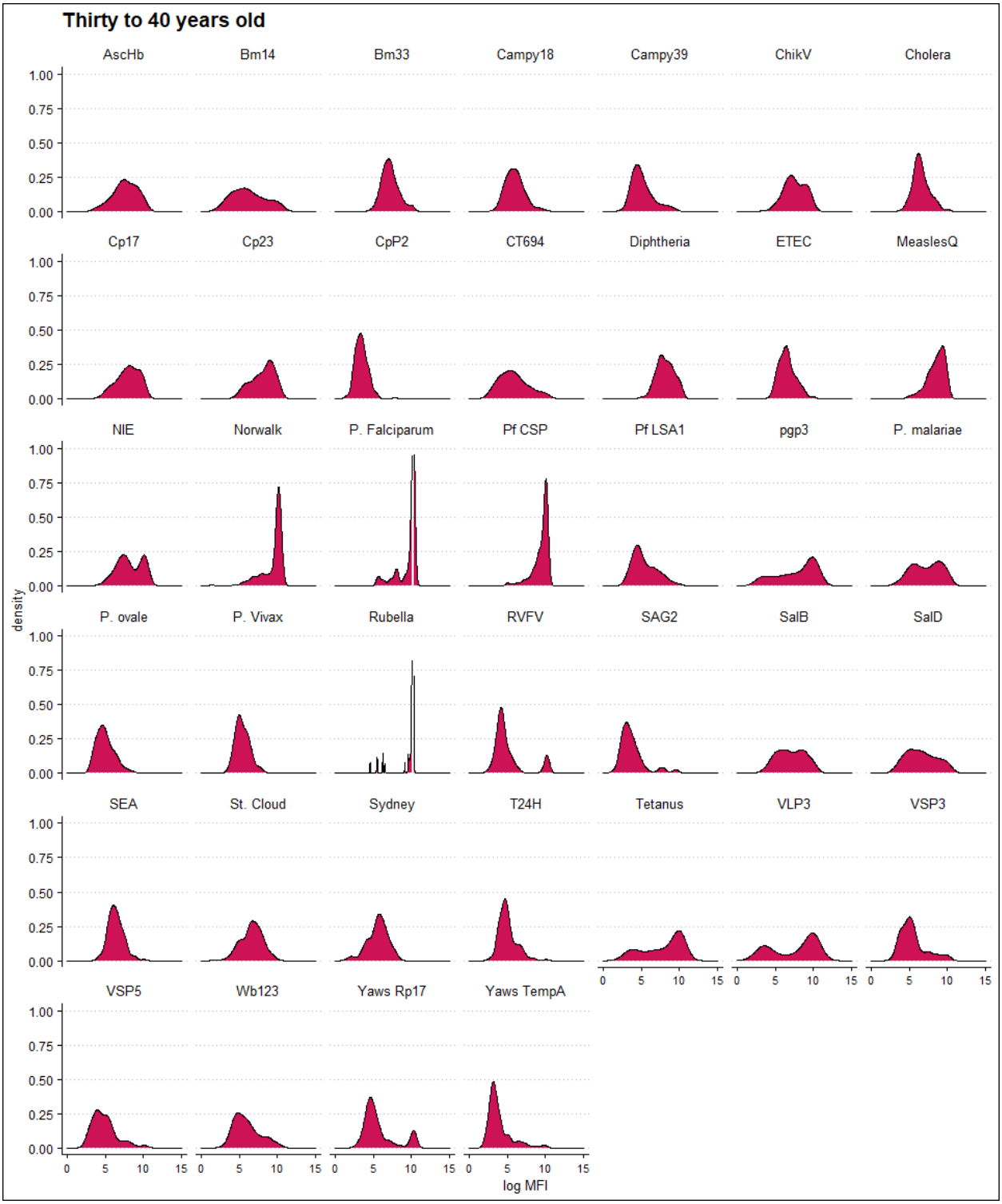


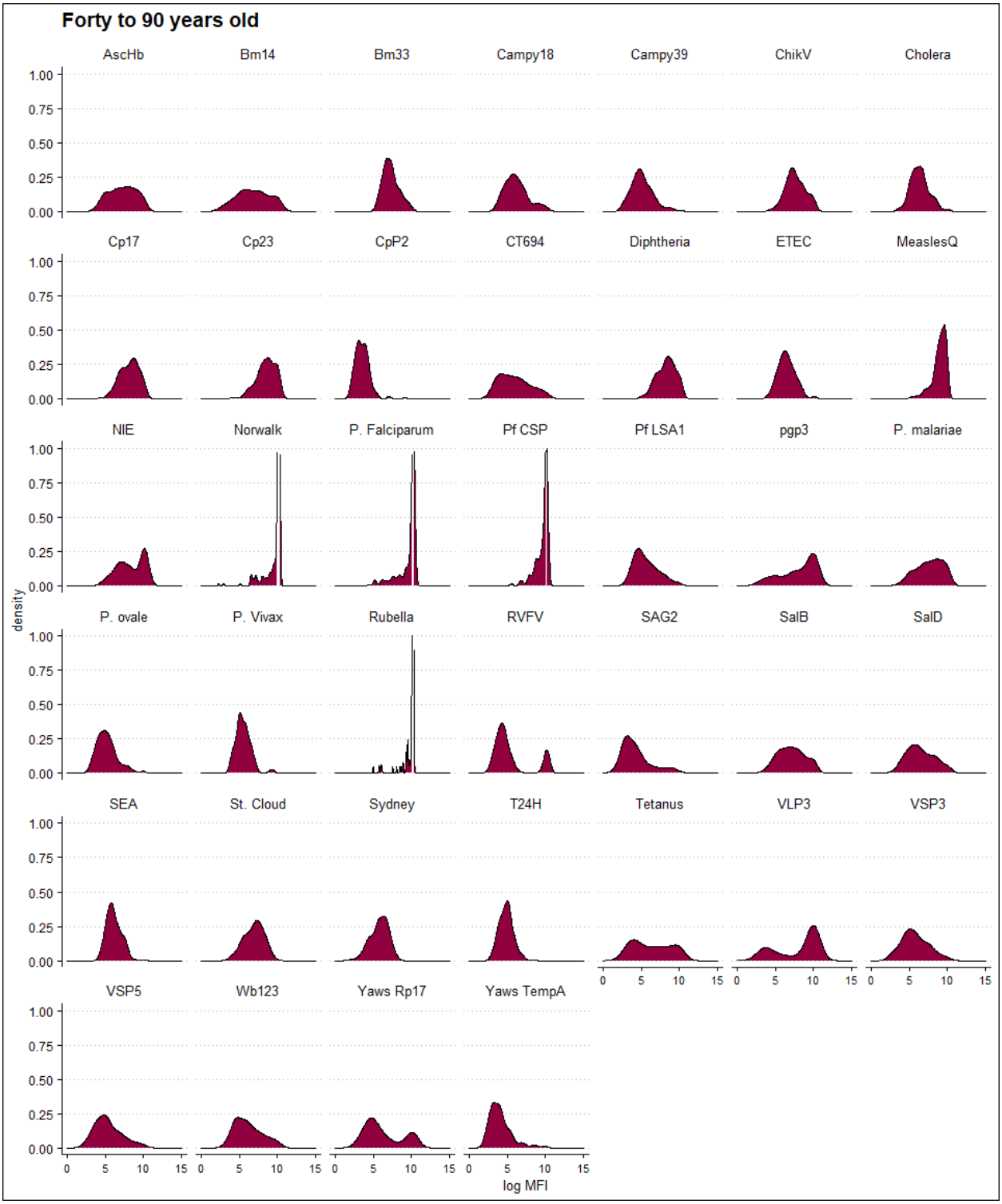

Five to ten years old
AscHb
Bm14
Bm33
Campy18
Campy39
ChikV
Cholera
Cp17
Cp23
CpP2
CT694
Diphtheria
ETEC
MeaslesQ
NIE
Norwalk
P. Falciparum
Pf CSP
Pf LSA1
pgp3
P. malariae
P. ovale
P. Vivax
Rubella
RVFV
SAG2
SalB
SalD
SEA
St. Cloud
Sydney
T24H
Tetanus
VLP3
VSP3
VSP5
Wb123
Yaws Rp17
Yaws TempA
density
1.00
0.75
0.50
0.25
0.00
0
5
10
15
log MFI


Ten to 14 years old
AscHb
Bm14
Bm33
Campy18
Campy39
ChikV
Cholera
Cp17
Cp23
CpP2
CT694
Diphtheria
ETEC
MeaslesQ
NIE
Norwalk
P. Falciparum
Pf CSP
Pf LSA1
pgp3
P. malariae
P. ovale
P. Vivax
Rubella
RVFV
SAG2
SalB
SalD
SEA
St. Cloud
Sydney
T24H
Tetanus
VLP3
VSP3
VSP5
Wb123
Yaws Rp17
Yaws TempA
density
log MFI
0.00
0.25
0.50
0.75
1.00
0
5
10
15


Fourteen to 20 years old
AscHb
Bm14
Bm33
Campy18
Campy39
ChikV
Cholera
Cp17
Cp23
CpP2
CT694
Diphtheria
ETEC
MeaslesQ
NIE
Norwalk
P. Falciparum
Pf CSP
Pf LSA1
pgp3
P. malariae
P. ovale
P. Vivax
Rubella
RVFV
SAG2
SalB
SalD
SEA
St. Cloud
Sydney
T24H
Tetanus
VLP3
VSP3
VSP5
Wb123
Yaws Rp17
Yaws TempA
density
log MFI
0.00
0.25
0.50
0.75
1.00
0
5
10
15


Twenty to 30 years old
AscHb
Bm14
Bm33
Campy18
Campy39
ChikV
Cholera
Cp17
Cp23
CpP2
CT694
Diphtheria
ETEC
MeaslesQ
NIE
Norwalk
P. Falciparum
Pf CSP
Pf LSA1
pgp3
P. malariae
P. ovale
P. Vivax
Rubella
RVFV
SAG2
SalB
SalD
SEA
St. Cloud
Sydney
T24H
Tetanus
VLP3
VSP3
VSP5
Wb123
Yaws Rp17
Yaws TempA
density
log MFI
0.00
0.25
0.50
0.75
1.00
0
5
10
15


Thirty to 40 years old
AscHb
Bm14
Bm33
Campy18
Campy39
ChikV
Cholera
Cp17
Cp23
CpP2
CT694
Diphtheria
ETEC
MeaslesQ
NIE
Norwalk
P. Falciparum
Pf CSP
Pf LSA1
pgp3
P. malariae
P. ovale
P. Vivax
Rubella
RVFV
SAG2
SalB
SalD
SEA
St. Cloud
Sydney
T24H
Tetanus
VLP3
VSP3
VSP5
Wb123
Yaws Rp17
Yaws TempA
density
log MFI
1.00
0.75
0.50
0.25
0.00
0
5
10
15


Forty to 90 years old
AscHb
Bm14
Bm33
Campy18
Campy39
ChikV
Cholera
Cp17
Cp23
CpP2
CT694
Diphtheria
ETEC
MeaslesQ
NIE
Norwalk
P. Falciparum
Pf CSP
Pf LSA1
pgp3
P. malariae
P. ovale
P. Vivax
Rubella
RVFV
SAG2
SalB
SalD
SEA
St. Cloud
Sydney
T24H
Tetanus
VLP3
VSP3
VSP5
Wb123
Yaws Rp17
Yaws TempA
density
log MFI
0.00
0.25
0.50
0.75
1.00
0
5
10
15

Supplemental Table 3.1: Molecular markers of resistance and association with treatment outcome, artemether lumefantrine arms, DRC therapeutic efficacy study, 2017

	All samples	Reinfection Day 0	Recrudescence + Reinfection Day of recurrence	p-value
pfk13				
Successfully sequenced	87/90 (97%)	42/43 (98%)	45/47 (96%)	-
Wild type	85 (98%)	41 (98%)	43 (96%)	Ref
Synonymous[a]	3 (3%)	1 (2%)	2 (4%)	1
Non-synonymous	0 (0%)	0 (0%)	0 (0%)	NA
pfmdr1[a]				
Successfully sequenced	81/85 (95%)	36/38 (95%)	45/47 (96%)	-
N86	15 (19%)	9 (25%)	6 (13%)	Ref
86N/Y	13 (16%)	6 (17%)	7 (16%)	0.725
86Y	53 (65%)	21 (58%)	32 (71%)	0.268
Y184	49 (60%)	22 (61%)	27 (60%)	Ref
184Y/F	14 (17%)	6 (17%)	8 (18%)	1
184F	18 (22%)	8 (22%)	10 (22%)	1
D1246	51 (63%)	22 (61%)	29 (64%)	Ref
1246D/Y	12 (15%)	5 (14%)	7 (16%)	1
1246Y	18 (22%)	9 (25%)	9 (20%)	0.817
NYD	17 (21%)	9 (25%)	8 (18%)	Ref
NFD	18 (22%)	8 (22%)	10 (22%)	0.870
NFY	4 (5%)	1 (3%)	3 (7%)	0.662
NYY	5 (6%)	3 (8%)	2 (4%)	1
YFD	69 (85%)	27 (75%)	42 (93%)	0.446
YFY	5 (6%)	1 (3%)	4 (9%)	0.436
YYD	40 (49%)	17 (47%)	23 (51%)	0.663
YYY	26 (32%)	13 (36%)	13 (29%)	1
pfcrt[b, c]				
Successfully sequenced	85/85 (100%)	38/38 (100%)	47/47 (100%)	-
M74	8 (9%)	6 (16%)	2 (4%)	Ref
74M/I	2 (2%)	2 (5%)	0 (0%)	1
74I	75 (88%)	30 (79%)	45 (96%)	0.128
N75	10 (12%)	8 (21%)	2 (4%)	Ref

75N/E	1 (1%)	0 (0%)	1 (2%)	0.546
75E	74 (87%)	30 (79%)	44 (94%)	**0.042**
K76	9 (11%)	8 (21%)	1 (2%)	Ref
76K/T	1 (1%)	0 (0%)	1 (2%)	0.4
76T	75 (88%)	30 (79%)	45 (96%)	**0.013**
CVMNK	9 (11%)	8 (21%)	1 (2%)	Ref
CVIET	75 (88%)	30 (79%)	45 (96%)	**0.013**
CVMNT	1 (1%)	0 (0%)	1 (2%)	0.400
CVINK	3 (6%)	2 (6%)	1 (2%)	0.909

[a]*pfmdr1* haplotype constructed according to amino acids at positions 86, 184, and 1246; mixed infections included in numerator for each haplotype
[b]*pfcrt* haplotype constructed according to amino acids at positions 72, 73, 74, 75, and 76; mixed infections included in numerator for each haplotype
[c]All samples were wildtype for positions 72 (C) and 73 (V)

Supplemental Table 3.2: Molecular markers of resistance and association with treatment outcome, artesunate-amodiaquine arms, Democratic Republic of the Congo therapeutic efficacy study, 2017

	All samples	Reinfection Day 0	Recrudescence + Reinfection Day of recurrence	p-value
Pfk13				
Successfully sequenced	87/90 (97%)	42/43 (98%)	45/47 (96%)	-
Wild type	85 (98%)	41 (98%)	43 (96%)	Ref
Synonymous[a]	3 (3%)	1 (2%)	2 (4%)	1
Non-synonymous	0 (0%)	0 (0%)	0 (0%)	NA
Pfmdr1[b]				
Successfully sequenced	81/85 (95%)	36/38 (95%)	45/47 (96%)	-
N86	15 (19%)	9 (25%)	6 (13%)	Ref
86N/Y	13 (16%)	6 (17%)	7 (16%)	0.725
86Y	53 (65%)	21 (58%)	32 (71%)	0.268
Y184	49 (60%)	22 (61%)	27 (60%)	Ref
184Y/F	14 (17%)	6 (17%)	8 (18%)	1
184F	18 (22%)	8 (22%)	10 (22%)	1
D1246	51 (63%)	22 (61%)	29 (64%)	Ref
1246D/Y	12 (15%)	5 (14%)	7 (16%)	1
1246Y	18 (22%)	9 (25%)	9 (20%)	0.817
NYD	17 (21%)	9 (25%)	8 (18%)	Ref
NFD	18 (22%)	8 (22%)	10 (22%)	0.870
NFY	4 (5%)	1 (3%)	3 (7%)	0.662
NYY	5 (6%)	3 (8%)	2 (4%)	1
YFD	69 (85%)	27 (75%)	42 (93%)	0.446
YFY	5 (6%)	1 (3%)	4 (9%)	0.436
YYD	40 (49%)	17 (47%)	23 (51%)	0.663
YYY	26 (32%)	13 (36%)	13 (29%)	1
Pfcrt[c, d]				
Successfully sequenced	85/85 (100%)	38/38 (100%)	47/47 (100%)	-
M74	8 (9%)	6 (16%)	2 (4%)	Ref
74M/I	2 (2%)	2 (5%)	0 (0%)	1
74I	75 (88%)	30 (79%)	45 (96%)	0.128
N75	10 (12%)	8 (21%)	2 (4%)	Ref

75N/E	1 (1%)	0 (0%)	1 (2%)	0.546
75E	74 (87%)	30 (79%)	44 (94%)	**0.042**
K76	9 (11%)	8 (21%)	1 (2%)	Ref
76K/T	1 (1%)	0 (0%)	1 (2%)	0.4
76T	75 (88%)	30 (79%)	45 (96%)	**0.013**
CVMNK	9 (11%)	8 (21%)	1 (2%)	Ref
CVIET	75 (88%)	30 (79%)	45 (96%)	**0.013**
CVMNT	1 (1%)	0 (0%)	1 (2%)	0.400
CVINK	3 (6%)	2 (6%)	1 (2%)	0.909

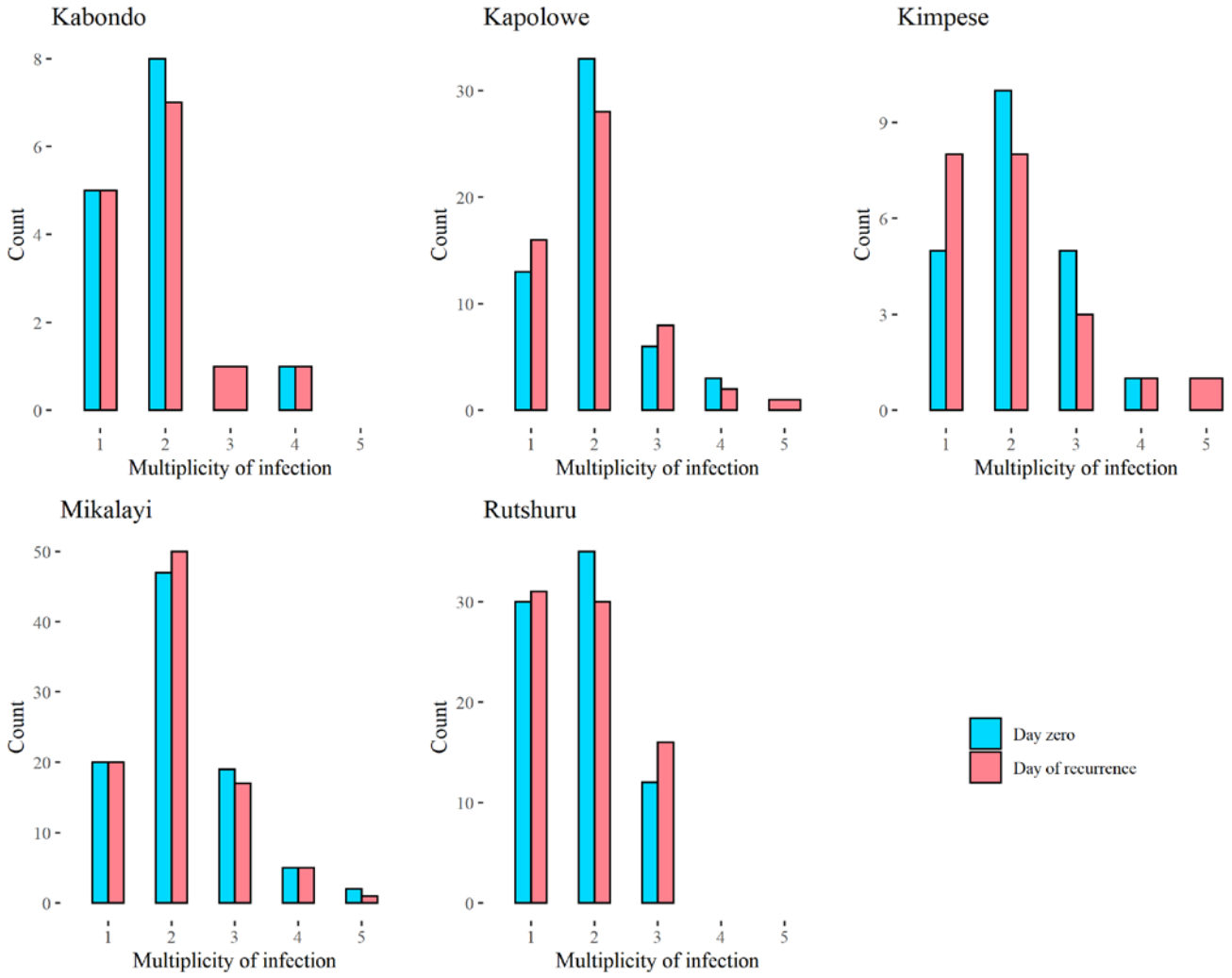
[a]Synonymous mutations include P417P, C469C, R471R, S477S, T478T, G496G, Y511Y, R539R, S576S,
[b]*pfmdr1* haplotype constructed according to amino acids at positions 86, 184, and 1246; mixed infections included in numerator for each haplotype
[c]*pfcrt* haplotype constructed according to amino acids at positions 72, 73, 74, 75, and 76; mixed infections included in numerator for each haplotype
[d]all samples were wildtype for positions 72 (C) and 73 (D)

Supplemental Table 3.3: Molecular markers of resistance and association with treatment outcome, dihydroartemisinin-piperaquine arms, Democratic Republic of the Congo therapeutic efficacy study, 2017

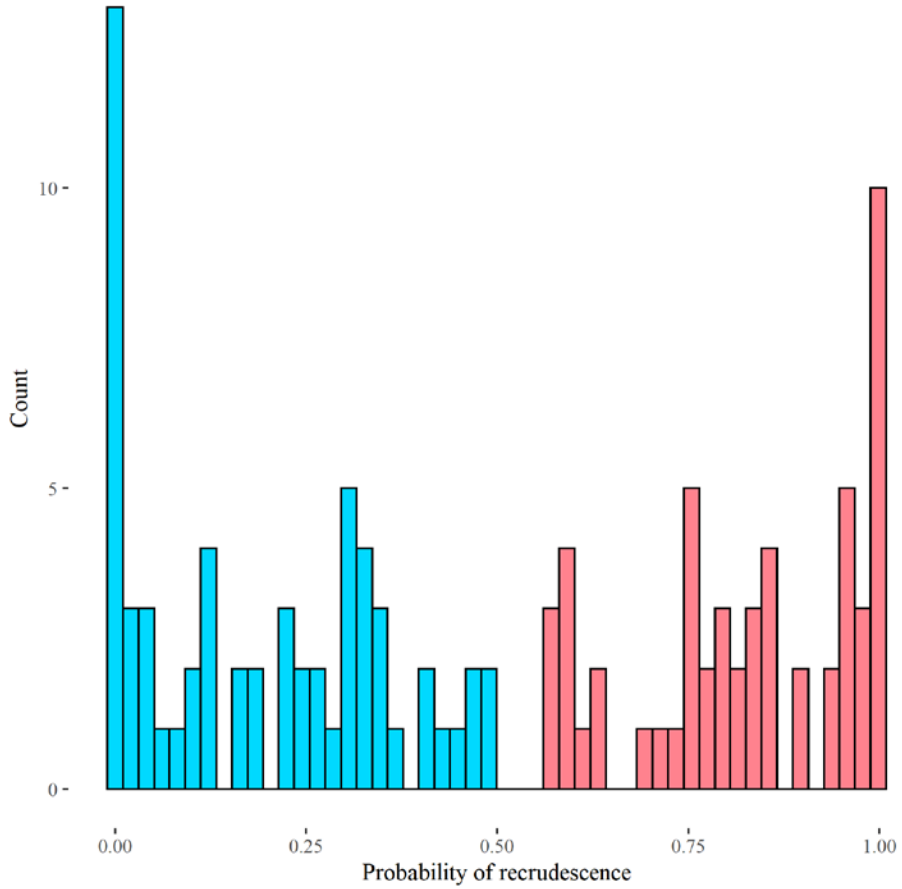
	All samples	Reinfection Day 0	Recrudescence + Reinfection Day of recurrence	*P*
pfk13				
Successfully sequenced	208/224 (93%)	99/102 (97%)	109/122 (89%)	-
Wild type	199 (96%)	96 (97%)	103 (95%)	Ref
Synonymous	8 (4%)	0 (0%)	5 (5%)	0.796
Non-synonymous	1 (0.5%)	0 (0%)	1 (0.9%)	1
pfmdr1[a]				
Successfully sequenced	223/223 (100%)	101/101 (100%)	122/122(100%)	-
N86	127 (57%)	57 (56%)	70 (57%)	Ref
86N/Y	33 (15%)	16 (16%)	17 (14%)	0.859
86Y	63 (28%)	28 (28%)	35 (29%)	1
Y184	113 (51%)	58 (57%)	55 (45%)	Ref
184Y/F	60 (27%)	22 (22%)	38 (31%)	0.092
184F	50 (22%)	21 (21%)	29 (24%)	0.353
D1246	188 (84%)	84 (83%)	104 (85%)	Ref
1246D/Y	7 (3%)	2 (2%)	5 (4%)	0.662
1246Y	28 (13%)	15 (15%)	13 (11%)	0.497
NYD	118 (53%)	53 (52%)	65 (53%)	Ref
NFD	81 (36%)	35 (35%)	46 (38%)	0.927
NFY	11 (5%)	4 (4%)	7 (6%)	0.828
NYY	11 (5%)	5 (5%)	6 (5%)	1
YFD	71 (32%)	34 (34%)	37 (30%)	0.805
YFY	9 (4%)	3 (3%)	6 (5%)	0.755
YYD	59 (26%)	25 (25%)	34 (28%)	0.874
YYY	28 (13%)	15 (15%)	13 (11%)	0.538

[a]*pfmdr1* haplotype constructed according to amino acids at positions 86, 184, and 1246; mixed infections included in numerator for each haplotype

Supplemental Figure 3.1: Multiplicity of infection of day 0 and day of recurrence samples from treatment failures as determined by the maximum number of alleles detected among seven neutral microsatellite markers, Democratic Republic of the Congo therapeutic efficacy study, 2017

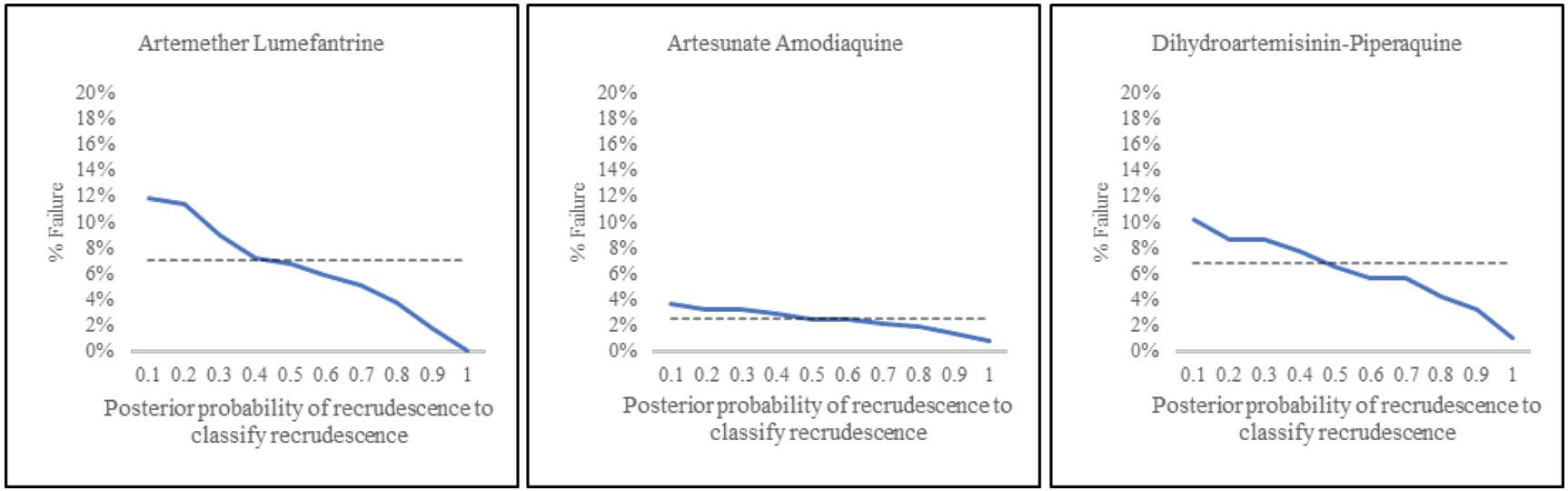


Supplemental Figure 3.2: Histogram of posterior probability of recrudescence of all late recurrences for which the probability of recrudescence is over 0% (n=114)



The sum posterior probability of recrudescence was 57. There were 54 samples with a posterior probability of recrudescence of .5 or higher (and labeled as a recrudescence). The mean posterior probability of recrudescence was 0.04 (standard deviation= 0.17). Among late failures for which the posterior probability of recrudescence was >0 (n=114, 42.5%), the mean posterior probability of recrudescence was 0.49 (standard deviation= 0.37)

Supplemental Figure 3.3: Assessment of the use of different cutoffs of posterior probability of recrudescence derived using a Bayesian algorithm for interpreting microsatellite data for molecular correction, Democratic Republic of the Congo therapeutic efficacy study, 2017



Failure rate estimates obtained using the Bayesian analysis algorithm for artemether lumefantrine, artesunate amodiaquine, and dihydroartemisinin piperaquine. The failure rate derived by summing the posterior probability of recrudescence for each arm is denoted in each plot by a horizontal gray line. The cutoff for posterior probability at which a recurrence was classified as a recrudescence varied between ≥0.1 and 1.