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Elucidating barriers to malaria elimination: An analysis of the role of low-density and imported *Plasmodium falciparum* infections to onward malaria transmission

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Abstract

Elucidating barriers to malaria elimination: An analysis of the role of low-density and imported *Plasmodium falciparum* infections to onward malaria transmission

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Despite yearly investments of more than US $4.1 billion dollars and extensive efforts, malaria continues to be one of the greatest contributors to morbidity and mortality in endemic areas. To continue to reduce the malaria burden, new control strategies, therapeutics, and vaccines are needed. While the malaria burden has declined since the last decade, the rate of change has plateaued in recent years, calling for new and innovative strategies to continue to drive malaria elimination. To prioritize resource allocation, accurately assess the effectiveness of candidate vaccines and therapeutics, and develop effective control strategies, it is essential to better understand the drivers of *Plasmodium* transmission, identify malaria hotspots, and develop new methodologies that allow for a more in-depth understanding of the true burden of disease.

There are several threats to malaria elimination efforts. Among these include a large reservoir of low-density *Plasmodium* infections in endemic areas and importation of cases. Low-density infections are highly prevalent in endemic areas and can contribute to onward transmission. However, since at low densities, they do not cause immediate clinical illness and are below the limit of detection (LoD) of standard field diagnostics, there is a paucity of data on their natural history of infection, which limits our understanding of the necessity of targeting these types of infections. Imported *Plasmodium* infections, which can be further transmitted, and introduce new strains or lines of resistance, are of rising concern, especially with increasing human mobility. This dissertation utilizes advanced epidemiologic methods and novel laboratory techniques to study low-density and imported *Plasmodium* infections.

In the first analysis, household clustering of subpatent infections around rapid diagnostic test (RDT) positive infections on Bioko Island, Equatorial Guinea was analyzed. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) for *Plasmodium* 18S rRNA was used to identify *Plasmodium falciparum (Pf)* parasite infections that were not detected by RDT (referred to as subpatent infections) on a subset of household samples from the 2015 Malaria Indicator Survey (MIS). The association between living in a household with an RDT(+) individual and having a subpatent infection was evaluated using multivariate hierarchical logistic regression models with inverse probability weights for selection. To evaluate possible modification of the association by potential importation of the RDT(+) case, the analysis was repeated among strata of matched sets based on the reported eight-week travel history of the RDT(+) individual(s). The adjusted prevalence odds of subpatent infection were 2.59-fold greater (95% CI: 1.31, 5.09) for those in a household with an RDT(+) individual compared to individuals in a household without RDT(+) individuals. When stratifying by travel history of the RDT(+) individual, the association

between subpatent infections and RDT(+) infections was stronger in the strata in which the RDT(+) individual(s) had not recently travelled (adjusted Prevalence Odds Ratio (aPOR) 2.95; 95% CI:1.17, 7.41), and attenuated in the strata in which recent travel was reported (aPOR 1.76; 95% CI: 0.54, 5.67).

In the second analysis, data collected before and after a travel moratorium imposed in response to COVID-19 was leveraged to estimate the impact of imported *Plasmodium* infections on prevalence in areas of historically high travel prevalence on Bioko Island. A difference in differences approach was used to estimate the change in odds of *Pf* infection for individuals living in historically high travel areas following the travel moratorium relative to those living in historically low travel areas. A survey generalized linear model with robust standard errors and a logit link function was fit with an interaction term between travel area and year to estimate the impact of the travel moratorium on risk of *Plasmodium* infection. Comparing the change from 2019 to 2020 in high travel areas to low travel areas, the adjusted odds of infection were 39% lower in high travel areas (aOR: 0.61; 95% CI: 0.43, 0.88) than would have been expected in the absence of a travel moratorium.

Finally, in a third study, the feasibility of using daily at-home dried blood spot (DBS) collection as a method to study the natural history of asymptomatic, low-density infections was assessed in a small pilot study in Katakwi district, Uganda. One hundred and two adults and 29 children who were RDT-negative and asymptomatic for malaria at screening were invited to self-collect DBS daily for 28 days. Venous blood samples and clinic-collected DBS were taken at enrollment and at four weekly clinic visits as well. Participant opinions about the DBS collection process were solicited through daily Diary Cards and a 5-point Likert scale survey on acceptability administered at the final clinic visit. The DBS and venous blood were analyzed by qRT-PCR. The

number of participants completing the study, the total number of DBS collected, and the opinions of the process and any reported pain were analyzed to determine compliance and acceptability of the study procedure. The human internal control mRNA and *Plasmodium* 18S rRNA were evaluated for the at-home versus clinic-collected DBS and venous blood to assess quality of the at-home collected samples and evaluate the accuracy of DBS as a parasite detection tool. At-home DBS collection was found to be a feasible approach to studying low-density infections. Almost all participants (92%) completed the study, and only three individuals withdrew due to pain or inconvenience of the study procedure. Overall, 97% of participants collected ≥ 16 of 24 home DBS, and 87% of all spots had ≥ 40 µL of blood. The procedure was well tolerated and viewed favorably by participants. At-home collected DBS were acceptable for qRT-PCR and showed only slightly lower concentrations of human control mRNA compared to clinic-collected DBS (human internal control TBP mRNA cycle threshold 0.8 cycles earlier for clinic-collected DBS; 95%CI: 0.65, 0.98), though this latter difference was not clinically impactful. Correlation between *Pf* 18S rRNA from paired whole blood and DBS samples was high (R=0.93)

Conclusions: The results of these studies provide new evidence on the contribution and characteristics of low-density and imported *Plasmodium* carriage to malaria transmission. In addition, a new method for studying *Plasmodium* infections over time that is particularly well suited for the study of low-density infections has been validated. At-home DBS collection should be widely implemented for epidemiological surveillance, clinical trial baseline surveys and parasite monitoring, as well as for in-depth analyses of parasite dynamics, immune responses, and genetics. The increased understanding of low-density infections that will come from the utilization of this evaluation strategy will improve control strategies and development of new therapeutics and vaccines to combat malaria.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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Chapter 1. Introduction

Despite increased funding and global efforts, malaria continues to cause illness and death in areas where it is endemic. In 2020, malaria remained endemic in 85 countries and territories, and there were 241 million estimated cases and 627,000 deaths reported, concentrated largely in sub-Saharan Africa (1). As more countries work towards malaria elimination, new control strategies, therapeutics and vaccines are needed.

The complex morphological changes that the parasite undergoes in both the mosquito and human host, the diversity of strains, and continued high burden of infection makes malaria very difficult to combat. There are several threats to the progress towards malaria elimination, including increased drug resistance by some strains, mutation in mosquitoes that leads to insecticide resistance, areas that still lack health infrastructure and access to treatment, increased human mobility, and many asymptomatic, often low-density infections that are difficult to detect but may drive continued parasite transmission (2–4).

To continue to reduce malaria burden and make progress towards elimination, cost-effective, tailored control strategies that drive down transmission are needed. This requires a deeper understanding of what is driving that transmission and how to best target and clear parasite reservoirs in the population. In addition to control strategies, progress continues to be made on new therapeutics and vaccine candidates that could help aid in transmission reduction (5). The efficacy of these products may be largely dependent on the underlying burden of malaria in an area and the infection history of an individual. Evidence is strong to suggest there is a large proportion of *Plasmodium* infections that are asymptomatic and not captured by standard field diagnostics (6). However, little is known about the natural history of these infections because they are difficult and costly to study. Without a better understanding of the trajectories of these infections, it is challenging to make policy decisions on how and whether to target these types of infections.

This dissertation explores the epidemiology of low-density and imported *Plasmodium* infections. **Chapter 2** and **Chapter 3** explore the roles of low-density infections and importation to malaria prevalence on Bioko Island. **Chapter 4** evaluates the feasibility of at-home dried blood spot (DBS) collection as a tool to study low-density *Plasmodium* infections. Key terms used in the literature and throughout this dissertation are defined in **Table 1.1** below.

Table 1.1. Description of key terms used through this dissertation

Term	**Description**
Field diagnostics	Include microscopy and rapid diagnostic tests (RDT)
Rapid diagnostic test (RDT)	Unless specified, refers to traditional RDTs, not ultra-sensitive RDTs
Molecular methods	Any nucleic acid amplification technique, primarily polymerase chain reaction (PCR)
PCR	Molecular methods that amplify DBA targets
Reverse transcription PCR (RT-PCT)	Molecular methods that amplify RNA targets
qPCR & qRT-PCR	Quantitative molecular methods that allow for the estimation of parasite densities based on copy numbers
Low-density infections	Infections with densities below ~100,000 parasites/mL, which is near the LoD of many widely available RDTs
Subpatent infections	Infections that are detected by PCR or RT-PCR, but not detected by field diagnostics. Most, but not all, subpatent infections are low-density.
Parasites/mL	Parasite densities will be expressed as parasites per microliter (mL). In older literature, densities were expressed as parasites per microliter (µL) before more sensitive diagnostics were widely used.

1.1 MALARIA BIOLOGY AND LIFECYCLE

Malaria is caused by the single-cell Apicomplexan *Plasmodium* parasite. In humans, there are at least seven species of *Plasmodium* known to cause illness and the most common and deadly is *Plasmodium falciparum* (*Pf*) (5). *Pf* is transmitted through the bite of an adult female *Anopheles* mosquito. The complex life cycle of the parasite in the human and mosquito, as well as the immune response generated against the parasite, results in inadequate long-term protection against the infection: individuals can be infected with malaria multiple times throughout their lifetime (7).

Human *Pf* infection begins when a female *Anopheles* mosquito deposits sporozoites from their salivary glands into a human host during a blood meal. The sporozoites quickly travel through the blood stream to the liver, where they infect hepatocytes. Over the course of 7 to 10 days, the parasite multiplies and differentiates into 10,000 to 30,000 mature merozoites inside a schizont (8). Upon maturation, the schizont ruptures, releasing the merozoites into the blood stream, where they infect erythrocytes (red blood cells, or RBCs). Inside the cytoplasm of an RBC, the merozoites further mature into early and then late stage trophozoites, undergo schizogony within a parasitophorous vacuole (9) . At the end of the replication cycle, the merozoites rupture out of the RBC to infect additional RBC. Typically, the *Pf* erythrocytic asexual reproduction occurs over a 48-hour period. Each maturation produces between 8-24 merozoites per infected RBC, leading to exponential growth in parasite density over time (8,10). A small proportion (~5-10%) of erythrocytic cycles are programmed for sexual reproduction which leads to the creation of gametocytes (11), a process that has been shown to begin early in the parasite lifecycle, even when asexual parasite densities are low (12). There are five stages of gametocyte development, with each stage lasting about two days. Stages I-IV occur while the infected RBC is sequestered in the bone marrow, and Stage V gametocytes circulate in the peripheral blood, where they can be taken up by a mosquito (11,13). Gametocytes often comprise <5% of the total *Pf* parasite biomass (14), but can vary based on length of infection (15), seasonality (15), age of the individual infected (16), symptoms (17,18), and treatment given (18) with ranges from 1.8% to 18% depending on age of the individual infected; adults tend to have higher proportion of gametocytes compared to children (19).

During the erythrocytic stage, the RBC undergoes remodeling as a result of proteins released by the parasite (5). If these RBCs pass through the spleen, the deformities are recognized, and they are removed. To avoid splenic clearance before the erythrocytic cycle is complete, the parasite releases proteins that modulate the surface of RBCs and allow them to adhere to the walls of endothelial cells (5,10,20). This process of sequestration allows infected cells to complete their replication cycle before being removed by the spleen. Additionally, this process of sequestration results in significant fluctuations in parasite levels in the peripheral blood over the course of infection, which can impact detection of the parasites depending on the day and time that a sample is taken (21,22). In a small study in Mali in which adult males with asymptomatic infections were sampled three times a day, all subjects had at least one negative sample by microscopy during the thirteen day study period, and on average, about one-third of all samples were negative by microscopy (21). A small study that sampled asymptomatic children daily in Tanzania also found that a proportion of all infections over a 14 day period were not detected even when using a higher sensitivity PCR diagnostic method (22).

1.2 IMMUNE RESPONSE AND SYMPTOMATIC AND ASYMPTOMATIC MALARIA

Symptoms associated with clinical malaria occur during the erythrocytic stage of parasite development (7,20,23). When the erythrocytes burst, merozoites are released along with toxic byproducts from the parasite (23). In response to this, there is a strong pro-inflammatory immune response, and heightened production of interferon gamma (IFN-ɣ), interleukin-12 (IL-12), and tumor necrosis factor alpha (TNF-α) (5,8,23). These pro-inflammatory cytokines aid in clearing the parasite from the bloodstream, and are pyrogenic mediators, signaling for fever production. As parasite densities increase and more toxins are released into the bloodstream, the immune response and risk of fever increases (23,24). In malaria-naïve populations, the pyrogenic threshold is variable, but has been estimated to occur between parasite densities of 10,000 to 200,000,000 parasites/mL, dependent on strain, intensity, and host age and sex (25). In endemic regions, the pyrogenic threshold often is not reached until much higher parasite densities (>10,000,000 parasites/mL), and is highly dependent on transmission intensity, multiplicity of infection, circulating strains, age, and sex (5,23,26–28). Fever threshold tends to be associated with higher parasite densities in high transmission areas, and lower densities in areas with less transmission (5,28–32).

The relationship between parasite density and fever threshold also partly explains the high frequency of asymptomatic infections in endemic areas. As individuals are continually exposed to *Plasmodium* parasites, they are less likely to present with clinical symptoms. One reason for this is a shift in the immune response to one that is more anti-inflammatory during subsequent infections. Increases in cytokines such as IL-10, IL-27, and tissue growth factor beta (TGF-β) are more prevalent in individuals who have been exposed to *Plasmodium* parasites multiple times (8,33). Release of these cytokines reduce disease severity, but also suppress parasite clearance (33). In addition to a shift in the immune response, individuals previously exposed to *Plasmodium* develop levels of protective immunity, thought to be mediated through IgG antibodies against surface proteins on the sporozoite and merozoite (5), as well as T cell responses to the liver- and blood-stage infections (7). Together, these responses typically decrease the density of circulating parasites but do not clear them quickly, while simultaneously limiting pyrogenic and other systemic immune responses, which leads to the development of asymptomatic infections. While an anti-inflammatory immune response controls the amount of symptomatic malaria, it also inhibits parasite clearance. As such, asymptomatic infections can exist for long periods of time. A recent modeling study estimated that the duration of infection for untreated asymptomatic infections was between 87 and 536 days, varying by age and sex (34). Asymptomatic infections can be classified into two categories: patent infections - those with parasite densities high enough to be detected by standard field diagnostics, and subpatent infections – those with low-density parasites below the LoD of most field diagnostic tools.

1.3 MALARIA DIAGNOSTIC TOOLS

There are three main malaria diagnostic tools used for clinical diagnosis and research: microscopy, rapid diagnostic tests (RDT), and nucleic acid amplification tests (NAAT). Microscopy was long hailed as the gold standard of malaria diagnosis. A small amount of blood, usually taken from finger prick, is spread on a glass slide, dried, sometimes fixed, then stained and evaluated under a microscope by a trained microscopist. Any parasite stage that circulates in the peripheral blood can be visualized by this method. Microscopy can produce an estimate of parasite density by counting the total number of parasites in the field and multiplying by a known conversion factor (35) or by counting the number of parasites relative to the number of leukocytes (35). Highly trained microscopists can detect infections down to ~20,000 parasites/mL in thick blood smears (TBS) , but commonly the LOD of TBS in field conditions is higher, varying between 100,000 and 500,000 parasites/mL (36). Thin blood smears, which are used to identify the species of the infecting parasite, have an even higher LoD because less blood is examined under the microscope (37).

Microscopy requires working microscopes, slides, electricity, and trained staff and takes upwards of two hours to return results. As such, many endemic areas have shifted to utilizing RDTs for diagnosis and treatment, as well as surveillance. The use of RDTs is favorable due to their low cost, ease of use, and quick result time. Tests can be administered in the field and results are available within 15 minutes An RDT is especially favored for conducting surveillance studies because they can be done by non-clinical staff in field settings.

RDTs are lateral flow tests that detect malaria antigens in the peripheral blood. The most common RDT for detecting *Pf* targets the *Pf* histidine rich protein II (*Pf*HRP2). *Pf*HRP2 is a highly expressed, water-soluble protein released during the erythrocytic cycle of parasite development (38–40). At higher parasite densities, there is strong correlation between *Pf*HRP2 concentration and parasite densities; the correlation is less strong at lower parasite densities (41). Some RDTs also contain an antibody target for *Plasmodium* lactate dehydrogenase (pLDH), a lower abundance protein expressed by all *Plasmodium* species. Therefore, RDTs with both *Pf*HRP2 and pLDH targets can identify whether an individual has a *Pf* infection, a non-*Pf* infection, or a mixed infection with *Pf* and another *Plasmodium* species, depending on the parasite density. Because *Pf*HRP2 is secreted from RBCs, it can be detected even during sequestration (40). However, results are qualitative only, and the sensitivity of the RDT rapidly declines with parasite densities below ~100,000 parasites/mL (36). In addition, *Pf*HRP2 antigens can circulate in the blood following parasite clearance, resulting in some false positive results from this diagnostic method (36,38). Finally, with the increased frequency of *hrp2* gene deletions in some *Pf* parasites, RDTs fail to detect a proportion of infections, even with high densities (42).

Newer ultra-sensitive *Pf*HRP2 RDTs (uRDTs) have been designed to detect *Pf*HRP2 concentrations 10-fold lower than traditional RDTs (41), which could make them powerful tools for low-density, asymptomatic malaria detection. However, given that the relationship between *Pf*HRP2 and parasite density is weaker as densities decrease and *Pf*HRP2 can remain in the blood after infection clearance, uRDTs are likely to generate more false positive results at low densities, an issue that becomes more prominent if tests are read after the manufacturer's recommend 20 minute processing time (43).

The most sensitive diagnostics for malaria are nucleic acid amplification tests (NAAT), which amplify DNA or RNA targets of the *Plasmodium* parasite. These techniques can detect infections ~20 parasites/mL and produce very few false positive results. RNA-focused NAATs (e.g., qRT-PCR or nucleic-acid based sequence amplification) work by amplifying *Plasmodium* 18S rRNA, an extremely stable rRNA expressed at ~5,000-10,000 copies per ring-stage parasite (44). DNA-focused NAATs (e.g., qPCR) instead detect the genomic DNA encoding the 18S rRNAs (44–49). Typically, NAATs are done on venous blood, but can also be used on DBS (48). While NAATs are highly sensitive, most are complex laboratory assays requiring a large amount of infrastructure, equipment, and reagents, which limit their usability in field sites.

1.4 LOW-DENSITY *PLASMODIUM FALCIPARUM* INFECTIONS

There is growing evidence that there is a large proportion of low-density infections in malaria endemic areas. A 2015 meta-analysis compared lateral flow RDTs to DNA PCR-based tests (6). The RDT LoD was 100,000-200,000 parasites/mL compared to 1,000-5,000 parasites/mL for PCRs. On average, RDTs detected less than half of all PCR-detectable *Pf* infections in cross-sectional population surveys (6). RDTs detected the fewest number of PCR-positive infections in adults, suggesting that this age category comprises the highest proportion of low-density carriers, especially in low transmission settings. In areas where PCR prevalence was 5-20%, 87% of all infections in individuals >15 years were undetectable by RDT.

In general, higher parasite densities correlate with greater transmission probabilities because there are more gametocytes circulating in the blood. However, several factors influence gametocyte carriage and infectivity potential, including symptoms, duration of infection, treatment, transmission reducing activity induced through immune responses, age, seasonality, and transmission intensity (16,18,50–53), making it difficult to directly estimate infectivity potential of different infections. A recent meta-analysis estimated that the relative infectiousness of patent infections is 2.87 times that of subpatent infections (4), but when considering the abundance of these infections and likely longer duration, a modeling study using data from several infectivity studies estimated that subpatent infections account for 20-40% of the infectious reservoir in moderate transmission settings (54), and may be more in lower transmission areas (4).

1.5 MALARIA IMPORTATION

With the increase of air travel, human mobility has increased, and the world is more interconnected than ever before. With the movement of people also comes the importation and spread of infectious diseases (55,56). New advances in technology and expanded use of mobile phones and social networks have allowed more accurate tracking of spatio-temporal human movements, and in turn have advanced the understanding of how human networks contribute to disease spread, including malaria (57–60). As malaria requires both an infected human host and a viable mosquito vector to transmit disease, the threat of malaria importation is most prominent in areas that have eliminated or nearly eliminated malaria, as they have conditions conducive for onward transmission (61–63). Several strategies are recommended and deployed by countries trying to limit malaria importation, including reactive case detection (64–66), vector control strategies to reduce receptivity (67), and enhanced case detection at border crossing (68,69). Many of these strategies rely on the detection of symptomatic infections, or those with high enough densities to be detected by a screening RDT. However, these approaches may miss a large proportion of cases. A 2015 screening study at the Cambodian borders with Thailand, Laos, and

Vietnam found the sensitivity of RDTs to detect infections was very low compared to PCR, and only 20% of those infected were febrile (69).

Importation may also be problematic in higher density areas and contribute to sustaining malaria prevalence. Furthermore, importation can introduce new malaria genotypes, which may result in higher levels of clinical malaria, or introduce strains resistant to antimalarial treatment. As such, there is an increased urgency to study the importation of malaria cases across a variety of endemic settings.

1.6 DISSERTATION AIMS

To continue to drive the incidence of malaria towards elimination, novel research strategies and improved data analyses are needed to better understand both the role of imported and low-density *Plasmodium* infections to sustained malaria transmission and better categorize where these infections are occurring. Most studies of low-density carriers are cross-sectional, leading to a paucity of data on the natural history of these infections. Low cost and simple strategies are needed to longitudinally study low-density infections, and additional analytic strategies that utilize currently available data are important to better understand the role of these infections in onward malaria transmission.

This dissertation aims to further elucidate the role of low-density and imported *Plasmodium* infections in two regions with elevated malaria prevalence. The dissertation evaluates associations between low-density and imported infections to malaria prevalence on Bioko Island, Equatorial Guinea, and tests the feasibility of a novel home testing strategy in Katakwi, Uganda, to study the natural history of *Plasmodium* infections. This work is described in the following chapters:

Chapter 2 aims to evaluate the possible household clustering of subpatent infections with RDT(+) infections on Bioko Island, Equatorial Guinea. The research used data and stored DBS from the 2015 Bioko Island Malaria Indicator Survey (MIS).

Chapter 3 aims to quantify the impact of imported infections to malaria prevalence in areas of historically high off-island travel on Bioko Island, Equatorial Guinea. The analysis uses data from the 2019 and 2020 MIS, and leverages a travel moratorium that occurred between the two surveys in a difference in differences model.

Chapter 4 aims to evaluate feasibility of a daily at-home DBS collections as a malaria epidemiology investigative tool for qRT-PCR through a pilot study in Katakwi, Uganda.

1.7 STUDY AREAS

1.7.1 *Bioko Island, Equatorial Guinea*

Bioko Island is a small land mass located in the Gulf of Guinea. Prior to 2004, Bioko Island had some of the highest reported malaria transmission intensities in the world, with estimated entomologic inoculation rate ranging from 281 to 787 infectious bites per year (70). As a result of investments from the Government and Corporate Social Responsibility programs from U.S. oil companies working in the country, an extensive malaria control program began in 2004 under the National Malaria Control Programme (NMCP) and continues today (71–73). Due to the extensive control efforts, the prevalence declined rapidly in the first years of the program (72,73), but then plateaued in recent years. Several analyses suggest that the constant movement of individuals between Bioko Island and the mainland of the country on continental Africa (where prevalence is much higher) may contribute to the persistent prevalence on the island (74–76). Bioko Island therefore offers a unique opportunity to evaluate several factors of malaria transmission, because they have extensive data collection, yearly malaria indicator surveys, and extensive travel data. I had the privilege of working with the Bioko Island Malaria Control Program (BIMCP) from 2012 to 2015, and as such, have a deep knowledge of the malaria transmission dynamics, which allowed for in-depth analyses.

1.7.2 *Katakwi district, Uganda*

Katakwi district is in northeast Uganda, an area of moderate to high malaria transmission (77). The region has a malaria clinic run by Med Biotech Laboratories, a collaborator of the University of Washington. The area conducts several trials of malaria control strategies and treatments, so there is a sensitized and willing study population. The area also has an extensive network of Village Health Team members who assist with research and ensure the safety and well-being of study participants. The Murphy Laboratory began collaborating with Med Biotech Laboratories in 2016, and this dissertation project was the first jointly planned study between the two organizations.

Chapter 2. Clustering of subpatent infections in households with asymptomatic rapid diagnostic test-positive cases in Bioko Island, Equatorial Guinea independent of travel to regions of higher malaria endemicity: a cross-sectional study

Citation: (78) Hergott, D.E.B., Balkus, J.E., García, G.A. et al. Clustering of subpatent infections in households with asymptomatic rapid diagnostic test-positive cases in Bioko Island, Equatorial Guinea independent of travel to regions of higher malaria endemicity: a cross-sectional study. *Malar J* **20**, 313 (2021). https://doi.org/10.1186/s12936-021-03844-6

2.1 ABSTRACT

Background: Prevalence of *Plasmodium falciparum* (*Pf*) malaria on Bioko Island remains high despite sustained, intensive control. Progress may be hindered by high proportions of subpatent infections that are not detected by rapid diagnostic tests (RDT) but contribute to onward transmission, and by imported infections. Better understanding of the relationship between subpatent infections and RDT detected infections, and whether this relationship is different with imported versus locally acquired infections, is imperative to better understand the sources of infection and mechanisms of transmission to tailor more effective interventions.

Methods: Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed on a subset of samples from the 2015 Malaria Indicator Survey to identify subpatent infections. Households with RDT(+) individuals were matched 1:4 with households with no RDT(+) individuals. The association between living in a household with an RDT(+) individual and having a subpatent infection was evaluated using multivariate hierarchical logistic regression models with inverse probability weights for selection. To evaluate possible modification of the association by potential importation of the RDT(+) case, the analysis was repeated among strata of matched sets based on the reported eight-week travel history of the RDT(+) individual(s).

Results: There were 142 subpatent infections detected in 1400 individuals (10.0%). The prevalence of subpatent infections was higher in households with versus without an RDT(+) individual (15.0% vs. 9.1%). The adjusted prevalence odds of subpatent infection were 2.59-fold greater (95% CI: 1.31, 5.09) for those in a household with an RDT(+) individual compared to individuals in a household without RDT(+) individuals. When stratifying by travel history of the RDT(+) individual, the association between subpatent infections and RDT(+) infections was stronger in the strata in which the RDT(+) individual(s) had not recently travelled (adjusted

Prevalence Odds Ratio (aPOR) 2.95; 95% CI:1.17, 7.41), and attenuated in the strata in which recent travel was reported (aPOR 1.76; 95% CI: 0.54, 5.67).

Conclusions: There is clustering of subpatent infections around RDT(+) individual(s) when both imported and local infection are suspected. Future control strategies that aim to treat whole households in which an RDT(+) individual is found may target a substantial portion of infections that would otherwise not be detected.

2.2 INTRODUCTION

Despite 17 years of intensive malaria control efforts funded at more than three times the regional average, the prevalence of *Pf* malaria on Bioko Island, Equatorial Guinea, as determined through RDTs in cross-sectional surveys, remains high at 17% (79). The prevalence is highly heterogenous (80), with some areas reporting prevalence <5% while others >25% (72). Such heterogeneity is perplexing; some of the highest burdens are reported in urban areas where improved housing conditions and fewer mosquito breeding sites typically result in lower malaria prevalence (59,81–83). To move Bioko Island towards malaria elimination, it is imperative to better understand the sources of infection and mechanisms of transmission to tailor more effective interventions.

Both clinical and active case detection strategies for malaria diagnosis and treatment typically rely on RDTs and/or microscopy. While these diagnostic approaches are highly sensitive for higher density infections (higher concentration of parasites per unit of blood), they fail to capture lower density infections that are below the limit of detection (6). Such subpatent infections are highly prevalent in endemic areas (6) and contribute to onward transmission (4). More sensitive molecular techniques, such as PCR and RT-PCR, are more costly and require laboratory infrastructure not routinely available in endemic areas, making their widespread use for front-line diagnostics improbable. However, if such tools can be used to identify relationships and patterns between subpatent and patent infections, it may enable the development of intervention strategies to target subpatent infections without use of complex testing. For example, a recent meta-analysis with data from 41 malaria endemic countries showed that household clustering of subpatent infections around symptomatic or asymptomatic patent infections exists in areas where local malaria transmission occurs depending on site's overall endemicity (84).

The malaria transmission dynamics on Bioko Island are complicated by the high frequency of travel between the island (relatively low prevalence) and the mainland portion of the country (much higher prevalence, >30%) (76,85). Importation of malaria cases from higher burden neighbors threatens elimination programs in several endemic areas (57,61,62,86,87). A recent analysis of Bioko Island suggested that the high prevalence in urban areas was mainly attributed to importation of cases, rather than local transmission, and thus control measures targeting travelers were proposed (76,88). However, it is unclear if household clustering of subpatent infections exists in areas that both have low receptivity to onward transmission and a high proportion of imported cases. For *Plasmodium* infection to propagate in a community, there must be a certain level of receptivity at that locale (86), which is dependent on the presence of available mosquitoes and human reservoirs. Therefore, network clusters of infections are expected in areas where local transmission is occurring. Local transmission can either be propagated from other locally occurring infections (Route 1 of **Figure 2.1**) or from an imported infection (Route 2 of **Figure 2.1**). Control strategies targeting travelers will be most successful in reducing the overall malaria burden in areas where imported infections contribute substantially to sustained transmission. However, these strategies may be less impactful on reducing island-wide transmission if such imported infections occur in areas where the conditions are not conducive to onward transmission (Route 3 of **Figure 2.1**). The association between subpatent infections, RDT(+) infections, and importation in Bioko Island, is unknown.

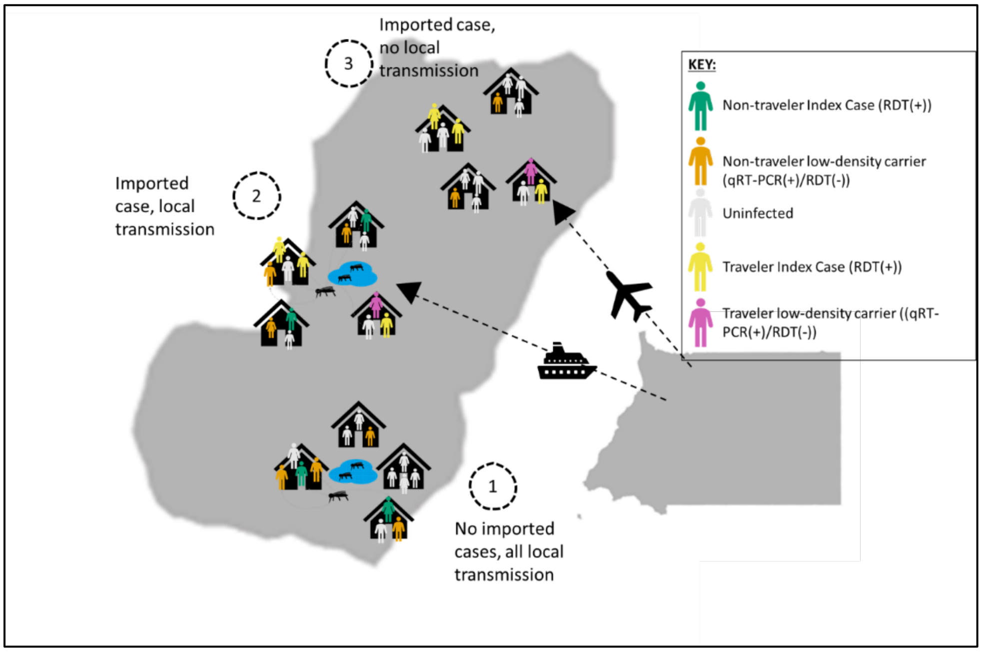


Figure 2.1. Schematic diagram showing the three possible transmission profiles hypothesized to exist on Bioko Island.

Given the potential role of both subpatent infections and travel on malaria transmission dynamics, this study utilized samples and data from the annual Malaria Indicator Survey (MIS) to evaluate whether subpatent infections are more common in Bioko Island households with an RDT(+) individual than those without an RDT(+) individual, and whether the association was modified by the potential importation of the RDT(+) infection in a traveler. It was hypothesized that the greatest proportion of subpatent infections would be found in households with RDT(+) individuals with no recent travel history.

2.3 METHODS

2.3.1 *Study setting and original data collection*

Bioko Island, the largest island of Equatorial Guinea, is approximately 2,000 km^2 and has ~335,000 inhabitants as of 2015, the majority of whom (80%) live in the urban district of Malabo in the northern region of the island (89). Malaria transmission occurs year-round. Since 2004, the NMCP has launched an intensive malaria control strategy in collaboration with the BIMCP, which includes indoor residual spraying (IRS), distribution of long-lasting insecticidal nets (LLINs), distribution of free antimalarials for uncomplicated and complicated malaria, intermittent preventive treatment in pregnancy (IPTp), entomological monitoring, a behavior change communication program, and enhanced malaria testing strategies (72,73,90).

The MIS is carried out annually on Bioko Island to track malaria prevalence; the present analysis uses data and samples collected during the 2015 MIS. The sampling strategy and study design were previously described (72). In brief, households were randomly selected by an independent statistician from each of the 284 communities on the island that had at least 20 households registered in the 2015 census (91). A trained enumerator interviewed the head of household to gather demographic information on all household members, household and individual characteristics, as well as malaria knowledge and household behaviors relating to malaria prevention. All members of the household who were present at the time of the survey and provided consent were invited to be tested for malaria parasitemia and two DBS were collected on filter paper and stored at ambient temperature in desiccant for later analysis. Participants with malaria parasitemia were provided artemisinin-combination therapy (ACT) by a Ministry of Health and Social Welfare (MoHSW) nurse per the national malaria treatment policy. Those with hemoglobin < 8 g/dL or who were febrile were referred to a local clinic for appropriate follow-up and treatment.

2.3.2 *Laboratory Methods*

Malaria parasitemia in the MIS was assessed using a CareStart Malaria HRP2/pLDH RDT (Access Bio, Somerset, NJ, USA). For this analysis, we only considered *Pf* parasitemia, indicated by the presence of the HRP2 band, either with or without the pLDH band. Subpatent *Pf* infections were detected using a method derived from a validated, highly sensitive qRT-PCR shown to have a limit of detection of 20 parasites/milliliter (p/mL) (92). Total nucleic acid extraction was performed using the semi-automated NucliSENS easyMAG instrument (bioMerieux, Marcy l'Etoile, France), according to the manufacturer's instructions. Briefly, 50 µL DBS were hand cut with sterile scissors and added to 2 mL of NucliSENS lysis buffer, and the mixture was incubated for 30 minutes at 55°C. The lysed sample was then transferred to the well of a plastic vessel with 70 µL of NucliSENS magnetic silica followed by automatic magnetic separation on the easyMAG to yield 40 µL of eluted nucleic acids.

Amplification and multiplex qRT-PCR were performed using the Applied Biosystems Quant Studio 5 Real-Time PCR System (Waltham, MA, USA). The thermal profile used for qRT-PCR was as follows: RT for 10 minutes (min) at 48°C, denaturation for 2 min at 95°C, and then 45 cycles of 5 seconds at 95°C for melting and 35 seconds 50°C for annealing. Each reaction contained 7.5 µL of template total-nucleic-acid and a 17.5 µL reaction master mix containing 2x SensiFAST Probe Lo-ROX Master Mix (Bioline/Meridian) with 0.5 µM of each primer and 0.25 µM of each probe. The pan-*Plasmodium*- and *Pf*-specific probes and primers were previously described (92). All qRT-PCR assays were run with appropriate controls including malaria positive, malaria negative, and non-template controls. For quantification, copies/mL of whole blood were determined based on an absolute RNA calibrator curve and were also converted to estimated parasites/mL of whole blood by dividing by the per-parasite copy number conversion factor of 7.4×10^3 18S rRNA copies/parasite as previously reported (92). If parasite densities were <20 parasites/mL, qualitative results were reported: "low-positive" for 10 to <20 parasites/mL, and "not detected" for any density lower than 10 p/mL or negative result.

2.3.3 *Study design, exposures, and outcomes*

A subset of samples and data from the 2015 MIS were analyzed using a cross-sectional study design to evaluate whether subpatent *Pf* infections were more common in households that included an RDT(+) individual compared to those with no RDT(+) individuals. There were 135 households randomly selected in which at least one member was RDT(+), and randomly matched them 1:4 to households with no RDT(+) individuals in the same district to balance the underlying prevalence in the sample. Households were eligible for selection into the analysis if at least two people were tested by RDT and at least one individual tested was RDT(-). Given this sample size and assuming that 15% of individuals in households without an RDT(+) individual had a subpatent infection, at alpha=0.05, there was 80% power to detect a minimum prevalence odds ratio (POR) of 1.82 in the primary analysis. Not all samples with an RDT result in the MIS had a viable DBS for qRT-PCR analysis. As such, only matched sets in which each household had at least one qRT-PCR were included in the final analysis.

The main exposure of interest, living in a household with an RDT(+) individual, was generated using the RDT results from all individuals who were tested during the survey. A household was classified as having an RDT(+) individual if at least one member tested positive for *Pf* malaria by RDT during the MIS, indicated by the presence of the HRP2 band. For the primary analysis, it was assumed that individuals who lived in the household but who were not present during the time of testing were RDT(-), and therefore would not influence the classification of the household. Individuals were defined as having a subpatent infection, the main outcome of interest, if they were RDT(-) for *Pf* but *Pf* qRT-PCR positive, independent of the estimated parasite density.

2.3.4 *Statistical analysis*

The association between living in a household with an RDT(+) individual and having a subpatent infection was evaluated using multivariate hierarchical logistic regression models (93) that incorporated inverse probability weights (IPW) to account for possible selection bias in household sampling. Models accounted for correlation at the household and community level as random effects and were adjusted for individual and household level covariates collected during the MIS that were considered *a priori* as potential confounders. Individual characteristics included a categorical variable for age, sex, recent travel to the mainland of Equatorial Guinea, and whether the individual reported sleeping under a bed net the previous night. Household factors included household size and the presence of open eaves. District was also included in the model as it was used to match households during selection.

Using data from all individuals in the full dataset, the probability that each individual was sampled by RDT was estimated using a generalized regression model accounting for categorical age, sex, off and on island travel history, and reported illness in the previous 14 days. Weights in the final model were the inverse of the selection probability for each included individual. All analyses were performed in R studio v.12.5033. Regression analyses were performed using the *lme4* package with the *optimx* or *bobyqa* optimizers. For each analysis, a minimally adjusted hierarchical model with no IPW, in which only district, the matching variable, was included, was evaluated. POR from this model as well as the inverse probability weight adjusted prevalence odds ratios (IPW aPOR) and associated 95% confidence intervals (CI) for each analysis are reported. To better understand the impact of suspected imported RDT(+) infections on household transmission, associations were further analyzed based on the travel histories of the RDT(+) individual(s). Matched household sets were stratified according to the reported travel history of the RDT(+) individuals. Those in sets in which an RDT(+) individual had reported traveling off the island in the past eight weeks were analyzed separately from the household sets in which the RDT(+) individual did not report recent travel (See **Figure 2.2)**. All reported travel in the past eight weeks was to the mainland of Equatorial Guinea. The same analytic approaches were repeated in both strata.

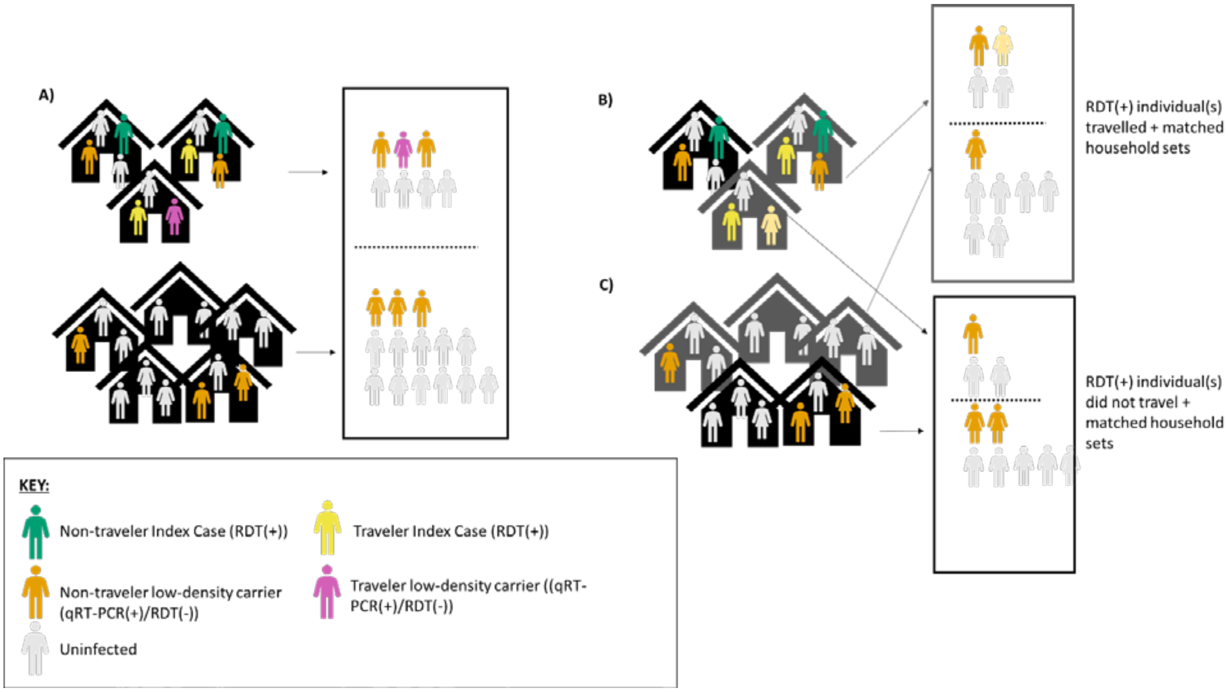


Figure 2.2. Schematic showing individuals included in the main (Panel A) and stratified analyses (B and C). For the stratified analyses, individuals who live in a household with an RDT(+) individual who reports recent travel and their matched household sets (Panel B) are analyzed separately from individuals in households where the RDT(+) individual(s) did not report recent travel and their matched household sets (Panel C).

To evaluate the robustness of the assumptions regarding individuals who were not present in the home at the time of sample collection, several sensitivity analyses were conducted. In the primary analysis, it was assumed that individuals who were not present during the time of testing were RDT(-) and their presence would not change the exposure status of the household. To test this assumption, the RDT(+) probability for each individual missing an RDT test result in the sample was predicted using a hierarchical logistic regression prediction model. Using data from all individuals in the full MIS dataset with an RDT result, a predictive model that accounted for clustering at the household level and included a categorical variable for age, reported travel both on and off island in the past eight weeks, bed net use the previous night, presence of eaves in the household, household size and district was built. This model was then applied to the individuals in the sample dataset who had no RDT result to generate an RDT(+) predicted probability for each individual. Individuals with the highest predicted probabilities were assigned to be RDT(+) to achieve an overall RDT prevalence in the missing individuals of 5%, 10%, and 20%. These values were chosen to simulate a prevalence in the missing individuals that was similar to that seen in those tested (10%), similar to the maximum prevalence seen in the population (20%), or lower than what was seen in those tested (5%). The imputed RDT values were included with the observed RDT data to re-assign the exposure status of households as needed, and the full regression models were repeated with the imputed data sets.

Finally, to determine the impact of utilizing the highly sensitive qRT-PCR method compared to commonly used, less sensitive methods, an exploratory analysis was performed in which individuals with parasite densities <1,000 parasite/mL, the approximate LoD for newer ultra-sensitive RDTs (41), were reclassified as not infected.

2.4 RESULTS

Of the 5,163 households sampled in the 2015 MIS, 3,737 met the inclusion criteria for selection into the analysis (**Figure 2.3**). Initially, 135 of the 1,162 households with an RDT(+) individual and 540 matched households with no RDT(+) individuals were selected, which corresponded to 489 and 1,530 individuals, respectively. After excluding individuals that had no qRT-PCR result (n=308), and any matched household sets that did not have qRT-PCR results from all households in the set, the final dataset consisted of 246 individuals from 92 households with an RDT(+) infection, and 1,154 individuals from 368 households with no RDT(+) infections.

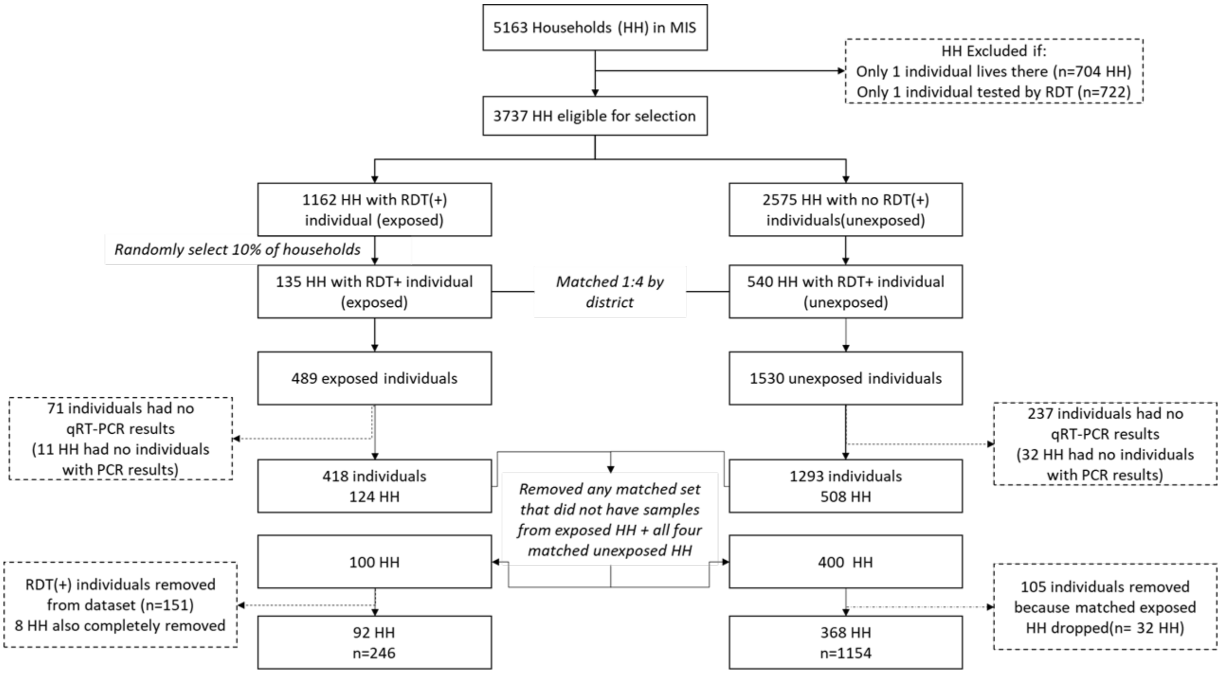


Figure 2.3. Flow chart showing how the final sample selection for analysis was determined. (HH=households; MIS=malaria indicator survey, RDT= rapid diagnostic test, qRT-PCR=quantitative reverse transcriptase polymerase chain reaction)

Select characteristics of households and individuals by household RDT status are summarized in **Table 2.1**. A higher proportion of households with an RDT(+) individual had open eaves and had a slightly larger household size compared to households without an RDT(+) individual. Individuals in households with an RDT(+) individual were slightly younger, with a higher proportion reporting travel in the past eight weeks, and a lower proportion reporting that they slept under a bed net the previous night compared to individuals in households with no RDT(+) individuals.

Of the 145 samples from individuals who were RDT(+), 108 (74.5%) were also positive by qRT-PCR (See **Table 2.3**). Overall, there were 142 subpatent infections (10.0%) in the sample, with a mean density of 80,500 parasites/mL (range: 10 – 43.7*10^5 p/mL). Of note, five such infections had high qRT-PCR densities, well above the limit of detection of an RDT. A greater proportion of individuals in households with an RDT(+) individual had subpatent infections than those in households with no RDT(+) infections (15.0% vs. 9.1%). Among those with subpatent infections, there was no difference in the mean parasite densities between the two groups (50,800 p/mL vs. 91,000 p/mL, p=0.52). In the minimally adjusted, unweighted model, those living in households with an RDT(+) individual were 1.9 times as likely to have a subpatent infection than those who did not live with an RDT(+) individual (POR 1.93, 95% CI: 1.19, 3.14). In the fully adjusted model with IPW, the odds of having a subpatent infection were 2.6 times greater (aPOR 2.59; 95% CI: 1.31, 5.09) among those who lived in a household with an RDT(+) individual compared to individuals who lived in a household without RDT(+) individuals (**Table 2.2**).

Table 2.1. Comparison of general characteristics of individuals who were RDT(-) during the MIS, and either lived in a household (HH) with an RDT(+) individual (94 HH) or did not (361 HH)

	RDT(+) in HH (n=246)	**No RDT(+) in HH (n=1154)**	**All (n=1400)**
Individual Characteristics			
Age (years)	17.3 (15.5); 0.1-76	19.1 (16.9); 0-88	18.8 (16.7); 0-88
Hemoglobin (mm Hg)	12.0 (1.8); 5.7-16.9	12.3 (1.6); 5.5-18.9	12.2 (1.7); 5.5-18.9
Slept under net the previous night	122 (49.6%)	688 (59.6%)	810 (57.9%)
Was sick in past 14 days	12 (4.9%)	74 (6.4%)	86 (6.1%)
Travelled off-island in past 8 weeks	50 (20.3%)	114 (9.9%)	164 (11.7%)
Male sex	96 (39.0%)	495 (42.9%)	591 (42.2%)
Household Characteristics			
Household size^2	7.0 (2.9); 2-15	5.5 (2.2); 2-12	5.8 (2.4); 2-15
Proportion of household tested	0.7 (0.2); 0.1-1	0.7 (0.2); 0-1	0.7 (0.2); 0-1
Household prevalence by RDT	0.3 (0.2); 0-2	0.0 (0.0); 0-0	0.1 (0.2); 0-2
No screened windows	234 (95.1%)	1101 (95.4%)	1335 (95.4%)
Eaves are open	93 (37.8%)	266 (23.1%)	359 (25.6%)
Community characteristics			
Urban	193 (78.5%)	868 (75.2%)	1061 (75.8%)
Malabo (Urban) District	167 (67.9%)	783 (67.9%)	950 (67.9%)
Malabo Periphery	26 (10.6%)	85 (7.4%)	111 (7.9%)
Baney District	15 (6.1%)	70 (6.1%)	85 (6.1%)
Luba District	26 (10.6%)	156 (13.5%)	182 (13.0%)
Riaba District	12 (4.9%)	60 (5.2%)	72 (5.1%)

Table 2.2. Inverse probability weighted adjusted prevalence odds ratios (POR) of a subpatent infection among individuals sampled in the 2015 Malaria Indicator Survey in Bioko Island.

	All Households (n=1400)		**Travel Strata (n=329)**		**No Travel Strata (n=1071)**	
	Minimally adjusted POR [95% CI]	**IPW aPOR [95% CI]**	**Minimally adjusted POR [95% CI]**	**IPW aPOR [95% CI]**	**Minimally adjusted POR [95% CI]**	**IPW aPOR [95% CI]**
Household characteristics						
Living in HH with RDT(+) individual	1.93 [1.19,3.14]	2.59 [1.31,5.09]	1.92 [0.84,4.39]	1.76 [0.54,5.67]	1.97 [1.1,3.54]	2.95 [1.17,7.41]
Having open eaves	--	1.74 [0.91,3.31]	--	2.33 [0.6,8.97]	--	1.61 [0.71,3.66]
Household size	--	0.98 [0.87,1.1]	--	0.96 [0.74,1.23]	--	1 [0.85,1.16]
Individual Characteristics						
Slept under bed net previous night	--	1.13 [0.71,1.81]	--	0.92 [0.39,2.19]	--	1.2 [0.66,2.18]
Travelled off island in past 8 weeks	--	2.03 [1.14,3.6]	--	2.45 [0.92,6.57]	--	1.9 [0.85,4.22]
Male sex	--	1.52 [1.03,2.23]	--	1.31 [0.6,2.85]	--	1.72 [1.08,2.75]
Age Under 5	--	Ref	--		--	
Age 5-14 years	--	3.19 [1.46,6.94]	--	1.7 [0.46,6.24]	--	4.76 [1.68,13.46]
Age 15-44 years	--	9.16 [4.4,19.07]	--	2.95 [0.92,9.47]	--	17.53 [6.34,48.47]
Age 45+ years	--	4.19 [1.68,10.45]	--	4.16 [0.95,18.19]	--	4.61 [1.37,15.51]
Community Characteristics						
Malabo (urban)	Ref	Ref	Ref	Ref	Ref	Ref
Baney	0.45 [0.16,1.28]	0.42 [0.11,1.54]	--	--	0.42 [0.14,1.25]	0.37 [0.08,1.68]
Luba	0.44 [0.21,0.92]	0.35 [0.14,0.88]	0.69 [0.09,5.47]	0.53 [0.04,6.26]	0.4 [0.18,0.89]	0.33 [0.11,1.01]
Riaba	1.22 [0.53,2.82]	0.95 [0.29,3.1]	0.87 [0.11,7]	0.61 [0.05,7.42]	1.27 [0.49,3.3]	1.09 [0.24,4.94]
Malabo (periphery)	0.86 [0.41,1.81]	0.79 [0.3,2.11]	1.52 [0.18,13.21]	1.44 [0.08,26.19]	0.78 [0.34,1.79]	0.69 [0.21,2.25]

Table 2.3. Concordance between RDT *Pf* positivity and qRT-PCR *Pf* positivity, for select samples from the MIS from Bioko Island, Equatorial Guinea, 2015 (n=1,650). Results include individuals from all households tested, before exclusions were made to reach the final analytic sample.

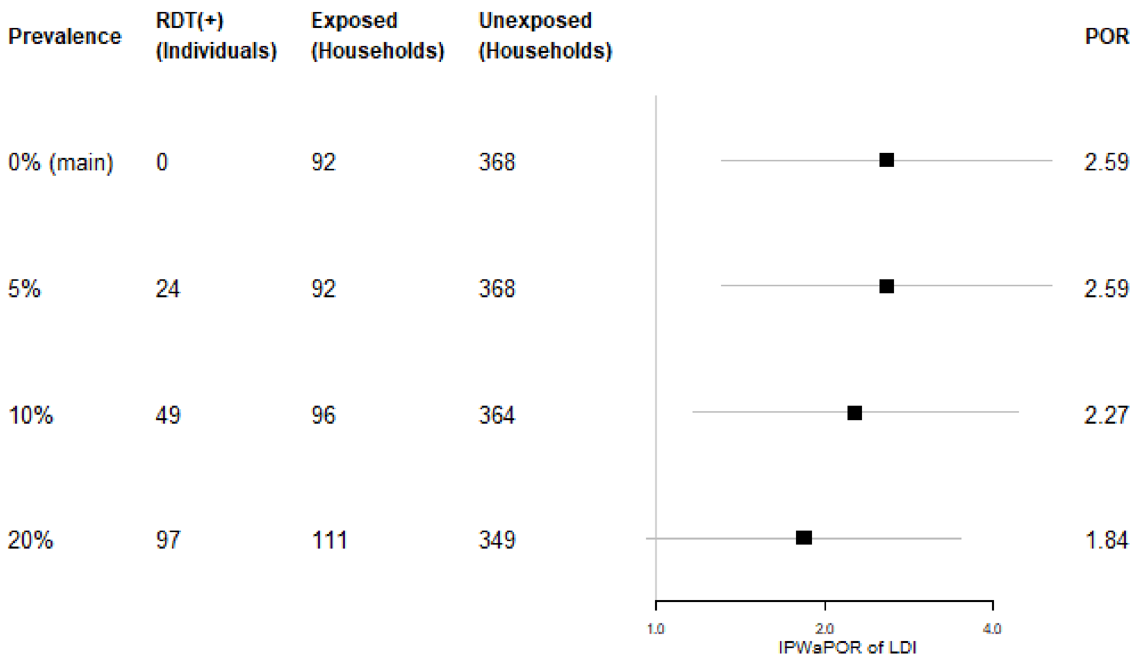
		RDT	
		***Pf*(+)**	***Pf*(-)**
qRT-PCR	***Pf*(+)**	108	160
	***Pf*(-)**	37	1345
	TOTAL	**145**	**1505**

Analysis by travel history to the mainland of Equatorial Guinea was conducted based on the travel status of individuals who were RDT(+). There were 145 individuals in 92 households who were RDT(+). Of these, 40 individuals (27.6%) from 27 households reported travel to the mainland of Equatorial Guinea in the past eight weeks. Individuals in households with RDT(+) travelers (n=57) were analyzed separately from those in households with RDT(+) non-travelers (n=189), along with their matched household sets. Among the strata in which an RDT(+) individual had recently travelled, 15.8% of individuals in households with RDT(+) individuals had a subpatent infection compared to 8.8% in households without an RDT(+) individual. In the minimally adjusted, unweighted model, those living in households with an RDT(+) individual were 1.92-times as likely to have a subpatent infection than those who did not live with an RDT(+) individual; however, the result was not significant (95% CI: 0.84, 4.93). In the fully adjusted model with IPW, the odds of having a subpatent infection was 1.76-fold that of those living in homes with an RDT(+) individual compared to those in homes without RDT(+) individual, but the result was again not significant (95% CI: 0.54, 5.67) (**Table 2.2**).

Among the strata in which an RDT(+) individual had no reported recent travel, 14.8% of individuals in households with RDT(+) individuals had a subpatent infection compared to 9.2% in households without an RDT(+) individual (minimally adjusted POR 1.97, 95% CI: 1.1, 3.54). In the fully adjusted model with IPW, the odds of having a subpatent infection in those living in a household with an RDT(+) individual was 2.95-fold greater than in homes without RDT(+) individuals (95% CI: 1.17, 7.41) (**Table 2.2**). These results suggest possible modification of the relationship between subpatent and patent infections by the recent travel history of the RDT(+) individual in the household, as the association is stronger in households in which there is no history of travel.

The primary analysis assumed that individuals who were absent during the time of the survey were RDT(-). To test the robustness of this assumption, a series of sensitivity analyses were conducted using imputed RDT values for the individuals who were missing test results. Various scenarios of plausible ranges of RDT positivity in those who were missing were evaluated. There were 497 individuals from the households in the primary analysis who did not have an RDT result (22% of all individuals). Of these, 113 were from households with an RDT(+) individual and 384 were from households with no RDT(+) individuals. Compared to individuals who were tested, those who were not tested were older (mean age 29.0 years vs. 17.7 years), had a higher proportion who were male (60.6% vs. 41.9%), and reported more off-island travel (24.0% vs. 12.0%). **Figure 2.4** presents the modeling results based on imputed RDT(+) values in those with missing data at various levels of prevalence. As the presumed prevalence of infection in those missing RDT results increased, there was a slight decrease in the association between subpatent infection and living in a household with an RDT(+) individual. Only when 20% prevalence was assumed in those who were missing did the effect estimate fail to reach significance (POR 1.84, 95% CI: 0.96, 3.51).

Finally, to evaluate whether a similar association would be detected using other detection methods that are more sensitive than conventional RDT but less sensitive than the qRT-PCR method, a model was run to evaluate whether the association held when individuals with subpatent infections with densities below 1,000 parasites/mL (approximate LoD of uRDTs) were classified as RDT(-). There were 35 individuals with infections < 1,000 p/mL who were reclassified from subpatent infection to negative infection, 9 from households with RDT(+) individuals and 26 from households with no RDT(+) individuals. With this new classification, 11.4% of individuals who lived in households with an RDT(+) individual had a subpatent infection, compared to 6.8% of individuals who lived in a household without an RDT(+) individual. The adjusted IPW POR were similar to that of the main analysis, but the confidence interval was wider (aPOR 2.42, 95% CI: 1.02, 5.74).



Prevalence	RDT(+) (Individuals)	Exposed (Households)	Unexposed (Households)	POR
0% (main)	0	92	368	2.59
5%	24	92	368	2.59
10%	49	96	364	2.27
20%	97	111	349	1.84

Figure 2.4. Inverse probability weight adjusted prevalence odds ratios (IPWaPOR) from sensitivity analyses which assumed various numbers of individuals missing from the main analysis were RDT(+), to achieve a range of plausible prevalences in the missing group.

Abbreviations: POR= prevalence odds ratio; LDI= subpatent infection; HH= household; RDT= rapid diagnostic test

2.5 DISCUSSION

In this analysis of the relationship between subpatent *Pf* and RDT(+) *Pf* infections from the 2015 MIS data on Bioko Island, living in a household with an RDT(+) household member was associated with increased odds of having a subpatent *Pf* infection compared to living in a household with no RDT(+) household members. When stratifying by the travel history of the RDT(+) individual, the association was strongest among households in which the RDT(+) individual did *not* recently travel, while the association was attenuated and non-significant in the strata in which the RDT(+) individual travelled.

The results of the main analysis are similar to those observed in the highlands of Kenya, an area of low, but heterogeneous transmission (94). In that area, where *Pf* malaria prevalence by microscopy was estimated to be 6%, subpatent infections were 1.7 (95% CI: 1.6-1.8) times as likely to be in found in a household with a sentinel case, defined as an RDT(+) symptomatic adult or child or an RDT(+) asymptomatic child. In Zanzibar, where transmission is at pre-elimination levels, the association was even greater; the odds of having a subpatent infection were 7.4-times as great (95% CI: 2.8-19.9) for individuals living in a household with an RDT(+) individual compared to those living >1000 m from the index case (95). A recent meta-analysis of data from 17 different studies utilizing a mass test and treat strategy reported significant clustering of subpatent infections in households with RDT(+) individuals, and the degree of clustering increased linearly with decreasing prevalence (84). These studies, along with data from the present analysis, suggest that household clustering is important to consider for malaria control strategies across a broad range of transmission settings.

Imported infections are a threat to malaria elimination efforts (57,61,62,86,87), especially if they are imported to areas conducive to onward transmission. When household sets were stratified based on the travel history of the RDT(+) individual, the association between subpatent infection and RDT(+) individuals was strongest in the strata in which there was no recent travel by the RDT(+) individuals. While an association was still suggested in households where importation of the infection was suspect, the association was attenuated and no longer significant. If it is assumed that reported travel in the past eight weeks is an indicator that the infection was imported, the attenuated relationship observed in the travel strata adds support to the hypothesis that imported cases may be returning to areas less receptive to propagation of infection, and therefore there may be little onward transmission of those infections (88). Interestingly, the proportion of subpatent infections in households with and without RDT(+) cases was similar in the travel and non-travel strata, but after adjusting for relevant covariates, including travel status of the RDT(-) individuals, the association was stronger in the non-travel strata and attenuated in the travel strata. The most likely explanation for this is the high amount of travel in individuals with subpatent infections in households with RDT(+) travelers. Of nine individuals with subpatent infections in households with an RDT(+) traveler, seven (78%) also reported recent travel, compared to 21% of subpatent infections in households without an RDT(+) individual (n=24). There was no difference in reported travel among individuals with subpatent infections in the non-travel strata. This may suggest that the clustering of subpatent infections in households with travelers is the result of multiple imported infections, while subpatent infections in households without recent travel are primarily due to local acquisition and transmission. A similar pattern was seen in a recent study in Zanzibar, an area with very low transmission. In that analysis, the odds of subpatent infection in household members of symptomatic RDT(+) cases who had recently traveled were 1.4 times that of clusters around locally acquired symptomatic RDT(+) infections; however, this association was largely driven by increased risk of subpatent infection of co-travelers of the index case (66). In households where the index case was imported, the odds of subpatent infection was 2.5-times as great among co-traveling household members compared to non-traveling members. Given the study design and sample size, it was not possible to further delineate the patterns of transmission, and further studies designed to evaluate this are warranted.

Mass test and treat (MTAT) programs in which RDT(+) infections are actively identified and treated in communities have shown to have minimal impact on patent malaria prevalence in both low (96) and high (97) transmission settings, which is thought to be due to the high prevalence of subpatent infections that are missed with this type of strategy. Additionally, these strategies rely on individuals being present at the time of testing, which pose logistical challenges, especially in urban areas, and may miss frequent travelers. A presumptive household treatment strategy in which all members of a household of an RDT(+) infection are provided treatment, independent of their RDT measured infection status, may better target subpatent infections and more effectively interrupt transmission (84). In Bioko Island, this type of strategy may be most beneficial during localized outbreaks, in which a quick response is needed following an uptick in malaria cases (81). Further, in areas of low-transmission where risk of malaria importation is high, strategies that aim to treat all household members of index patients who have recently traveled have been suggested (66). This analysis suggests that a program like this carried out in an area of moderate malaria transmission, such as Bioko Island, may have an impact on the overall parasite burden in the area, and would be beneficial independent of the recent travel status of the index case. In an area such as Bioko, officials might also consider providing treatment to all family members of travelers who are screened with an RDT on return and test positive. Even with measures like this, it should be noted that a number of subpatent infections in households without RDT detectable infections were also observed in this study, which may not be targeted with this type of approach.

In addition to living in a household with an RDT(+) individual, this analysis suggested that males and older individuals, especially those between 15 and 44 years of age, were most likely to have a subpatent infection, which is similar to what has been seen in other transmission areas (6). It has been suggested that this group is most likely to have subpatent infections because of behaviors that increase likelihood of exposure, such as employment or greater time spent outdoors at night (98–100). These factors may increase their cumulative exposure to malaria, which increases the likelihood they have built up a sufficient antibody response to control recent infections and keep parasite densities low (101). Interestingly, a recent prospective analysis of *Pf* infections in Uganda challenged this theory by showing that females cleared subpatent chronic infections twice as quickly as males (34). Given that most data that compares subpatent and patent infections, including this study, come from cross-sectional studies, if there is a true difference in the rate of parasite clearance between males and females, it is possible that the increased risk reflects length-biased sampling instead of changes to the immune response. Whether the association is due to a true increase in risk or a sampling bias, the associations may still be beneficial in devising strategies that can target subpatent infections without utilizing ultra-sensitive diagnostic tools.

This study has several strengths, including the use of a highly sensitive qRT-PCR detection method, and the use of inverse probability weighting to account for selection bias that occurs during MIS sampling. However, these findings should be interpreted in the context of several limitations. While IPW was used to account for the missing data for subpatent infections, it was not possible to account for the possibility that individuals who were absent during testing were

RDT(+), which may influence the exposure status of the household. In sensitivity analyses, the RDT prevalence in individuals missing from the survey would have to be >20% before association between subpatent infections and RDT(+) cases disappeared. Given that the RDT prevalence in the sample was 8.5%, and the RDT prevalence estimated for the survey was 12.7% (95%CI: 12.0-13.4%), it is unlikely that the RDT prevalence in individuals who were not surveyed would approach 20%. While some RDT positivity in missing individuals attenuated the association, it was not substantial enough to alter the conclusions.

Another limitation is the utilization of travel history as a proxy for an imported infection. In the present study, travel history was based upon reported travel to the mainland of Equatorial Guinea in the past eight weeks. While this measure has been used to evaluate imported infections in other settings (63), there are several limitations that should be considered. First, given that a *Pf* infection, on average, takes 12 days to be detected in blood in malaria-exposed populations (102), and the average time to naturally clear an infection is estimated between 87 and 200+ days (34), recent travelers could experience either higher or lower density infections, depending on date of their return and the date they acquired the infection in relation to the time of the survey. In an attempt to account for this possibility, recent travel of the RDT(-) individuals was controlled for, but it is still possible that some imported infections were missed. Secondly, given the large time span of travel, it is not possible to definitively determine if someone’s infection was acquired outside of the island or locally with the present study design. Frequency of travel and the amount of time spent in higher prevalence areas, as well as protective measures that were taken while traveling all might impact the probability that the RDT infection was imported. The time since return to the island and malaria risk behaviors exhibited at home might impact the risk of a locally acquired infection in a traveler since their return. Finally, in addition to travel to the mainland of the country, within-island travel is also common in Bioko, and increases the likelihood that individuals become infected outside of their communities. Without the use of parasite DNA sequencing or a prospective study design, some RDT infections may have been incorrectly classified in the stratified analyses. It is most likely that this misclassification would be non-differential and therefore correction of the misclassification would only strengthen the results. However, it is possible that there is differential misclassification, but without additional variables it is unclear the impact on the results.

Finally, the study defined a positive case based on the result of an RDT. However, qRT-PCR results showed that one-quarter of RDT(+) infections had no detectable parasitemia, most likely the result of persistent HRP2 antigenemia following clearance of an infection. While it is possible that inclusion of these individuals could impact the results, only one of these infections was in a household without another RDT(+) infection with detectable parasitemia, and therefore exclusion of these individuals would not change the exposure status of the households in this analysis. There were also five individuals who were RDT(-) but had qRT-PCR defined parasitemia above the limit of detection of RDTs. A study in the mainland of Equatorial Guinea confirmed that *hrp2* deletion is present in the country (103) and may be the source of false negatives in our study population. While three of these infections were in individuals who did not live in a household with an RDT(+) individual, and therefore, the exposure status of the household may have been impacted if a different RDT was used, the small number of false positives would not be expected to impact our results. Given that RDTs are most commonly used for detection of infection in the study setting, our results represent the associations that are seen with these widely deployed diagnostic tools.

2.6 CONCLUSIONS

Subpatent infections of *Pf* infections are more prevalent in households with an RDT(+) individual in Bioko Island, Equatorial Guinea. The association is present both when local and imported RDT(+) infections are suspected, albeit stronger in households where local acquisition is suspected. These findings support the need for possible malaria control strategies that treat household members of RDT(+) individuals to target subpatent infections and decrease the *Pf* infectious reservoir.

Chapter 3. Impact of 6-month COVID-19 travel moratorium on *Plasmodium falciparum* prevalence on Bioko Island, Equatorial Guinea: A difference in differences analysis

3.1 ABSTRACT

Background: Imported *Plasmodium* infections may sustain prevalence in areas with high amounts of travel. However, data are lacking that definitively quantify the impact of imported infections on malaria prevalence. On Bioko Island, Equatorial Guinea, travel between the island and mainland was suspended between March and September 2020 in response to the COVID-19 pandemic, ostensibly also halting the importation of *Plasmodium falciparum (Pf)* infections to the island. This provided a natural experiment to evaluate the contribution of imported infections to *Pf* prevalence on Bioko Island.

Methods: Data from 2019 and 2020 malaria indicator survey (MIS) were used to estimate the change in odds of *Pf* infection for those living in high travel areas following the travel moratorium relative to those living in low travel areas (based pre-moratorium travel prevalence). Enumeration areas with the highest quartile of historical travel prevalence (n=28) were compared to those with the lowest historical travel prevalence (n=28). A survey generalized linear model with robust standard errors and a logit link function was fit with an interaction term between travel area and year to estimate the impact of the travel moratorium on risk of malaria infection. Models were adjusted for spray coverage and whether an individual went inside before 7PM.

Results: In 2019, the *Pf* prevalence in low travel areas was 7.5% (95% CI: 4.5, 10.5), and 13.8% in high travel areas (95% CI: 12.5, 15.1). In 2020, prevalence increased in low travel areas to 12.8% (95% CI: 6.9, 18.7) but decreased in high travel areas to 11.9% (95% CI: 10.3, 13.5). The adjusted odds of infection comparing 2020 to 2019 in high travel areas was 0.75 (95% CI: 0.64, 0.89), while the adjusted odds of infection in low travel areas over the same period was 1.22 (95% CI: 0.913, 1.633). Relative to low-travel areas, the odds of *Pf* infection after the travel moratorium were 39% lower in high travel areas (aOR: 0.61; 95% CI: 0.43, 0.88).

Conclusions: In the absence of imported infections, high travel areas saw a reduction in malaria prevalence compared to low travel areas. This finding provides direct evidence that importation is a significant contributor to malaria prevalence on Bioko Island and control strategies that target travelers should be considered.

3.2 INTRODUCTION

Despite increased efforts and control strategies, *Pf* malaria transmission remains endemic in 85 countries and territories (104). The increased frequency in which individuals move between and within countries has created added challenges for areas that have recently eliminated malaria, as well as those working towards elimination (57,61,62,66,86–88,105). Bioko Island, Equatorial Guinea has seen a significant decrease in malaria burden over the past decade (72); however, there are several areas of the island, especially urban areas where prevalence is typically lower, that have not yet approached pre-elimination levels. These urban areas also tend to have a higher proportion of individuals who travel between Bioko Island and the mainland of Equatorial Guinea (74), where malaria prevalence is substantially higher (88). Several previous studies suggest that there is a high amount of importation of malaria to Bioko Island in returning travelers (76), and that these imported infections contribute to sustained prevalence in urban areas (75,88). However, previous analyses are based on retrospective reporting of travel history captured via cross-sectional surveys, and there have been no studies that have allowed for direct estimation of the impact of imported malaria cases to prevalence in high travel areas. Better estimates of the contribution of imported cases to malaria transmission are needed to inform malaria control measures.

In 2020, to address the COVID-19 pandemic and minimize local transmission of SARS-CoV-2, Equatorial Guinea imposed travel restrictions throughout the country, eliminating movement between the islands and the mainland from March to September 2020 (106). This, ostensibly, also eliminated the importation of *Plasmodium* infections. The travel restriction provides a natural experiment in which the impact of imported infections can be directly assessed. By comparing the risk of malaria infection before and after the travel restriction in areas that historically have had a high-volume of travel to areas of historically low volume of travel using a difference in differences analysis, this study directly assesses the impact of imported malaria infection on risk on Bioko Island. Findings from this analysis may be used by policy makers in support of decisions concerning control measures that may target these types of infections when travel resumes.

3.3 METHODS

3.3.1 *Study Setting*

Bioko Island is the largest island in Equatorial Guinea, with an estimated 300,000 people residing there with majority concentrated in Malabo, the capital city (107). Governmental institutions are located both in Malabo as well in two cities within the continental region of Equatorial Guinea. Normally, government officials have a high frequency of travel between the Bioko and the continental region (74). Since 2004, the NMCP has implemented a robust malaria control strategy under the BIMCP, including IRS, distribution of LLINs, distribution of free antimalarials for uncomplicated and complicated malaria, IPTp, entomological monitoring, a behavior change communication program, enhanced malaria testing strategies, and outbreak response (72,73,90,108).

3.3.2 *Malaria Indicator Survey*

The MIS is carried out annually on Bioko Island between July and August and has been previously described (72,78). Briefly, information on malaria risk factors, including off island travel in the previous eight weeks, is collected from selected households. Historically, the MIS utilized selected sentinel sites for sampling, but in 2015 the sampling frame was expanded to include all communities with at least 20 households (78). Beginning in 2019, the sampling units were changed from community to geographically defined enumeration areas (EAs); under this scheme all households were eligible for selection into the survey through a stratified, single-cluster survey design. To guide and track programmatic malaria activities, under the NMCP, Bioko Island has been divided into geographically defined "map areas" which are 1 km x 1 km squares (91). The 209 map areas that included households were used to define EAs for the MIS. If a map area had at least 100 households, it was its own EA; if there were fewer than 100 households in the map area, several map areas were combined (based on geographical proximity) to create an EA with at least 100 households. Before sampling, the EAs were then divided into two strata based on population density and estimated local residual transmission (LRT), which is amount of malaria transmission estimated to be from local sources (88). Stratum 1 includes EAs with generally lower population density and generally higher LRT, representing mostly rural populations, and stratum 2 includes EAs with generally higher population density and generally lower LRT, representing mostly urban populations. The division of EAs by stratum is shown in **Figure 3.1.** To select the sample for the MIS, within each EA, a simple random sample of households was taken using specified sampling fractions for each stratum: 24% for stratum 1, and 4.8% in stratum 2.

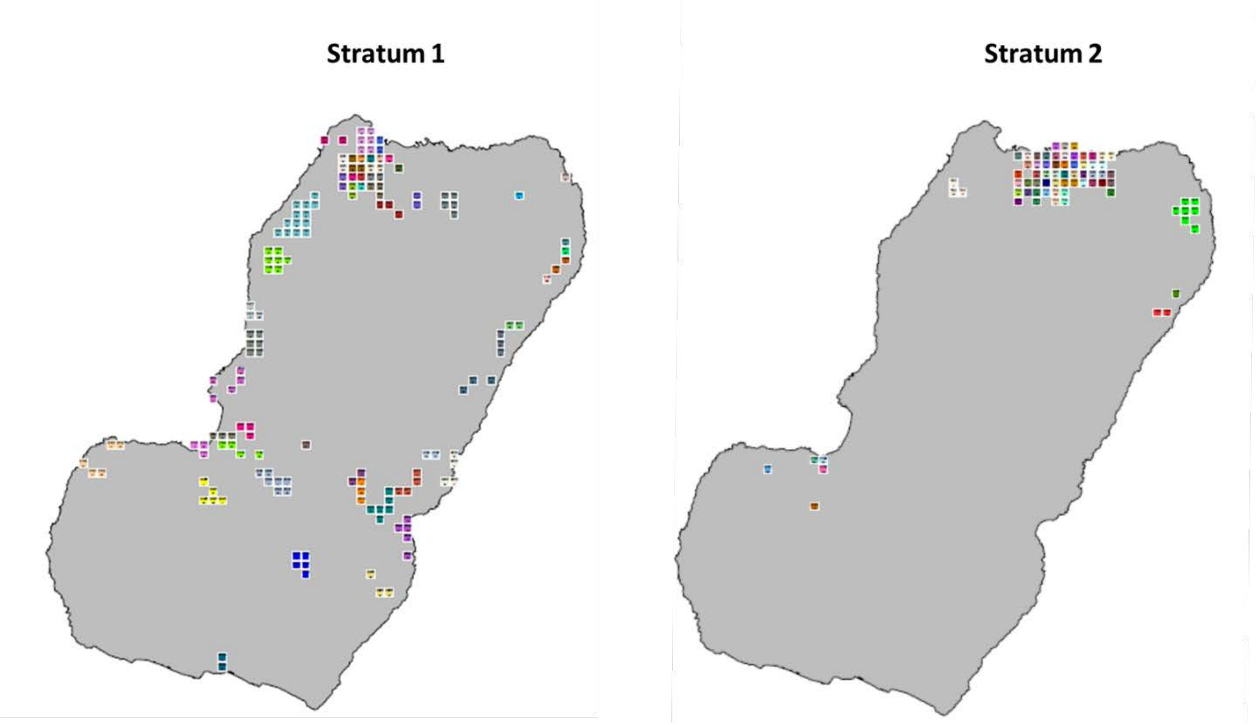


Figure 3.1. Depiction of the 109 enumeration areas utilized for sampling for the Malaria Indicator Survey. Stratum 1 includes EAs with generally lower population density and generally higher LRT and stratum 2 includes EAs with generally higher population density and generally lower LRT.

All adults provided written consent for testing, and the head of household consented for anyone under the age of 18. All consenting individuals who lived in a selected household and were present during the time of the survey were tested for *Plasmodium* malaria parasites using a CareStart Malaria HRP2/pLDH rapid diagnostic test (RDT) (Access Bio, Somerset, NJ, USA). Individuals who were positive for malaria by RDT were provided with artemisinin-combination therapy (ACT) by a Ministry of Health and Social Welfare (MoHSW) nurse per national policy, based on World Health Organization guidelines (109).

3.3.3 *Sample selection for analysis*

For this analysis, the estimated travel prevalence to the mainland of Equatorial Guinea, or the fraction of people surveyed who reported having travelled in the eight weeks prior to the MIS (88), for each EA prior to 2020 was utilized to define areas with high and low travel. Since 2015, information on where individuals in sampled households travelled in the eight weeks preceding the survey was collected as part of the MIS. Smoothed mainland travel prevalence for each map area was estimated using travel data from the 2015 to 2018 MIS, as per methods described in Guerra *et al* (88). For map areas with no estimates, the value from their nearest neighbor was utilized. If an EA was composed of multiple map areas, a weighted average was calculated from all map areas in the EA. The weight of each map area was equal to the number of households in that area out of the total number of households in the EA. After a travel prevalence was assigned to each EA, those in the top quartile of travel prevalence were labeled as “high travel” areas, and those in the bottom quartile of travel prevalence were labeled as “low travel” areas; EAs from the middle two quartiles were excluded from analysis.

3.3.4 *Statistical Analyses*

All analyses were conducted within the *survey* package of R studio v12.5033. The survey design dataset accounted for the stratified sampling weights of the original MIS as well as the non-independence of results within households and within EA. The main outcome of interest, *Pf* positivity, was coded as a binary variable. For each travel area, the survey mean prevalence was estimated from individual level data by year and are presented with a 95% CI. Difference in prevalence between 2019 and 2020 by group was evaluated by a survey t-test. An additional t-test was carried out to evaluate the difference in the change in prevalence between high and low travel areas.

To analyze the possible impact of the travel moratorium on malaria risk on Bioko Island, a difference in differences analysis was conducted to compare the change in odds of infection between 2019 and 2020 in historically high travel areas relative to the change in odds of infection in historically low travel areas during the same time. An unadjusted and adjusted survey generalized linear model with robust standard errors and a logit link function was fit. To determine variables to include in the adjusted model, values of several variables determined to be related to malaria risk *a priori* were compared between 2019 and 2020 within travel group. Any variable that had a meaningful difference between years within a travel group was included in the final model. To estimate how the odds of infection in high travel areas changed between 2019 and 2020 relative to the change in odds in low travel areas over the same period, the model included an interaction term between a binary variable for time, and travel. The general model is shown below:

$$\log(oddsRDT+) = \beta_0 + \beta_1 POST + \beta_2 hightravel + \beta_3 POST * hightravel + \beta_4 Covars + \varepsilon$$

Where β_0 is the log-odds of malaria infection in low travel areas in 2019, β_1 is the difference in log-odds comparing 2020 to 2019 in low travel areas, β_2 is the difference in log-odds of infection between low-travel and high-travel areas in 2019, and β_3 is the difference in differences of log-odds comparing the change in high travel areas between 2019 to 2020 to the change in low travel areas from 2019 to 2020. β_3 is the coefficient of interest to estimate the impact of the travel restrictions on prevalence. Coefficients and 95% CIs were exponentiated to interpret odds ratios of the different estimates.

Data from the 2018 MIS was used to assess the robustness of the parallel trends' assumption, by both visually assessing the trends from 2018 to 2019 in high and low travel areas, as well as by plotting the mean residuals of a linear model regressing *Pf* prevalence over time (110). For the analysis of parallel trends, non-survey weighted prevalences were calculated each year, as sample selection in 2018 was not done in the same manner as subsequent years.

There were three EAs known to have had large land use changes over the study period. As there was not a reliable way to measure land use change in all areas during the study period, a sensitivity analysis was conducted, in which the main analysis was repeated with a data set that excluded the three EAs that were known to have had land use changes over the study period.

3.4 RESULTS

In 2019 and 2020, there were 109 EAs sampled in the MIS; 53 were in stratum 1, and 56 in stratum 2. There were 64 EAs that were composed of a single map area, and 45 EAs made up of two or more map-areas. The range of travel prevalence to the mainland of Equatorial Guinea in each EA ranged from 1.5% to 39.9% (**Figure 3.2**). The EAs in the top quartile of the travel distribution (≥12.2%) were classified as high travel areas, and those in the bottom quartile of the travel distribution (≤4.3%) were classified as low travel areas, resulting in 56 EAs classified as high or low travel areas included in this analysis (**Figure 3.3**). The distribution of households and individuals in each of the travel classifications is shown in **Table 3.1.** Consistent with the construction of the stratum, a greater proportion of EAs in high travel areas were from stratum 2 (82.1%), while a greater number of EAs in the low travel areas were from stratum 1 (60.7%). Low travel areas also had fewer individuals sampled compared to high travel areas, consistent with the population densities of these areas.

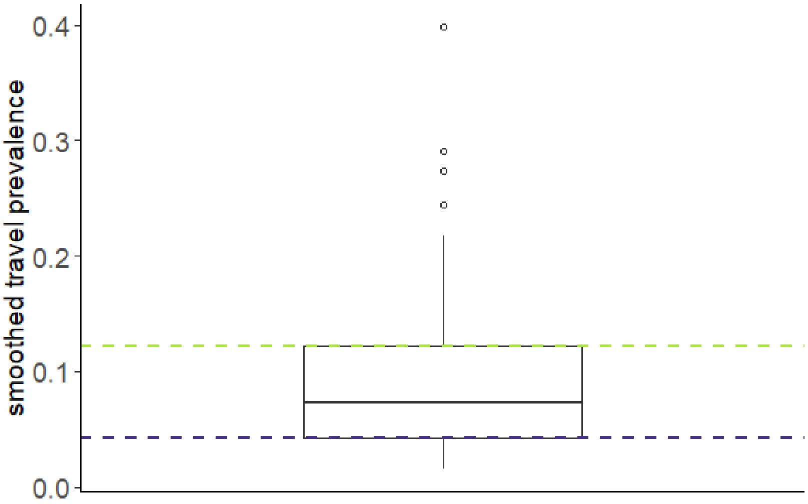


Figure 3.2. Distribution of travel by Enumeration Area (EA), based on smoothed travel prevalence estimates from 2015-2018 (88). EAs in the top quartile of travel distribution (above the green line) were classified as high travel areas, and those in the bottom quartile of travel distribution (below the purple line) were classified as low travel areas.

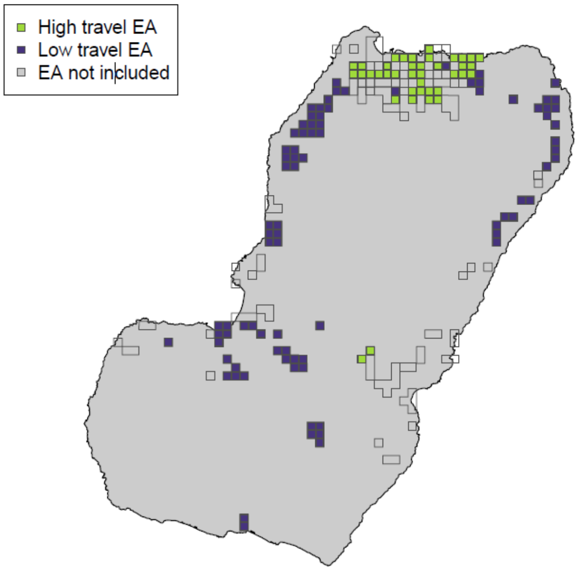


Figure 3.3. Enumeration areas (EAs) on Bioko Island, Equatorial Guinea selected for the difference in differences analysis. EAs in green are those in the top quartile of historical smoothed travel prevalence. EAs in purple are those in the bottom quartile of historical smoothed travel prevalence.

Table 3.1. Total enumeration areas (EA), households, and individuals sampled in the Bioko Island Malaria Indicator Survey and included in the difference in differences, stratified by travel area and stratum for 2019 and 2020.

	Low Travel Areas			**High Travel Areas**		
	Stratum 1 n (%)	**Stratum 2 n (%)**	**Total n**	**Stratum 1 n (%)**	**Stratum 2 n (%)**	**Total n**
Enumeration Areas	17 (61)	11 (39)	**28**	5 (18)	23 (82)	**28**
2019						
Households Sampled	498 (62)	305 (38)	**803**	179 (12)	1356 (88)	**1535**
Individuals Sampled	1334 (57)	1021 (43)	**2355**	457 (10)	4079 (90)	**4536**
2020						
Households Sampled	468 (64)	268 (36)	**736**	147 (11)	1226 (89)	**1373**
Individuals Sampled	1110 (57)	848 (43)	**1958**	450 (10)	3896 (90)	**4346**

The prevalence of malaria in 2019 was 7.5% in low travel areas (95% CI: 4.5, 10.5), and 13.8% in high travel areas (95% CI: 12.5, 15.1). In 2020, prevalence in low travel areas increased to 12.8% (95% CI: 6.9, 18.7) while decreasing to 11.9% in high travel areas (95% CI: 10.2, 13.5), representing a prevalence difference of 5.3% (95% CI: 0.3, 10.2), and 1.9% (95% CI: -3.3, -0.6), respectively**(Table 3.2)**. Assuming parallel trends in high and low travel areas, the *Pf* prevalence difference in high travel areas was 7.2% lower than would have been expected in the absence of the travel moratorium (95% CI: -12%, -2.2%).

Table 3.2. Number of individuals tested and prevalence estimate by year and travel group. Difference in prevalence compares 2020 to 2019 prevalence by travel area. The differences in differences compares to two prevalence differences.

Group and Year	**Individuals Tested (n)**	***Pf* Prevalence (95% CI)**	**Difference in Prevalence (95% CI)**	**Difference in Differences (95% CI)**
Low travel - 2019	3182	7.5% (4.5, 10.5)	5.3% (0.3, 10.2)	-7.2% (-12, -2.2)
Low travel - 2020	1178	12.8% (6.9, 18.7)		
High travel - 2019	7070	13.8% (12.5, 15.1)	-1.9% (-3.3, -0.6)	
High travel - 2020	1674	11.9% (10.3, 13.5)		

Results from the comparison of odds of infection between years and travel areas are presented in **Figure 3.4.** In the unadjusted analysis, the odds of infection in 2020 in high travel areas were 26% lower than the odds of infection in 2019 (OR= 0.84, 95% CI: 0.75, 0.95) **[Figure 3.4-B, gray line**]. Conversely, in low travel areas, the odds of infection in 2020 were 80% higher compared to 2019 (OR= 1.8, 95% CI: 1.17, 2.76) **[Figure 3.4-A]**. A variety of factors known to be related to malaria risk were compared between 2019 and 2020 within each of the travel groups (**Table 3.3**). Of those evaluated, only the time that individuals went indoors and the insecticide spray coverage showed significant differences between years and were included in the final model. The proportion of individuals who reported going inside their house before 7pm doubled from 2019 to 2020 in both low travel areas (22.5% to 47.2%) and high travel areas (28.1% vs. 50.4%). The proportion of households sprayed with insecticide decreased in 2020 (35.1%) compared to 2019 (46.9%) in low travel areas, whereas the spray coverage in high travel areas was higher in 2020 compared to 2019 (57.2% versus 28.3%).

When adjusting for spray coverage and going inside before 7pm, the odds ratio in high travel areas shifted away from the null (OR=0.75; 95% CI: 0.64, 0.89) **Figure 3.4-B**, black line] while the odds ratio in low travel areas shifted towards the null (OR=1.22; 95 % CI: 0.91, 1.63) [**Figure 3.4-A**, black line]. In 2019, the adjusted odds of infection in high travel areas were 50% higher than the odds of infection in low-travel areas (aOR=1.53; 95% CI 1.17, 2.01) [**Figure 3.4-E**]. However, in 2020, there was no difference in the odds of infection comparing the two travel areas (aOR=0.94; 95% CI: 0.68, 1.3) [**Figure 3.4-D**]. Comparing the change from 2019 to 2020 in high travel areas to low travel areas, the unadjusted odds of *Pf* infection after the travel moratorium were 51% lower in high travel areas (aOR= 0.49, 95% CI: 0.30, 0.73) [**Figure 3.4**-**F**] than would be expected. Following adjustment, the difference was slightly diminished, but odds of infection were still 39% lower in high travel areas compared to what would have been expected based on trends in low travel areas (aOR=0.61; 95% CI: 0.43, 0.88)

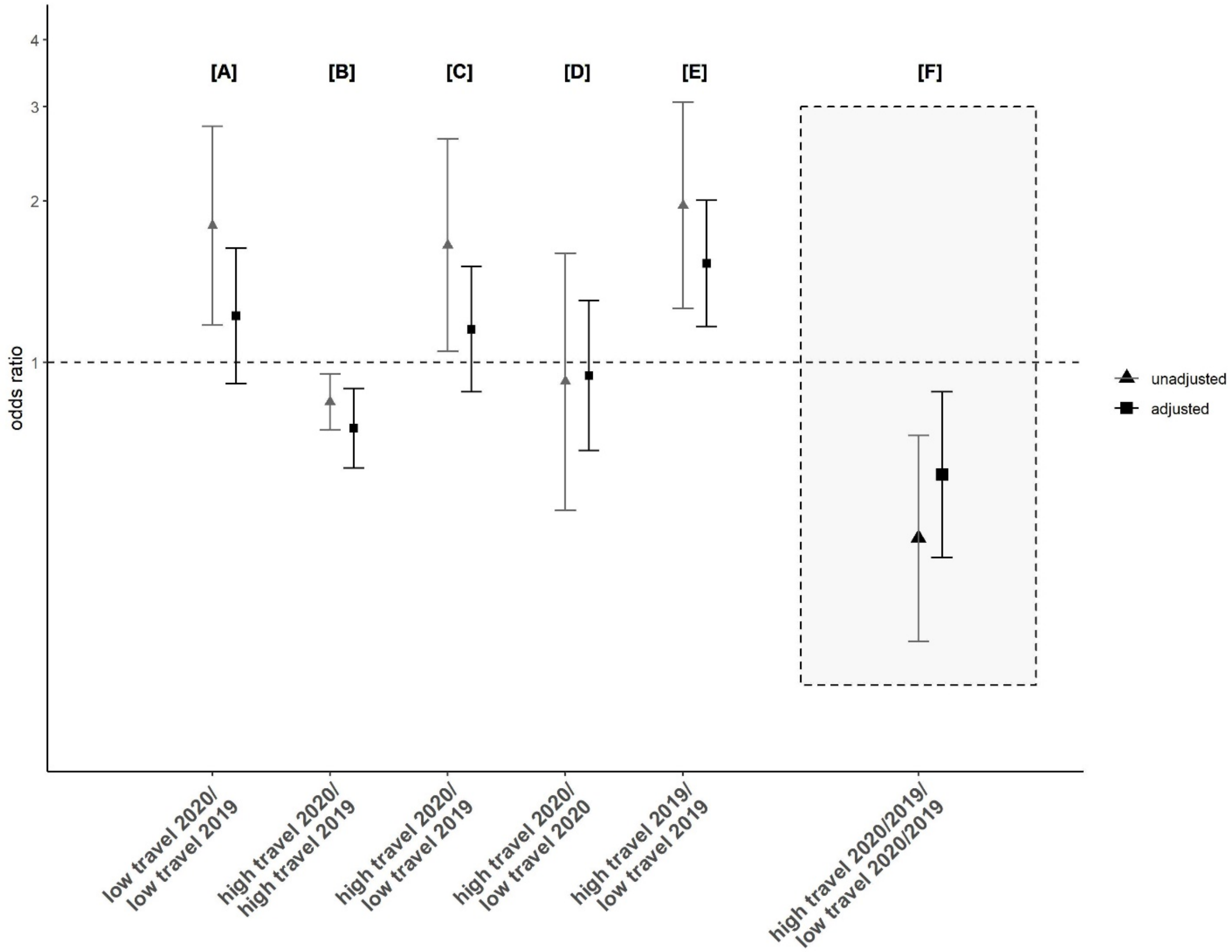


Figure 3.4. Comparison of odds of infection in different years and travel areas (high or low) on Bioko Island [panels A-E]. Adjusted odds ratios (grey triangles) are from models adjusted for spray coverage and whether an individual went inside before 7PM. The ratio of ratios comparing the change in odds in high travel areas between 2019 and 2020 relative to the change in low areas is shown in the gray box [panel F].

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Table 3.3. Comparison of select individual and household characteristics by year and travel area. Characteristics were selected *a priori* because of their relationship with malaria transmission.

	Low Travel EAs		**High Travel EAs**		**All**	
Variable	**2019**	**2020**	**2019**	**2020**	**2019**	**2020**
Individual Variables (sample size)	n ind = 2355	n ind = 1958	n ind = 4514	n ind = 4345	n ind = 6891	n ind = 6304
Age (years)	25.1 (21.6); 0.1-110	26.5 (21.8); 0.1-90	21.4 (18.0); 0.1-110	21.9 (18.1); 0.1-95	21.8 (18.1); 0.1-110	22.0 (18.1); 0.1-95
Average trips off island	0.0 (0.1); 0-1	0.0 (0.0); 0-1	0.1 (0.2); 0-1	0.0 (0.1); 0-1	0.1 (0.2); 0-1	0.0 (0.1); 0-1
Female	1209 (51.3%)	994 (50.8%)	2353 (52.1%)	2369 (54.5%)	3635 (52.7%)	3386 (53.7%)
Permanent resident of household	2124 (90.2%)	--	4269 (94.6%)	--	6518 (94.6%)	--
Is employed	549 (38.7%)	493 (42.7%)	1002 (37.8%)	994 (40.0%)	1596 (38.6%)	1449 (40.1%)
Travelled off-island in past 8-weeks	56 (2.4%)	5 (0.3%)	330 (7.3%)	36 (0.9%)	514 (7.5%)	60 (1.0%)
Slept in household previous night	2262 (96.1%)	1789 (97.1%)	4379 (97.0%)	4029 (97.4%)	6666 (96.7%)	5860 (97.3%)
Slept under a bednet previous night	1008 (44.6%)	736 (41.2%)	1686 (38.5%)	1507 (37.5%)	2606 (39.1%)	2154 (36.8%)
Sick in past two-weeks	262 (11.2%)	166 (9.0%)	462 (10.3%)	314 (7.6%)	736 (10.7%)	457 (7.6%)
Go inside before 7pm	**511 (22.5%)**	**812 (47.2%)**	**1208 (28.1%)**	**1953 (50.4%)**	**1755 (26.9%)**	**2865 (51.0%)**
Go to sleep before 9pm	618 (28.6%)	495 (29.6%)	850 (20.9%)	749 (20.0%)	1256 (20.4%)	1106 (20.3%)
Household Variables (sample size)	n hh = 825	n hh = 813	n hh = 1696	n hh = 1674	n hh = 2521	n hh = 2487
Household size	3.9 (2.9); 1-16	3.3 (2.4); 1-18	4.2 (2.8); 1-16	3.9 (2.6); 1-16	4.1 (2.8); 1-16	3.7 (2.6); 1-18
Number of visitors in house	0.1 (0.2); 0-1	0.1 (0.2); 0-1	0.0 (0.2); 0-1	0.1 (0.2); 0-1	0.0 (0.2); 0-1	0.1 (0.2); 0-1
Has the following item:						
Sofa	569 (69.1%)	600 (73.8%)	1425 (84.0%)	1426 (85.2%)	1994 (79.1%)	2026 (81.5%)
Table	634 (76.8%)	664 (81.7%)	1355 (79.9%)	1392 (83.2%)	1989 (78.9%)	2056 (82.7%)
Armoire	365 (44.2%)	358 (44.0%)	1108 (65.4%)	1000 (59.7%)	1473 (58.5%)	1358 (54.6%)
Air conditioning	59 (7.2%)	65 (8.0%)	555 (32.7%)	644 (38.5%)	614 (24.4%)	709 (28.5%)
Stove	417 (50.5%)	442 (54.4%)	1375 (81.1%)	1418 (84.7%)	1792 (71.1%)	1860 (74.8%)
Washing Machine	145 (17.6%)	149 (18.3%)	549 (32.4%)	626 (37.4%)	694 (27.5%)	775 (31.2%)
Car	129 (15.6%)	127 (15.6%)	567 (33.5%)	568 (33.9%)	696 (27.6%)	695 (27.9%)

Household sprayed in past 6-months	**358 (46.9%)**	**274 (35.1%)**	**418 (28.3%)**	**897 (57.2%)**	**776 (34.6%)**	**1171 (49.8%)**
Own bednets	181 (46.2%)	196 (43.9%)	259 (26.1%)	341 (34.6%)	440 (31.8%)	537 (37.5%)
Housing type:						
Social housing	11 (1.3%)	13 (1.6%)	200 (11.8%)	161 (9.6%)	211 (8.4%)	174 (7.0%)
Barracks	125 (15.2%)	152 (18.7%)	594 (35.0%)	617 (36.9%)	719 (28.5%)	769 (30.9%)
Apartment	19 (2.3%)	14 (1.7%)	136 (8.0%)	114 (6.8%)	155 (6.1%)	128 (5.1%)

There were three areas with known land use changes over the study period that may have impacted risk of malaria transmission. Analysis of the estimated prevalence in 2019 and 2020 in these three areas showed that in two areas (Baticopo and Basupu), the observed prevalence difference between years was greater than the average difference in the low travel group (**Table 3.4)**. Removing these three EAs from the analysis, resulted in *Pf* prevalence difference in high travel areas was 5.2% lower than would have been expected in the absence of the travel moratorium (95% CI: -8.9%, -1.4%) (**Table 3.5**). Comparing the relative change from 2019 to 2020 in high travel areas to low travel areas, the adjusted odds of *Pf* infection after the travel moratorium were 32% lower in high travel areas (aOR= 0.68, 95% CI: 0.44, 0.92).

Table 3.4. *Plasmodium falciparum (Pf)* prevalence in 2019 and 2020 for three enumeration areas with known land use changes during the study period.

		Prevalence	
EA	**Community**	**2019**	**2020**
502	Basupu	17%	45%
792	Baticopo	23%	33%
1028	Riaba	8%	8%

Table 3.5. Number of individuals tested and prevalence estimate by year and travel group with a data set that excludes three EAs in the low-travel group with known land use changes during the study period. Difference in prevalence compares 2020 to 2019 prevalence by travel area. The differences in differences compares to two prevalence differences.

Group and Year	**Individuals Tested (n)**	***Pf* Prevalence (95% CI)**	**Difference in Prevalence (95% CI)**	**Difference in Differences (95% CI)**
Low travel -2019	2786	6.2% (3.4%, 8.8%)	3.3% (-0.37, 6.9%)	-5.2% (-8.9%, -1.4%)
Low travel -2020	819	9.5% (6.2%, 12.7%)		
High travel -2019	7070	13.8% (12.5%, 15.1%)	-1.9% (-3.3, -0.6)	
High travel -2020	1674	11.9% (10.3%, 13.5%)		

For the results of a difference in differences model to be valid, the parallel trends assumption must hold (111). Using data from 2018, the mean prevalence in high and low travel EAs was calculated and plotted with unweighted data from 2019 and 2020. **Figure 3.5** indicates that the trend from 2018 to 2019 was similar in low and high travel groups, and then diverged in 2020 when travel was stopped. A plot of the mean residuals of a basic linear model including only *Pf* positivity and time also confirmed trends were parallel (**Figure 3.6**).

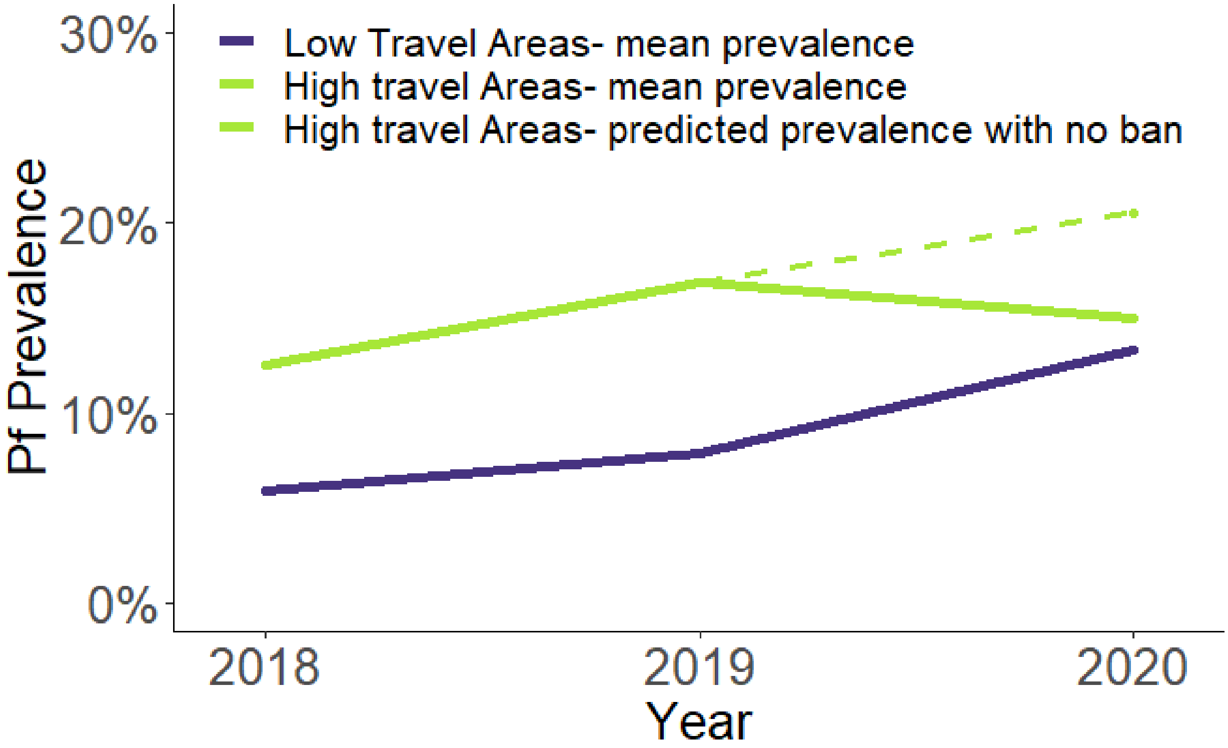


Figure 3.5. Test of parallel trends assumption. Estimated mean *Pf* prevalence in low travel (purple) and high travel (green) areas in 2018, 2019 and 2020. The dashed line represents predicted prevalence in 2020 in the absence of a travel moratorium. Prevalence estimates are non-weighted in this graph.

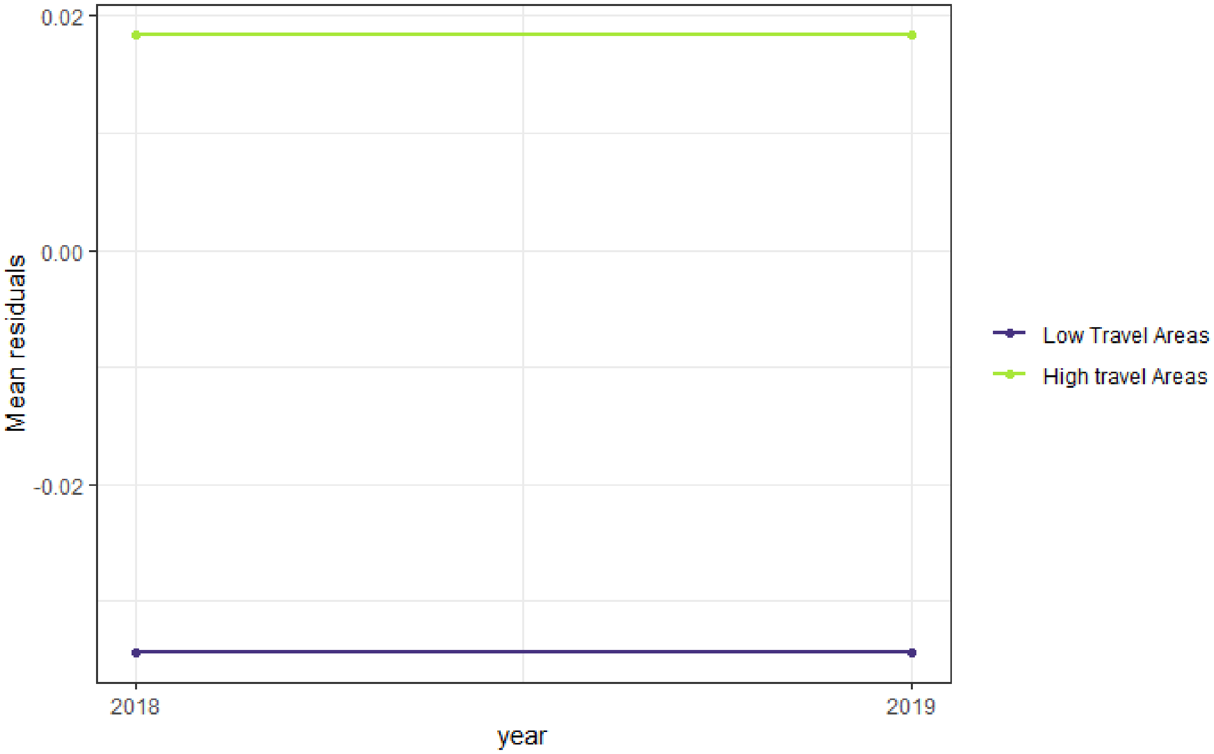


Figure 3.6. Plot of mean residuals of linear regression of *Pf* infection over time to test the robustness of the parallel trends assumption.

3.5 DISCUSSION

Simulation models suggest there are areas of Bioko Island in which high proportions of malaria prevalence can be attributed to infections acquired while travelling to higher burden areas (75,76,88). A recent model suggested that drastically reducing the number of travelers between Bioko Island and the mainland could significantly reduce prevalence in areas with a high proportion of travelers (75). However, prior to 2020, there were, understandably, no intervention studies nor other data to definitively support the model simulations. Travel restrictions imposed as a measure to control the spread of SARS-CoV-2 in 2020 provided an opportunity to directly evaluate the impact of imported infections, and it was observed that in the absence of travel, prevalence in historically high travel areas decreased by 2%, while prevalence in low travel areas increased by 5% over the same period. This suggests that, assuming parallel trends, in the absence of the travel moratorium, one would have expected *Pf* prevalence to be 7% higher in high travel areas than what was actually observed.

In 2019, prior to travel restrictions put in place to control the spread of COVID-19, odds of malaria infection were two to three times higher in areas of Bioko Island with a historically high proportion of travelers, relative to areas of historically low travel. This finding is similar to a 2013 analysis that showed infection risk was greater in children living in areas with the highest proportion of travelers (76). In 2020, when the movement of individuals was restricted, there was no difference in risk of malaria infection observed in high travel areas compared to low travel areas. This observation both further supports the hypothesis that a significant fraction of the *Pf* prevalence observed in high travel areas could be explained by imported infections (88), while also suggesting that malaria risk in these areas is not solely driven by importation. Previous analyses have suggested there may be areas where malaria prevalence is solely attributable to imported infection (88), as several of these locations are in urban centers where there is generally improved infrastructure and fewer mosquitoes. If this were true, it would have been anticipated that the risk in high travel areas would be substantially less relative to low travel areas once importation was eliminated. However, this analysis showed no difference in malaria risk in the absence of travel was observed. One explanation for this finding is that there was still residual travel occurring, even with the travel moratorium, which allowed infections to continue to be imported during 2020. While a small percentage of individuals did report travel in the past 8 weeks in the 2020 MIS (1%), it seems unlikely that this would sustain the observed prevalence in the population of high travel areas. Another explanation is that infections in high travel areas were acquired through travel to other areas of the island. There is often frequent travel within the island, especially between Malabo and areas in the periphery, where the force of infection is significantly higher (74,75). In 2019, residents in both high and low travel areas reported an average of one trip to another part of the island in the past eight weeks (range: 1-5 trips), which did not substantially change in 2020. Therefore, it is possible that the remaining prevalence in high travel areas is from within-island parasite movement. However, there was no significant difference seen between prevalence in those who reported within island travel and those who did not in 2020 (**Table 3.6**). A final explanation is that local transmission continues to occur within high travel areas, and levels of endemic transmission persist when infections are removed. This is supported by a recent incidence study in Malabo suggesting local transmission occurring in peri-urban areas in Malabo district (112). While travelers tended to be more likely to have an infection, the study identified no incident infections related to travel, supporting the hypothesis that there is local transmission occurring, even in areas where travel is common. In addition, recent entomological monitoring in urban Malabo using human landing collections have confirmed the presence of anopheline vectors showing varying levels of human biting rates (unpublished data). Therefore, the results of this analysis suggest that control strategies that aim to reduce the malaria burden in travelers, either by reducing the burden in the areas where they travel, and/or by treating returning travelers, would impact the overall prevalence in several communities in Bioko. Additionally, control measures that aim to reduce local transmission, such as IRS, distribution of LLINs, and larval source management should be continued, even if additional interventions that target imported infections are introduced. Further analyses are needed to better understand the role of importation and local transmission at a more granular level.

Table 3.6. *Plasmodium falciparum* (*Pf)* prevalence by year and travel area in individuals who indicated travelling to the mainland in the past 8-weeks compared to those who did not report recent travel.

		Pf* prevalence *(95% CI)		
Year	**Travelled on Island**	**Overall**	**High travel areas**	**Low travel areas**
2019	No	13.3% (12.2, 14.4)	13.9% (12.5, 15.2)	7.2% (4.3, 10.0)
	Yes	13.5% (11.5, 15.6)	12.8% (10.1, 15.6)	9.3% (4.2, 14.6)
2020	No	11.8% (10.5, 13.1)	11.6% (9.8, 13.5)	12.4% (7.3, 17.6)
	Yes	13.1% (10.6, 15.4)	13.9% (10.6, 15.4)	13.8% (3.1, 24.5)

The interpretation of our results depends on several assumptions. First, it is assumed that the change in prevalence in low-travel areas is a valid estimate of the change we would expect in the high-travel areas in the absence of imported infections. That is, the two areas had parallel trends prior to the elimination of travel (111). While it is difficult to definitively verify this assumption, comparing data from the 2018 MIS to that from 2019 and 2020 suggested that prior to the halt in travel, there were similar trends over time in the high and low travel groups. Additionally, the final model adjusted for measured variables that impact malaria transmission and changed over time within travel groups (time variant factors). However, it is possible that there are unmeasured factors that were not accounted for in the model. Most notable would be changes in the ecological landscape. For example, an outbreak occurred in 2019 in a low-travel area in the south of the Island, because of recent construction that created additional breeding sites (108). There was no precise measure of land use over the study period, so it was not possible to adjust for this variable in the analysis. However, there were three areas known to have major changes in land use, including the one where the 2019 outbreak occurred. Two of these three areas showed very large prevalence differences from 2019 to 2020, suggesting there may have been additional transmission due to the changes in the ecological landscape. When these EAs were removed from the analysis, the difference in differences and ratio of ratios were slightly attenuated, but still of similar magnitude and significance. Therefore, if it were possible to precisely measure changes in the ecological landscape and include them in the model, we may expect a slightly lower prevalence difference, but the conclusions would remain the same. Another assumption of a difference-in-differences model is that secular trends are consistent over time and have the same impact on both travel areas. While there have been changes in the monthly amount of rainfall overtime on Bioko, there have been increases both in high and low travel areas, and the assumption is that this would equally impact malaria transmission potential in these areas.

The emergence of SARS-CoV-2 in early 2020 disrupted health systems around the world. As countries closed borders, limited movement, and restricted activities to curtail the initial spread of COVID-19, other public health programs were impacted. This is especially true for many malaria endemic countries, in which COVID-19 restrictions and global supply chain issues resulted in disruptions in the distribution of long-lasting insecticide nets, application of insecticides, and availability of anti-malarial medicines (106,113,114). The World Health Organization modeled the potential impact of disruptions to malaria interventions and estimated these disruptions could increase cases by upwards of 20% and deaths by greater than 50%, especially in scenarios where access to treatment was disrupted (115). Similar impacts were seen during the Ebola outbreak in 2014-2015 when health systems were disrupted (116–119). However, in these models and analyses, the potential impact of reducing importation and movement of *Plasmodium* infections was not considered (120,121). This analysis shows that on Bioko Island, where malaria control interventions remained largely uninterrupted during the pandemic (106) travel restrictions resulted in a decrease in malaria prevalence in areas with high travelers. It is possible that other areas with high proportions of imported infections may also have seen these decreases because of the travel restrictions, despite other interruptions to the health care system. This analysis suggests that the impact of COVID-19 on malaria burden may be underestimated in areas with a high prevalence of travelers. As malaria endemic countries continue to adapt their malaria control strategies in midst of the ongoing pandemic, the impact of imported infections, and possible reintroduction of these infections as travel restrictions are lifted, should be considered as part of control strategy discussions.

3.6 CONCLUSIONS

Travel restrictions initiated to limit the spread of COVID-19 allowed for a direct quantification of the impact of imported *Plasmodium* infections to malaria risk on Bioko Island. This analysis demonstrated that there is a substantial proportion of malaria risk that is attributed to the importation of travel. However, even without imported infections, there is evidence of local transmission. Therefore, strategies that aim to reduce local transmission as well as limit the number of imported infections should be considered to continue to drive the reduction of malaria burden on Bioko Island.

Chapter 4. Feasibility of community at-home dried blood spot collection combined with pooled reverse transcription PCR as a viable and convenient method for malaria epidemiology studies

4.1 ABSTRACT

Background: A significant proportion of *Plasmodium* infections in endemic regions are below the limit of detection of standard field diagnostic tools. These infections threaten control efforts and have the potential to impact vaccine and therapeutic efficacy studies. Efforts to understand the natural history of asymptomatic submicroscopic parasitemia is to a large extent curtailed by the associated high cost and logistical complexity of current strategies to evaluate such infections. The extent of the impact is highly dependent on the natural history, but a paucity of strategies to evaluate such infections has left gaps in our understanding. Simple, cost-effective ways to study low-density *Plasmodium* infections over-time are needed. Self-collected DBS analyzed using pooled and individual qRT-PCR techniques for *Pf* 18S rRNA provide such a solution. Here, the feasibility and acceptability of daily at-home DBS collections as a tool to study parasite dynamics of low-density infections was assessed.

Methods: RDT-negative individuals in Katakwi district, northeastern Uganda, were recruited between April and May of 2021 to collect daily at-home DBS for 28 days. Venous blood samples and clinic collected DBS were taken at enrollment and at four weekly clinic visits. Participants were trained in DBS collection and asked to collect six DBS per week between clinic visits. Participant opinions about the DBS collection process were solicited through daily Diary Cards and a 5-point Likert scale acceptability survey given at the final study visit. DBS and venous blood were analyzed by qRT-PCR. The number of participants completing the study, the total DBS collected, and the acceptability of the process and any reported pain were analyzed to determine compliance and acceptability of the study procedure. The human internal control mRNA and *Plasmodium* 18S rRNA were evaluated for the at-home versus clinic-collected DBS and venous blood to assess quality of the at-home collected samples and evaluate the accuracy of DBS as a parasite detection tool.

Results: One-hundred two adults and 29 children were enrolled, and 96 and 26 completed the study, respectively. Three individuals withdrew due to pain or inconvenience of study procedures. Overall, 97% of participants collected ≥16 of 24 at-home DBS, and 87% of all spots had ≥40 µL of blood. The procedure was well tolerated and viewed favorably by participants. At-home collected DBS were acceptable for qRT-PCR and showed slightly lower concentrations of human control mRNA compared to clinic-collected DBS (human internal control TBP mRNA cycle threshold 0.8 cycles earlier for clinic-collected DBS; 95%CI: 0.65, 0.98). Correlation between *Pf* 18S rRNA from paired whole blood and DBS samples was high (r=0.93, 95% CI: 0.91, 0.95).

Conclusions: At-home DBS collection is a feasible, acceptable, and robust method to collect blood to evaluate the natural history of low-density *Plasmodium* infections using *Pf* 18S rRNA qRT-PCR.

4.2 INTRODUCTION

Over the past decade, substantial progress has been made to reduce malaria morbidity and mortality. However, despite significant investment and robust national anti-malaria programs, malaria remains endemic in over 85 countries and territories. In 2020, there were 241 million estimated cases and 627,000 deaths (1). To continue to reduce malaria burden, new control strategies, therapeutics and vaccines are needed. To design the most effective control strategies and accurately assess efficacy of candidate vaccines and therapeutics, it is essential to understand the true malaria burden in endemic populations (122). Recent analyses show that a large proportion of *Plasmodium* infections in endemic regions are both asymptomatic and low-density (6), so they are not detected by standard field diagnostic tools. While these infections may not lead to clinical illness and death in the individuals who harbor them, they can contribute to onward transmission (4), and may result in immunomodulation (33) that impacts the effectiveness of vaccines and therapeutics. The impact of these infections on malaria transmission dynamics and extent of immunomodulation depends largely on the natural history of infection, but there is a lack of data and limited tools to evaluate parasite dynamics over time in large populations.

The few studies that have looked at natural infection of asymptomatic persons have shown that parasite densities are highly dynamic (21,22,123,124), and trajectories vary widely between study participants. While these studies are informative, samples were collected in clinics by trained staff, and therefore are limited in size and scope because of logistical and financial constraints. Additionally, infections in these studies were analyzed using microscopy (21) or DNA-based PCR (22,123,124), which are not as sensitive as molecular methods now available. To better understand how low-density infection impacts malaria transmission and malaria interventions on a large scale, more studies are needed with analytically sensitive methods and in a large variety of endemic areas. Unfortunately, many areas where malaria is endemic have limited access to health care resources. Even in areas with sufficient resources, daily travel to a clinic to provide samples is inconvenient and burdensome. Without methods to address these limitations, there will continue to be gaps in the understanding of low-density infections. Innovative techniques that are acceptable to participants are needed to allow frequent, accurate, and cost-effective sampling of low-density infections across a variety of settings.

DBS are a convenient, minimally invasive blood collection technique that does not require a clinic or phlebotomist. Blood collected on DBS cards is highly stable and can be stored and shipped at room temperature, making them an appealing option in areas with limited or infrequent electricity and lower the cost of shipping and storage. DBS have been used for malaria detection in a variety of research studies, and a recent meta-analysis concluded that DBS were non-inferior to venous blood samples for qualitative parasite detection across a variety of settings (48). Most studies that utilize DBS prepared them from capillary or venous blood drawn in a clinic setting, which limits their widespread utilization. However, self-collected DBS samples have been successfully used for the detection of hepatitis C (125), HIV (126,127), HbA1c levels (128), and monitoring of various drug or vitamin levels (129–134). This same technique could be applied to study malaria, making it more accessible in areas without health clinics, and allowing for more frequent sampling.

Most previous studies that utilized DBS for parasite detection relied on quantitative PCR of the *Plasmodium* 18S rRNA coding genes (49,135–143), but DNA-based DBS tests are much less sensitive than qRT-PCR for the actual *Plasmodium* 18S rRNAs, which means some infections may be missed (144). DBS have been used for 18S qRT-PCR(41,144,145), and methodology was recently adapted for pooling DBS (146), which allows for cost-effective, highly sensitive detection of malaria parasites.

Given the ease of DBS collection, stability of DBS analytes over time, and the suitability for pooling, self-collected DBS-based studies might be a feasible way to evaluate and monitor low-density *Plasmodium* infections. Here, the feasibility of daily at-home DBS collections as a tool to study *Plasmodium* dynamics was studied over a 28-day period in Katakwi district, Uganda.

4.3 METHODS

4.3.1 *Study Area*

The study was carried out at Med Biotech Laboratories Malaria Clinic in St. Anne Health Center III, Katakwi district, northeastern Uganda. The catchment area of the clinic includes seven villages in Usuk subcounty; participants were recruited from two of these villages. The classical rainy season in Katakwi district is March to November, with marked peaks in April-May and August-October. Transmission is high during the rainy season, peaking in July (72.0 cases/1000 people/month), and lower during the dry season (16.2 cases/1000 people in February) (77).

4.3.2 *Study Design*

This was a longitudinal cohort study to assess the feasibility of DBS collection over a 28-day period. Target sample size was 100 adults (18 to 60 years) and 30 children (8 to 17 years). The study was approved by the National HIV/AIDS Research Committee (NARC) of the Uganda National Council for Science and Technology (UNCST) [Approval #: ARC 228] as well as the University of Washington (UW) Institutional Review Board [STUDY00009434]. All adult participants provided written consent, while children provided assent along with written consent of a parent or guardian, per UNCST guidelines. After providing informed consent, study staff administered a 10-question assessment of understanding to potential adult participants (and/or parents of children), and only those who answered at least 80% correctly were permitted to continue with screening and enrollment.

After consenting, prospective participants were evaluated for eligibility and basic demographic information (age, sex, occupation) were collected. Participants answered questions about malaria prevention behaviors and were assessed for malaria signs. All data was captured through interviewer administered questionnaire. Forehead temperature, weight, height, blood pressure and pulse were measured, and all participants were offered a malaria rapid diagnostic test (RDT). Healthy volunteers who met inclusion criteria, including being asymptomatic for Grade 2 or higher malaria-related symptoms and negative for *Plasmodium* parasites by SD Malaria Ag P.f./Pan rapid diagnostic test (RDT, Standard Diagnostics Inc, Republic of Korea), were sequentially enrolled into the study until the sample size per each age category was attained. Full inclusion and exclusion are presented in **Table 4.1.**

Table 4.1. Full inclusion and exclusion criteria for participants participating in the daily at-home DBS collection study in Katakwi District, Uganda.

Inclusion Criteria	• Males and female children aged 8-17 years old (with assent procedures) and male and female adults 18-60 years • Asymptomatic for Grade 2 or higher malaria-related signs and symptoms and afebrile (<38.0°C) at baseline • Lives in one of the seven villages in Katakwi District, Uganda • Does not plan to move out of the study area for one month following enrollment. • Willing to self-collect a daily blood spot sample for 28-days (+/- 2 days). • Willing and able to return to the study clinic on a weekly basis to turn in DBS cards and provide 5 mL (adult) or 1 mL (child) of blood through venous draw at each visit. • Reliable access to the clinical sites and availability to participate for duration of study. • Able to fully understand the implications of study participation and provide informed consent. • Able to provide assent (for child participants) • Agreement to come to the study clinic if study participant experiences febrile illness or prick site infection during the study period. • Agreement not to take anti-malarial medications unless through the Malaria Clinic at St. Anne Health Center III. • Agreement not to take other medications without informing the study team. • Absence of any significant chronic disease.
Exclusion Criteria	• Being pregnant at enrollment or planning to get pregnant during the study period. • Currently taking antimalarial treatment(s). • RDT-positive at screening • Any other finding that, in the judgment of the investigator, would interfere with, or serve as a contraindication to, protocol adherence, assessment of safety, or a subject's ability to give informed consent, or increase the risk of having an adverse outcome from participating in the study.

Eligible participants attended an enrollment visit and were then invited to attend four weekly clinic visits and collect a single DBS each day of the week when they did not come into the clinic. They were trained in DBS collection by study staff at enrollment and retrained at subsequent clinic visits if necessary. Weekly, participants were provided with all materials to collect at-home DBS for the following week. One DBS card was designed to collect DBS for three days, placed on the first, third, and fifth spots on the card (**Figure 4.1)**. After DBS collection, participants were asked to fill out a daily Diary Card, indicating which spot they put the blood, if they had signs of fever or other symptoms, if they were willing to continue, and if they slept under a bednet the previous night. In addition, they were asked to rate the level of pain they felt after the blood prick, on a scale from 0 to 5 ("no pain" to "great pain"). Due to delays in obtaining a version of the Diary Card in the local language at the study start, information on the Diary Card was recorded retrospectively by clinic staff during weekly visits for the first week for all participants, as well as the second week for the first 11 participants enrolled. Once translated versions of the Diary Cards were obtained, participants filled them out at home, and they were reviewed with study staff at weekly visits. Examples of the Diary Card and Instructional placemat for DBS collection are provided in the **Appendix.**

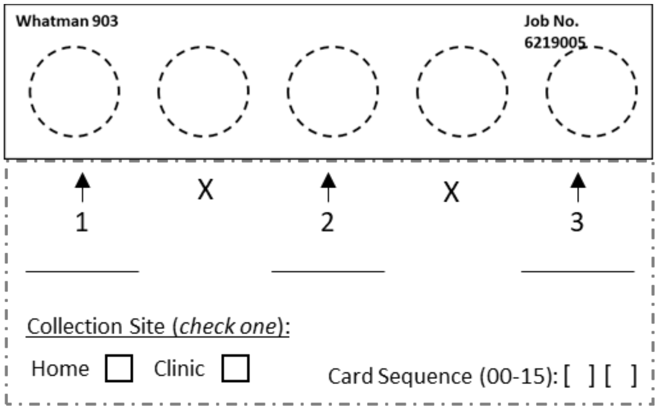


Figure 4.1. Example of labeled dried blood spot (DBS) card provided to participants. Numbers and arrows indicated where blood should be spotted.

During clinic visits, participants turned in collected DBS cards and received supplies for the following week. In addition, at each visit, two DBS were collected by clinic staff, a venous blood sample was taken, forehead temperature was record, and malaria signs and symptoms in the past 24 hours and malaria prevention behaviors in the past week were captured through a structured interviewer-administered questionnaire. Any participant with malaria signs and symptoms during the study period was tested using a RDT. Anyone positive by RDT was treated with antimalarials as per the Ministry of Health-Uganda guidelines (109) and subsequently withdrawn from further DBS participation. Acceptability of the study was assessed using a 5-point Likert scale questionnaire administered by study staff at the final clinic visit. The questionnaire assessed participants' feelings on the painfulness of the procedure, ease in collecting DBS, likelihood of collecting DBS for a longer period of time, and whether participants would prefer to come to the clinic over collecting DBS at home. Participants were compensated equivalent to $10 USD the screening/enrollment visit, and then $5 USD for each week's completed DBS card and $5 USD for subsequent study visits.

4.3.3 *Sample collection*

At each clinic visit, 4 mL of venous blood was drawn from adults and 1 mL from children into K_2-EDTA vacutainer tubes (BD-Themo Fisher Scientific Inc, Sweden). Within 24 hours, 50 µL of whole blood were transferred into two 2 mL screw top tubes containing 1 mL of pre-aliquoted NucliSENS lysis buffer (bioMerieux, Marcy-l'Etoile, France) and gently mixed by inverting the tube. The samples were immediately stored at -20^{o}C for a maximum of seven days at the field site before transfer to the central laboratory, where they were stored at <-70^{o}C. An additional 1 mL (adult) or 0.5 mL (child) of whole blood was transferred to a 2 mL tube and stored at ≤ -20 ^{o}C. The remaining blood was centrifuged, and the plasma supernatant was transferred to clean tubes and stored at ≤-20 ^{o}C.

DBS were collected on Protein Saver 903 DBS cards (Global Life Sciences Solutions, Buckinghamshire, UK) using automatic lancets (Accu-Chek Safe-T-pro Uno 1.5mm/MM 28G, Roche, Germany). Cards were stored in gas-impermeable plastic bags, with desiccators and a moisture indicator. After collection, cards were dried for at least four hours but no more than 12 hours, and then stored at room temperature. DBS cards were stored (five to six months) and shipped at room temperature until processing.

4.3.4 *Laboratory Methods*

Prior to cutting, the volume of blood on each spot to the nearest 10 μL was estimated by comparing the spot to a Protein Saver card with known volumes of pipetted blood. DBS spots were then laser cut using contact-free methods and deposited into 2 mL NucliSENS lysis buffer as described (147). Pools were constructed by combining 0.2 mL of lysis buffer from each of the 10 different tubes from the same participant (within-participant pooling). Extraction and qRT-PCR were then performed on the Abbott m2000 sp/rt systems using a triplex assay targeting *Pf* 18S rRNA, pan-*Plasmodium* 18S rRNA, and the human TATA binding protein (TBP) mRNA control as previously described (92,146). Initial runs for DBS were conducted using within-participant pools of up to 10 samples per pool. If the pool was negative, all samples were reported as negative. If the pool was positive, samples were deconvoluted and re-run individually. Venous blood samples were processed using methods previously described (92).

4.3.5 *Statistical Analyses*

To analyze the feasibility of DBS collection as tool to evaluate the daily dynamics of *Plasmodium* infections we assessed compliance, acceptability, and quality of the spots collected. Analyses were stratified by age category (adults or children) and sex (in adults only). Study data was captured on paper case report forms (CRFs) and then input and managed using REDCap electronic data capture tools hosted at University of Washington (148,149). Verification of data transcription by a second reviewer was performed on 10% of all entries. All statistical analyses were performed in RStudio v12.5033 (Boston, MA)

Characteristics of screened and enrolled participants were summarized using descriptive statistics. The number of participants who withdrew each week and reasons for withdraw are presented. Compliance was evaluated by summarizing the number of total home DBS that were collected throughout the study period. As there was no standard for what was expected, categories were created based on what were assumed to be reasonable patterns of compliance: 'Excellent' compliers were those that missed no more than two spots during the entire study (22-24 spots), 'good' compliers missed no more than 1-2 spots per week or one whole week of collection (16-22 spots), 'fair' compliers missed 2-3 slots per week or two collection weeks (11-14 spots), and 'poor' compliers missed more than half of the samples each week (<11 spots). The number and proportion of excellent and good compliers (≥16 DBS) and fair and poor compliers (<16 DBS) is presented. Individuals who were withdrawn because they developed malaria prior to Day 18 of the study were excluded, as they did not have the opportunity to collect at least 16 samples during their participation.

Acceptability was assessed through descriptive analyses of the final visit opinion survey answers as well as an evaluation of reported pain scores over time, collected via Diary Card. Mean daily pain scores and 95% CI were calculated and plotted to assess the overall pattern throughout the study. Weekly average pain scores were compared using a paired t-test. Mean values were summarized based on available data and missing dates were not imputed. As a secondary analysis, patterns of pain over time were categorized among individuals who had complete pain data reported, defined as a pain rating for each blood spot that was collected.

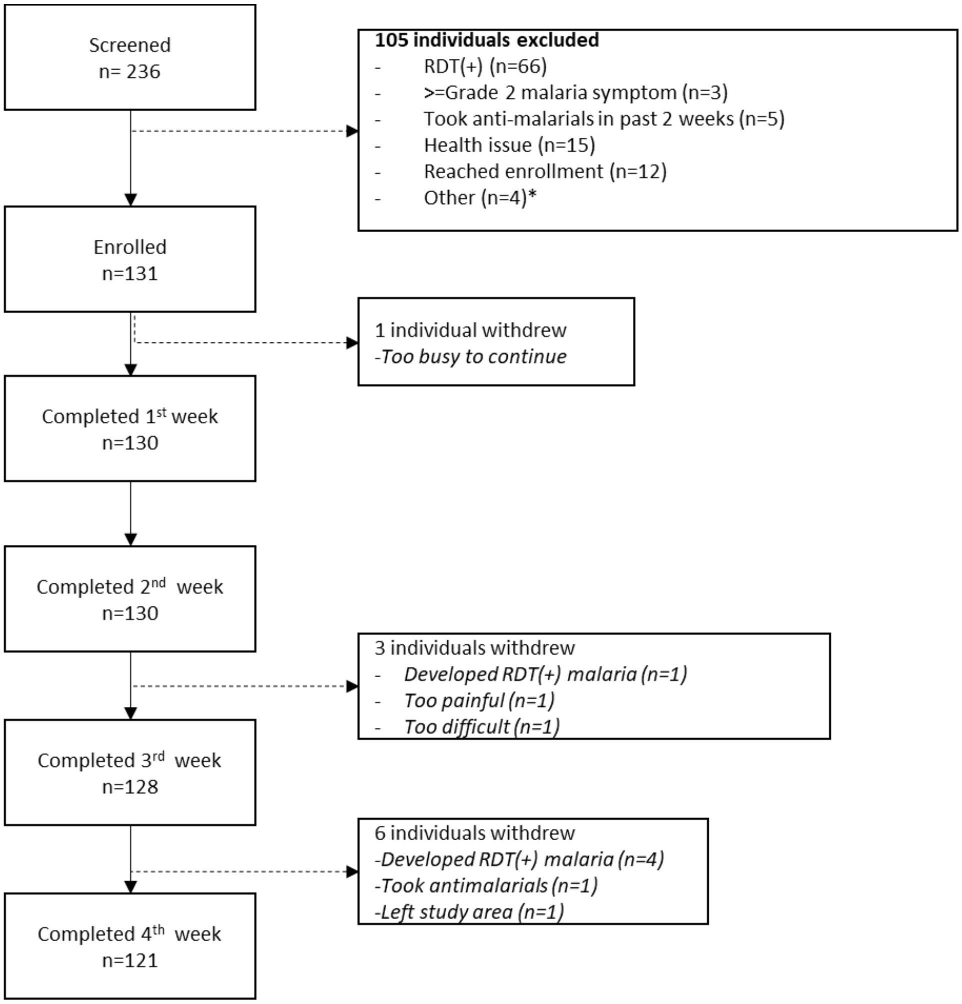
Quality of the DBS spot was evaluated by summarizing the volume of blood collected by participants each day, and the presence of the TBP quality control signal in each sample. The number and proportion of samples in each 10 µL volume category (10-50 µL) during each week of collection was calculated and compared as a measure of quality of DBS collection. To evaluate the integrity of the DBS and analyze whether there was possible degradation of samples collected, the number and proportion of total spots that failed quality control (had no TBP cycle threshold (CT)) were summarized. For each individual with deconvoluted samples, the mean TBP CT value and SD of all their samples was calculated. The proportion of individuals with TBP SD <1 cycle and >1 cycle was summarized. To analyze whether samples collected at home may have been of poorer quality than those collected at the clinic, the mean TBP CT values of clinic-collected DBS versus at-home-collected DBS samples were compared using Fischer's exact t-test. Mean TBP CT values and 95% CI of samples collected on the first day or each week were compared to those collected on the final day of sampling each week using Fischer's exact t-test to determine whether those collected earlier in the week, and therefore exposed to more air, may have been subject to further degradation.

Finally, to assess the accuracy of DBS as a parasite detection tool for low-density infections, the correlation between estimated parasite copy numbers detected from paired clinic collected liquid and DBS samples was estimated using Pearson's correlation coefficient. As the limit of detection is slightly lower for venous blood samples (10 parasites/mL) compared to DBS (20 parasites/mL), venous blood samples positive below the DBS LoD were excluded from the analysis. Additional sensitivity analyses were conducted using volume adjusted *Pf* 18S rRNA copy numbers for DBS, as well as restricting analyses to include only DBS with 50 µL of blood.

4.4 RESULTS

4.4.1 *Study enrollment and demographics*

This study recruited and enrolled 102 adults (median age 33 years; range 18-59) and 29 children (median age 13 years, range 8-17) from Opoyongo and Oleroi villages between April 9 and April 22, 2021. The final study visit occurred on May 20, 2021. Characteristics of those screened and enrolled and study flow are shown in **Table 4.2** and **Figure 4.2**, respectively. In both children and adults, a higher proportion of males were screened than enrolled, and a higher proportion of those enrolled reported sleeping under a bednet the previous night compared to those screened. There were no notable differences in other demographic or malaria behavior characteristics between those enrolled and not enrolled. The main reason for exclusion was being RDT(+) at screening: 29 children (42.0%) and 37 adults (22.2%) tested positive for *Pf* during the screening visit**,** for an overall prevalence of RDT(+) malaria of 28% (95% CI: 22.6, 34.0) in the screened population.



*other reasons for exclusion: too old (1), plans to leave study area (1), fear of pricking (1), judged to not be able to handle pain (1)

Figure 4.2. Study disposition showing the number of adults and children screened, enrolled, and continuing in the study by week.

Table 4.2. Demographic, health, malaria prevention behaviors and malaria symptoms in adults and children screened and enrolled for the 28-day at-home DBS feasibility study in Katakwi, Uganda.

	Children		**Adults**		**Overall**	
	Not Enrolled (N=40)	**Enrolled (N=29)**	**Not Enrolled (N=65)**	**Enrolled (N=102)**	**Not Enrolled (N=105)**	**Enrolled (N=131)**
Demographics and Health						
Age (years)	12 (± 2.6) [8.0, 17]	13 (± 2.6) [8.0, 17]	34 (± 13) [18, 64]	33 (± 12) [18, 59]	25 (± 15) [8.0, 64]	29 (± 13) [8.0, 59]
Male sex (%)	24 (60.0%)	12 (41.4%)	44 (67.7%)	42 (41.2%)	68 (64.8%)	54 (41.2%)
Weight (kgs)	34 (± 9.6) [19, 63]	39 (± 11) [22, 59]	57 (± 6.9) [41, 73]	58 (± 8.7) [41, 85]	48 (± 14) [19, 73]	54 (± 12) [22, 85]
Height (cm)	34 (± 9.6) [19, 63]	39 (± 11) [22, 59]	170 (± 23) [75, 200]	170 (± 16) [82, 200]	170 (± 25) [54, 200]	170 (± 20) [50, 200]
Febrile at Screening	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Village						
Opoyongo	32 (80.0%)	19 (65.5%)	33 (50.8%)	45 (44.1%)	65 (61.9%)	64 (48.9%)
Oleroi	8 (20.0%)	10 (34.5%)	32 (49.2%)	57 (55.9%)	40 (38.1%)	67 (51.1%)
Occupation						
Peasant Farmer	2 (5.0%)	0 (0%)	60 (92.3%)	96 (94.1%)	62 (59.0%)	96 (73.3%)
Not applicable	37 (92.5%)	27 (93.1%)	5 (7.7%)	5 (4.9%)	42 (40.0%)	32 (24.4%)
Other	1 (2.5%)	2 (6.9%)	0 (0%)	1 (1.0%)	1 (1.0%)	3 (2.3%)
Malaria Prevention Behaviors						
Slept under bednet previous night	32 (80.0%)	25 (86.2%)	55 (84.6%)	94 (92.2%)	87 (82.9%)	119 (90.8%)
Slept under net in past week	32 (80.0%)	25 (86.2%)	56 (86.2%)	94 (92.2%)	88 (83.8%)	119 (90.8%)
House has open eaves	39 (97.5%)	29 (100%)	64 (98.5%)	102 (100%)	103 (98.1%)	131 (100%)
Principal wall material						
Mud	38 (95.0%)	29 (100%)	64 (98.5%)	99 (97.1%)	102 (97.1%)	128 (97.7%)
Brick	2 (5.0%)	0 (0%)	1 (1.5%)	3 (2.9%)	3 (2.9%)	3 (2.3%)
House sprayed with insecticide in past 6 months	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
House smeared with insecticide-treated soil	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Spends more than 2 hours outside of house between dusk and dawn	40 (100%)	29 (100%)	65 (100%)	101 (99.0%)	105 (100%)	130 (99.2%)
Grade 2 or Higher Malaria Symptoms reported						
Chills/Rigors	1 (2.5%)	0 (0%)	0 (0%)	0 (0%)	1 (1.0%)	0 (0%)
Headache	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Fatigue/malaise	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Myalgia	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Low back pain	0 (0%)	0 (0%)	1 (1.5%)	0 (0%)	1 (1.0%)	0 (0%)
Vomiting	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Diarrhea	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Abdominal pain	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Arthralgia	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Chest Pain	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

4.4.2 *Compliance*

Ten of the 131 participants (8%) did not complete the full study. One adult withdrew in the first week, citing being too busy to continue. Three participants withdrew between the second and third weeks of collection; two cited the procedure as too painful or too difficult, and one had RDT(+) malaria. Six participants were withdrawn during the final week of collection; four were withdrawn because they developed symptomatic, RDT(+) malaria, one left the study area, and one took antimalarial medications (**Figure 4.2 and Table 4.3**).

Table 4.3. Summary of compliance in dried blood spot collection over the 28-day study period for adults and children. A total of 24 home blood spots were possible for individuals that completed the entire study.

	Adult Females	**Adult Males**	**Children**	**All**
Excellent or Good compliers (>=16 samples collected)$^{+}$	60/60 (100%)	36/38 (95%)	27/28 (96%)	122/126 (97%)
Fair or poor compliers (<16 samples collected)$^{+}$	0/60 (0%)	2/38 (5%)	1/28 (4%)	3/126 (2.4%)
Developed RDT(+) *Pf* malaria during study*	2/60 (3.3%)	2/42 (4.8%)	1/29 (3.4%)	5/131 (3.8%)
Discontinued in week 1*	0/60 (0%)	1/42 (2.4%)	0/29 (0%)	1/131 (0.8%)
Discontinued in week 2*	0/60 (0%)	0/41(0%)	0/29 (0%)	0/130 (0%)
Discontinued in week 3*	0/60 (0%)	1/40 (2.5%)	1/29 (3.5%)	2/129 (1.6%)
Discontinued in week 4*	1/58 (1.7%)	1/38 (2.6%)	0/26 (0%)	2/122 (1.6%)

$^{+}$ Denominator includes DBS samples received at University of Washington laboratory, and excludes 1 individual (adult male) who developed malaria before Day 18 of study.

* Denominator includes all participants enrolled in study. Excludes any individuals who discontinued because of malaria.

Of the 131 enrolled individuals, DBS cards were received at the University of Washington for 99/101 adults and 28/29 children and are included in the assessment of compliance. Of the 126 individuals who did not develop RDT(+) malaria by Day 18 of the study, 97% of individuals were good or excellent compliers **(Table 4.3)**. Only one adolescent (4%) and two adults, both of whom were males, collected <16 samples during the study.

4.4.3 *Acceptability*

Acceptability of the study procedure was high (**Figure 4.3)**. Almost all (93%) participants indicated that pricking their finger was a simple task, and only 7% of participants indicated they would prefer to come to the clinic to have someone else collect the DBS for them. While most participants viewed the process positively, about one-quarter (21%) said they would not participate in this type of collection for a longer period. Survey reliability was determined by congruency between questions designed to elicit opposite responses from participants. For example, when comparing the responses to “pricking my finger was a simple task” and “it was difficult to collect a blood spot each day”, congruency between questions was 81% (95% CI: 73%-87%). Congruency was 75% (95% CI: 67%-82%) when comparing responses to “pricking my finger produced minimal pain’ and “pricking my finger was painful”.

The mean pain score and 95% CI by day and age group is shown in **Figure 4.4,** and the average weekly pain scores and 95%CI are presented in **Figure 4.5.** Most participants reported little to no pain from the procedure throughout the study duration. While the average weekly pain scores were always below 1, there was a significant difference in the pain reported in week 2 compared to week 1 (0.14 vs. 0.08, p=0.004), and weeks 3 and 4 (0.08 and 0.083, respectively). Complete data reporting for pain was available for 82 participants (62%), 67 adults and 15 children. Among those, two-thirds of participants reported no pain from the finger prick during the entire study. Adult males were more likely to report no pain compared to adult females and children (80% vs. 59% and 60%, respectively). Among those who reported pain, 12% only reported pain in the first two weeks of the study, while an additional 15% reported pain during the second study week only.

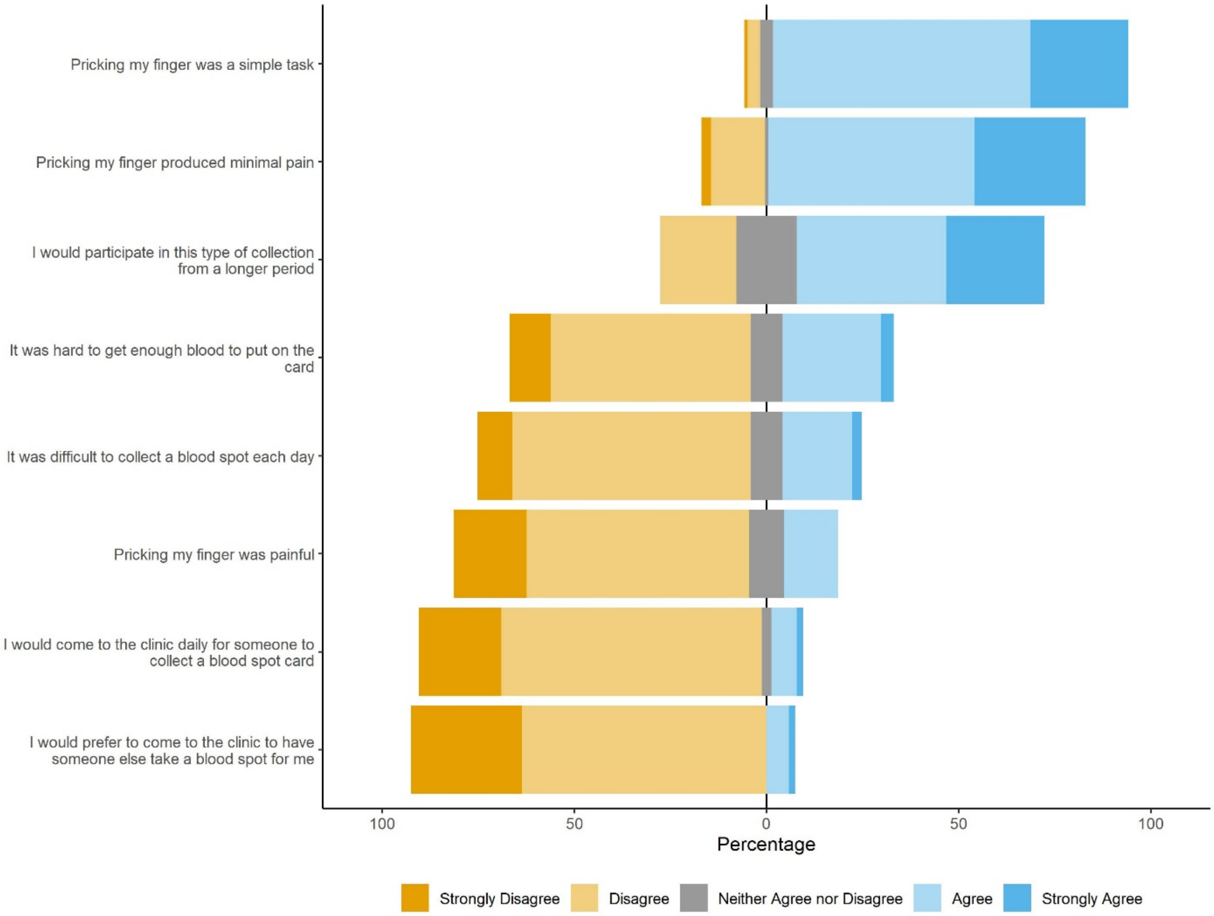


Figure 4.3. Responses from 123 participants to the Likert-scale questionnaire soliciting opinions about the DBS procedures.

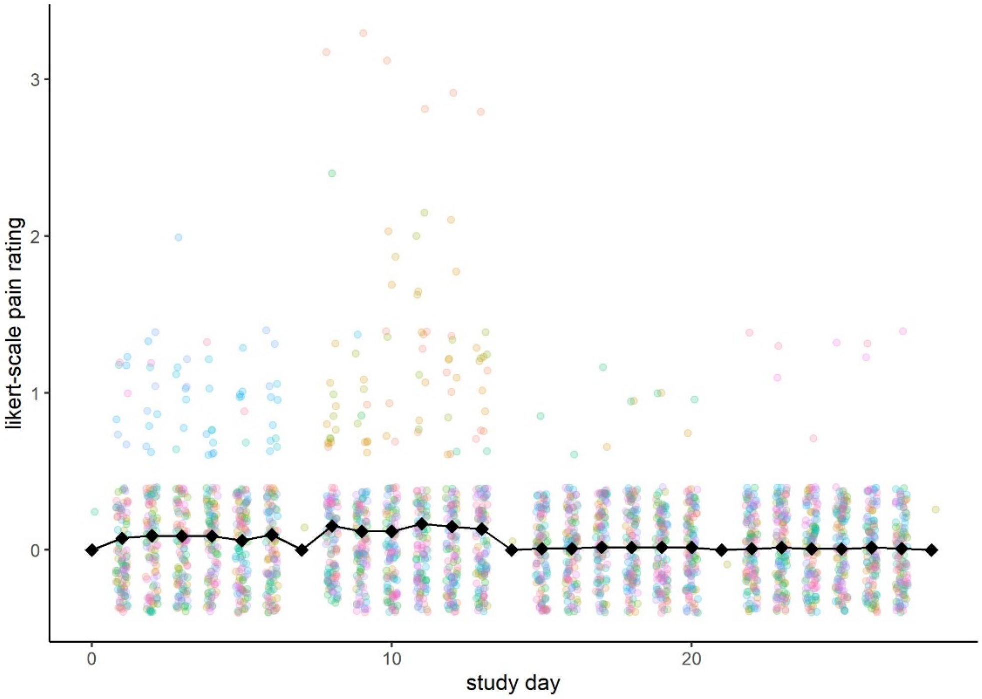


Figure 4.4. Reported pain of finger prick procedure by day of study. 0=no pain to 5=great pain. Children and adults presented together.

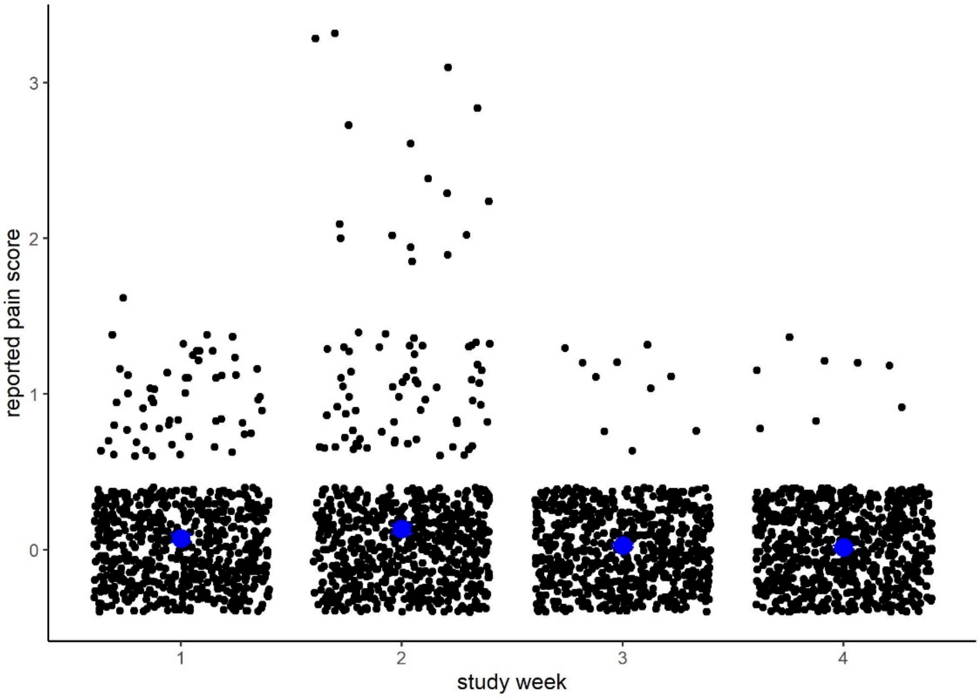


Figure 4.5. Reported pain by study week. Blue dots represent the mean weekly score, black dots represent individual responses.

4.4.4 *DBS quality*

The volumes of 2,955 individual DBS were estimated. Overall, 73% had ≥50 µL, and 87% of samples had ≥40 µL of blood, volumes considered optimal for the qRT-PCR assay (**Table 4.4**). There were 44 individuals (35%) who had sufficient blood on every spot collected during the study period. Blood volume was lowest in week one when only 48% of samples had ≥50 µL of blood. In subsequent weeks, nearly all DBS were of sufficient volume.

There were 76 individuals who had *Plasmodium* parasites detected at some point during the 28-day follow-up period. From these individuals, there were 1,731 individual DBS that were deconvoluted and available for the analysis of human TBP mRNA quality. The mean TBP CT for these spots was 34.97±1.22 cycles (range: 28.69 – 39.23). Individually, the standard deviation (SD) among all samples collected was less than one cycle for 55 participants (72%), while 21 participants (28%) had SD of greater than one cycle. The maximum SD of any participant was 1.62 cycles.

Table 4.4. Number and proportion of total samples that were estimated to certain volumes by study week.

Spot volume	**Week 1 (n= 747)**	**Week 2 (n=756)**	**Week 3 (n=743)**	**Week 4 (n=708)**	**All (n= 2955)**
≥50 µL	357 (0.48)	589 (0.78)	613 (0.83)	612 (0.86)	2171 (0.73)
40 µL	126 (0.17)	108 (0.14)	108 (0.15)	78 (0.11)	420 (0.14)
30 µL	114 (0.15)	38 (0.05)	21 (0.03)	15 (0.02)	189 (0.06)
20 µL	87 (0.12)	16 (0.02)	1 (0.00)	3 (0.00)	107 (0.04)
10 µL	41 (0.05)	3 (0.00)	0 (0.00)	0 (0.00)	44 (0.01)
<10 µL	22 (0.03)	2 (0.00)	0 (0.00)	0 (0.00)	24 (0.01)

Among deconvoluted samples, there were 305 spots collected at the clinic and 1427 spots collected at home. The TBP CT for clinic-collected spots was slightly earlier than for at-home collected spots (mean clinic-collected TBP CT 0.85 cycles earlier than at-home DBS, 95% CI: 0.71, 0.99). When comparing DBS collected on the first day of weekly home collection (n=237) to the last day of weekly home collection (n=237), there was no difference in TBP CTs in these samples (35.13 versus 35.12), suggesting that samples stored longer at home and removed more frequently from bags did not degrade any more than those collected at the end of each week. There was also no difference in the average TBP CT values of samples collected during each week of the study (**Table 4.5**). In-clinic collected DBS had average TBP CTs that were 2.59 cycles later than for venous blood (95% CI: 2.43 – 2.72 cycles), indicating that there is some loss of the human mRNA control target on DBS compared to venous blood.

Table 4.5. Comparison of mean TBP CT values for DBS collected at various times and locations

Group	n	Mean TBP CT (SD)	Difference (95% CI)
Home spots	1427	35.1 (1.2)	*(ref)*
Clinic spots	305	34.3 (1.1)	0.85 (0.71 , 0.99)
Week 1	362	35.1 (1.4)	*(ref)*
Week 2	367	35.1 (1.1)	-0.06 (-0.12, 0.24)
Week 3	358	35.2 (1.1)	0.02 (-0.20, 0.16)
Week 4	339	35.1 (1.2)	-0.05 (-0.14, 0.24)
First day of week	237	35.1 (1.1)	*(ref)*
Last day of week	237	35.1 (1.2)	-0.01 (-0.21, 0.22)

There were 617 paired venous blood and DBS samples taken at the clinic from 128 participants. There was very high correlation between the *Pf* 18S rRNA copy numbers from paired DBS and venous blood samples (Pearson’s R=0.93, 95% CI: 0.92, 0.94) (**Figure 4.6**). Of the 617 sample pairs, 136 were positive in both venous blood and DBS samples, 451 were negative in both samples, 11 were positive by DBS but negative in venous blood, and 19 were positive in venous blood and negative in DBS. Amongst the 11 DBS-positive/venous-negative discrepant samples, the median copy number was 5.8 [IQR: 5.7 – 6.1]. Amongst the 19 DBS-negative/venous-positive discrepant samples, the copy median number was 5.7 [IQR: 5.5-6.2]. There was no difference in correlation when restricting analyses to DBS with 50 µL of blood or when using volume adjusted copy numbers for DBS samples (**Figure 4.7)**, suggesting that this technique is highly robust, even when the target ≥40 µL volume of blood is not obtained.

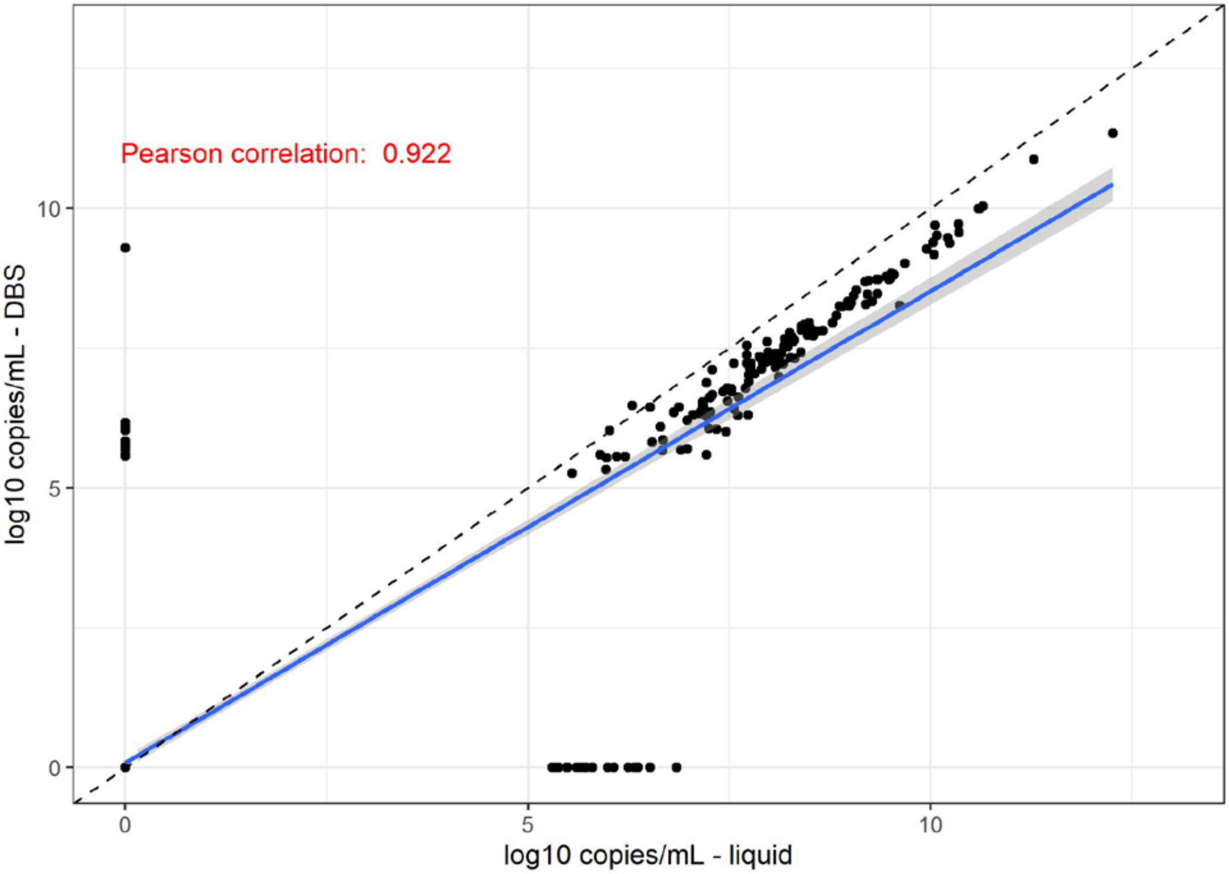


Figure 4.6. Correlation between $\log_{10}$ copy numbers of *Pf* parasites estimated by paired venous and DBS samples collected in Katakwi district, Uganda at weekly clinic visits.

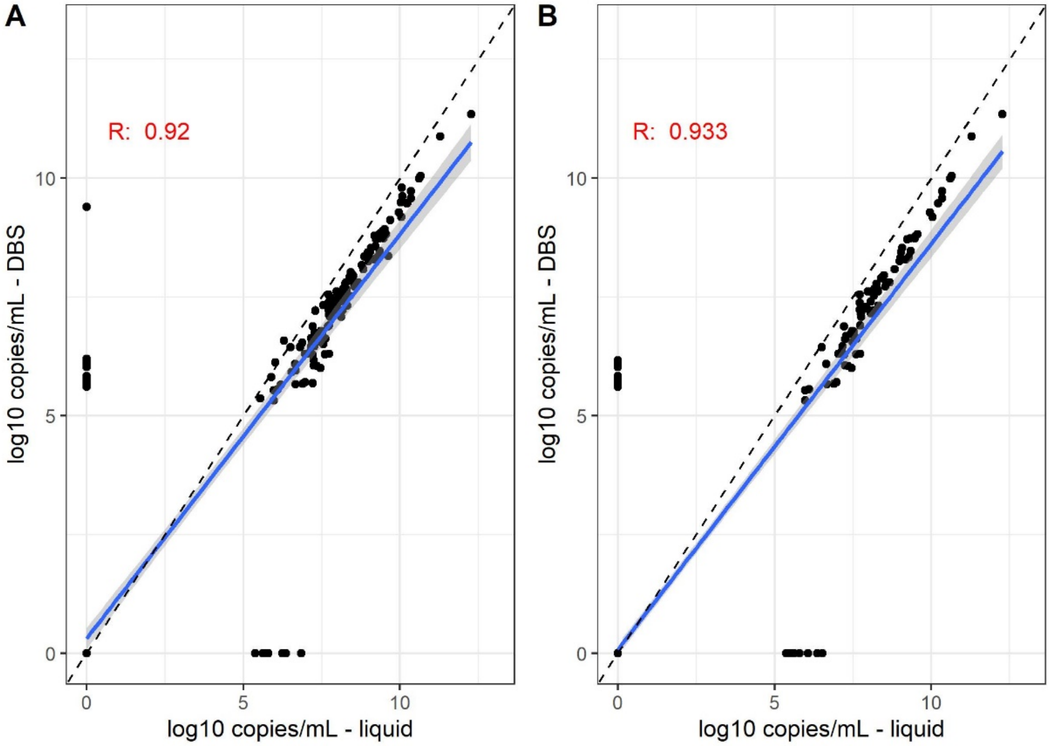


Figure 4.7. Correlation between venous blood and DBS samples collected during weekly clinic visits. **(A**) includes all sample pairs but uses volume-adjusted copy numbers for DBS; (**B**) restricts analysis only to pairs in which the DBS had 50 µL of blood.

4.5 DISCUSSION

This study demonstrates that daily, at-home DBS collection is a feasible and acceptable method to study the natural history of low-density *Plasmodium* infections over time. The within-participant pooling strategy also provided substantial cost savings. In addition to this analysis on the feasibility and acceptability of at-home DBS for malaria studies, the dynamics of these asymptomatic infections provided important epidemiological insights and will be the subject of another forthcoming publication.

Compliance with at-home DBS collection was extremely high. Only five (4%) participants withdrew from the study early due to pain or inconvenience of the collection procedures. Among those who stayed in the study, there was a high rate of compliance, and 85% of participants collected all DBS during the study period. Compliance in this study is similar or higher than compliance in previous studies that looked at longitudinal dynamics through clinic sampling. In a study of adult males in Mali who were sampled two or three times a day for 12-13 consecutive days, 98.5% of all planned samples were collected (21). In a study of afebrile Mozambican men, 28% of participants missed one follow-up visit at the clinic (123). Both of these studies were either for a shorter overall duration or required fewer samples over the same time period. It is a strength of the current study technique that there was such high compliance with the longer time and frequency of sampling. About one-third of participants in the current study indicated they would not want to continue in daily sampling for a longer period of time, suggesting that rates of discontinuation may be greater if such studies were extended for a longer time. Additional qualitative and quantitative analyses are needed to determine the optimal sampling frequency and duration.

Participants found the at-home sampling to be a simple and low-pain process; no participant reported pain >3 on a 5-point Likert-scale, and the average weekly pain score was always below one. The highest pain scores were reported in week 2 of the study. There are several possible explanations for this. First, several participants had to be retrained after the first week of blood collections because there was insufficient blood on their week 1 DBS cards. Insufficient blood can be a result of not pricking the finger deeply enough or not massaging the finger sufficiently to get blood. Therefore, it is possible that participants were not pricking themselves correctly during week one, and when they were retrained in proper technique, there was greater penetration and as a result, slightly more pain. Another explanation for the heightened pain in week 2 could be because participants were pricking their finger for the second time. If the prick site had not fully healed, it may have been more painful the second time. However, if this were the explanation, we would have expected pain scores to stay increased through week 3 and 4 when additional repeat pricking was performed, but that was not reported. Finally, it is possible that the increased pain scores in week 2 are a result of reporting biases. Information on pain for week 1 was captured retrospectively at the clinic. It is possible that participants were fearful to report pain to study staff because they wanted to continue in the study, and therefore pain scores in week 1 reflect social desirability bias more than true reflection of the pain of the procedure. Even if there was some bias in the reporting, the overall pain scores throughout the study were low, and pain did not seem to be a barrier to completing the 28-day collection for most participants.

In addition to being feasible to collect, our analyses showed that the DBS collected were of high quality and produced comparable readings to venous blood collections. Comparison of mean human TBP mRNA CTs from DBS collected at the clinic versus home showed a small reduction in mRNA signal in at-home collected DBS, but both at-home and in-clinic DBS were sufficient to obtain quality qRT-PCR results. Unsurprisingly, the TBP CT values in venous blood samples were earlier than in the DBS collected in-clinic on the same day. However, there was minimal difference seen in the quantification of *Pf* parasites in these samples, supporting the high stability of the 18S rRNA malaria target in the sample (92,146). Similar results have been seen in other studies comparing venous to DBS samples; a recent meta-analysis of seven such studies concluded there was no qualitative difference in parasite events detected from DBS compared to whole blood (48). Amongst the *Pf* 18S rRNA discrepant samples (DBS-positive/venous blood negative and vice versa) seen in the current study, most discrepant samples were in the estimated parasite density range whereby discrepancies could be explained by the Poisson distribution for rare events. The qRT-PCR approach was recently shown to reliably detect a single parasite in a 50 µL sample when limiting Poisson distribution sampling was overcome (Matsubara 2022, in press).

Given the collection, storage, and shipping benefits of DBS over venous blood, the current analysis adds to the evidence that there is little quality difference in the results from each samples type.

Surprisingly, there was no difference in the quality of sample, as measured by human TBP mRNA, when comparing DBS collected at the beginning of a sample week to those collected at the end. Home collected samples, especially the first spot on each card, were subjected to greater handling, removal from desiccant, and exposure to the air than samples collected at the end of the week or at the clinic. Therefore, one might expect more degradation in these samples. Since this was not observed, the data supports a strategy whereby a single DBS card can be used to reliably collect multiple samples at different points in time without sacrificing the quality of the sample. This study was the first to show that self-collected DBS spots can be used to quantitatively study *Plasmodium* infections over time. The simplicity, stability, and cost-effectiveness of this method will allow for large-scale evaluations of low-density infections in malaria-exposed populations. Home DBS collection overcomes several logistical difficulties encountered in previous studies because such DBS do not require clinic staff nor travel time for participants. Participants in this study overwhelmingly preferred at home collections to clinic collections, and several of them commented on the convenience and time saving nature of the home collection approach. Home collection could be made even more convenient if participants could either mail in samples, as has been done with previous self-collected DBS for other diseases (126), or if DBS could be collected by village health workers or other Ministry of Health networks that work routinely in communities. Thus, the use of DBS has the potential to expand malaria surveillance into areas that are typically hard to reach due to lack of health infrastructure, improve clinical trial designs, and deeply enhance our understanding of the natural history of low-density *Plasmodium* infections.

4.6 CONCLUSIONS

At-home DBS collection for analysis by qRT-PCR using within subject pooling is a feasible, cost-effective strategy to study dynamics of low-density *Plasmodium* infections. This technique should be utilized to improve current understanding of the contribution of low-density parasite infections to the infectious reservoir, and the possible impact of these infections on therapeutic and vaccine efficacy studies.

Chapter 5. Discussion

5.1 FUTURE DIRECTIONS FOR MALARIA CONTROL ON BIOKO ISLAND

Low-density *Pf* infections and imported malaria cases threaten malaria reduction and elimination efforts. In **Chapter 2,** it was confirmed that there are many subpatent infections on Bioko Island, Equatorial Guinea. The prevalence of these infections was greater in households with individuals with an RDT-detectable infection than in those with no RDT-detectable infections. This pattern of clustering could be leveraged when designing control strategies targeting these infections. Utilizing a household treatment strategy may target a large proportion of these infections (84), but would require the use of more expensive, higher sensitivity diagnostics. When the analysis was stratified by the travel history of the RDT(+) individual, the associations were stronger in households with no previous travel but were also still present in those with recent travelers. This suggests that there may be continued onward transmission of imported infections on the island.

This hypothesis was further strengthened by the analysis in Chapter 3**.** Leveraging a travel moratorium in response to COVID-19, it was possible to directly estimate the impact of imported infections to malaria prevalence on Bioko Island using a difference in differences analysis. This analysis suggested that the *Pf* prevalence observed in areas with historically high rates of travel would have been 7% higher than what was seen if importation of infections had continued. Furthermore, when importation was eliminated, there was no difference in the odds of infection comparing high and low travel areas, suggesting that levels of endemic transmission persist even in the absence of importation.

Combined, the analyses from these two Chapters support continuing current control strategies in Bioko Island, while adding additional measures that target imported infections to further reduce prevalence. Two such options could be testing and treating at ports of entry or periodic mass drug administration in communities with high proportions of travelers. An MTAT strategy is appealing because it specifically targets individuals with confirmed parasitemia, which may increase adherence to treatment. MTAT involves minimal daily staff and provides easy access to the at-risk population. This strategy would target inhabitants of Bioko Island returning from travel to higher endemicity areas, as well as visitors coming from those areas, which may better mirror the impact that was observed during the travel restrictions. However, this strategy may prove to be logistically difficult depending on the volume of flights and ferries arriving each day and the number of people that would need to be tested. This strategy may also miss infections that were newly acquired, as well as those below the LoD of the selected RDT. Targeted mass drug administration (MDA) in those communities identified to have the greatest proportion of travelers could periodically reduce parasite burden in these areas and reduce overall prevalence, similar to what was seen with the travel moratorium in **Chapter 3**. However, MDA programs historically are met with suboptimal compliance, and raise concerns about increasing drug resistance, especially if individuals do not complete treatment (150). This strategy would also not target infections imported with travelers not residing on the island, so may be less impactful overall.

5.2 FUTURE DIRECTIONS FOR UTILIZING AT-HOME DBS COLLECTION

The best control strategies to target imported and low-density infections may depend on better understanding of the longitudinal history of these types of infections. In **Chapter 4**, it was shown that at-home DBS collection is a feasible and accurate way to analyze *Plasmodium* infections over time. This methodology can be widely deployed to increase the understanding of malaria epidemiology across a wide range of endemicities. A recent publication by Drakeley and colleagues (3) described several theoretical trajectories of asymptomatic infection which differentially affect our understanding of asymptomatic infection, carriage, and clearance. The Drakeley team identified several gaps in asymptomatic malaria research and three key metrics that need to be determined to understand how asymptomatic infections contribute to ongoing transmission:

(i) the proportion of asymptomatic individuals that later develop symptoms and seek treatment;

(ii) the distribution of the duration of asymptomatic infections in populations; and

(iii) the relative infectivity of asymptomatic infections.

Using the at-home DBS collection protocol that was validated in **Chapter 4,** studies can be designed to answer these questions. Large-scale epidemiologic surveillance studies can be deployed that collect weekly or bi-weekly blood samples from individuals and track symptoms overtime to help answer questions one and two. Additional modeling studies with the data from **Chapter 4** are planned to help determine the ideal frequency of sampling. Less frequent sampling may overcome barriers to longer sampling times, but could also lead to more missed sampling, as it becomes less routine. Adherence could be improved through the utilization of SMS reminders or village health units.

Additionally, self-sampling DBS collection could also be utilized to enhance clinical trial designs for vaccines and therapeutics. Data from **Chapter 4** revealed many low-density infections and varying transmission dynamics within those infections over a 28-day period. The in-depth analyses of these transmission dynamics will be presented in a future publication, but warrant mention here. There were also several days in which parasite densities in infected individuals dipped below the limit of detection of qRT-PCR; implying that some infections may be missed with single point-in-time sampling, even when using highly sensitive diagnostics. As even low-density infections can impact immune responses (151), it seems imperative that the true *Plasmodium* infection status and recent history of trial participants be characterized. Instead of testing participants at a single screening visit, more frequent samples could be taken in the weeks or month prior to enrollment, providing a more accurate assessment of the infection status of participants. A recent analysis utilized DBS to identify antibody signatures of asymptomatic infections (151). This analysis used a sample from a single point in time. The methodology could be expanded to allow for longitudinal sampling and the development of serologic and parasite markers that may allow the classification of the natural history of infection from fewer samples in the future.

Finally, self-collected DBS present a solution for malaria surveillance during times of health system interruption, as was seen with the Ebola outbreak in West Africa in 2014 to 2016 and recently with the global COVID-19 pandemic. During times of non-malaria disease outbreaks, individuals are less likely to access health care (116,117), malaria prevention strategies are interrupted, and even when they continue, are often met with lower acceptance in the communities (106). In addition, recommended strategies often shift away from testing and move towards presumptive or universal treatment (152). While these strategies provide immediate solutions to control morbidity and mortality, it is difficult to fully assess their impact and do not allow for continued disease surveillance. Self-collected DBS could be combined with these strategies to allow for continued monitoring of malaria epidemiology during these times of disruption, without necessitation the population to enter health clinics or have lengthy interactions with field staff.

5.3 CONCLUSIONS

Enormous strides have been made to increase our understanding of *Plasmodium* infections and improve strategies to target infected individuals and reduce burden. However, much remains unknown, especially regarding the impact of subpatent, asymptomatic infections and imported infections. Identifying patterns and characteristics of subpatent infections as was done in **Chapter 2** can help countries create control strategies that target these infections without the need for sensitive diagnostics. While importation can be difficult to study, travel restrictions imposed to slow the spread of COVID-19 allowed for the direct evaluation of the contribution of parasite importation to malaria prevalence on Bioko Island in **Chapter 3**. As restrictions are lifted, countries and regions should consider strategies that reduce the movement of parasites. While insights can be gained from cross-sectional studies, more longitudinal data is needed to better understand the impact of low-density and imported *Plasmodium* infections. In **Chapter 4**, we showed that at-home daily DBS collection is one such approach to gather this data. At-home DBS was feasible and well accepted, and this approach should be expanded to study low-density infections on a greater scale. Together, these analyses support that future malaria control strategies should consider both subpatent and imported infections, and additional epidemiology studies should be done using at-home DBS to better understand the dynamics of these infections over time.

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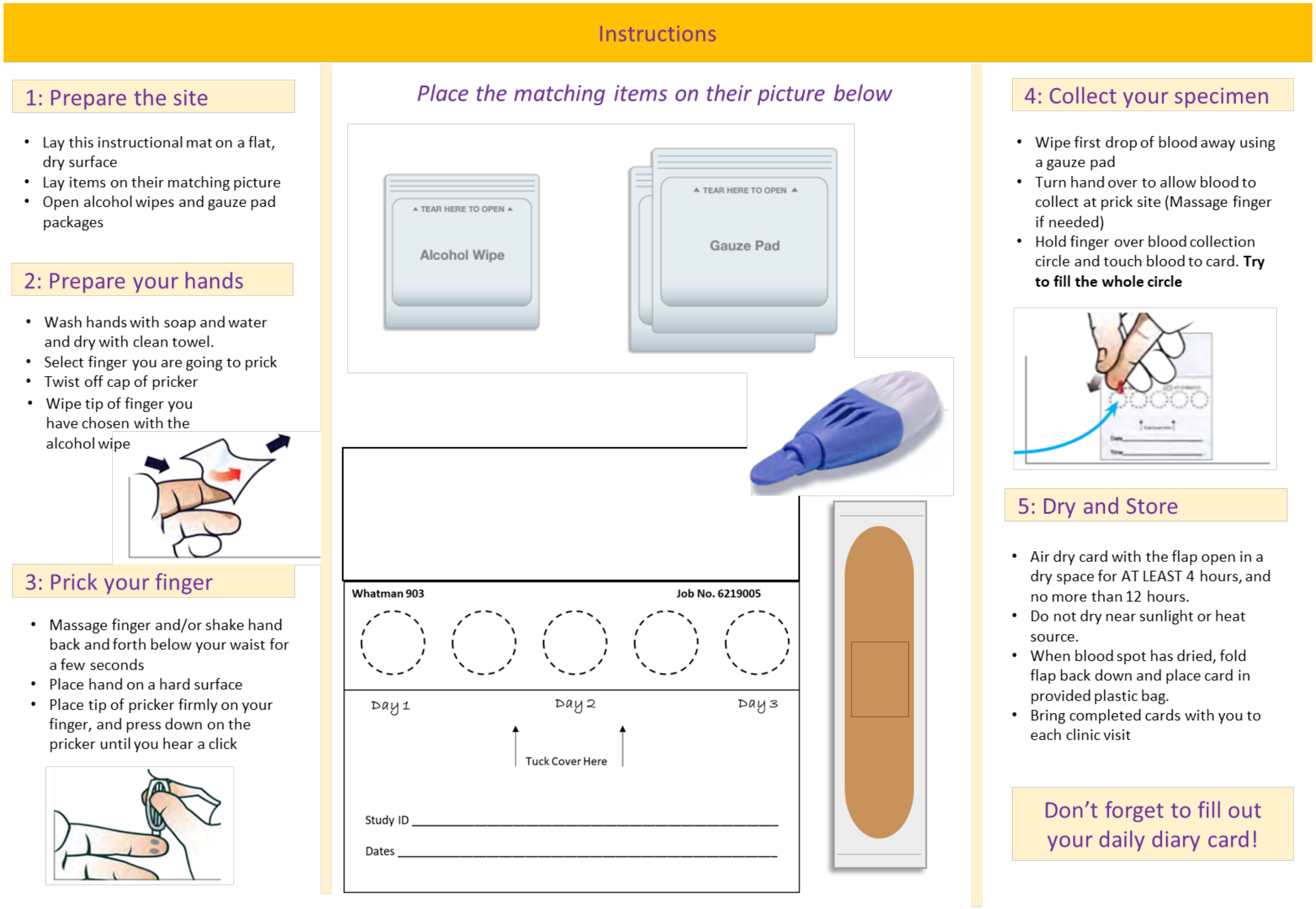
APPENDIX A

Instructional placard and Diary Card provided to participants to guide the daily collection of dried blood spots (DBS). Participants received versions translated into the local language.



Instructions

1: Prepare the site

- Lay this instructional mat on a flat, dry surface
- Lay items on their matching picture
- Open alcohol wipes and gauze pad packages

2: Prepare your hands

- Wash hands with soap and water and dry with clean towel.
- Select finger you are going to prick
- Twist off cap of pricker
- Wipe tip of finger you have chosen with the alcohol wipe

3: Prick your finger

- Massage finger and/or shake hand back and forth below your waist for a few seconds
- Place hand on a hard surface
- Place tip of pricker firmly on your finger, and press down on the pricker until you hear a click

Place the matching items on their picture below

Whatman 903 **Job No. 6219005**

Day 1 Day 2 Day 3

Tuck Cover Here

Study ID ______________________________

Dates ______________________________

4: Collect your specimen

- Wipe first drop of blood away using a gauze pad
- Turn hand over to allow blood to collect at prick site (Massage finger if needed)
- Hold finger over blood collection circle and touch blood to card. **Try to fill the whole circle**

5: Dry and Store

- Air dry card with the flap open in a dry space for AT LEAST 4 hours, and no more than 12 hours.
- Do not dry near sunlight or heat source.
- When blood spot has dried, fold flap back down and place card in provided plastic bag.
- Bring completed cards with you to each clinic visit

Don't forget to fill out your daily diary card!

Diary Card for MMDL-001 Study

Participant ID: _______________

Dates of Collection: ___________ (dd/mmm/yyyy) to ___________ (dd/mmm/yyyy)

Circle dates: Week 1 (Days 1-6) / Week 2 (Days 8-13) / Week 3 (Days 15-20) / Week 4 (Days 22-27)

Please place a sticker on the circle corresponding to the location of your blood spot on the card. If you did not take a blood spot on a given day, ***do not*** **place a sticker, only fill in the information.**

CARD A (1st, 2nd, and 3rd at-home collections of the week)

Participant ID: _______________					
	Day 1	(BLANK)	Day 2	(BLANK)	Day 3
Day of week					
Date (dd/mm/yyyy)					
Spot collected?	YES / NO	YES / NO	YES / NO	YES / NO	YES / NO
Feverish?	YES / NO	YES / NO	YES / NO	YES / NO	YES / NO
Other symptoms?					
Willing to continue?	YES / NO	YES / NO	YES / NO	YES / NO	YES / NO
Level of pain? (0=none,5=great pain)					
Did you sleep under a bednet last night?	YES / NO	YES / NO	YES / NO	YES / NO	YES / NO

CARD B (4th, 5th, and 6th at-home collections of the week)

Participant ID: _______________					
	Day 4	(BLANK)	Day 5	(BLANK)	Day 6
Day of week					
Date (dd/mm/yyyy)					
Spot collected?	YES / NO	YES / NO	YES / NO	YES / NO	YES / NO
Feverish?	YES / NO	YES / NO	YES / NO	YES / NO	YES / NO
Other symptoms?					
Willing to continue?	YES / NO	YES / NO	YES / NO	YES / NO	YES / NO
Level of pain? (0=none,5=great pain)					
Did you sleep under a bednet last night?	YES / NO	YES / NO	YES / NO	YES / NO	YES / NO

Weekly diary received by clinic site staff: _______________ Staff member initials

_______________ Date (dd/mmm/yyyy)