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Sarah Kuehl April 15, 2015

Exploration of relationship between multiplicity of infection and transmission intensity in malarial infections in Ghana

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
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Bachelor of Sciences with Honors

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Abstract

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The malaria parasite *Plasmodium falciparum* has evolved partial or complete resistance to all available antimalarial drugs. *P. falciparum* infections often comprise multiple genetically distinct strains that are suggested to compete for resources within a host. Competition may have important effects on the spread of resistance due to the potential fitness cost associated with resistance as well as suppression of resistant parasites by drug-sensitive competitors. Therefore, the average number of clones in an infection, termed the multiplicity of infection (MOI), could be of great significance. Studies have shown a positive correlation between transmission intensity and MOI on a broad geographic scale, which could potentially explain the surprising pattern of antimalarial drug resistance emerging in low-transmission settings and spreading to high-transmission settings. To explore the link between transmission intensity and MOI on a smaller scale, PCR and microsatellite genotyping were used to assess the MOI of four regions in Ghana that differ in transmission intensity, sampled over a ten-year period. The results indicate no consistent differences in MOI distribution between sites but significant change in MOI over time for all but one site. Further investigations are needed to elucidate the important determinants of MOI on various spatial and temporal scales.

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Acknowledgements

I would like to acknowledge all members within the Division of Parasitic Diseases and Malaria at the CDC for their help in my research, with a special thanks to principal investigator Venkatachalam Udhayakumar for letting me conduct research in his lab as well as Mary Bushman, for closely working with me throughout this project, and Ira Goldman. Additionally, I would like to recognize principal investigator Jacobus de Roode in the Emory Department of Biology for providing me with the opportunity to join his lab and for serving as my mentor in research for the past two years. Finally, I would like to extend my thanks to all individuals involved with the therapeutic efficacy studies in Ghana, in particular Dr. Nancy Duah and Dr. Kwadwo Koram of the Noguchi Memorial Institute of Medical Research, University of Ghana for their assistance and provision of the samples used in this study.

Table of Contents

Abstract

The malaria parasite *Plasmodium falciparum* has evolved partial or complete resistance to all available antimalarial drugs. *P. falciparum* infections often comprise multiple genetically distinct strains that are suggested to compete for resources within a host. Competition may have important effects on the spread of resistance due to the potential fitness cost associated with resistance as well as suppression of resistant parasites by drug-sensitive competitors. Therefore, the average number of clones in an infection, termed the multiplicity of infection (MOI), could be of great significance. Studies have shown a positive correlation between transmission intensity and MOI on a broad geographic scale, which could potentially explain the surprising pattern of antimalarial drug resistance emerging in low-transmission settings and spreading to high-transmission settings. To explore the link between transmission intensity and MOI on a smaller scale, PCR and microsatellite genotyping were used to assess the MOI of four regions in Ghana that differ in transmission intensity, sampled over a ten-year period. The results indicate no consistent differences in MOI distribution between sites but significant change in MOI over time for all but one site. Further investigations are needed to elucidate the important determinants of MOI on various spatial and temporal scales.

Introduction

According to the 2014 World Malaria Report, an estimated 198 million clinical cases of malaria occurred in 2013, accompanied by 584,000 deaths (World Health Organization 2014). Currently, 3.3 billion people are at risk of infection and funding needs exceed $5 billion to combat this disease (World Health Organization 2014). *Plasmodium falciparum* is the most virulent human malaria parasite, responsible for 90% of human malaria infections and nearly all deaths (World Health Organization 2014). While great progress has been made in malaria control, with global mortality dropping 47% since 2000 (World Health Organization 2014), antimalarial drug resistance is a problem that threatens to negate these improvements. High prevalence of resistance to chloroquine and sulphadoxine-pyrimethamine therapies led the World Health Organization to endorse the use of artemisinin-based combination therapies as a first-line treatment for falciparum malaria in nearly all countries endemic to malaria, but spreading resistance to this treatment has been confirmed as well (Bosman & Mendis 2007; Ashley et al. 2014;). With the looming threat of multi-drug resistant malaria, understanding the factors surrounding emergence and spread of drug resistance is of paramount importance.

Transmission intensity, or the frequency with which malaria infections are transmitted, varies geographically. Studies reveal that drug resistance has repeatedly emerged in low-transmission settings in Southeast Asia and South America and then spread to high-transmission settings in Africa (Payne 1987; Roper et al. 2004; Ashley et al. 2014). The origin of resistance in low-transmission regions is surprising, given the simple logic that more opportunities for resistance to evolve are present in high transmission regions due to higher prevalence of infection and

substantial antimalarial drug use. One plausible explanation of this paradoxical observation is that parasites experience higher levels of competition in high-transmission regions.

P. falciparum exhibits substantial genetic diversity (Walliker et al. 1987) and *P. falciparum* infections frequently contain multiple, genetically distinct strains (Babiker & Walliker 1997, Anderson 2000). The proposed link between transmission intensity, competition, and emerging drug resistance partially stems from an observed positive correlation between transmission intensity and average multiplicity of infection (MOI) (Arnot 1998, Anderson et al. 2000). MOI refers to the number of strains detected in a given infection. These various strains are believed to occupy similar ecological niches within the human host, with total parasite density potentially limited by factors such as available red blood cells or glucose levels (Jakemen et al. 1999; Menendez et al. 2000; Li et al. 2001). If limiting resources drive ecological competition within the host, total parasite density should remain constant, while the densities of individual strains are reduced. This is significant because in infections with both drug-sensitive and resistant strains, drug-sensitive strains may competitively suppress drug-resistant strains, inhibiting their spread to new hosts. Experimental models in mice support this concept (de Roode et al 2004). Furthermore, mouse models have demonstrated the within-host expansion of resistant parasite densities following the removal of drug-sensitive competitors, an occurrence termed competitive release (de Roode et al 2004); this expansion may facilitate increased transmission of drug-resistant parasites following treatment.

Of additional importance are findings that suggest that resistance carries a fitness cost (Babiker, Hastings, & Swedberg 2009). Competition can sometimes exaggerate fitness differences, thus competition may increase the strength of selection against resistant strains, potentially accelerating the decline of resistant strains in the absence of drug-mediated selection. Consequently, competition may have two important effects in that: (1) it may lower within-host resistant-strain frequencies and thus decrease resistance transmission and (2) increase negative selection pressure against resistant strains due to the predicted fitness cost of resistance.

Competition is thought to be less severe with low MOI because there are fewer strains competing in each host; thus, in areas of low transmission with low MOI, transmission of resistant parasites is less strongly inhibited. Mathematical models support the potential link between MOI and the emergence of drug resistance in a region (Klein et al. 2012, Hastings 1997).

In regions forced to retire certain antimalarials due to high rates of treatment failure, studies that tracked the prevalence of resistance following drug retirement further support the hypothesis that competition inhibits spread of resistant parasites. Multiple studies have found that the frequency of resistant alleles significantly dropped following a drug's retirement (Hailemeskel et al. 2013; Laufer et al. 2006; Mwai et al. 2009; Zhou et al. 2008), providing that resistance has not reached fixation within a population (McCollum et al. 2007). Furthermore, in countries with low transmission, resistance prevalence appears to decline at a slower rate (Liu et al. 1995). While the links between transmission intensity, MOI,

and drug resistance have been explored on a global level, there is a need to explore whether these links are observed at a more regional level.

This study explores a previously noted (unpublished) trend regarding drug resistance decline in Ghana. In the course of ongoing antimalarial efficacy studies, patient samples were collected from four sites in Ghana (Figure 1, Table 1) between 1999 and 2010. Midway through this period in early 2005, the antimalarial drug chloroquine was retired due to markedly low efficacy (Ghana Health Service 2004), providing the opportunity to monitor the subsequent decline in drug resistance at all four sites. Interestingly, while all four sites demonstrated a significant decline in resistance prevalence ($p=1E-8$), there also appeared to be differences between the sites in the rates of decline of resistance prevalence (Figure 2). Of additional interest is that the four regions sampled vary in transmission intensity (Figure 1, Table 1). Consequently, this study provides the opportunity to explore the links between transmission intensity, MOI, and drug resistance decline on a smaller geographic scale. Using microsatellite genotyping to examine multiplicity of infection at all four sites throughout the sample collection period, this study investigated the hypothesis that high transmission sites experienced faster rates of drug resistance decline due to higher MOI and thus increased competition.

Methods

Study Sites Ecology.

This study used samples from four sites in Ghana (Figure 1, Table 1), obtained in the course of studies monitoring the efficacy of antimalarial drugs in Ghana. Two sites, Navrongo and Yendi, are rural and located in the northern belt of Ghana, which is primarily savannah. The other two sites, Hohoe and Sunyani, are urban and located in the middle belt of Ghana, which is primarily forested. Navrongo is the capital of the Kassena Nakana district in the Upper East region of Ghana, with an estimated human population of 25,470. Malaria transmission here is highly seasonal, with most infections occurring during a rainy period from June to November (Duah et al. 2007). Yendi is the capital of the Yendi Municipality in northern Ghana, with an estimated human population of 40,336. Transmission is very seasonal and mostly occurs between June and November (Duah et al. 2007). Hohoe is the capital of the Hohoe Municipality in the Volta region, with an estimated human population of 40,303. Malaria occurs here year round, but with increases shortly following its main rainy season from June-October (Duah et al. 2007). Sunyani is the capital of the Sunyani Municipality in the Brong-Ahafo region, with an estimated human population of 80,245. Transmission is perennial, with peaks following the major rains in June-October (Duah et al. 2007).

Study Sites Transmission Intensity.

There are different methodologies for measuring transmission intensity in a region, but the measurement that best incorporates exposure of the human population to the infectious stages of the malaria parasites, termed sporozoites, is the entomological inoculation rate (EIR). EIR is calculated as the product of the

measured sporozoite prevalence in a mosquito population multiplied by the estimated mosquito human biting rate. (Shaukat, Breman, & McKenzie 2010).

Between the year 2001 and 2002, the recorded mean EIR in Navrongo was 418 infective bites per person per year (Appawu et al. 2004). No estimates of EIR for Yendi have been published in the last ten years, but the EIR can be assumed to be similar to Navrongo because it lies in the same ecological region of Ghana. The closest site to Hohoe with a published estimate of EIR is Kpone (approximately 200 kilometers away), with a reported EIR of 62.1 infective bites per person per year (Nancy Duah, pers. comm.). The closest site to Sunyani with a published estimate of EIR is Kintampo (approximately 125 kilometers away), with a reported EIR of 269 infective bites per person per year (Derry et al. 2010,). There are different methods of estimating EIR that may yield different results (Shaukat, Breman, & McKenzie 2010), so the EIR values cited in the literature should be viewed on a relative, not absolute scale because they were estimated using different methods. The Malaria Atlas Project map provides a depiction of the transmission intensity differences between the four sites that can be used for a relative comparison as well (Figure 1). Based upon both the literature (Table 1) and the Malaria Atlas Project estimates, the sites' transmission intensities increase: Hohoe<Sunyani<Yendi<Navrongo.

Sample Collection.

Samples were collected at approximately two-year intervals between 1999 and 2010. Finger-prick blood samples, absorbed onto Whatman filter paper, were taken from children aged 6-59 months presenting symptoms of uncomplicated

malaria or with a history of fever during the past twenty-four hours. Further inclusion criteria for children entailed infection with only *P. falciparum* detected by microscopy and parasitemia levels between 2000 and 200,000 parasites per µl (e.g. Koram et al. 2005). All samples were acquired prior to any treatment (see Table 2 for sample sizes).

DNA Isolation and Genotyping Methods.

DNA was extracted from blood spots on filter papers using the TE buffer extraction method (Bereczky et al. 2005) with the QIAmp DNA Mini Kit (Qiagen).

Five putatively neutral microsatellites (Table 3) were amplified using PCR. Hemi-nested PCRs were used for TA1, Polyα, PfPK2, and 2490, with cycling conditions and primers as described in Anderson et al. (1999). The primary reaction contained 1 µl template, 8.5 µl PCR 2X Master Mix (Promega), 0.6 µl forward primer, 0.6 µl of reverse primer, and 4.3 µl of water, for a total volume of 15 µl. The secondary reaction contained the same concentrations, using primary reaction product as the template. Nonnested PCR was used for C3M69, with cycling conditions as described in Nair et al. (2003) and primers as described in McCollum et al. (2007). The reaction mixture contained 2 µl template, 7.5 µl 2X Master Mix, 0.6 µl forward primer, 0.6 µl reverse primer, and 4.3 µl of water, for a total volume of 15 µl. Parasite strains 3D7, Dd2, HB3, and 7G8 were used as positive controls for microsatellite genotyping.

Microsatellite Analysis.

PCR products were genotyped by fragment electrophoresis on a capillary sequencer (ABI 3130 xl Genetic Analyzer). ROX 350 ladder (Applied Biosystems) was used to set the size comparison standard for the alleles and was mixed with HiDi (Applied Biosystems) in a 1:24 ratio, respectively. 10.5 µl of this mixture was added to 1.5 µl of PCR product in each well. Microsatellite fragment analysis was performed using the Geneious Pro R8 microsatellite plugin. Calling peaks (deciding whether to include a peak as an allele or discard as noise or artifact) was performed manually for improved discrimination and flexibility. For consistency in calling peaks, rules were established: for all loci, additional peaks had to be at least 25% of the height of the tallest peak. Each locus had a unique, characteristic shape, so additional rules were established for each locus (see Supplemental Information).

All loci examined had a repeat unit of three, so the software sorted alleles according to size into "bins" three bp apart. Each allele was considered a distinct genotype. Due to the haploid state of *Plasmodium* in erythrocytic stages, the number of alleles found in a sample corresponds to the number of parasite genotypes found in the sample. It is important to note that due to limitations in PCR sensitivity to multiple parasite strains, the number of genotypes determined to be in a sample is a lower bound (minimum) for MOI and most likely an underestimate.

Data Analysis

The proportions of samples from Ghana positive for chloroquine-resistant parasites were analyzed using a generalized linear model with binomial errors, with time as a continuous variable and location (a proxy for transmission intensity) as an

ordered factor (Hohoe<Sunyani<Yendi<Navrongo). Chloroquine resistance prevalence decreased significantly with time (p=1E-8) and was significantly associated with transmission intensity overall (p=0.0002). In addition, the rate of decline of chloroquine resistance was significantly associated with transmission intensity (time x location interaction effect, p=0.018; Figure 2). The single and interaction effects of transmission intensity were observed with quadratic orthogonal contrasts.

In this study, for each sample, the number of alleles at each locus was determined and the largest of these values was used as the estimate of MOI for the sample. Kruskal-Wallis tests were used to compare the MOI distributions between sets of samples from various locations and sampling periods. Additionally, the proportions of sample populations with mixed-strain infections (MOI>1) were examined. Fisher's exact tests were used to assess whether location or sample period affected the prevalence of mixed-strain infections. A chi-squared test was used in place of a Fisher's exact test when variation across all time periods was examined due to larger sample numbers. Allelic richness, or the total number of alleles observed at a particular locus, was examined for every microsatellite except C3M69 because its peak shape prevents accurate estimation of amplicon size (see Supplemental Information).

Results

Mean MOI

The MOI for all samples in each region was calculated to test the prediction that MOI would be higher in regions with increased transmission intensity. To match this expectation, MOI was expected to follow the pattern: Hohoe<Sunyani<Yendi<Navrongo, due to their corresponding increasing transmission intensities. Across the study, MOI ranged from zero to six and its mode was two (Figure 3, Table 4). Mean MOI was calculated as an estimate of each region's overall MOI and compared between sites to assess if the expected pattern was followed. Values in Table 4 demonstrate that no correlation appeared to emerge between transmission intensity and mean MOI. The distribution of MOI did not vary significantly by location across all time periods, but did show significant variation between sites for the sample periods 1999-2000, 2007-2008, and 2010 ($H=13.53$, $df=3$, $p<0.01$; $H=32.52$, $df=3$, $p<1E-6$; $H=12.22$, $df=2$, $p<0.01$, respectively; Table 5a). MOI distribution significantly varied across sample periods for all locations except Yendi ($H=31.89$, $df=3$, $p<1E-6$; $H=31.42$, $df=2$, $p<1E-6$; $H=18.06$, $df=4$, $p=0.001$; for Hohoe, Sunyani, and Navrongo respectively; Table 5b).

Proportion of mixed-strain infections

The proportion of infections harboring multiple strains (those with MOI>1) was examined as another way to test whether higher transmission regions had more mixed-strain infections. Proportion with MOI>1 was estimated by dividing the number of samples with MOI>1 by the total sample size for a particular time period and location. Across all locations and time periods, proportion MOI>1 had a lower limit of 0.59 and an upper limit of 1.0 (Table 4). When compared between sites,

proportion with MOI>1 values did not follow the expected trend: Hohoe<Sunyani<Yendi< Navrongo (Table 4, 6a). A chi squared test assessed if location impacted the proportion of infections with MOI=1 to MOI>1 across all time periods. While proportion of mixed-strain infections varied significantly by location across all sample periods (X^2=12.80, p<0.01), when periods were examined individually, Fisher's exact tests indicated that only the time intervals 1999-2000, 2005-2006, and 2007-2008 significantly varied by location (p<0.001; p=0.05; p=0.003, respectively; Table 6a). Fisher exact tests also evaluated if time period impacted mixed-strain proportions. It seems that in all locations except Yendi, the proportion of mixed-strain infections varied significantly by year (p<0.001; p<0.002; p=0.001, for Navrongo, Sunyani and Hohoe, respectively; Table 6b).

Allelic Richness

Allelic richness (number of alleles per locus) was investigated to determine if increased richness resulted in increased MOI. Among the four microsatellites examined, the maximum number of alleles was twenty-four and the minimum was four. Allelic richness was consistently highest in Polyα and lowest in 2490, with TA1 and PfPK2 in the middle range (Figure 4). In a regression analysis, no correlation was seemed to exist between MOI and allelic richness (Figure 6). Generally, allelic richness did not appear to significantly change over time in any of the locations (Figure 5), nor did location seem to have an effect on allelic richness (Figure 4), with two notable exceptions. The first was that Polyα allelic richness increased from thirteen to nineteen in Hohoe from 1999-2000 to 2003-2004. To determine if this

influenced MOI, changes in mean MOI (based on all loci) as well as MOI for the single locus Polyα were examined for these two time periods in Hohoe. Mean MOI for all loci increased from 1.71 to 2.32 (Table 4) and MOI for Polyα increased from 1.11 to 1.46. Consequently, the increased MOI for both Polyα and all loci between 1999-2000 and 2003-2004 could partially result from a corresponding increase in allelic richness between these time periods. The second noteable change in allelic richness was that TA1 richness dropped from twelve to eight after 2007-2008 in Yendi (Figure 4a, 5d). Between 2007-2008 and 2010 in Yendi, mean MOI dropped from 2.10 to 2.02 (Table 4) and TA1 MOI dropped from 1.38 to 1.37. Due to these very minor changes in MOI, for these time periods in Yendi, changes in allelic richness do not seem likely to have influenced MOI.

Discussion

The results indicate overall that MOI and mixed-strain proportions were highly variable. Under assumed stable transmission intensities, MOI and mixed-strain proportions varied significantly between time periods for all sites but one, indicating they may be under important additional influences beyond a region's transmission intensity (Table 5b, 6b). Additionally, for some time periods there was significant variation between sites, while for others, MOI as well as mixed-strain proportions appeared to be similar (Table 5a, 6a). This again suggests that MOI is subject to other variables beyond a region's transmission intensity.

Surprisingly, mean MOI and proportion of mixed-strain infections in the four regions did not follow the expected trend of increasing transmission intensity with increasing MOI. The expected pattern for both (Hohoe<Sunyani<Yendi<Navrongo) was not observed (Table 5a, 6a).

Allelic richness did not seem to influence MOI because it generally was similar between sites and appeared to remain consistent (Figure 4, 5). Additionally, no correlation was found between MOI and allelic richness for the four loci (Figure 6).

The lack of positive correlation between transmission intensity and mixed-strain infections could suggest that (1) MOI is under additional influences beyond annual transmission intensity or (2) measurement errors pertaining to transmission intensity or MOI may have influenced these results.

One possibility for the first suggestion is that MOI is sensitive to short-term variability in transmission intensity. This study ranked the transmission intensities of the four sites in Ghana based upon the annual entomological inoculation rates (EIR) as predicted by models from the Malaria Atlas Project as well as annual EIR numbers previously published in the literature. However, studies indicate there is significant temporal variation in transmission intensity (Kasasa et al. 2013, Appawu et al. 2004, Rumisha et al. 2014). Kasasa et al. (2013) found EIR to significantly vary by month and to be influenced by climate, land use, and environmental factors. Additionally, these regions experience increases in transmission and infection prevalence following their rainy seasons. Consequently, because all samples for this study were collected during these rainy seasons, an annual EIR value may not be an

accurate representation of the true EIR in the four regions when the samples were collected. Additionally, in one three-year study, annual EIR in Navrongo ranged from 157 to 1132 infective bites (Kasasa et al. 2013). Because of the high degree of both monthly and yearly EIR variation, a study in which monthly EIR values are recorded and then compared to corresponding monthly MOI values would be beneficial to further investigate the link between MOI and transmission intensity.

Another possible reason for the observed indistinct relationship between MOI and transmission intensity in the Ghana samples could be that the previously noted relationship between MOI and transmission intensity is not as strong in high transmission regions. In a published meta-analysis by Arnot (1998), there clearly was a positive correlation between MOI and EIR. However, there was noticeably more variation in average MOI for regions with EIR >100. In this study, all sample sites except Hohoe had transmission intensities predicted to exceed 100 (EIR [≈]62.1, [≈]269, [≈]418, [=]418 for Hohoe, Sunyani, Yendi, and Navrongo, respectively). Additionally, the estimated EIR for Hohoe (62.1) is still substantially greater than EIR estimates (<1) for most of Southeast Asia and South America (Gething et al. 2011). Consequently, it is plausible that MOI differs markedly between areas with extremely low and high transmission, but after a certain level of transmission intensity is reached, further increases are not significant with respect to MOI and possible within-host competition.

The lack of significant differences between sites could also be due to an idea that Hastings and Watkins (2005) proposed. They noted that in the Anderson et al. (2000) study that linked increased prevalence of infection to higher MOI, there was

a disproportionately high number of infections that had two or more strains in areas of extremely low transmission in Brazil. They suggested that based upon a commonly observed finding that a small portion of a population suffers a disproportionate burden of overall infection (Woolhouse et al. 1997), decreasing transmission intensity lowers disease prevalence by protecting individuals only mildly at risk, while MOI remains high in the overly burdened portion of the population. Thus, in low-transmission regions, a minority of infections may have high MOI, yet these minority cases are more likely to be symptomatic and thus tested. With this notion, while the prevalence of infections in a region is low, the MOI of all infections would still be high. This concept could be important in regions with all relatively high transmission intensities (EIR>50) and could potentially reveal why MOI did not seem to significantly vary between sites.

Regarding measurement error, there are several problems with EIR as a measured proxy for transmission intensity in a region. EIR is the product of the measured sporozoite prevalence in a mosquito population multiplied by the estimated mosquito human biting rate. Estimates of these components are cumbersome and the sensitivity of each goes down with declining transmission. Multiple methods can be used to calculate EIR, with each resulting in slightly different end EIR values. For example, to calculate the human biting rate component of EIR, human landing catches, pyrethrum spray catches, exit trap collections, and CDC light traps can all be used to capture mosquitoes (World Health Organization 1975). Because of this, there is an issue of comparability between values provided in the literature. Therefore, while numbers from the literature were given, they can

only be used as a general indicator of the transmission intensity in a region. The models that predicted EIR were also useful only in providing transmission intensities of sites in relation to one another. It is possible that the EIR differences between sites are less than predicted, which could explain the apparent overall lack of differences in MOI between sites.

Measurement error in MOI is likely to have occurred as well due to a sensitivity threshold of the PCR and capillary electrophoresis methodology used. It is believed that these techniques have a limited ability to detect minority strains in multiple-strain infections. Strains low in absolute parisitemia or low in frequency relative to other strains in an infection are likely to remain undetected (Contamin et al 1995). Much debate surrounds the implications of this PCR sensitivity issue. On the one hand, PCR and capillary electrophoresis provides MOI values that can be compared on a relative scale and thus, lack of sensitivity could be deemed irrelevant. However, MOI values could also be significantly greater than what PCR can detect and consequently, MOI differences of one or two might be insignificant. In this study, we assumed the former prediction that MOI can be viewed on a relative scale; however, if the latter idea proved to be more accurate, our results would be dramatically skewed.

Sample collection was also a limitation to this study due to the fact that all samples were acquired from children presenting symptoms of uncomplicated malaria. MOI has been shown to vary based upon age group and symptoms. Asymptomatic malaria patients have repeatedly been shown to have lower MOI than symptomatic patients from the same area (Smith et al. 1999). Additionally,

adults seem to tend to have lower MOI than children (Smith et al. 1999). Consequently, this study provides a limited depiction of MOI variation across the entire population.

If the measuring caveats are considered to be unimportant, further explorations are needed to elucidate the association between transmission intensity and MOI. This relationship may only be important at large geographical scales (e.g. sub-Saharan Africa versus South-East Asia) and when comparing regions with drastically different transmission intensities (e.g. EIR<10 versus EIR >100). This study examined their relationship at a regional scale, but still at a large temporal time scale. Additional small-scale geo-temporal studies are needed. Additionally, to continue to explore factors behind the differing rates of drug decline in Ghana, a separate investigation examining the population structure of each site regarding genetic differentiation (for example through Fst) between sites would be helpful. It is possible that regions varied due to distinct resistant genotypes that were better adapted than others in competing against other strains rather than due to differences in MOI. Additionally, if it appeared that all sites were genetically similar, it would suggest that all parasites in Ghana could be viewed as one large population, which is useful knowledge for drug policy implementation.

Exploring this link between transmission intensity and MOI aims to deepen our current understanding of the potential role competition plays in drug resistance emergence and survival. The unexpected variability of MOI demonstrated in this study reveals that these links may not be as simple as originally suggested. More studies are needed before drug policies can incorporate this knowledge. Ultimately,

better comprehension surrounding MOI and its determinants will aid new and better policies for managing drug resistance.

Figures and Tables

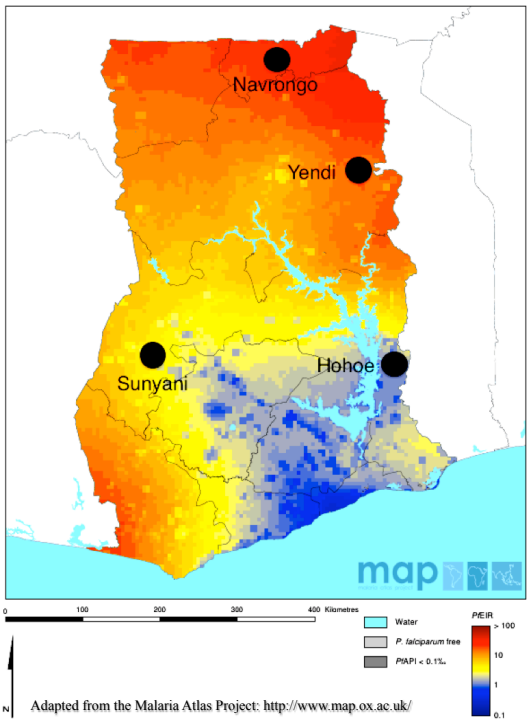

Figure 1. *P. falciparum* transmission intensity in Ghana. Red=high; blue=low. Sites ranked by EIR, high to low: Navrongo>Yendi>Sunyani>Hohoe. Estimates of *P. falciparum* EIR *(*PfEIR) were made using a model that incorporated data on estimated parasite prevalence rates.

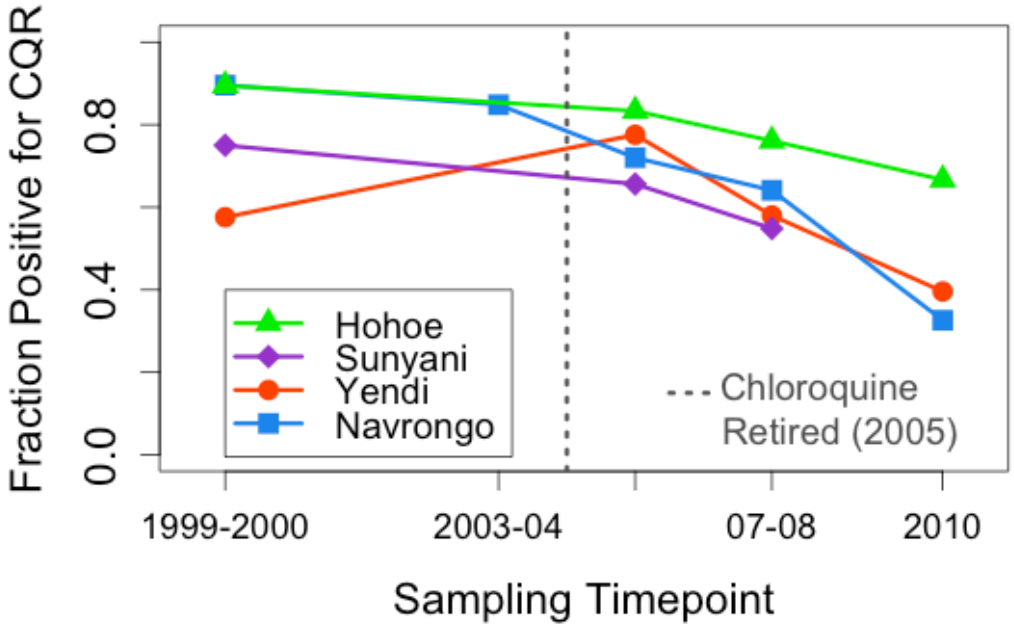

Figure 2. Fraction of samples from Ghana positive for chloroquine-resistant (CQR) genotype. Dotted gray line shows the year (2005) that chloroquine was retired as a first-line treatment for malaria. CQR prevalence decreased significantly with time for all sites (p=1E-8) and was significantly associated with transmission intensity overall (p=0.0002). Rate of decline of CQR was significantly associated with transmission intensity (time x location interaction effect, p=0.018).

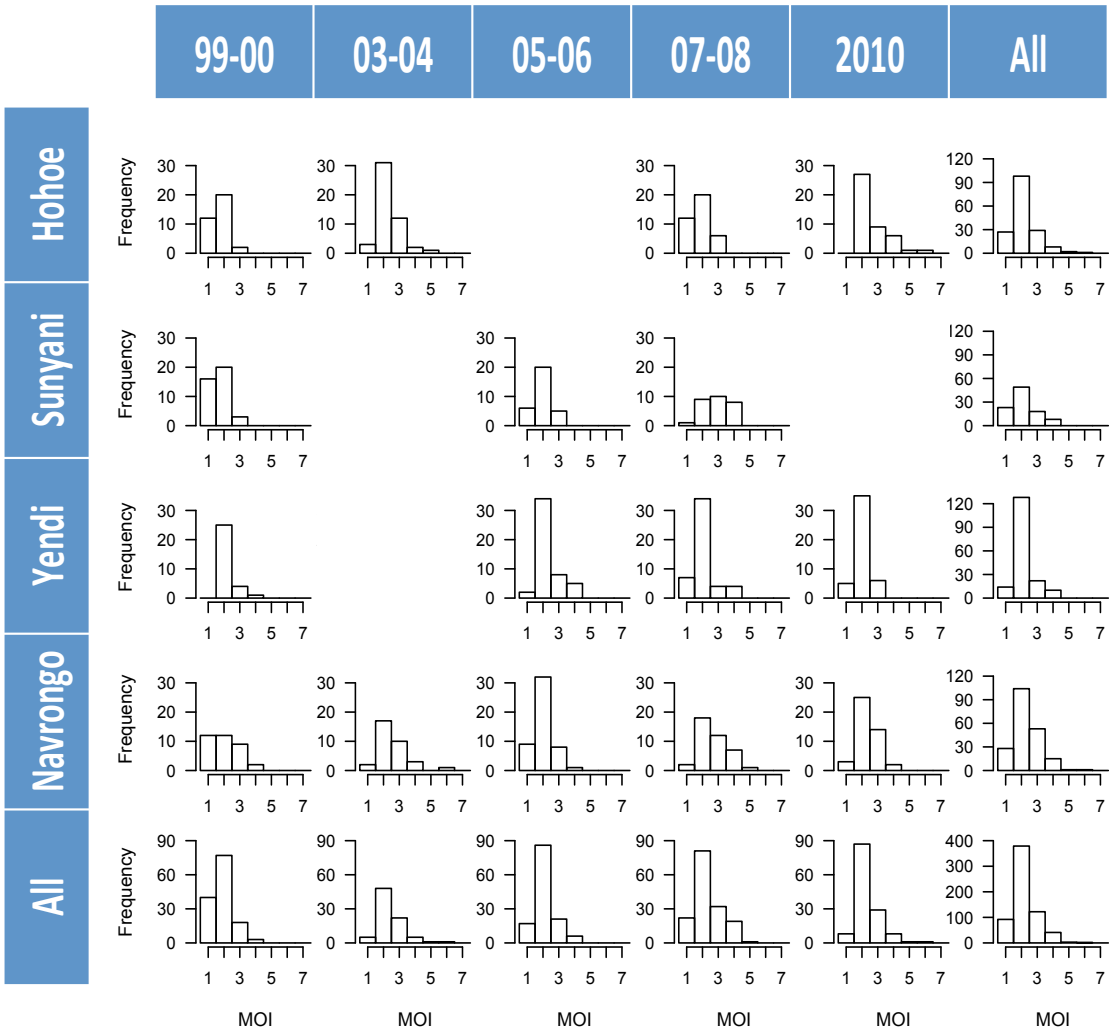

Figure 3. Histograms displaying distribution of MOI for samples stratified by location and sample period. Sample periods are listed in consecutive order from left to right; locations are listed by row in order of increasing transmission. Blank areas indicate that samples were not collected in that location during that specific time period. Note that histograms that encompass all locations or all sample periods have a greater Y-axis limit than the other graphs.

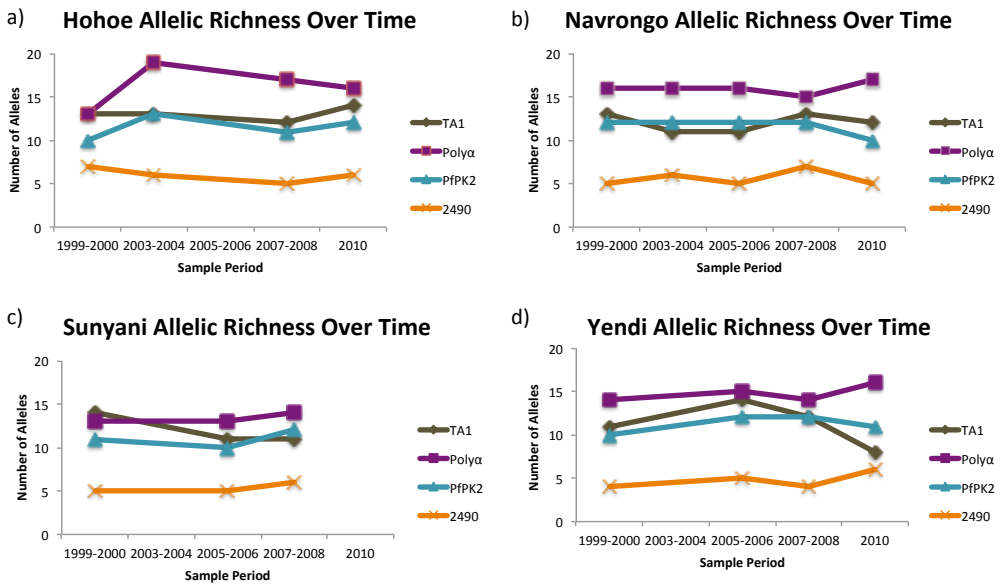

Figure 4. Plots of allelic richness over time for each of the four sample sites.

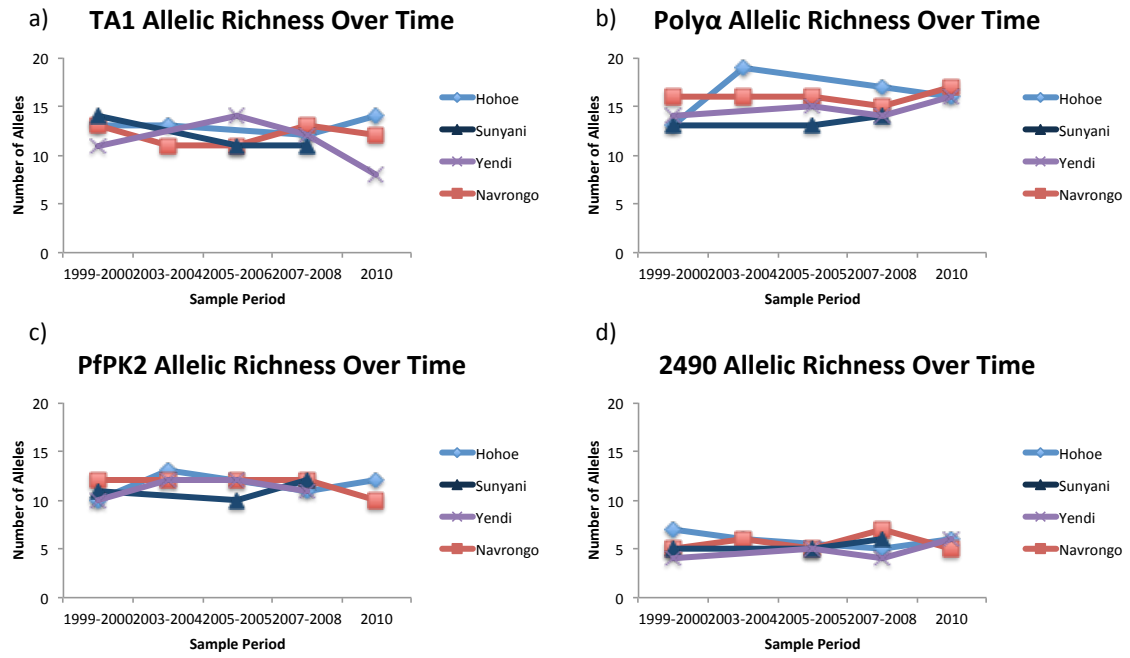

Figure 5. Plots of allelic richness over time for each of the four microsatellite loci.

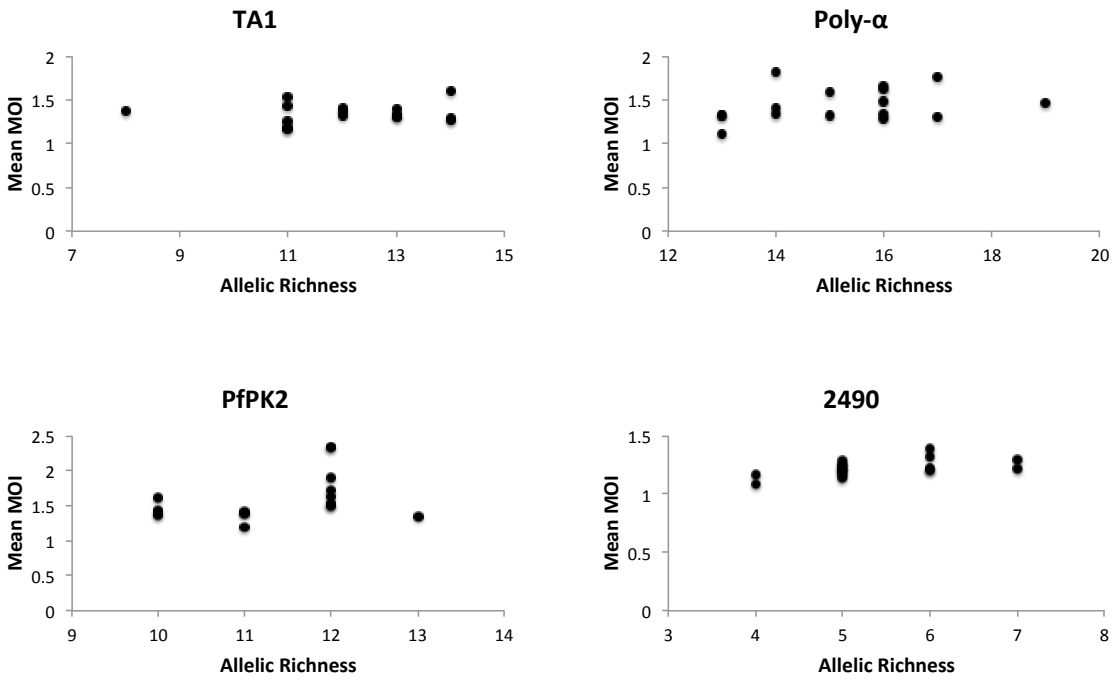

Figure 6. Plots of average MOI and allelic richness for four loci. Each point represents the locus's average MOI for a particular time period and location.

Table 1. Description of four sample sites. Entomological inoculation rate (EIR) is a measurement of transmission intensity, calculated as the human biting rate multiplied by the sporozoite rate of the mosquito population.

	Hohoe	Sunyani	Yendi	Navrongo
Ecology	Urban; forested	Urban; forested	Rural; savannah	Rural; savannah
Population size	40,303	80,245	40,336	25,470
Transmission	Perennial	Perennial	Seasonal	Seasonal
EIR	≈62.1 (of site 200 km away)	≈269 (of site 125 km away)	Similar to Navrongo	≈413

Table 2. Sample sizes for study sites Hohoe, Sunyani, Navrongo, and Yendi. Values without parentheses indicate samples with all five loci successfully genotyped. Values in parentheses include total number of samples collected at each site for each time period. The restricted sample set was used for all MOI analyses; the more inclusive sample set was used to examine allelic richness. NA indicates that samples were not collected in that particular year and location.

*Sunyani sample period was from 1999-2001

	Hohoe	**Sunyani**	**Yendi**	**Navrongo**
1999-2000	34 (45)	*39 (48)	30 (40)	33 (50)
2003-2004	49 (50)	NA	NA	33 (34)
2005-2006	NA	31 (36)	49 (49)	50 (50)
2007-2008	38 (49)	28 (32)	49 (49)	40 (45)
2010	44 (50)	NA	46 (48)	44 (50)
All	165 (194)	98 (116)	174 (186)	200 (229)

Table 3. List of microsatellite regions amplified with their respective chromosome and PCR technique.

Locus	*Chromosome*	*PCR Technique*
TA1	6	Hemi-nested
Poly**α**	4	Hemi-nested
PfPK2	12	Hemi-nested
2490	10	Hemi-nested
C3M69	3	Non-nested

Table 4. *P. falciparum* multiplicity of infection stratified by location and time period. From left to right, locations are listed in order of increasing EIR. Values without parentheses indicate samples with all five loci successfully genotyped. Values in parenthesis include total number of samples collected at each site for each time period. The restricted sample set was used for all MOI analyses; the more inclusive sample set was used to examine allelic richness.

All Sample Periods

MOI	Hohoe	Sunyani	Yendi	Navrongo
1	27 (37)	23 (31)	14 (21)	28 (39)
2	98 (109)	49 (53)	128 (134)	104 (112)
3	29 (36)	18 (23)	22 (22)	53 (59)
4	8 (9)	8 (9)	10 (10)	15 (17)
5	2 (2)	0 (0)	0 (0)	1 (1)
6	1(1)	0 (0)	0 (0)	1 (1)
Total	165 (194)	98 (116)	174 (187)	202 (229)
Mean	2.17	2.11	2.16	2.31
Percent with MOI >1	83.6	76.5	91.9	86.1

1999-2000

MOI	Hohoe	Sunyani	Yendi	Navrongo
1	12 (18)	16 (21)	0 (7)	12 (20)
2	20 (21)	20 (22)	25 (28)	12 (17)
3	2 (6)	3 (5)	4 (4)	9 (11)
4	0 (0)	0 (0)	1 (1)	2 (2)
5	0 (0)	0 (0)	0 (0)	0 (0)
6	0 (0)	0 (0)	0 (0)	0 (0)
Total	34 (45)	39 (48)	30 (40)	35 (50)
Mean	1.71	1.67	2.2	2.03
Percent with MOI >1	64.7	59	100	65.7

2003-2004

MOI	Hohoe	Navrongo
1	3 (3)	2 (2)
2	31 (31)	17 (18)
3	12 (13)	10 (10)
4	2 (2)	3 (3)
5	1 (1)	0 (0)
6	0 (0)	1 (1)
Total	49 (50)	33 (34)
Mean	2.32	2.55
Percent with MOI >1	93.9	93.9

2005-2006

MOI	Sunyani	Yendi	Navrongo
1	6 (9)	2 (2)	9 (9)
2	20 (22)	34 (34)	32 (32)
3	5 (5)	8 (8)	8 (8)
4	0 (0)	5 (5)	1 (1)
5	0 (0)	0 (0)	0 (0)
6	0 (0)	0 (0)	0 (0)
Total	31 (36)	49 (49)	50 (50)
Mean	1.97	2.33	2.02
Percent with MOI >1	80.6	95.9	82

2007-2008

MOI	Hohoe	Sunyani	Yendi	Navrongo
1	12 (15)	1 (1)	7 (7)	2 (2)
2	20 (26)	9 (9)	34 (35)	18 (19)
3	6 (8)	10 (13)	4 (4)	12 (15)
4	0 (0)	8 (9)	4 (4)	7 (8)
5	0 (0)	0 (0)	0 (0)	1 (1)
6	0 (0)	0 (0)	0 (0)	0 (0)
Total	38 (49)	28 (32)	49 (50)	40 (45)
Mean	1.84	2.89	2.1	2.68
Percent with MOI >1	68.4	96.4	85.7	95

2010

MOI	Hohoe	Yendi	Navrongo
1	0 (1)	5 (5)	3 (6)
2	27 (31)	35 (37)	25 (26)
3	9 (9)	6 (6)	14 (15)
4	6 (7)	0 (0)	2 (3)
5	1 (1)	0 (0)	0 (0)
6	1 (1)	0 (0)	0 (0)
Total	44 (50)	46 (48)	44 (50)
Mean	2.64	2.02	2.34
Percent with MOI >1	100	89.1	93.2

Table 5. Summary of results for mean MOI. a) Examination of significant (P values ≤0.05) between site variation in mean MOI. Expected trend was mean MOI would increase as: Hohoe<Sunyani<Yendi<Navrongo. b) Examination of significant (P values ≤0.05) between sample period variation.

a)

Time Period	Vary by location?	Expected trend?
1999-2000	Yes	No
2003-2004	No	NA
2005-2006	No	NA
2007-2008	Yes	No
2010	Yes	No
All periods	No	NA

b)

Sample Site	Vary by time?
Hohoe	Yes
Sunyani	Yes
Yendi	No
Navrongo	Yes

Table 6. Summary of results for proportion of mixed-strain infections. a) Examination of significant (P values ≤0.05) between site variation. Expected trend was proportion of mixed-strain infections would increase as: Hohoe<Sunyani<Yendi<Navrongo. b) Examination of significant (P values ≤0.05) between sample period variation.

a)

Time Period	Vary by location?	Expected trend?
1999-2000	Yes	No
2003-2004	No	NA
2005-2006	No	NA
2007-2008	Yes	No
2010	No	NA
All periods	Yes	No

b)

Sample Site	Vary by time?
Hohoe	Yes
Sunyani	Yes
Yendi	No
Navrongo	Yes

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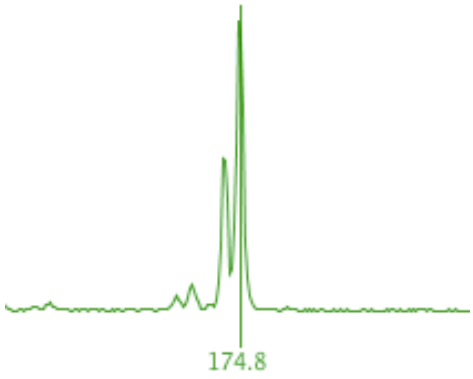
Supplemental Information

Peak Identification. The five microsatellites are listed below with information pertaining to peak shape as well as rules established for calling peaks. These rules were created after viewing the entire set of samples and assessing characteristics typical of each locus.

TA1.

Locus information: range: 125 to 210 bp; repeat unit: 3; dye: HEX

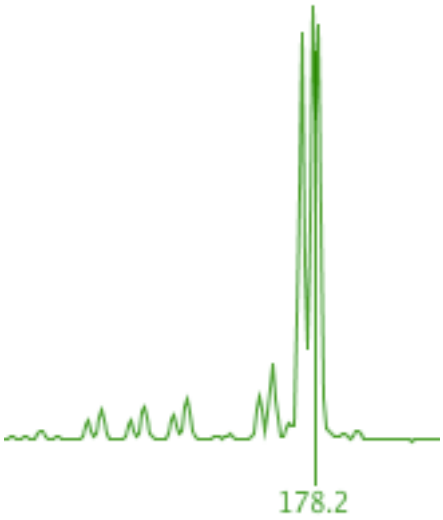
Peak shape: double peak; often has smaller peak three units before taller double peak



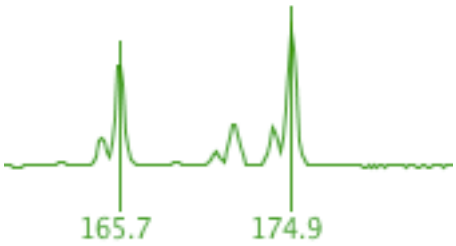
Rules for calling peaks:

1) Additional peaks should be at least 25% height of tallest peak
2) Exclude peaks 3 units before taller peaks unless they are at least 50% height of subsequent peak

Examples:



a. =>only one peak called; other peaks are less than 25% height of tallest peak on far right

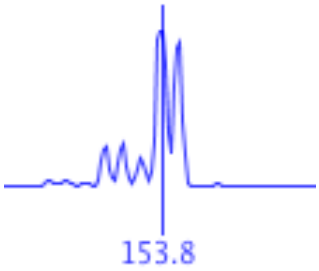


b. =>middle peak not called because it is less than 50% height of peak 3 units to its right

Polyα.

Locus information: range: 100 to 196 bp; repeat unit: 3; dye: FAM

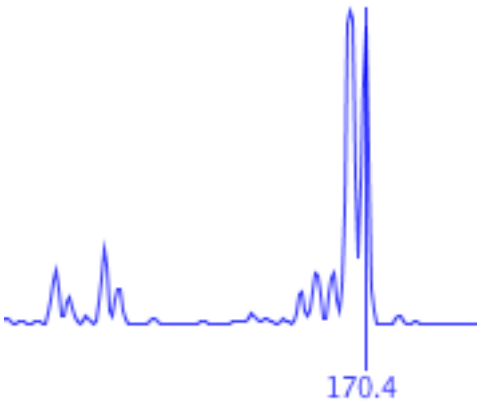
Peak shape: three short peaks preceding a taller double peak



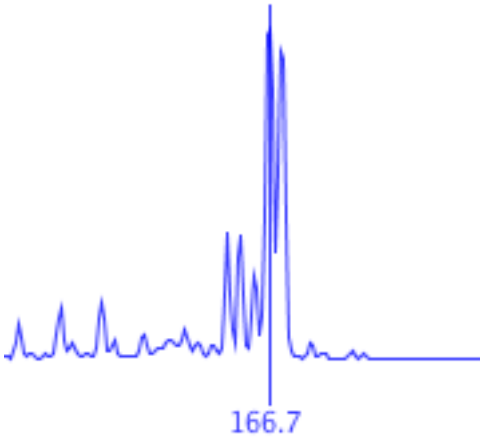
Rules for calling peaks:

1) Additional peaks should be at least 25% height of tallest peak
2) Exclude the three preceding peaks as a separate peak unless it is 75% height or greater of consecutive double peak
3) Shape of peak is extremely important; do not call peaks that lack the characteristic triple peak followed by a taller double peak

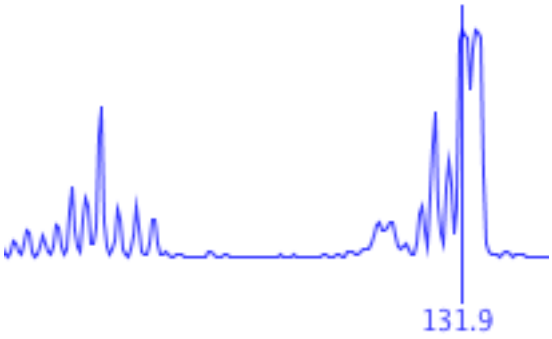
Examples:

a. 

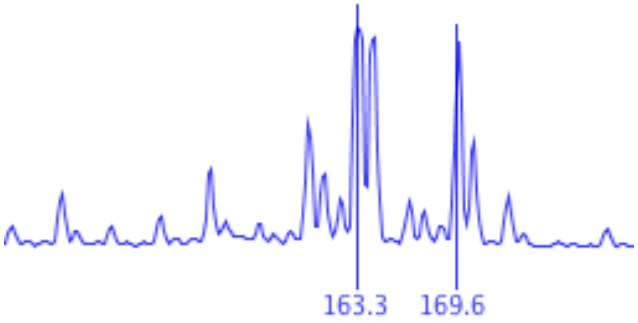
=>peaks on left not called because they are not at least 25% height of tallest peak



b. =>2 taller peaks before main double peak not called because they are less than 75% height of subsequent taller peaks

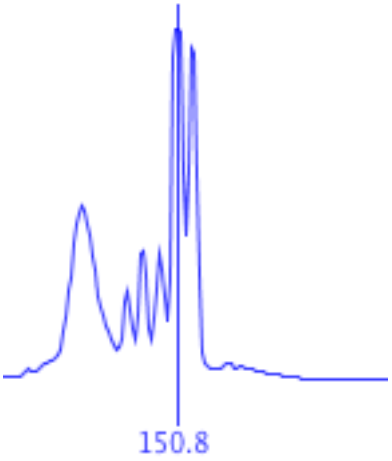


c. =>peak on left not called because it lacks the distinct tall double peak with three shorter peaks and noise surrounds it



d. =>peaks on left not called because they lack the characteristic double peak shape

Additional Notes:

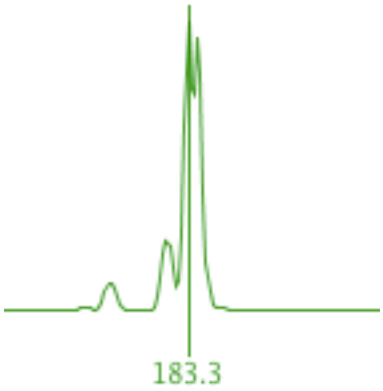


a. =>this large rounded peak was classified as noise

PfPK2.

Locus information: range: 138 to 196 bp; repeat unit: 3; dye: HEX

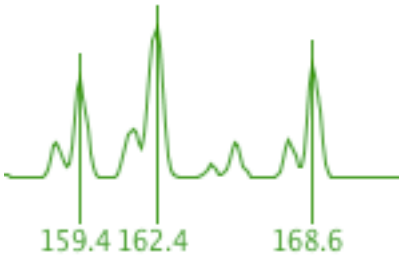
Peak shape: single peak; often a small peak three units before main peak



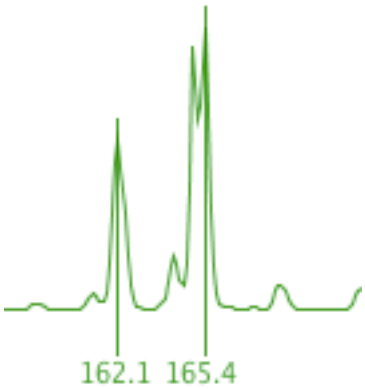
Rules for calling peaks:

1) Additional peaks should be at least 25% height of tallest peak
2) Exclude peaks 3 units before taller peaks unless they are at least 75% height of subsequent peak

Examples:

a. 

=>middle right peak not called because it is less than 25% height of tallest peak

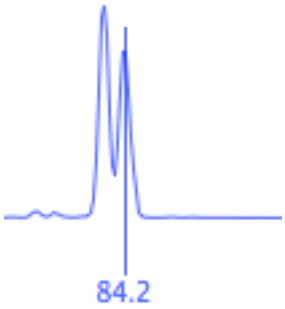
b. 

=>left peak called because it is at least 75% height of taller peak to its right

2490.

Locus information: range: 70 to 98 bp; repeat unit: 3; dye: FAM

Peak shape: Double peak



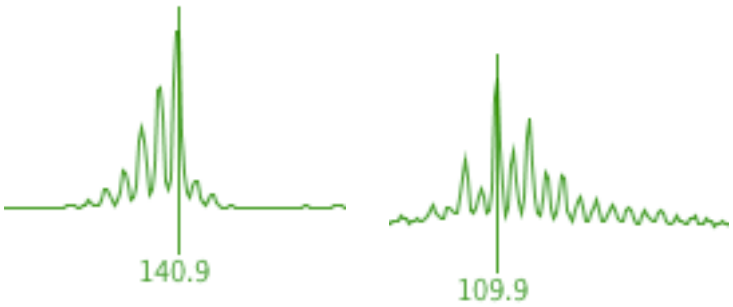
Rules for calling peaks:

1) Additional peaks should be at least 25% height of tallest peak
2) If low level of noise, minimum peak height is 150

C3M69.

Locus information: range: 80 to 180 bp; repeat unit: 3; dye: HEX

Shape of peak: series of peaks that build to highest peak then taper

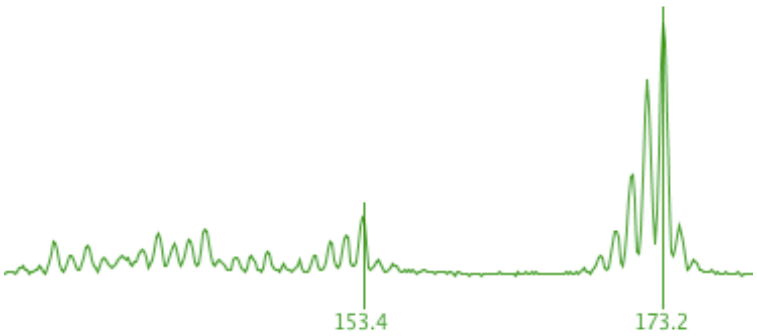


or

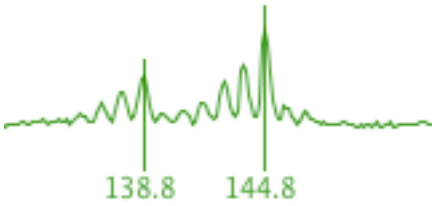
Rules for calling peaks:

1) Additional peaks should be at least 25% height of tallest peak; with this locus, this rule is extremely important to follow due to difficulty in discerning noise from peaks. For consistency, all areas with peaks at least 25% height of tallest peak were called and peak shape was ignored

Examples:



a. =>one additional peak called because it was the only peak at least 25% height of main peak



b. =>two peaks called because there are two distinct regions with the building and tapering shape