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Jessica Bailey Walters Cassin, Master of Science, 2012

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Abstract: Although great strides have been made in the reduction of the worldwide burden of malaria disease, a better understanding of the epidemiology of malaria is needed to continue the fight against the disease. Specifically, insight into the transmission of malaria within households might offer new targets of malaria intervention, and policy changes aimed at the control of the spreading antimalarial resistance. To this end, this study examines the relationship between malaria infection and household exposure in Blantyre, Malawi. Blood samples were collected from children and their caregivers for analysis using six neutral, unlinked microsatellite markers. Parasites within infections were genotyped and the infections in children were compared to infections in their caregivers to determine the number of microsatellites shared between the two infections, a marker for the genetic relationship of the two infections. The comparison of genotype between infections, allows a specific infection to be tracked through the human-vector-human transmission cycle. The mean proportion of parasite alleles shared for individuals residing in the same house was compared to that of individuals residing in different households. Overall, children had infections that were more similar to their parents than to that of other caregiver in the population (p value =.036). This indicates that intra-household malaria infections are more similar than inter-household malaria infection and suggests that individuals in the household are a source of malaria infection within the household. For half of the occurrences of shared infections, the parasite was found in both caregiver and child at the same time, a

synchronous exposure indicating a shared exposure either within the area or travel outside of the Blantyre area. The results provide encouraging indications that future research may yield new information that will be influential in reducing the burden of malaria disease worldwide through policy decisions regarding parasite control and the prevention of the spread of antimalarial resistance. Further research is needed to assess the intra-household source of infection, and validate the study in other populations.

Malaria Transmission in Households in Blantyre, Malawi

Jessica Bailey Walters Cassin

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List of Abbreviations

ACT: Artemisinin-combination therapy

ARDS: Acute respiratory distress syndrome

CDC: Center for Disease Control

EIR: Entomological inoculation rate

PCR: Polymerase chain reaction

qPCR: Quantitative PCR

SNP: Single nucleotide polymorphism

SP: Sulfadoxine-pyrimethamine

WHO: World Health Organization

I. Introduction

Malaria is an urgent public health problem. It is endemic in many parts of the developing world because of poverty, lack of access to preventative and control measures, and a widening spread of resistance (both vector resistance to insecticides as well as parasite resistance to antimalarial medication). It is one of the leading causes of illness among children in sub-Saharan Africa. Half of the world's population remains at risk. Of the estimated 655,000 yearly malaria deaths, the majority are young children (WHO, 2011).

Malaria is caused by a parasitic infection of one of five species of *Plasmodia* that infects humans. *Plasmodium falciparum* is the most deadly, and is the main malaria species throughout Africa. *P. vivax* is the second most common infection worldwide and the most frequent parasite detected outside of Africa. Three other species P. *malariae, P. ovale,* and *P. knowlesi* (which was recently shown to cause human disease), contribute less significantly to the burden of malaria worldwide (WHO, 2011).

Malaria is transmitted to humans via the bite of an infected female *Anopheles* mosquito vector. While the infected anopheles is taking a blood meal, malaria parasite sporozoites are released from the mosquito's salivary glands into the host. The sporozoites travel to the liver where they invade hepatocytes. During the next 1-2 weeks in the liver, the parasites develop into schizonts, which rupture releasing merozoites. The merozoites leave the liver and reenter the circulatory system, invading erythrocytes. *P. vivax* and *P. ovale* form hypnozoites form and remain dormant in the liver for several months generating new blood stage infections long after the initial bite. In the

bloodstream, the parasites continue to grow in number via asexual reproduction. The merozoites mature into trophozoites and divide in the schizont stage, to produce many merozoites. The merozoites eventually cause the infected erythrocytes to lyse, releasing more merozoites and instigating the clinical symptoms that occur during malaria infection. The time from the initial sporozoite infected bite to the development of clinical symptoms is from 7 to 15 days for *P. falciparum* infection. Some merozoites mature into gametocytes (the sexual erythrocytic stage) which are responsible for transmission to the mosquito and can be taken up by the next *Anopheles* to draw a blood meal from the infected human. When the gametocytes have entered the gut of the mosquito, they develop into mature sex cells, or gametes. The respective male and female gametes fuse to form diploid zygotes. In this stage the parasite undergoes sexual reproduction producing the abundance of genetic diversity seen in *Plasmodium*. The zygotes then nest in the wall of the mosquito gut, forming oocysts. After 2 weeks, the oocysts burst, releasing haploid sporozoites, which will move to the salivary glands for delivery during the mosquito's next blood meal (WHO, 2011); (Mackintosh, Beeson, & Marsh, 2004).

Clinical malaria is classified in two categories: uncomplicated and severe. Uncomplicated malaria symptoms include: paroxysmal fevers, rigors, headache, malaise, cough, aches, vomiting, or mild GI symptoms. Severe malaria is characterized by significant organ damage due to the sequestration or accumulation of *P. falciparum* infected erythrocytes in small blood vessels. Severe malaria typically presents with one or more of the following manifestations: cerebral malaria, metabolic acidosis, severe anemia, hypoglycemia, hemoglobiuria, acute renal failure, acute respiratory distress syndrome (ARDS), or acute pulmonary edema. Severe malaria is fatal in 1/10 of treated

cases and has much higher rates of death in untreated cases. Individuals may be asymptomatic, having detectable levels of parasites circulating in their blood stream but without the clinical symptoms of malaria. They may still harbor gametocytes, allowing for continued infection of the mosquito vector, despite their lack of symptoms (WHO, 2011).

Malaria is diagnosed in the field by clinical diagnosis and parasitological diagnosis. Clinical diagnosis is a diagnosis of clinical symptoms alone. The criteria for diagnosis usually include fever within the last 24 hours and the presence of anemia. The symptoms of uncomplicated malaria are non-specific, thus diagnosis through clinical symptoms can lead to misdiagnosis and the overuse of antimalarial drugs. Clinical diagnosis should be confirmed through the use of parasitological diagnosis methods such as light microscopy which is currently considered the gold standard of malaria detection. Light microscopy can detect as few as 5-10 parasites/μl of blood when performed by a skilled technician. The method begins with a finger-stick or venous blood sample. A technician then spreads thick and thin smears of blood across a slide, stains it, and examines it to determine if parasites are present (WHO, 2011).

In the lab, polymerase chain reaction (PCR) can be used to amplify and detect parasite DNA. Specific, highly conserved regions of the parasite genome are amplified with PCR and used in a variety of ways such as: quantitative-PCR (qPCR). The results of which can be applied to next-generation sequencing, and mutation analysis. These methods are generally restricted to the lab because of the specialized equipment and reagents necessary for the assays. With PCR, species differentiation and antimalarial susceptibility detection is also possible.

The WHO outlines the proper treatments and precautions for protection against malaria. Prescribed control strategies include: vector control (with impregnated bed nets, and indoor residual spraying), intermittent preventative therapy (IPT) for vulnerable populations such as pregnant women and young children, accurate diagnosis of malaria, and timely and appropriate antimalarial treatment when diagnosis of malaria is confirmed. Currently, in most countries, the first line of treatment for uncomplicated malaria is an ACT (artemisinin-based combination therapy). ACTs were introduced after the first generations of antimalarial drugs (chloroquine and sulfadoxine-pyrimethamine, SP) showed widespread resistance and high levels of treatment failure. Concern is also growing over the spreading resistance to artemisinin along the Thai-Burma border. The spread of artemisinin resistance would lead to increased treatment failures and the loss of another effective antimalarial (Noedl et al., 2008) (Dondorp et al., 2009).

Background Evidence

Great strides have been made in the reduction of the disease burden (33% reduction of malaria related deaths since 2000 in the WHO African Region), and the development of promising vaccine candidates (The RTS,S Clinical Trials Partnership,2012), but gaps in the basic knowledge of malaria epidemiology remain. One such gap of knowledge involves the transmission of malaria within households. A study by Michael et al. indicates that mosquitoes introduce infection into a house from a bloodmeal taken in the outside community (Michael et al., 2001). Malamba et al. showed that malaria transmission within households is complex, as exampled by the conveyed protection that treatment of one individual extends to other members of the household (Malamba et al., 2006). If reducing potential sources of infection within the household,

(i.e. treating individuals in the household with chemoprophylaxis) reduces the infection in other household members, it is possible that individuals with circulating gametocytes contribute to the incidence of malaria within their household by potentiating infection of mosquito vectors within the household. If mosquito vectors remain in a household where malaria is present, there is a continued intra-household source of gametocytes to infect mosquitos for the infectious cycle to persist and be spread to the family. This would make a shared household an exposure of interest.

Although there had been multiple attempts made at understanding the biting patterns of the *Anopheles*, until the introduction of DNA genotyping it had been difficult to successfully study mosquitoes in the wild. Michael et al. investigated this by focusing on the human blood contained in the mosquito after feeding. Mosquitoes were collected from each household participating in the study and the human blood taken up during feeding was genotyped using PCR amplification and microsatellite analysis. The researchers found that the mosquitoes contained blood from the household members as well as those from the surrounding community. Over 13% of mosquitoes biting within households contained blood from two or more individuals living in the household and 27% of the mosquitoes caught within a household had fed on people not living in that particular household (Michael et al., 2001).

More recently, Nkhoma et al. examined the genetic heterogeneity within and between infections in order to examine the mechanism by which a polyclonal infections arise. Malaria infections can be monoclonal (single parasite genotype present) or polyclonal (multiple parasite genotypes present). Previously, the hypothesis was that polyclonal infections were the result of multiple infectious bites over time. To determine

if this hypothesis was supported by evidence Nkhoma and colleagues collected samples from the Thai-Burma border in Thailand (low transmission setting) and from Blantyre, Malawi (moderate transmission setting). Researchers reasoned that because polyclonal infections are more common in areas of higher transmission (like Malawi) due to higher quantities of infectious bites, they would be more likely to have higher levels of intra-infection variability if the hypothesis was correct. On the other hand, individuals from Thailand should have homogenous parasite genotypes within an infection. Using 316 single nucleotide polymorphisms (SNPs) spread across the *P. falciparum* genome they created haplotype structures and mapped patterns of parasite relatedness within and between infections. The authors found that in both Malawian and Thai samples, parasites were more similar within an infection than across infections. Parasites within an infection were related at the half-sibling level or greater level in 66.7% and 47.4% of infections in Malawi and Thailand respectively. Between infections, only 2.6% and 3.4% are half-sibling or greater. The authors concluded that polyclonal infections are the result of limited parasite genotypes taken up in a bloodmeal that undergo genetic recombination within the mosquito producing the genetic diversity observed in polyclonal infections, rather than by repeated inoculation of the human host from multiple mosquito vectors (Nkhoma et al., 2012).This finding is important for two reasons. First, it offers evidence that despite the genetic diversity produced during the sexual reproduction stage it is possible to trace an infection from one individual to another. Second, it shows that intra-infection variability is low compared with inter-infection variability. Thus, it is reasonable to assume that high a concordance rate between two individuals suggests a common source of infection.

In 2006, Malamba et al. examined the effect of using co-trimoxazole as a chemoprophylaxis in HIV-infected patients on the spread of SP resistance in a rural Ugandan community. Both co-trimoxazole and SP have the same mechanism of action, leading to fears that widespread use of co-trimoxazole would lead to increased rates of SP resistance. At the time, SP was used as a first line of treatment of malaria. The threat of increased resistance in an area that already had high levels of SP resistance was alarming. Researchers collected samples from the households of both co-trimoxazole treated and co-trimoxazole untreated HIV infected individuals and compared the rates of SP resistance in each group. The researchers not only found no evidence of increased levels of SP resistance in the household members of those treated with co-trimoxazole, but those residing with HIV patients had lower rates of malaria overall (Malamba et al., 2006). Their results indicate that treatment of one individual extends protection to other household members, suggesting that individuals within the household may provide a source of infection to other members of the household.

If this is indeed the case, that malaria is spread within households via human-vector-human transmission, it would be especially important to understand who the main source of infection within a household is. Malamba et al. showed that protection is conveyed to other household members by the use of chemoprophylaxis in one at-risk individual in the household, but it is possible that other individuals in the household might be contributing more significantly to intra-household malaria transmission.

Generally speaking, within a household there are two distinctly different populations that could be sources of infection to the household, adults and children. Due to acquired immunity (in endemic areas), adults are at low risk of symptomatic malaria

and may remain largely asymptomatic while still harboring malaria parasites including gametocytes, making them continually infectious to an *Anopheles* vector and a potential source of infection for their household. Children meanwhile have largely naïve immune systems and are often symptomatic, suggesting that they might provide a source of infection to the household due to the lack of immune suppression observed in adults. However, because they are frequently symptomatic, they may be treated more rapidly.

Currently, core of efforts are focused on endemic populations that are at higher risk for severe malaria disease and/or known disposition for adverse outcomes due to infection; namely young children and pregnant women. However, if individuals in the household are providing a source of infection to the others in the household, we might be a missing category of interventions that would confer greater protection to those most at risk. For example, if adults are the main source of infection within a household, might preventative treatment of adults offer protection to children? Additionally, a better understanding of the household origin of malaria infection may provide insight into spreading resistance. Are untreated asymptomatic adults a source of infection? Or are children, on which the strongest drug resistance pressures are conferred through frequent illness and treatment, the source of household infection? This study seeks to determine if intra-household infections are more genetically similar than inter-household infections, and if individuals in a household provided a continued source of infection within the household. Given the above is true, whether parents or children contribute more significantly to the burden of malaria infection within a household. A better understanding of the complex interplay of parasite transmission within a household could contribute to improve the prevention of malaria illness. Based on the evidence presented

above, we hypothesize that household exposure will contribute to rates of malaria, and that the most likely origin of household infection is from the asymptomatic adults.

II. Methods

The location of the study was Blantyre, Malawi, a country located in the southeastern corner of the African continent. The country's second largest city and commercial center is Blantyre in the southern region of the country. Blantyre has mild temperatures, averaging 13-21 Celsius, year round. Malaria transmission in Blantyre and the surrounding region is perennial with seasonal peaks during the rainy season, December through March (Mzilahowa, McCall, & Hastings, 2007).

Children were enrolled from 2007 through 2009 in Blantyre as part of the clinical trial by Laufer et al., *A longitudinal trial comparing chloroquine as monotherapy or in combination with artesunate, azithromycin or atovaquone-proguanil to treat malaria* (Laufer et al., 2012). Sample collection occurred at the Ndirande Health Centre in Blantyre, the only health facility in the Ndirande region. Children were eligible for participation in the study if they presented with uncomplicated symptomatic malaria illness as defined by clinical illness in addition to a *P. falciparum* positive smear, were between 6 months and 5 years, were willing to remain in the study area and attend follow-up visits, had no allergy to a study drug, and did not use a chemoprophylaxis (such as HIV patients). The results of the major clinical trial have been published previously (Laufer et al., 2012)

Although only children were enrolled in the clinical trial (mentioned in the paragraph above), their caregivers were invited to participate in a surveillance study for

detection and evaluation of malaria parasite relatedness between infections in the children enrolled in the clinical trial and their caregivers. Caregivers were eligible for enrollment in the surveillance study if they resided with the child enrolled in the clinical trial during their participation in the study, provided information about travel history and bed net use, and agreed to attend follow up visits for sample collection. The samples provided by children in the clinical trial were used in addition to samples provided by their caregivers that enrolled in the surveillance study. Samples were collected during regular visits or during unscheduled visits due to illness. Blood smears were taken and assessed at the collection site on the day of collection via light microscopy for diagnosis of malaria illness. Only one caregiver was enrolled per child. All samples provided by caregivers were included and for the children, samples from the first day of all episodes of malaria illness during the study period were included. Study protocol was approved by the University of Malawi College of Medicine Research and Ethics Committee and the Institutional Review Board of the University of Maryland, Baltimore. Written informed consent was provided before participation in the study.

In addition to smears taken for diagnosis via light microscopy, blood samples were also collected on filter paper and dried. Extractions of the filter paper smears were performed on all samples collected, regardless of microscopy results with the Dried Blood Spot Protocol from the QIAGEN QIamp DNA 96 Blood Kit for the QIAGEN Biorobot (Germantown, MD). After extraction, all caregiver samples were screened to detect the presence of *P. falciparum* using rtPCR (samples were run with a serial dilution of a laboratory strain of control parasite DNA, 3D7).

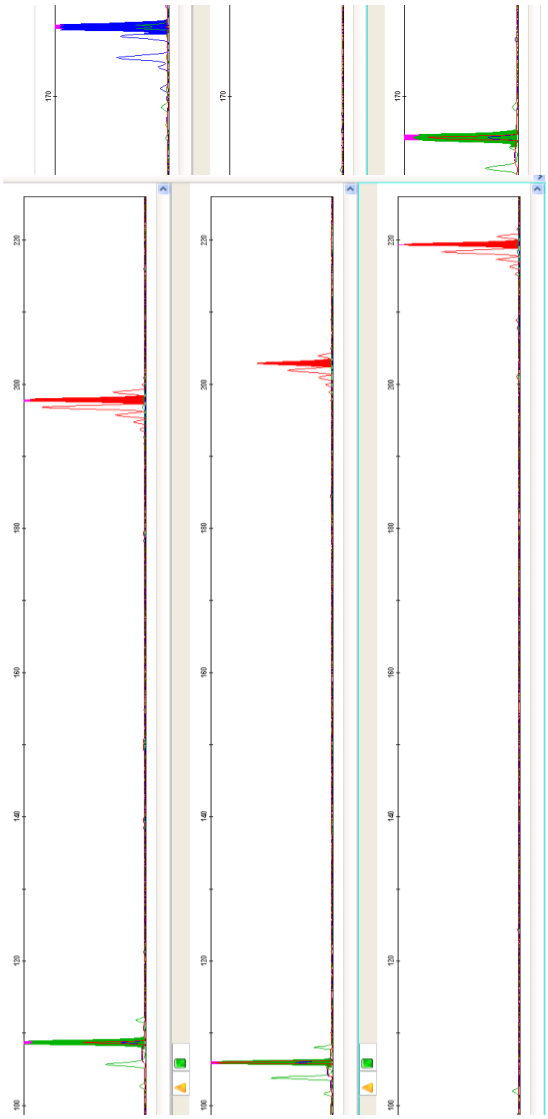
Caregiver samples identified as positive for *P. falciparum* and all samples collected from their children were amplified using a heminested, multiplexed PCR method. Markers used for amplification were the microsatellites Poly alpha, PfPK2, TA81, ARA2, TA87, and TA40. These markers are short repetitive (three base pair repeats) regions in the parasite genome that are neutral and unlinked. These six markers are the most polymorphic of the 12 microsatellites previously identified by Tim Anderson et al., which are sufficient to characterize the full spectrum of parasite diversity in the region (Anderson, Su, Bockarie, Lagog, & Day, 1999). The six microsatellites with PCR primers are provided in Table 1.

Table 1: Microsatellites. The table shows the six microsatellites with amplification primers and expected size ranges. The primers in color below represent the color with which they are labeled for use in the microsatellite genotyping (see Figures 1 and 2).

Multiplex	Locus (Anderson 1999)	primer 5' to 3'	Fluorescent Label	size range from Anderson 1999	Size in 3D7 reference genome
1	Poly α-R	ATCAGATAATTGTTGGTA	FAM	114-201	151 bp
	Poly α-F	AAAATATAGACGAACAGA			
	Poly α-3(IR)	GAAATTATAACTCTACCA			
1	PFPK2-3R	CCTCAGACTGAAATGCAT	HEX	159-192	172 bp
	PFPK2-F	CTTTCATCGATACTACGA			
	PFPK2-R	AAAGAAGGAACAAGCAGA			
1	TA81-3F	GAAGAAATAAGGGAAGGT	PET	112-142	122 bp
	TA81-R	TTTCACACAACACAGGATT			
	TAA81-F	TGGACAAATGGGAAAGGATA			
2	ARA2-3(F)	GTACATATGAATCACCAA	FAM	63-90	73 bp
	ARA2-R	GCTTTGAGTATTATTAATA			
	ARA2-F	GAATAAACAAAGTATTGCT			
2	TA87-3F	ATGGGTTAAATGAGGTACA	HEX	90-126	100 bp
	TA87-R	ACATGTTCATATTACTCAC			
	TA87-F	AATGGCAACACCATTCAAC			
2	TA40 Rev-1	GAAATTGGCACCACCACA	PET	217	217 bp
	TA40 For	AAGGGATTGCTGCAAGGT			
	TA40 Rev-2	CATCAATAAAATCACTACTA			

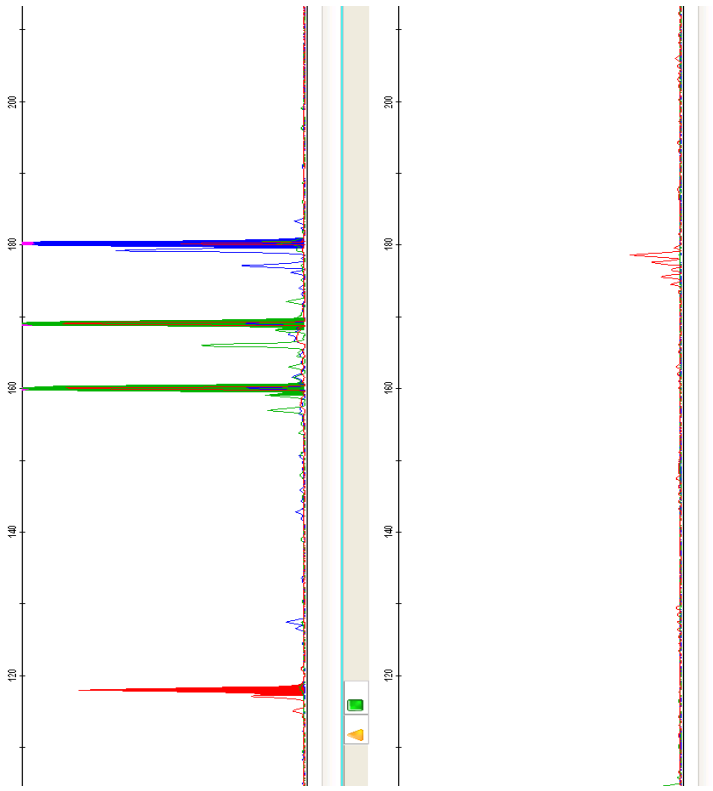
After PCR, samples were prepared for molecular genotyping by capillary electrophoresis with the Protocol for Microsatellite Genotyping by Unlinked Markers. (CVD Malaria Group, 2012). Genemapper 4.0 (Applied Biosystems, Foster City CA) was used to analyze the output file (called an electropherogram) and microsatellites were scored and recorded. Microsatellites were scored based on known peak morphology from laboratory control strains run in conjunction with research samples. For this study, the control strains 3D7, V1S, and HB3 at a concentration of 2.5pg/µl were used. The peak morphology for each of these strains is shown in Figure 1.

Figure 1: Control DNA Multiplex 1 and Multiplex 2. Control strains are from top: Multiplex1: V1S, 3D7, HB3 and Multiplex 2: V1S, 3D7, HB3. Three fluorescent labels were used, thus the PCR required two separate multiplexed reactions with three primers per Multiplex.



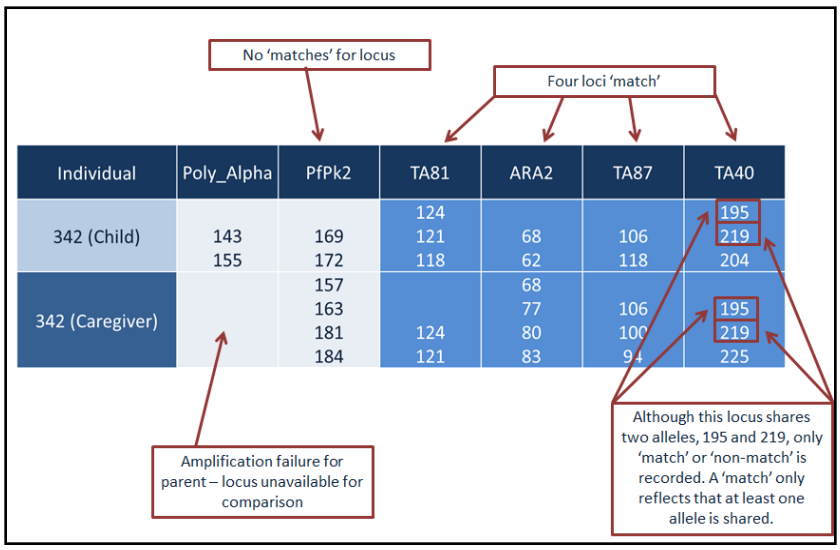
Samples in which any microsatellites failed to amplify were rerun. We considered less than four markers to be uninterpretable because it is insufficient to definitively distinguish a genotype. The presence of more than one allele at a given locus (Figure 2) indicates the presence of more than one parasite strain circulating in the blood, referred to as a polyclonal infection. The minimum number of parasite genotypes is indicated by the number of alleles at the locus with the highest number of different alleles. A maximum number of haplotypes cannot be inferred from microsatellite analysis because each microsatellite is amplified, sequenced, and scored independently within each sample. It is impossible to determine full haplotypes using this type of analysis.

Figure 2: Example of sample with multiple alleles per loci. Both Multiplex 1 and Multiplex 2 are show. In this example, the markers PfPK2, TA 87, and TA 40 have more than one allele. This is a polyclonal infection with a minimum number of two parasite haplotypes circulating in the blood during sample collection.



All scored samples were run through a custom PerlScript that correct the alleles of each microsatellite to the control markers which are of known size. Variation in the control, either larger or smaller than expected, is corrected in the samples. This minimizes variation between runs. Using the output from the program, the parasite genotype from the caregivers' samples were compared to the parasite genotype from their child's sample and a count of the total number of markers with shared alleles was recorded. A single microsatellite marker was called a match if at least one allele for that locus was shared between both individual's samples. A non-match was recorded for a locus if no alleles were shared by the parent and the child for that locus. If the caregiver or child had an amplification failure for a particular locus, that locus was unavailable for use in the comparison (Table 2). Only those comparison pairs in which four or more loci amplified and were available for comparison were included in the analysis. Additionally, only samples which were collected within two months of one another were included in this analysis. This stipulation to data analysis protocol was mandatory due to the nature of the parasite life cycle (see above in the introduction). Samples collection which occurred more than two months apart cannot be considered to be plausibly related. Sixty days is a more than sufficient timeframe to allow for the parasite's migration through human-host-human. The comparisons were made using another custom PerlScript. The script compared every scoreable child's sample to every scoreable sample from that child's parent (Table 2). All analyses were conducted in Microsoft Excel 2010 (Redmond, WA) or Mystat (Chicago, IL).

Table 2: Example of comparison scoring. Children's samples were compared to their parents sample to determine the number of microsatellites shared by both the caregiver and child across the infection. Comparisons were scored as follows: the marker Poly alpha did not amplify in the caregiver's sample, thus only five comparisons could be made. Although multiple alleles match on some loci, only match (the microsatellite is shared between the two infections) or non-match is recorded for each locus regardless of the total number of alleles shared per locus. This caregiver/child pair matches on four out of five loci, thus the proportion shared for this comparison pair is: 4 out of 5.



Individual	Poly_Alpha	PfPk2	TA81	ARA2	TA87	TA40
342 (Child)	143 155	169 172	124 121 118	68 62	106 118	195 219 204
342 (Caregiver)		157 163 181 184	124 121	68 77 80 83	106 100 94	195 219 225

Two infections were compared to each other to determine the total number of microsatellites matches out of the total number of microsatellite markers available for comparison and the proportion was recorded for each comparison pair. This number is the measure of genetic similarity between the two samples being compared. The comparison of each child's sample to their caregiver's sample provided the distribution and mean of the observed similarity given the exposure of interest, a shared household. Comparisons which fall under this category were considered the exposure group. In order to determine similarity when the exposure was not present, the control group, every child's sample was compared to every caregiver sample excluding samples from their own caregiver was used. An average proportion of shared alleles was taken for each group of comparisons, exposure and control, to determine if the individuals in the

exposure group shared more alleles than did individuals in the control group. To test if the difference in means between these two groups was significant, they were compared using a two-sample t test in Mystat. Next, to further evaluate the relationship between exposure and level of similarity between samples, within the exposure group were further divided into two groups. These groups, samples collected within two months of one another and samples taken at an interval greater than two months apart were compared. Just as described above, the proportion of shared alleles was recorded for all comparisons, and the average proportion of shared alleles for comparisons within each group was recorded. The difference between the two means was also compared using a two-sample t test to test for significance.

Another analysis involving the individual microsatellite diversity was performed. The six microsatellites used in this study had differences in relative diversity in the population so the mean alleles shared for each marker in all four comparison groups was also assessed. For this analysis, any sample in which a single microsatellite marker was available for comparison was used.

Those individuals from the exposure group who shared at least four alleles were reviewed to determine whether the child or the parent was infected first. The individual who first tested positive with the infection was considered the source of the infection. If the samples were collected within one week of each other they were determined to be synchronous. Such a short window of time does not allow for the full parasite life cycle to be completed. In these cases, it was assumed that the detected parasites originated from a single source and the individuals were likely infected on the same day or within a few days of one another.

III. Results

A total of 195 caregivers agreed to participate in the study and gave a combined total of 1321 samples. This allowed their 195 children to also be included in the study, providing a total of 323 samples. The distribution of samples given per individual enrolled in the study is given in Figure 3. Twenty percent of samples from caregivers were malaria positive and available for analysis for the study. After excluding rtPCR negative samples and samples with more than two microsatellite amplification failures, 127 samples from a total of 86 caregivers and children remained for analysis (Figures 3 and 4). A complete breakdown of sample information for both the exposure and control groups is given in Table 3.

Figure 3: Samples provided per individual. Total samples provided for the study (A). Total samples which remained for analysis: rtPCR positive and successful amplification of at least four loci (B).

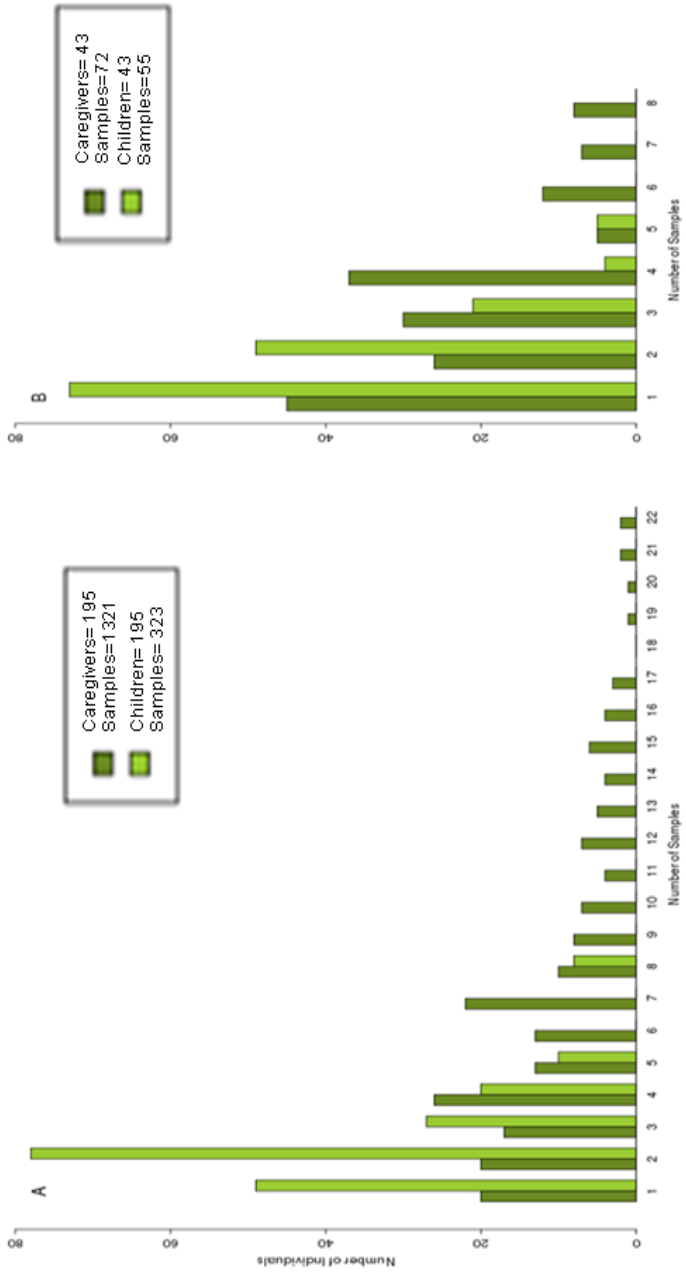


Figure 4: Amplification Success. Total samples available for microsatellite genotyping (x-axis) and the total number of microsatellites with amplification success (y-axis). Children had more successful amplification.

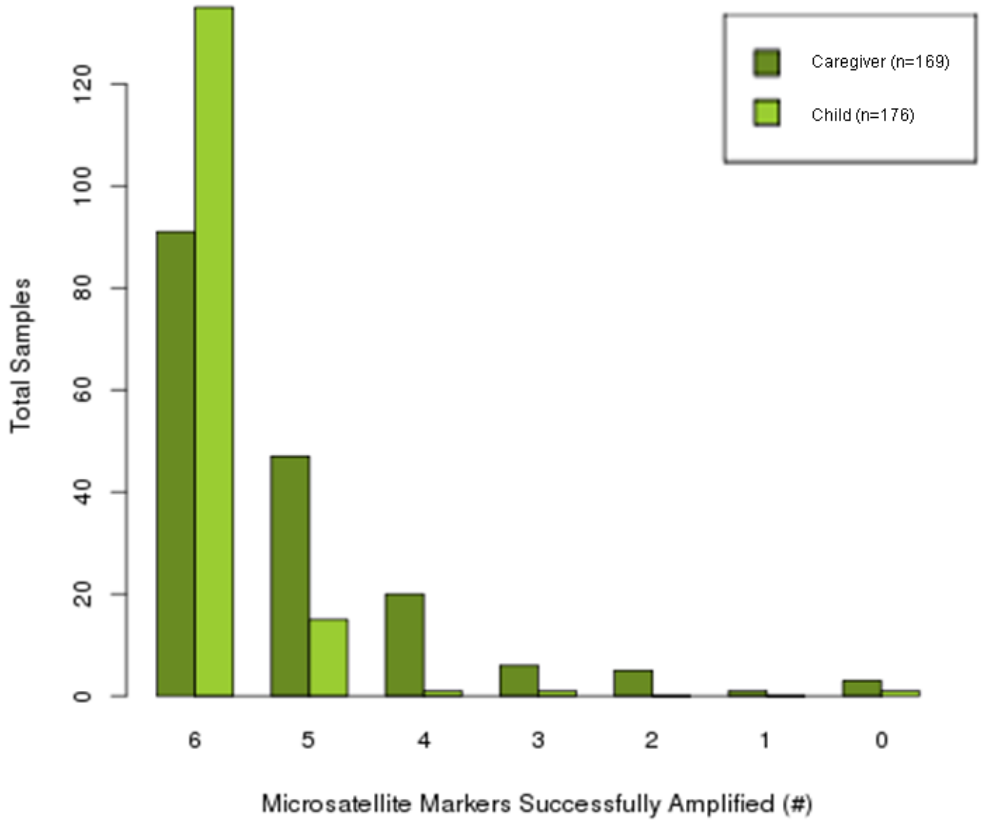


Table 3. Table of specimens available and analyzed for study.

	Caregivers	Children
Enrolled in Study		
Individuals	195	195
Samples Contributed	1321	323
rtPCR Positive		
Individuals	107	195
Samples	302	323
Amplification Success >4		
Individuals	81	107
Samples	152	161
Used in Comparison		
Individuals	43	43
Samples	72	55

Of the six microsatellite markers included in this study, Poly alpha was the most diverse and ARA2 was the least diverse. In general, there was an inversely proportional relationship between the diversity of the marker and common allele frequency in the population. The less diverse the marker, the higher the common allele frequency appeared in the population. There were 29 alleles for Poly alpha, and the four most common alleles appeared in less than half of the samples. ARA2 had 15 alleles, and nearly 75% of the population carried one of the four most common alleles. The allele frequency distributions were similar in adults and children (Figure 5). Despite the differences in allelic diversity, children consistently shared more alleles with their caregiver than they did with any other caregiver (Figure 6).

Figure 5: Allele frequency distribution. Figure shows the distribution of matches by microsatellite marker. This figure includes all comparisons from the exposure group in which samples were taken at less than two months apart, in which any one microsatellite was available for comparison.

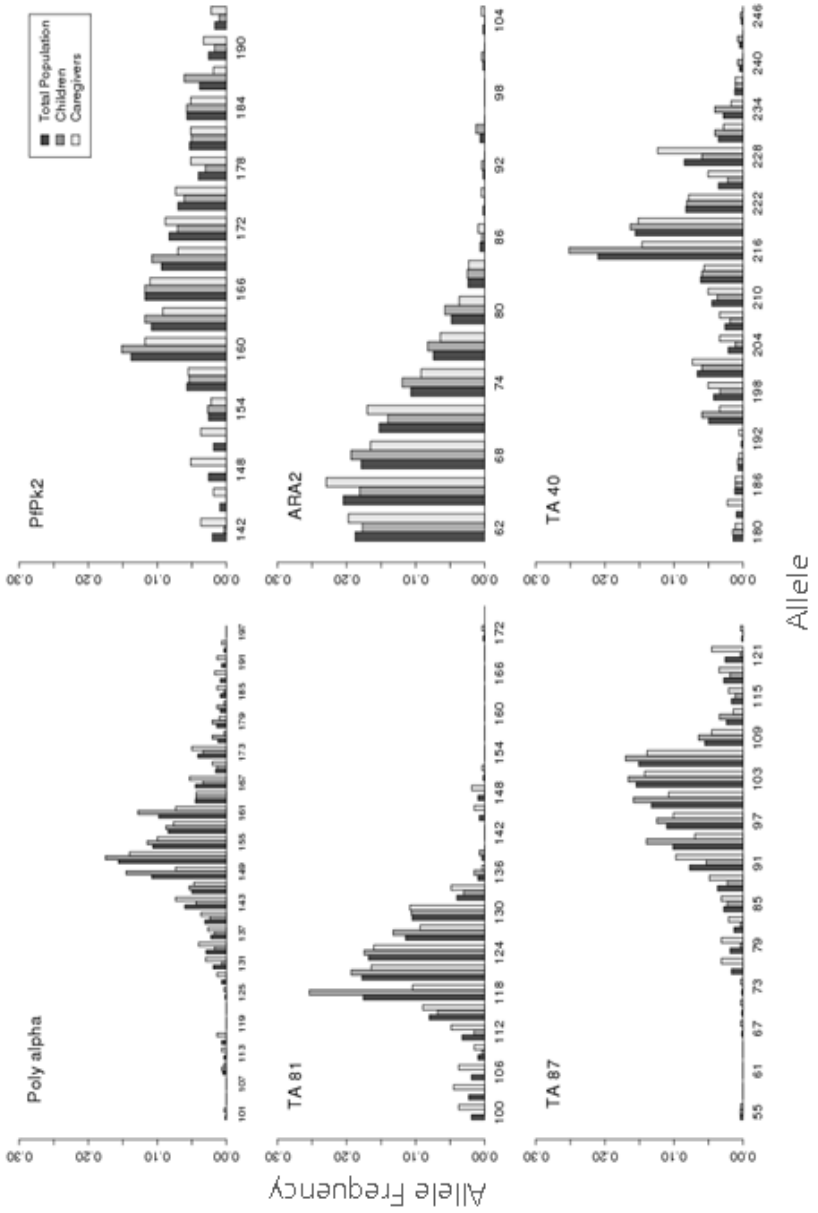
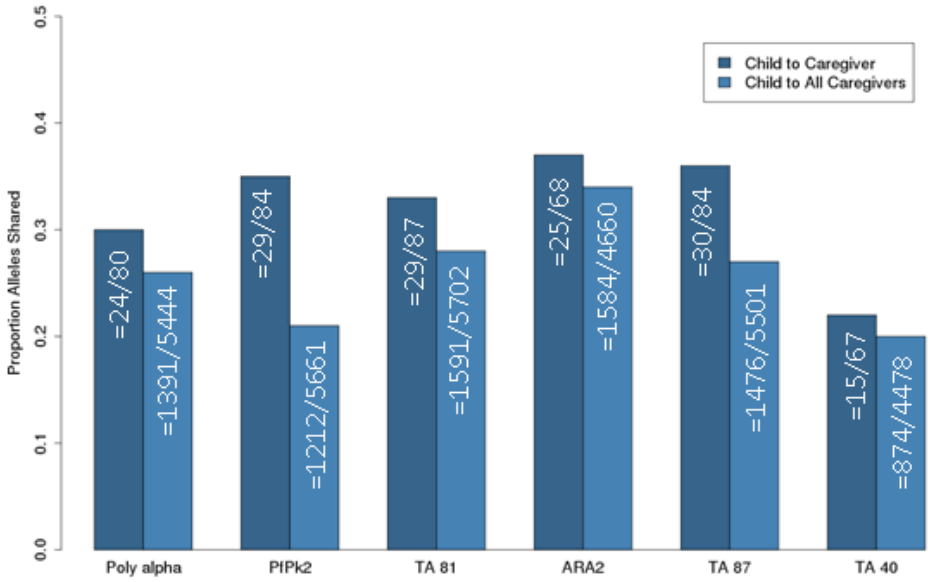


Figure 6. Proportion alleles shared by microsatellite marker for child-caregiver pairs and children compared to all caregivers. Numbers in columns indicate number of alleles shared between episodes per available comparison.



The exposure group had an average proportion of 0.31 (95% CI 0.25-0.40) shared alleles (Figure 7). In the control group, the average proportion of alleles shared was 0.26 (95% CI 0.25-0.27). The higher proportion of alleles shared between individuals residing in the same household compared to those living in a different household was statistically significant (p value = .036). Within the exposure group, samples collected less than two months apart were compared to samples collected greater than two months apart (Figure 8). The average proportion of alleles shared for those samples in which collection occurred more than two months apart, 0.31 (95% CI 0.31-0.32) was equivalent to the average of the control group. The greater than two months and the less than two months comparison was statistically significant (p value =.037). When restricted to only samples

with complete microsatellite success (all microsatellites had at least successfully one amplified allele), the difference between the mean proportion alleles shared was much greater 0.42 (95% CI 0.33-0.50) and 0.25 (95% CI 0.24-0.26). Almost no individuals from the greater than two month's group shared five or more alleles (Figure 9).

Figure 7: Mean alleles shared in child-caregiver pairs within a household vs. child-all caregiver pairs.

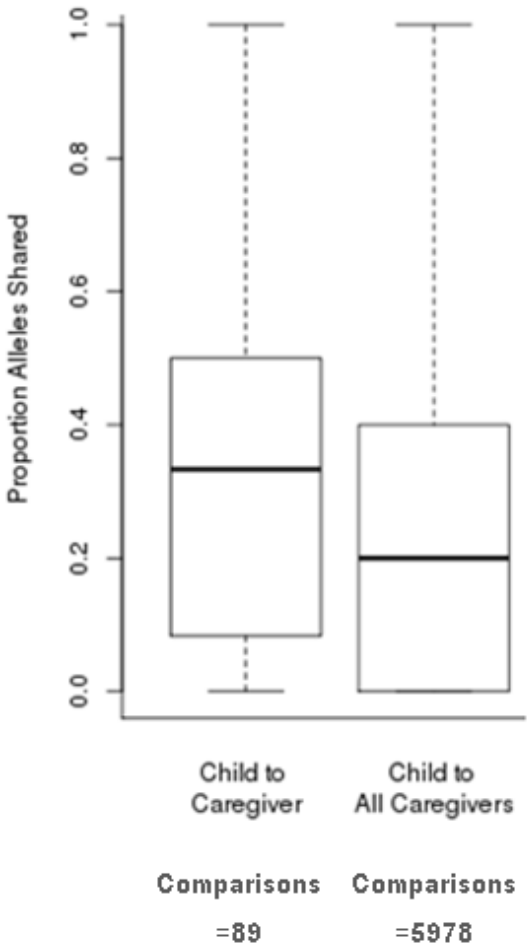


Figure 8: Mean alleles shared in caregiver-child pairs within a household at time intervals of less than two months vs. greater than two months.

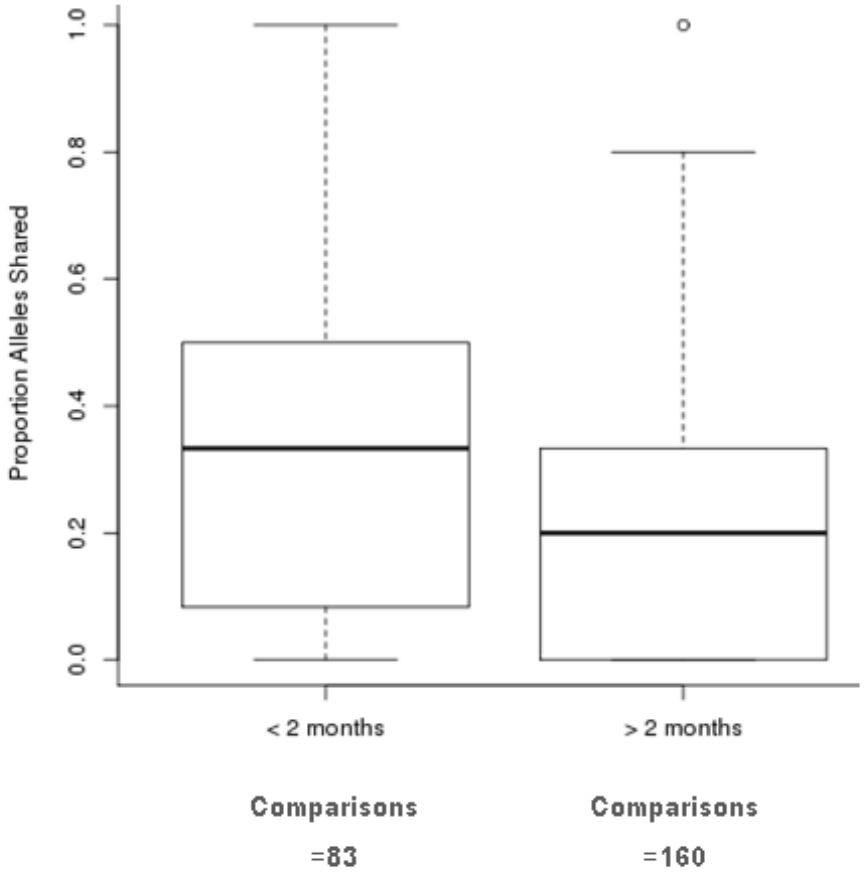
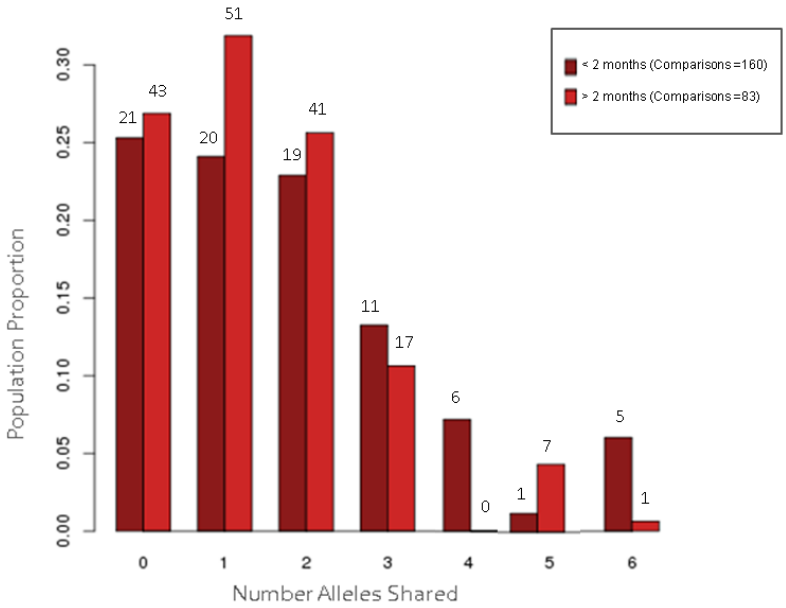


Figure 9: Distribution of alleles shared in caregiver-child pairs within a household at time intervals of less than two months vs. greater than two months.



Finally, comparisons from the exposure group which sample collection occurred less than two months apart were analyzed to determine in which individual the malaria parasite originated. Twelve comparisons shared more than four microsatellites. Of those 12, the infection originated in the caregiver in one instance, in the child in five instances. The remaining six instances were positive around the same time.

IV. Discussion

The results of this study support the hypothesis that intra-household malaria infections are genetically related. Further, the study suggests that most instances of individuals with genetically similar infections occurred synchronously. To the author's knowledge, this is the first study that looked at the genetic relatedness of malaria parasites within households.

As mentioned above, children compared to their caregiver had a higher average proportion of shared alleles than did children compared to all caregivers. Although the difference was statistically significant, it was only slightly so. The small difference between the two groups is most likely due to low levels of parasitemia (the quantitative content of parasitic load present in the host) in the caregiver's samples. Children, who tended to have much higher levels of parasitemia had more successful amplification of microsatellite markers. Not only does this allow for more possible comparisons, it increases the likelihood that less abundant alleles will be successfully amplified and sequenced. As such, when samples which had complete amplification success (all six microsatellites amplified for both samples) were compared, the difference between the exposure and control groups becomes more apparent. Using samples in which four or more microsatellites successfully amplified (as listed above in the results), the mean proportion alleles shared of the exposure and control groups, was 0.31 and 0.26, respectively. When restricted to only samples with complete microsatellite success (all microsatellites had at least successfully one amplified allele), the difference between the mean proportion alleles shared was much greater 0.42 and 0.25. This indicates that low levels of parasitemia may obscure the true similarity of individuals within the same household.

The analysis of the infection origin suggests that a common source transmission model is most likely the predominant transmission model. However, only 12 comparisons were available for this analysis. This is a small sample size, and might not be representative of the larger population. Additionally, sampling did not occur at regular enough intervals to be informative about the source of malaria within the household.

Finally, differences in sample collection time of children and caregivers makes it problematic to definitively assess which individual was positive with the infection first. Because most samples were collected during times symptomatic infection, the results might instead indicate which individual developed clinical symptoms first, which would expectedly be the child. In most comparisons in the exposure group, the child's sample was collected first, and is likely the result of systematic bias in the method of data collection. A caregiver might have transmitted an infection to a mosquito which is later transmitted to the child and back to the caregiver, but sample collection did not occur during the parent's first episode with the infection and only appears to have originated with the child.

Microsatellite Analysis

The use of microsatellites in this study provided many benefits, chiefly the ability to include a large number of samples due to the fact that the method is relatively inexpensive and efficient. Microsatellite analysis comes with some disadvantages as well. The nature of microsatellite scoring is more subjective than objective. The decision between expected peak morphology (which often includes other peaks), multiple alleles, or artifact of allele calling may be arbitrary for an untrained researcher. To reduce error due to the scoring of incorrect peaks, certain quality control measures were put into place. The use of three control strains broadens the expected peak morphology range and allows the researcher a better repertoire of possible peak morphology. Child and caregiver samples were run on separate plates. Occasionally, artifacts of the allele identification process can be interpreted as extraneous peaks. If this does occur, it is less likely that both plates experienced the same anomalies. All samples were scored by the same

individual so that no difference occurred between researchers. Finally, a small number of samples were analyzed by four different researchers to ensure concordance.

Although the precautions used in the analysis of the microsatellites to minimized bias and other confounding factors, one main concern of microsatellite genotyping remained: the inability to discern complete genotypes. The human-vector-human transmission process is complex. It is possible that even just one allele from one host will make it to the next host. Which alleles are transmitted is due to several factors. Genetic diversity is the result of the genetic recombination which occurs during the sexual reproduction stage in the mosquito. A random assortment of genotypes is delivered through salivary glands to the next individual. Finally, the immune system of the host may suppress certain parasite strains to which the host has been previously exposed to. Without complete genotype information, it may be unreasonable to assume that only those individuals who share four or more alleles are genetically related. For this reason, a method in which parasites can be definitively genotyped would allow for a more comprehensive picture of household exposure

One other possible confounder is the children's active participation in the clinical trial. However, these children were only treated when symptomatic, and there was no statistical difference in efficacy of treatment arms, so there should be no difference between the children in the study and the children in the general population. Also, healthcare is funded by the Malawian government, and provided by the same health center to both the general population and the study population. Thus, there is no evidence that children in the study were treated any differently than children who did not participate in the study.

Throughout this study, the parent has been referred to as the 'caregiver.' This is because the study allowed for inclusion of multiple caregivers residing with the child. However, in every instance, the child's mother brought the child to the health center and participated in the Caregiver Surveillance Study. While this allowed for a consistent picture of one individual's parasite population, it does not permit analysis of any other family members. Perhaps the father, older sibling, grandparent, or other household visitors contributed more heavily to the burden of household malaria than did the mother or the child.

Future Research

Suggestions to be incorporated in future research include the following. A large sample is needed to confirm what was found in this study. Regular collection time for all participants using short intervals would prevent any bias due to sample collection dates and should allow for a determination of the origin of intra-household malaria. Inclusion of other household members would further demonstrate which individuals provide a source of infection. Other statistical analysis such as bootstrapping or permutation analysis used to demonstrate the expected distribution of alleles shared under the null hypothesis could allow for stronger conclusions to be made. Finally, the data could be verified and strengthened through the use of sequencing technology that allowed for complete genotyping of samples, such as the limited dilution method used by Nkhoma et al.

V. Conclusions

This study examined the significant contribution of household exposure to malaria illness. These results support the recent expansion of bednet use beyond the recommendations for children as well as the continued use of indoor residual spraying to reduce the indoor mosquito population. Because the majority of infections were synchronous, the possibility of prophylaxis use during travel outside of the Blantyre area might be especially effective in the reduction of malaria disease. Further, the results also suggest that intra-household malaria transmission contributes to the burden of disease in the population and that more research is urgently needed to address gaps in our current understanding. Using this study as a proof of concept project, future research could address policy changes for treatment of vulnerable populations and the spread of drug resistance. These results provide encouraging indications that further research may yield new information that will be influential in reducing the burden of malaria disease worldwide.

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