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# Prevalence Of Asymptomatic Malaria Among Children In Kumasi, Ghana

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# Abstract

As the landscape of malaria in sub-Saharan Africa changes due to improved control efforts, there is increasing interest in elucidating the epidemiology of asymptomatic *P. falciparum* infection and its role in disease transmission, particularly in school age children. We conducted a cross-sectional survey in the summer of 2018 of children attending 4 primary/junior high schools within a 5 km radius of HopeXchange Medical Centre in Kumasi, Ghana, a city of 2.5 million inhabitants with year-round malaria transmission. Informed consent/assent and demographic information was obtained from 634 subjects (ages 5-17 years), after which measurements of height/weight/body temperature were recorded and a blood sample was obtained for evaluation by blood film microscopy, rapid diagnostic test (RDT) and nested polymerase chain reaction (PCR) for *P. falciparum*. Factors associated with malaria parasitemia were analyzed using a qualitative questionnaire covering socioeconomic factors, malaria prevention behaviors, and healthcare access. The overall prevalence of asymptomatic parasitemia varied by diagnostic test (microscopy: 5.5%; RDT: 11.8%; PCR: 23.4%). Agreement between methods was highest for samples with higher levels of parasitemia as measured by microscopy. Bivariate analysis showed that factors associated with a positive malaria test included school, lack of bed net usage and age. After controlling for relevant factors, school of attendance was the single greatest predictor of a positive malaria test in the study population. Across the 4 schools, use of PCR (n=555) increased the measured prevalence of asymptomatic malaria parasitemia when compared to the prevalence measured using microscopy (1.7 vs 7.6%; 2.8 vs 10.3%; 3.2 vs 28.4%; 12.2 vs 40.1%). These data are in agreement with previously described

higher sensitivity of PCR for detection of asymptomatic parasitemia. More importantly, these results capture the significant heterogeneity in asymptomatic malaria risk in Kumasi, underscoring the importance of characterizing the epidemiology of asymptomatic malaria, even across urban communities.

## Acknowledgements

I would like to thank the administration and staff at HopeXchange Medical Centre, especially the field study team and laboratory members for their role in sample collection and microscopy. I also thank all participants and administrators of participating schools for their cooperation.

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# Introduction

## Malaria in Urban West Africa

Despite recent improvements in malaria control, including wider distribution and use of insecticide-treated nets (ITN), malaria exerts a strong burden of disease in West Africa[1,2]. While the burdens of symptomatic and severe malaria are well-documented in African hospitals and medical clinics[3,4], total community prevalence of malaria in West African urban and peri-urban settings is less well described in the literature[5,6]. Kumasi, one of the most populous cities in Ghana with 2.5 million inhabitants[7], experiences year-round malaria transmission that peaks during the rainy season (Fig. 1 and 2)[8,2,9]. Studies suggest that urban settings such as Kumasi are less conducive to malaria transmission than rural settings due to reduced quality and number of breeding sites available to vector mosquito species, as well as improved access to health services[10,11]. However, one study suggests that urban malaria prevalence may be underestimated due to the high rate of self-medication among urban populations in West African cities such as Dakar, Senegal[10]. Indeed, some studies suggest that over 50% of urban African malaria cases are self-diagnosed and self-medicated[12,13], indicating that hospital or clinic-based prevalence data may not accurately represent community-wide urban prevalence.

Fig. 1: Average temperature and rainfall in Kumasi, Ghana from 1901-2015[8]

*Kumasi, Ghana experiences peak annual rainfall in June.*

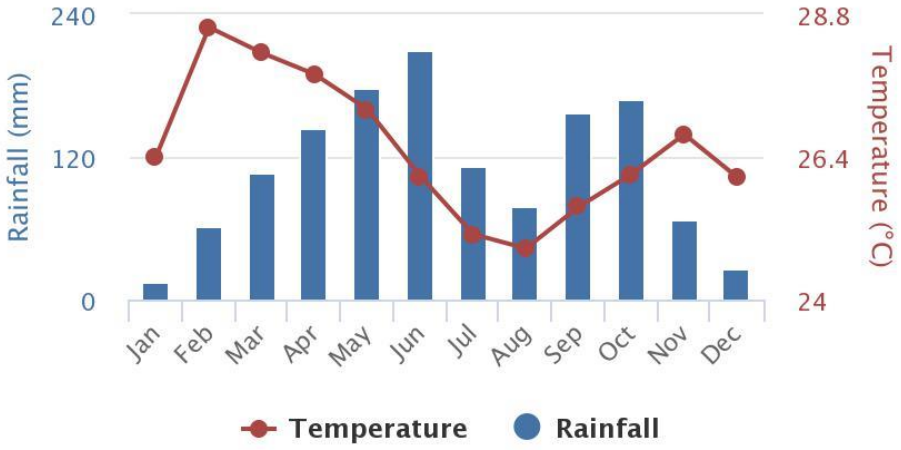
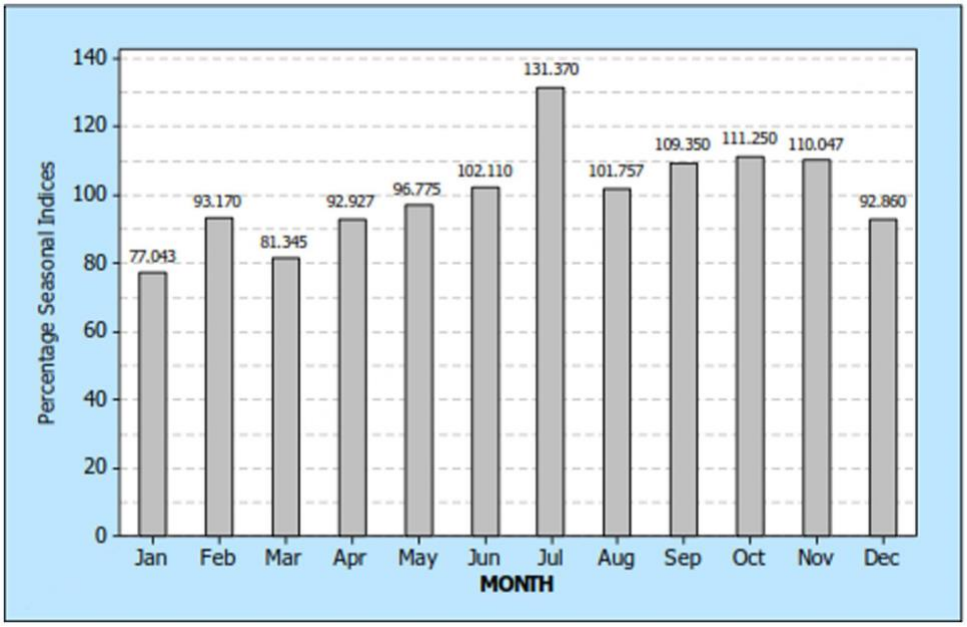



Fig. 2: Seasonal transmission pattern of malaria in Kumasi, Ghana[9]

*Data from an ARIMA model of malaria transmission data in Kumasi shows that a slight seasonal peak in transmission occurs in July.*



## Clinical Significance of Asymptomatic Malaria

Malaria parasitemia, regardless of symptoms, has been strongly associated with anemia and stunting among children in Kumasi; a study conducted in 2006 reported an average prevalence of malaria parasitemia among children under 5 in Kumasi at 8.6% using microscopy, with prevalence in some neighborhoods as high as 32.7% among children under 5 years[6]. There have been consistent associations between asymptomatic malaria and impaired cognitive function and educational performance[14], and increased risk of invasive bacterial disease, most often with non-typhoid *Salmonella* infections[14]. Among the most common sequelae of chronic asymptomatic malaria infection is anemia, which can be exacerbated by co-infection with parasite species whose distribution overlaps significantly with that of *P. falciparum*[15].

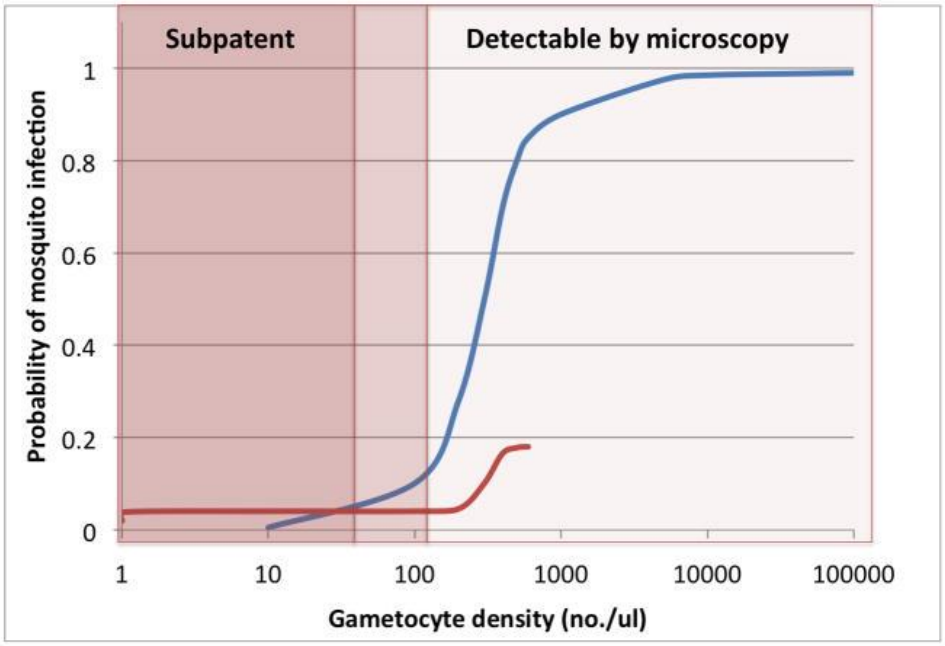
## Epidemiological Implications of Asymptomatic Malaria Among Children

Asymptomatic malaria parasitemia not only confers significant clinical risks to the infected individual, but also contributes to the malaria reservoir in endemic regions of sub-Saharan Africa[16]. A study in Ghana estimated a mean duration of 194 days, or roughly 6 months, for an asymptomatic *Plasmodium falciparum* infection[18]. One study suggests that when *Anopheles gambiae s.s.* mosquitoes are infected with *Plasmodium falciparum* from asymptomatic carriers, parasites develop greater oocyst density within the mosquito. This was thought to be due to both a greater infectivity and a higher density of gametocytes in the blood of asymptomatic versus symptomatic individuals,

potentially due to serum effects on gametocytes and cytokine activity in symptomatic hosts[17]. However, in another study, submicroscopic infections were shown to have a considerably lower probability of mosquito infection than infections of greater parasite density (Fig. 3)[19]; therefore it is doubtful that submicroscopic asymptomatic infections would contribute as significantly to this reservoir as asymptomatic infections above the threshold for detection via microscopy. School-age children are an especially significant contributor to this asymptomatic reservoir, given that their increased exposure and relatively modest immunological response to the parasite allow for repeated or chronic asymptomatic infections in this group[20].

Fig. 3: Submicroscopic gametocytemia and the mosquito infectivity threshold[19]

*Microscopy detects infections over 100 gametocytes / μL which correspond with a 10% chance or greater of mosquito infection, whereas submicroscopic infections carry less than a 10% probability of infecting a mosquito per encounter. The red curve represents data from a study of gametocyte infectivity among infected individuals in Burkina Faso and Kenya, and the blue curve represents a similar set of studies in West Africa.*

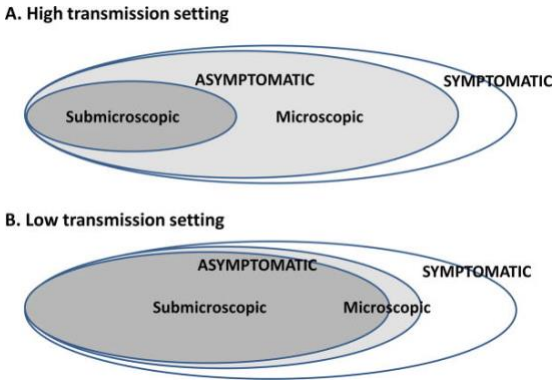


## Diagnostic Methods

*P. falciparum* is the predominant *Plasmodium* species in Ghana, causing 90-98% of malaria cases[21]. When compared with PCR as the reference standard, the sensitivity of microscopy in detecting *P. falciparum* infection has been measured to be quite low (44%), with high specificity (98%)[22]. A cross-sectional community survey in urban Dakar, Senegal reported 2.2% prevalence of asymptomatic malaria among children by microscopy, and 16.5% prevalence by PCR[10]; in Mali, asymptomatic malaria was detected by PCR in 46% of participants aged 6 months to 25 years old during a period of low transmission[23]. While PCR is currently the most sensitive available method of detecting malaria, its practical utility in routine identification of infections of any clinical or epidemiological significance is a subject of debate among experts in the field[24,25,19]. Additionally, the contribution of submicroscopic asymptomatic malaria parasitemia depends on the context, with submicroscopic parasitemia playing a larger role in low transmission settings than in high transmission settings(Fig. 4)[19].

Fig. 4: The contribution of microscopic vs. submicroscopic infections to the asymptomatic malaria reservoir[19]

*In high transmission settings, microscopic malaria plays a proportionally larger role in the asymptomatic malaria reservoir than in low transmission settings.*



## Malaria in the Community Served by HopeXchange Medical Centre

Malaria is the most prevalent diagnosis at HopeXchange Medical Center, with hospital census data reporting that 27.9% of 2,505 school-aged patients seeking care in the outpatient clinic were diagnosed with malaria over a 6-month period from July to December 2016. Malaria is the most common diagnosis among children evaluated in the outpatient clinic, indicating the need for reliable baseline statistics regarding malaria parasitemia among the patient population. The overall proportion of malaria parasitemia among school-aged children may be considerably greater than 27.9%, as most children are likely not presenting to clinics when they are asymptomatic.

In this study, we hypothesized that the prevalence of asymptomatic malaria parasitemia in children attending school within 5 kilometers of HopeXchange Medical Centre is comparable to that reported by similar surveys (>30% by PCR), which would suggest that the overall burden of malaria in this community is significantly greater than estimates derived based solely on those who present with clinical symptoms of infection. A reference proportion of approximately 30% prevalence, inferred from data on asymptomatic malaria prevalence reported in West Africa, provides a baseline for comparison[10,23].

Given limited data regarding the community prevalence of malaria in West African peri-urban populations and the unique problems presented by such a setting, a cross-sectional survey of asymptomatic malaria parasitemia at schools located within a 5 kilometer radius of HopeXchange Medical Center in Kumasi, Ghana, would provide

useful baseline data to inform the health needs of the catchment population of HopeXchange Medical Center. Additionally, comparison between various diagnostic methods would elucidate the effectiveness of each method of detecting asymptomatic infection[26].

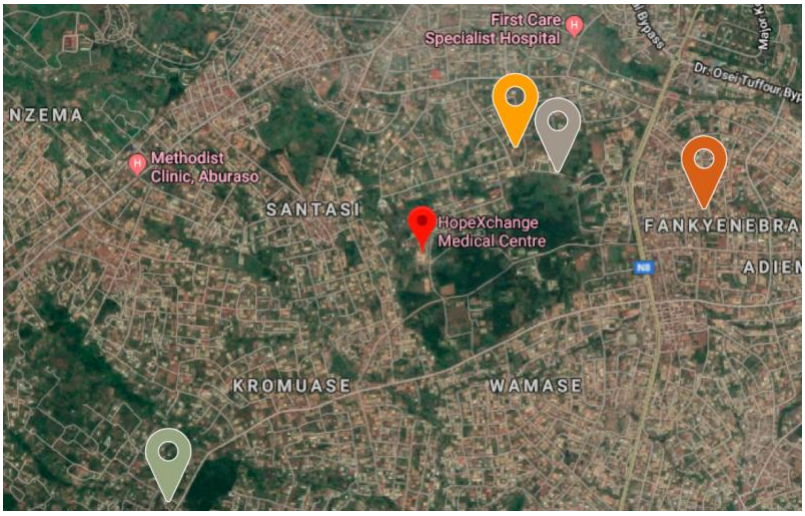
# Methods

## Subject Selection & Enrollment

The prevalence of asymptomatic malaria among the pediatric population served by HopeXchange Medical Center was measured with a prospective, cross-sectional survey among school-aged children attending primary and junior high schools within 5 kilometers of HopeXchange Medical Centre between June and July 2018 (Fig. 5). This time period coincided with the peak of the rainy season (Fig. 1). All afebrile (axillary temperature reading below 38 degrees Celsius) students between the ages of 5 to 17 years attending participating schools were considered eligible for enrollment. Informed consent was obtained from a parent or guardian for each participant, while assent was obtained from all children prior to enrollment.

Fig. 5: Geospatial distribution of study sites

*Opoku Ware, Fankyenebra, and Martyrs of Uganda/St. Lwanga are clustered to the northeast of HopeXchange Medical Centre, closer to the urban center of Kumasi. Darko lies to the southwest of HopeXchange Medical Centre in a more rural setting.*




*Darko* *Fankyenebra* *Opoku Ware* *Martyrs of Uganda*

## Demographic Data Collection

A questionnaire (see supplemental materials) was employed to define socio-economic status using World Bank indices for Ghana[27], malaria prevention behaviors such as ITN usage and indoor residual spraying in the home within the last year, and various indicators of healthcare access. The questionnaire was translated orally from English into the local language of Twi as needed by community health workers. Information regarding sex, age, weight, and vital signs (including temperature) were also measured during sample collection (see supplemental materials).

## Sample Collection for Diagnostic Methods

A dual-antigen Ag-*P.f*. HRPII/pLDH rapid diagnostic test (Alere™ , Standard Diagnostics, Inc., Republic of Korea) in addition to standard microscopy and nested PCR were used to determine the prevalence of malaria parasitemia in all consenting study participants. Safety lancets were used to draw blood from each subject's fingertip for capillary sampling[28].

## Rapid Diagnostic Test

Five µL of clean blood was applied to the RDT membrane using the inverted cup blood transfer device provided, along with four drops of buffer solution, following protocols recommended by Alere™ and WHO[29]. All RDT results were read and recorded within 15 minutes. All participants found positive for malaria by RDT were informed of their results and referred for medical evaluation according to Ghana Health Service recommendations.

## Microscopy

Thick blood films were prepared with 10% Giemsa stain[30] and analyzed with semi-quantitative microscopy methods[31] at the HopeXchange Medical Centre clinical laboratory. Parasitemia measurements were recorded within discrete ranges of parasites per µL of blood (Table 1). A more precise quantitative measurement of parasites per µL[30] was not feasible due to limitations of time and personnel.

Table 1[31]: Ranges of parasitemia as determined by light microscopy

*Microscopists at HopeXchange Medical Centre employed a semi-quantitative method that approximates the following ranges of gametocyte density observed on a blood film.*

| Parasitemia Level | Parasites per Field | Parasites/ µL |
|---|---|---|
| 0 (–) | No Parasites Found | 0 Parasites/ µL |
| 1 (+) | <10 Parasites per 100 Fields | 4-40 Parasites/ µL |
| 2 (++) | 11+ Parasites per 100 Fields | 40-400 Parasites/ µL |
| 3 (+++) | <10 Parasites in Every Field | 400-4,000 Parasites/ µL |
| 4 (++++) | 11+ Parasites in Every Field | >4,000 Parasites/ µL |

## PCR-based Detection of *P. falciparum*

Eighty µL of blood from each subject was collected directly from each subject using a 200 µL single channel micropipette and applied onto Whatman® FTA cards (Buckinghamshire, United Kingdom) for storage at room temperature[3] and transported to the Cappello Laboratory at the Yale School of Medicine (New Haven, Connecticut) to detect the presence of *P. falciparum*.

## DNA Extraction

DNA was extracted from the FTA cards using the method of Mas et al. 2007[32] by punching three 2.0 mm discs from each FTA card. Each set of punches was washed three times in 200 µL of Whatman® FTA purification reagent, then twice in 200 µL of TE buffer. Samples were dried for 10 minutes at 60 degrees Celsius. DNA was eluted by adding 51 µL pH 13 solution (0.1 N NaOH, 0.3 mM EDTA) and 99 µL pH 7 solution (0.1

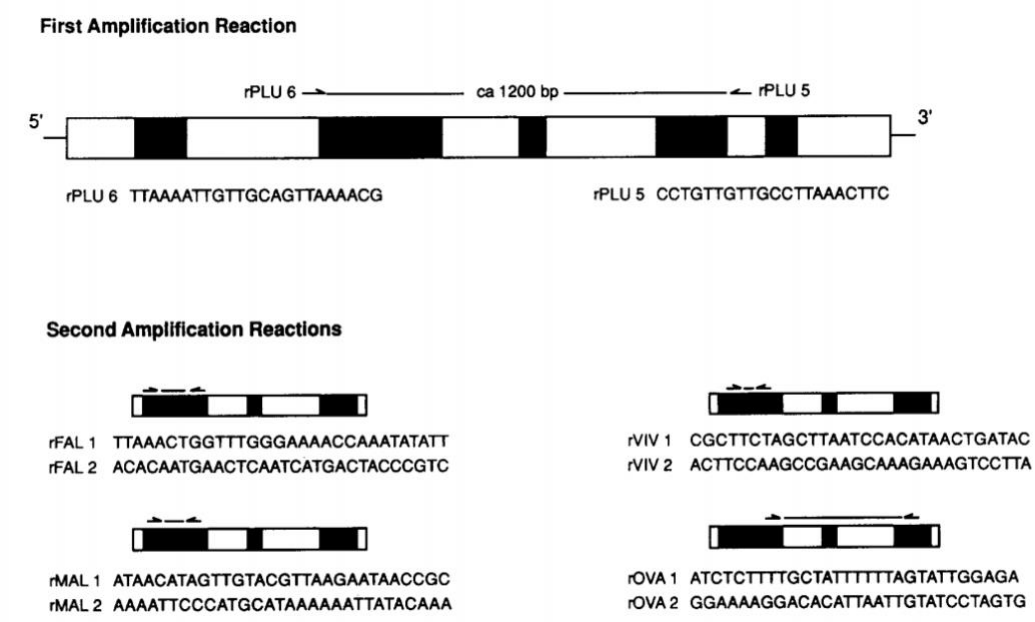
M Tris-HCl). The supernatant was transferred to sterile tubes and subsequently used for PCR. The following internal standard controls were used to maintain validity of results: Negative control (pure water), negative control with washed, no-sample punch (to ensure that the punch does not cause a positive result), positive control of a known *Plasmodium falciparum* 3D7 DNA standard solution (generously donated by Amy Bei and Choukri Ben Mamoun), the same *Plasmodium falciparum* 3D7 positive control standard added to a normally washed, no-sample punch (to ensure that the punch does not inhibit the reaction).

## Real Time PCR

Nested PCR for detection of *P. falciparum* genus- and species- specific DNA was conducted according to protocols outlined by Snounou et al. 1993[33]. This protocol allows for a first-round amplification reaction that binds *Plasmodium* genus-specific primers, then a subsequent second-round amplification reaction that binds *Plasmodium falciparum* species-specific primers. For each of these amplification reactions, 5 µL of supernatant from the DNA extraction protocol were added to 45 µL of a buffer solution containing 43 µL Invitrogen™ PCR SuperMix (Carlsbad, CA) and 1 µL of forward and reverse *Plasmodium* (first amplification reaction) and *P. falciparum* (second amplification reaction) primers (Fig. 6)[33]. Amplified second-round PCR products were analyzed in real time with qPCR[34] (Appendices). All samples collected on FTA cards are archived at the Cappello Laboratory for future analysis.

Fig. 6: Nested PCR primers identified by Snounou et al. 1993[33]

*This study employed a nested PCR protocol with a* Plasmodium *genus-specific first amplification reaction (rPLU6, rPLU5 primers) and a* P. falciparum *species-specific second amplification reaction (rFAL1 and rFAL2 primers).*




## Statistical Analysis

All data generated in this study were stored in Microsoft Excel (Redmond, Washington) and Qualtrics (Provo, Utah). Statistical analyses were completed using SAS 9.4 (Cary, North Carolina) and RStudio (Boston, Massachusetts). Given an overall sample size of 634, the power of the analysis to detect a statistically significant difference in prevalence is >99% calculated using SAS 9.4, given a reference proportion of 0.30, a sample mean of 0.12 and standard deviation of 0.32 at 95% confidence. Univariate analysis and linear regression were conducted with SAS 9.4 to determine the strength and direction of associations between asymptomatic malaria parasitemia and age. A logistic regression model determined which factors were predictive of positive RDT results in this population. A two-sided t-test ($\alpha$=0.05) detected any significant difference between

prevalence of asymptomatic malaria parasitemia among this study population and mean prevalence (30%) of asymptomatic parasitemia among study populations previously studied in other West African populations. The Fleiss' Kappa measure of agreement indicated the strength of inter-rater agreement between microscopy, RDT, and PCR diagnostic methods. Additionally, PCR was assumed as the gold standard for calculation of the sensitivity and specificity of microscopy and RDT performed during this study[35]. A principal components analysis was used to determine socio-economic scores based on questionnaire responses. BMI-for-age Z-scores were calculated from sex, height, and weight of each participant using methods for SAS 9.4 developed by the Centers for Disease Control Division of Nutrition, Physical Activity, and Obseity[36]. Socio-economic scores were calculated using ggbiplot and devtools packages for R[37]. Figures were created using GraphPad Prism 8 (San Diego, California).

## Results

Across the 4 schools, use of PCR (n=555) increased the measured prevalence of asymptomatic malaria parasitemia when compared to the prevalence measured using microscopy (1.7 vs 7.6%; 2.8 vs 10.3%; 3.2 vs 28.4%; 12.2 vs 40.1%). The overall prevalence of asymptomatic malaria varied by diagnostic test (microscopy: 5.5%; RDT: 11.8%; PCR: 23.4%). There was significant discordance in the results between RDT, microscopy, and PCR. When measured with a Fleiss Kappa statistic, Kappa = 0.3 between the three methods, indicating only "fair agreement"[38].

Both school location and socio-economic status were significantly associated with RDT, microscopy, and PCR outcomes independently of one another, and the odds of a positive microscopy and RDT result were lower among those who reported owning a bed net (regardless of reported compliance) (Tables 4, 5, and 6). Additionally, the odds of a positive RDT or PCR result among those who reported that their home had not been sprayed with insecticides within the last year were 4.2 (95% CI: 2.0, 8.9) and 2.1 (95% CI: 1.3, 3.6) times the odds of those who had reported that their home had been sprayed within the past year, respectively (Tables 5 and 6). In the adjusted model, only school location remained a significant factor in all three diagnostic outcomes (Tables 4, 5, and 6). For logistic regression models of microscopy, RDT, and PCR outcomes, the models with school as the only variable yielded the lowest Akaike Information Criterion (AIC) Score when compared with models including all variables of interest.

Results from 17 subjects were not included in the analyses due to missing temperature data and/or refusal of subjects to continue with sample collection. In total, 634 total subjects completed the study. The questionnaire was not administered for all subjects due to logistical challenges and therefore only 411 subjects have complete questionnaire response data available (86 responses from Martyrs of Uganda/St. Lwanga, 73 responses from Darko, 142 responses from Opoku Ware, and 110 responses from Fankyenebra).

Table 2: Description of the sample according to test results (compared between PCR and microscopy, without regard to RDT result).

*Among samples with results available for both microscopy and PCR, those which were positive by both PCR and microscopy were compared against those which were negative by both tests and those which were positive by PCR but negative by microscopy.*

| Characteristic | Negative by both tests (N=416)[b] | Positive by PCR, negative by microscopy (N =107)[b] | Positive by both tests (N =23)[b] | p[c] |
|---|---|---|---|---|
| Age (years) | 10.52 ± 2.89 | 9.97 ± 3.00 | 8.39 ± 2.54 | 0.0012 |
| SES Score | 0.10 ± 4.60 | -0.77 ± 3.43 | -0.44± 4.16 | 0.3160 |
| Sex | | | | 0.7624 |
| Female | 219(52.64%) | 60 (56.60%) | 12 (54.55%) | |
| Male | 197(47.36%) | 46 (43.40%) | 10 (45.45%) | |
| Bed Net | | | | 0.5364 |
| Owns & Uses | 127 (43.79%) | 28 (45.90%) | 5 (45.45%) | |
| Owns & Does Not Use | 84 (28.97%) | 12 (19.67%) | 2 (18.18%) | |
| Does Not Own | 79 (27.24%) | 21 (34.43%) | 4 (36.36%) | |
| Home Sprayed Within Past Year | | | | 0.0083 |
| Yes | 174 (60.84%) | 28 (43.08%) | 4 (33.33%) | |
| No | 112 (39.16%) | 37 (56.92%) | 8 (66.67%) | |
| School | | | | <0.0001 |
| Martyrs | 68 (16.35%) | 25 (23.36%) | 2 (8.70%) | |
| Opoku Ware | 108 (25.96%) | 8 (7.48%) | 1 (4.35%) | |
| Fankyenebra | 129 (31.01%) | 12 (11.21%) | 3 (13.04%) | |
| Darko | 111 (26.68%) | 62 (57.94%) | 17 (73.91%) | |
| BMI for Age Z-Score | | | | 0.8888 |
| Overweight | 10 (2.41%) | 3 (23.08%) | 0 (0.00%) | |
| Healthy | 365 (87.95%) | 94 (88.68%) | 19 (86.36%) | |
| Underweight | 40 (9.64%) | 9 (8.49%) | 3 (13.64%) | |

[a] Table values are mean ± SD for continuous variables and n (column %) for categorical variables.

[b] Numbers may not sum to total due to missing data, and percentages may not sum to 100% due to rounding.

[c] P-value is for t-test (continuous variables) or $\chi^2$ test (categorical variables) between all columns.

Table 3: Description of the sample according to school enrollment

*A chi-squared test detected statistically significant ($\alpha = 0.05$) heterogeneity between schools with respect to age, SES score, bed net ownership and usage, indoor residual spraying within the last year, BMI-for-age Z-scores, and results of all three diagnostic methods.*

| Characteristic | Darko (N=199 )[b] | Fankyenebra (N =150)[b] | Opoku Ware (N=177)[b] | Martyrs/Lwanga (N =108)[b] | p[c] |
|---|---|---|---|---|---|
| Age (years) | 10.51 ± 3.15 | 11.02 ± 3.13 | 10.99 ± 2.77 | 9.32 ± 2.54 | <0.0001 |
| SES Score | -1.80 ± 0.43 | 0.58 ± 5.69 | -0.18 ± 3.82 | 0.83 ± 4.48 | <0.0002 |
| Sex | | | | | 0.2791 |
| Female | 105(53.30%) | 73(48.67%) | 89 (50.28%) | 65(60.19%) | |
| Male | 92(46.70%) | 77(51.33%) | 88 (49.72%) | 43(39.81%) | |
| Bed Net | | | | | 0.0186 |
| Owns & Uses | 22(30.14%) | 58(50.88%) | 57 (37.01%) | 42 (46.67%) | |
| Owns & Does Not Use | 21 (28.77%) | 25 (21.93%) | 55 (35.71%) | 27(30.00%) | |
| Does Not Own | 30(41.10%) | 31 (27.19%) | 42(27.27%) | 21(23.33%) | |
| Home Sprayed Within Past Year | | | | | <0.0001 |
| Yes | 5 (6.58%) | 78 (65.00%) | 96 (67.13%) | 61 (65.59%) | |
| No | 71 (93.42%) | 42 (35.00%) | 47 (32.87%) | 32 (34.41%) | |
| Microscopy Result | | | | | <0.0001 |
| Negative | 175 (87.94%) | 145 (97.32%) | 172 (98.85%) | 103 (95.37%) | |
| Positive | 24 (12.06%) | 4 (2.68%) | 2 (1.15%) | 5 (4.63%) | |
| RDT Result | | | | | <0.0001 |
| Negative | 138 (69.70%) | 141 (94.00%) | 171 (96.61%) | 108 (100.00%) | |
| Positive | 60 (30.30%) | 9 (6.00%) | 6 (3.39%) | 0(0.00%) | |
| PCR Result | | | | | <0.0001 |
| Negative | 118 (59.90%) | 131 (89.73%) | 112 (92.56%) | 70(72.16%) | |
| Positive | 79(40.10%) | 15 (10.27%) | 9 (7.44%) | 27(27.84%) | |
| BMI for Age Z-Score | | | | | <0.0001 |
| Overweight | 0 (0.00%) | 1 (0.67%) | 6 (3.41%) | 6 (5.56%) | |
| Healthy | 184 (93.88%) | 139 (92.67%) | 138 (78.41%) | 96 (88.89%) | |
| Underweight | 12(6.12%) | 10 (6.67%) | 32 (18.18%) | 6 (5.56%) | |

[a] Table values are mean ± SD for continuous variables and n (column %) for categorical variables.

[b] Numbers may not sum to total due to missing data, and percentages may not sum to 100% due to rounding.

[c] P-value is for t-test (continuous variables) or $\chi^2$ test (categorical variables) between all columns.

Table 4: Bivariate and multivariable associations between study variables and having a positive microscopy result for asymptomatic malaria

*The unadjusted odds of a positive microscopy result were significantly higher among those with low SES scores, those who do not own bed nets, and those enrolled at Darko primary/junior high schools. In an adjusted model of all variables, enrollment at Darko was the only significant risk factor for a positive microscopy result.*

| Characteristic | N[a] | % Microscopy Positive | Unadjusted OR (95% CI) | Adjusted OR (95% CI)[b] |
|---|---|---|---|---|
| Age (years) | | | | |
| 5-7 | 113 | 11.50% | 1.00 | 1.00 |
| 8-10 | 201 | 6.97% | 0.90 (0.45, 1.79) | 0.48 (0.15, 1.58) |
| 11-13 | 189 | 4.23% | 1.00 (0.50, 1.99) | 0.26 (0.06, 1.08) |
| 14-16 | 122 | 0.00% | 0.65 (0.28, 1.48) | N/A |
| 17+ | 4 | 0.00% | N/A | N/A |
| SES Score | | | | |
| High | 123 | 1.63% | 1.00 | 1.00 |
| Low | 364 | 4.95% | 6.66 (2.06, 21.50) | 2.00 (0.22, 18.33) |
| Sex | | | | |
| Male | 329 | 4.56% | 1.00 | 1.00 |
| Female | 298 | 6.38% | 0.74 (0.46, 1.21) | 0.45 (0.15, 1.33) |
| Bed Net | | | | |
| Owns & Uses | 177 | 3.39% | 1.00 | 1.00 |
| Does Not Use | 125 | 4.80% | 1.43 (0.60, 5.03) | 1.37 (0.37, 5.02) |
| Does not Own | 124 | 4.84% | 2.25 (1.01, 5.03) | 1.70 (0.47, 6.13) |
| Home Sprayed Within Past Year | | | | |
| Yes | 237 | 2.95% | 1.00 | 1.00 |
| No | 190 | 6.84% | 2.41 (0.94, 6.18) | 0.64 (0.14, 2.83) |
| School | | | | |
| Martyrs/St. Lwanga | 108 | 4.63% | 2.76 | 4.26 (0.62, 29.29) |
| Opoku Ware | 174 | 1.15% | 1.00 | 1.00 |
| Fankyenebra | 149 | 2.68% | 1.82 (0.63, 5.23) | 1.50 (0.18, 12.39) |
| Darko | 199 | 12.06% | 12.39 (5.20, 29.54) | 12.80 (1.49, 109.55) |
| BMI for Age Z-Score | | | | |
| Healthy | 552 | 5.43% | 1.00 | 1.00 |
| Overweight | 13 | 0.00% | N/A | N/A |
| Underweight | 60 | 6.67% | 1.24 (0.42, 3.66) | 3.95 (0.84, 18.53) |

[a] Numbers may not sum to total due to missing data.

[b] For the fully-adjusted model, **N = 397.**

Table 5: Bivariate and multivariable associations between study variables and having a positive RDT for asymptomatic malaria

*The unadjusted odds of a positive RDT result were significantly higher among those with low SES scores, those who do not own bed nets, those who had not had their homes sprayed within the last year and those enrolled at Darko primary/junior high schools. In an adjusted model of all variables, enrollment at Darko was the only significant risk factor for a positive RDT result.*

| Characteristic | N[a] | % RDT Positive | Unadjusted OR (95% CI) | Adjusted OR (95% CI)[b] |
|---|---|---|---|---|
| Age (years) | | | | |
| 5-7 | 114 | 13.16% | 1.00 | 1.00 |
| 8-10 | 201 | 11.94% | 0.90 (0.45, 1.79) | 0.63 (0.22, 1.76) |
| 11-13 | 190 | 13.16% | 1.00 (0.50, 1.99) | 0.71 (0.25, 1.96) |
| 14-16 | 123 | 8.94% | 0.65 (0.28, 1.48) | 0.23 (0.02, 2.23) |
| 17+ | 4 | 0.00% | N/A | N/A |
| SES Score | | | | |
| High | 124 | 2.42% | 1.00 | 1.00 |
| Low | 367 | 10.63% | 6.66 (2.06, 21.50) | 0.93 (0.10, 8.71) |
| Sex | | | | |
| Male | 331 | 10.27% | 1.00 | 1.00 |
| Female | 299 | 13.38% | 0.74 (0.46, 1.21) | 0.78 (0.35, 1.76) |
| Bed Net | | | | |
| Owns & Uses | 178 | 6.18% | 1.00 | 1.00 |
| Does Not Use | 128 | 8.60% | 1.43 (0.60, 5.03) | 1.31 (0.47, 3.67) |
| Does not Own | 124 | 12.90% | 2.25 (1.01, 5.03) | 1.44 (0.55, 3.76) |
| Home Sprayed Within Past Year | | | | |
| Yes | 239 | 4.18% | 1.00 | 1.00 |
| No | 192 | 15.63% | 4.24 (2.02, 8.92) | 0.71 (0.21, 2.41) |
| School | | | | |
| Martyrs/St. Lwanga | 108 | 0.00% | N/A | N/A |
| Opoku Ware | 177 | 3.39% | 1.00 | 1.00 |
| Fankyenebra | 150 | 6.00% | 1.82 (0.63, 5.23) | 2.30 (0.52, 10.22) |
| Darko | 199 | 30.15% | 12.39 (5.20, 29.54) | 22.23 (4.41, 112. 06) |
| BMI for Age Z-Score | | | | |
| Healthy | 556 | 11.87% | 1.00 | 1.00 |
| Overweight | 13 | 0.00% | N/A | N/A |
| Underweight | 60 | 13.33% | 1.14 (0.52, 2.51) | 0.38 (0.04, 3.24) |

[a] Numbers may not sum to total due to missing data.
[b] For the fully-adjusted model, **N = 401**.

Table 6: Bivariate and multivariable associations between study variables and having a positive PCR result for asymptomatic malaria

*The unadjusted odds of a positive PCR result were significantly higher among those aged 5-7, those who had not had their homes sprayed within the last year, and those enrolled at Darko primary/junior high schools. In an adjusted model of all variables, enrollment at Martyrs of Uganda or St. Lwanga was the only significant risk factor for a positive PCR result.*

| Characteristic | N[a] | N(%) PCR Positive | Unadjusted OR (95% CI) | Adjusted OR (95% CI)[b] |
|---|---|---|---|---|
| Age (years) | | | | |
| 5-7 | 105 | 36 (34.29%) | 1.00 | 1.00 |
| 8-10 | 196 | 44 (22.45%) | 0.56 (0.33, 0.94) | 0.67 (0.32, 1.40) |
| 11-13 | 172 | 33 (19.19%) | 0.46 (0.26, 0.79) | 0.39 (0.17, 0.87) |
| 14-16 | 84 | 16 (19.05%) | 0.45 (0.23, 0.89) | 0.31 (0.06, 1.58) |
| 17+ | 4 | 1 (25.00%) | 0.64 (0.06, 6.37) | 1.83 (0.08, 42.83) |
| SES Score | | | | |
| High | 98 | 13 (13.27%) | 1.00 | 1.00 |
| Low | 326 | 69 (21.17%) | 1.76 (0.92, 3.33) | 1.51 (0.56, 4.04) |
| Sex | | | | |
| Male | 296 | 72 (24.32%) | 1.00 | 1.00 |
| Female | 263 | 56 (21.29%) | 1.19(0.80, 1.77) | 1.03 (0.56, 1.89) |
| Bed Net | | | | |
| Owns & Uses | 163 | 33 (20.25%) | 1.00 | 1.00 |
| Does Not Use | 104 | 14 (13.46%) | 0.61 (0.31, 1.21) | 0.47 (0.21, 1.04) |
| Does not Own | 105 | 25 (23.81%) | 1.23 (0.68, 2.22) | 0.94 (0.45, 1.94) |
| Home Sprayed Within Past Year | | | | |
| Yes | 211 | 32 (15.17%) | 1.00 | 1.00 |
| No | 163 | 45 (27.61%) | 2.13 (1.28, 3.55) | 0.70 (0.31, 1.59) |
| School | | | | |
| Martyrs/St. Lwanga | 97 | 27 (27.84%) | 1.00 | 1.00 |
| Opoku Ware | 121 | 9 (7.44%) | 0.21 (0.09, 0.47) | 0.13 (0.04, 0.38) |
| Fankyenebra | 146 | 15 (10.27%) | 0.30 (0.15, 0.60) | 0.24 (0.09, 0.61) |
| Darko | 197 | 79 (40.10%) | 1.74 (1.02, 2.94) | 2.26 (0.86, 5.95) |
| BMI for Age Z-Score | | | | |
| Healthy | 492 | 113 (22.97%) | 1.00 | 1.00 |
| Overweight | 13 | 3 (23.08%) | 1.01 (0.27, 3.72) | 2.42 (0.49, 11.97) |
| Underweight | 53 | 12 (22.64%) | 0.98 (0.45, 1.93) | 1.04 (0.30, 3.56) |

[a] Numbers may not sum to total due to missing data.
[b] For the fully-adjusted model, **N = 349**.

Table 7: Agreement of various microscopy levels against RDT and PCR results, n = 555

*At higher semi-quantitative levels of gametocyte density, agreement with RDT and PCR results was higher.*

| | RDT Positive | RDT Negative | PCR Positive | PCR Negative |
|---|---|---|---|---|
| Negative by Microscopy | 52 | 470 | 107 | 415 |
| Microscopy Level 1 | 11 | 11 | 13 | 9 |
| Microscopy Level 2 | 5 | 0 | 4 | 1 |
| Microscopy Level 3 | 6 | 0 | 6 | 0 |

Fig. 7: Visualization of comparative results of Microscopy, RDT, and PCR, n = 555

*Among samples across all schools for which microscopy, RDT, and PCR results were available.*

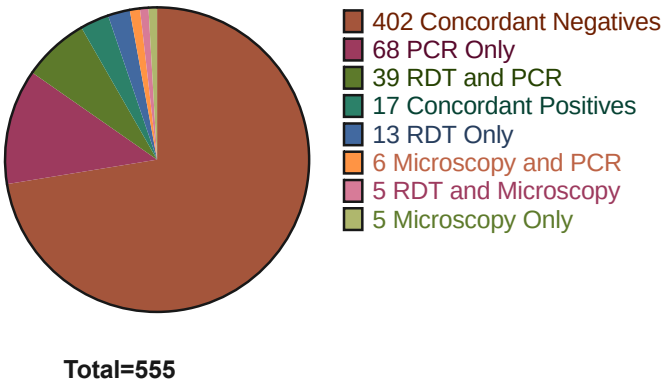



Fig. 8: Prevalence of asymptomatic malaria by method and school, n= 555

*Prevalence was measured using microscopy, RDT, and PCR for students at each of 4 schools.*

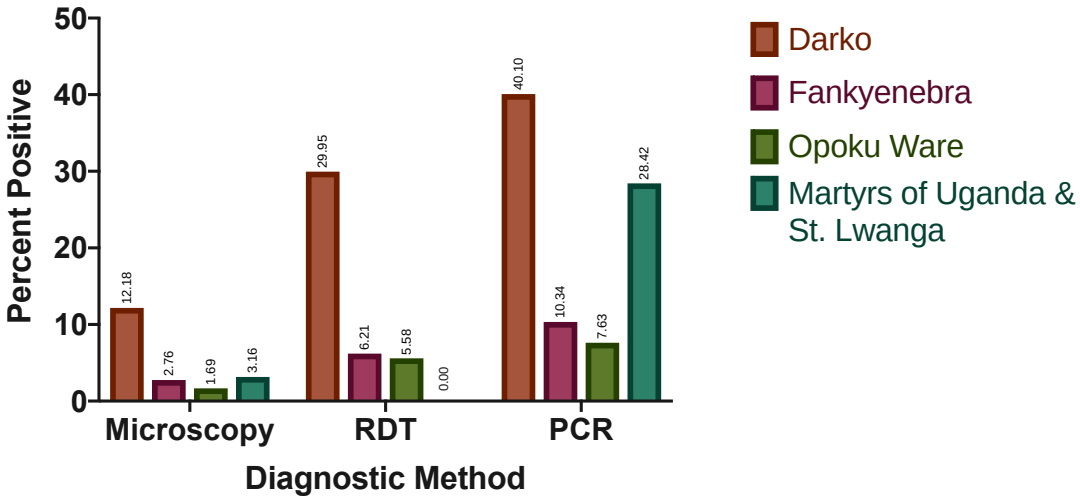


Table 8: Sensitivity and specificity of microscopy using PCR as the gold standard, n = 555

*The sensitivity of microscopy to detect asymptomatic malaria in children in this study was 17.69%, whereas the specificity of microscopy in this population was 97.65%.*

| | PCR Positive | PCR Negative |
|---|---|---|
| **Microscopy Positive** | 23 | 10 |
| **Microscopy Negative** | 107 | 415 |

Table 9: Sensitivity and specificity of RDT using PCR as the gold standard, n = 555

*The sensitivity of RDT to detect asymptomatic malaria in children in this study was 43.08% whereas the specificity of RDT in this population was 95.76%.*

| | PCR Positive | PCR Negative |
|---|---|---|
| **RDT Positive** | 56 | 18 |
| **RDT Negative** | 74 | 407 |

# Discussion

## Comparison Between Diagnostic Methods

The moderate kappa value of agreement between methods raises concern regarding the reliability of diagnostic methods used in this setting. However, agreement was highest for samples with higher parasitemia levels across all methods (Table 7), so it is likely that this disagreement between methods exists only at low densities of

parasitemia found in asymptomatic individuals. PCR was considerably more sensitive than the other measures, detecting 68 samples that RDT and microscopy methods both failed to detect (Fig. 7). It is possible that these results support the hypothesis that there may be a relatively high rate of false positives for many RDTs currently available due to interactions with rheumatoid factor, other immunological factors, or other non-*Plasmodium* infectious agents[39,40]. The RDT used in this study originally resulted in zero false positives during clinical trials, but in a later test did return false positives when exposed to non-*Plasmodium* infectious agents and is therefore not on the 2017 WHO Procurement List[40]. RDTs are also prone to react to circulating HRP2 antigen remaining after recent treatment[41], which may explain several false positives as well. Ultimately, the high sensitivity of PCR as a routine diagnostic or screening method could be interpreted as either an asset or a liability depending upon contextual and practical considerations. For the purposes of clinical screening, submicroscopic levels of infection may not be of interest, whereas for surveillance purposes PCR results may be of more value.

When comparing between the most sensitive method, PCR, and the most specific, microscopy (Table 8), we can see that there is a significant difference between samples which are negative by both PCR and microscopy, positive by PCR and negative by microscopy, and those which are positive by both PCR and microscopy with regards to age, SES score, and school attended (Table 2). If we assume that those samples which are positive only by PCR have lower densities of parasitemia than those which are

positive by both methods, then these results align with previous findings of age-dependent risk of asymptomatic malaria[42].

## Risk Factors Associated with Asymptomatic Malaria Parasitemia

This study identifies risk factors for asymptomatic infection that included school site, socio-economic status, bed net ownership, and indoor residual spraying (Tables 4, 5, and 6). The significant heterogeneity between schools with regards to bed net usage and malaria prevalence (Table 3) indicate either geospatial or socioeconomic differences in malaria prevention behaviors and outcomes. School site being the only significant risk factor in all three adjusted models (Tables 4, 5, and 6) may suggest that geographic location rather than socio-economic status is a primary determinant of asymptomatic malaria parasitemia among children in this community. The AIC values calculated confirm that school attended was the most informative parameter in modeling the outcomes of various diagnostic methods. Since Darko is in a considerably more rural location than the other three schools, this may be indicative of rural setting being a risk factor, or alternatively school location may serve as a more effective proxy for socio-economic status than the questionnaire employed in the study. This may also result from the fact that prevention behaviors and socio-economic status were collected based on self-report by parents of participants, which may have introduced interviewer and/or recall bias.

## Limitations

Sample collection took place during a limited time frame during the rainy season in Kumasi[8], which likely resulted in a higher prevalence of parasitemia than expected. It would be beneficial to collect additional data throughout the year for a more accurate representation of overall prevalence of asymptomatic parasitemia and to identify seasonal trends in transmission on a local level.

The sample consists of four school sites, with three of the sites clustered together and one site considerably farther from the hospital. It would be beneficial to collect additional data from other neighboring schools to explore the differences between rural and urban settings.

Due to limited time and personnel, semi-quantitative microscopy methods were employed to determine the level of parasitemia in each sample. While the WHO recommended protocol for malaria microscopy[30] would have been preferable, this method was sufficient for our purposes in determining binary outcomes with regards to the presence or absence of malaria parasites in each blood film.

Due to time constraints, complete PCR results are currently available from Darko and Martyrs of Uganda/St. Lwanga only, along with a subset of samples from Opoku Ware and Fankyenebra. Work is currently underway to complete the analysis of all available samples by PCR, at which time the analyses will be revised accordingly.

Questionnaires were not completed for all consenting participants and the results may have been biased by differential responses of parents who opted to fill out questionnaires themselves compared with those who were interviewed by community health workers.

## Future Directions

The WHO Global Technical Strategy for Malaria 2016-2030 calls for continuous monitoring of antimalarial medicine efficacy in order to direct treatment protocols and maintain progress toward malaria elimination[43]. Drug resistance may impact parasite virulence, with one study reporting a lower risk of symptomatic malaria among those with mutant *P. falciparum* strains than among those with wild-type strains[44]. As such, asymptomatic malaria carriers may act as a sentinel population for measuring the rise of drug resistance in the parasite population. At HopeXchange Medical center, a reported 1.6% treatment failure rate among patients treated for malaria indicates the potential for ACT drug resistance among the parasite population, but further study is needed. While a recent study indicated the presence of drug resistance genotypes among 89 children with severe malaria in Kumasi[45], the overall distribution of drug resistance genotypes has not been measured in this community. The samples collected in this study and stored at the Cappello Laboratory could be analyzed for the presence of resistance genotypes among the parasite population in this future.

Given that socio-economic status and prevention behaviors such as bed net usage and indoor residual spraying are consistently associated with malaria carriage according to the literature, it would be advisable to develop a questionnaire that better captures these factors in this particular setting than the one used in this study, which had been adapted from studies previously conducted in Kintampo, Ghana, a more rural region in the north of the country.

It would also be interesting for future studies to investigate prevalence of malaria at other school sites in Kumasi, especially given the heterogeneity in prevalence already observed between the few schools we enrolled.

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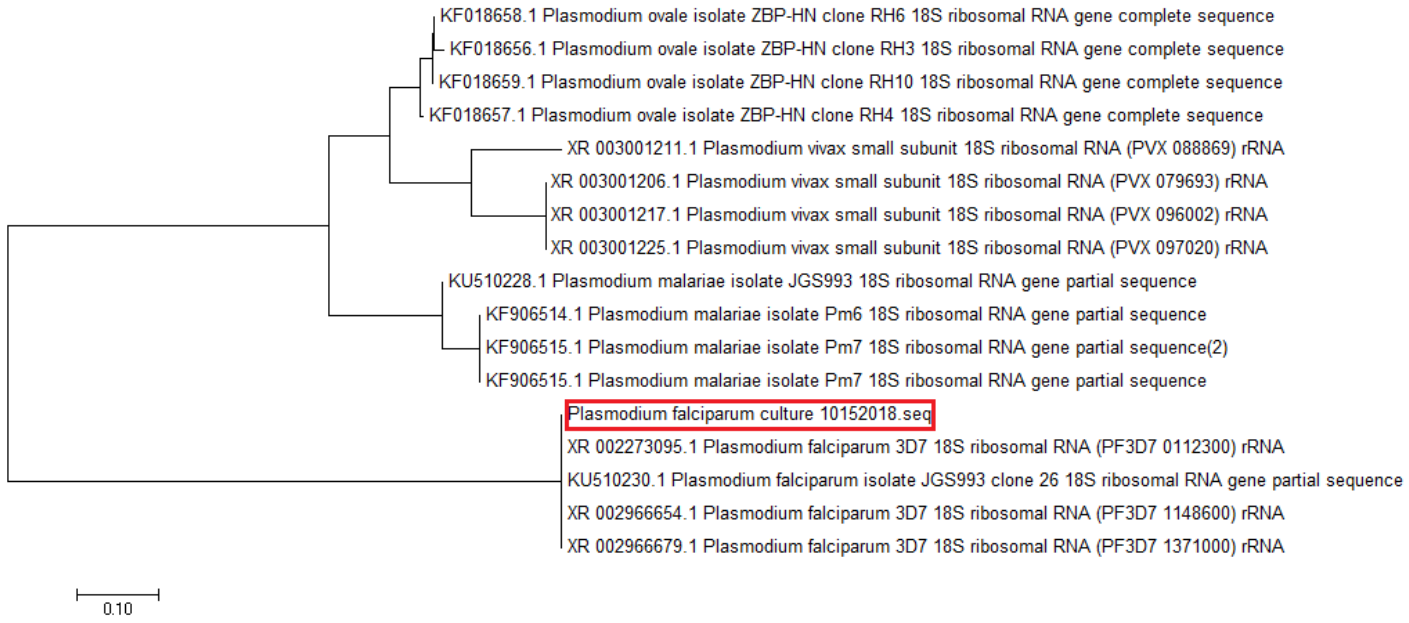
# Appendices

## Molecular characterization of *Plasmodium falciparum* positive control

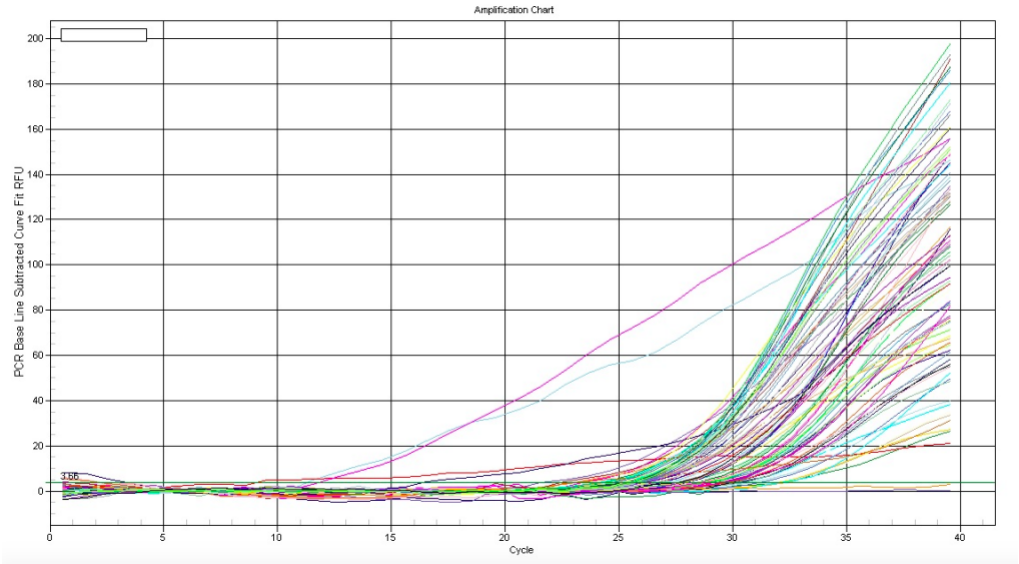
*The evolutionary history of the 3D7 and DD2 positive controls was inferred using the Neighbor-Joining method[46] and the evolutionary distances were computed using the Tamura-Nei method[47].*

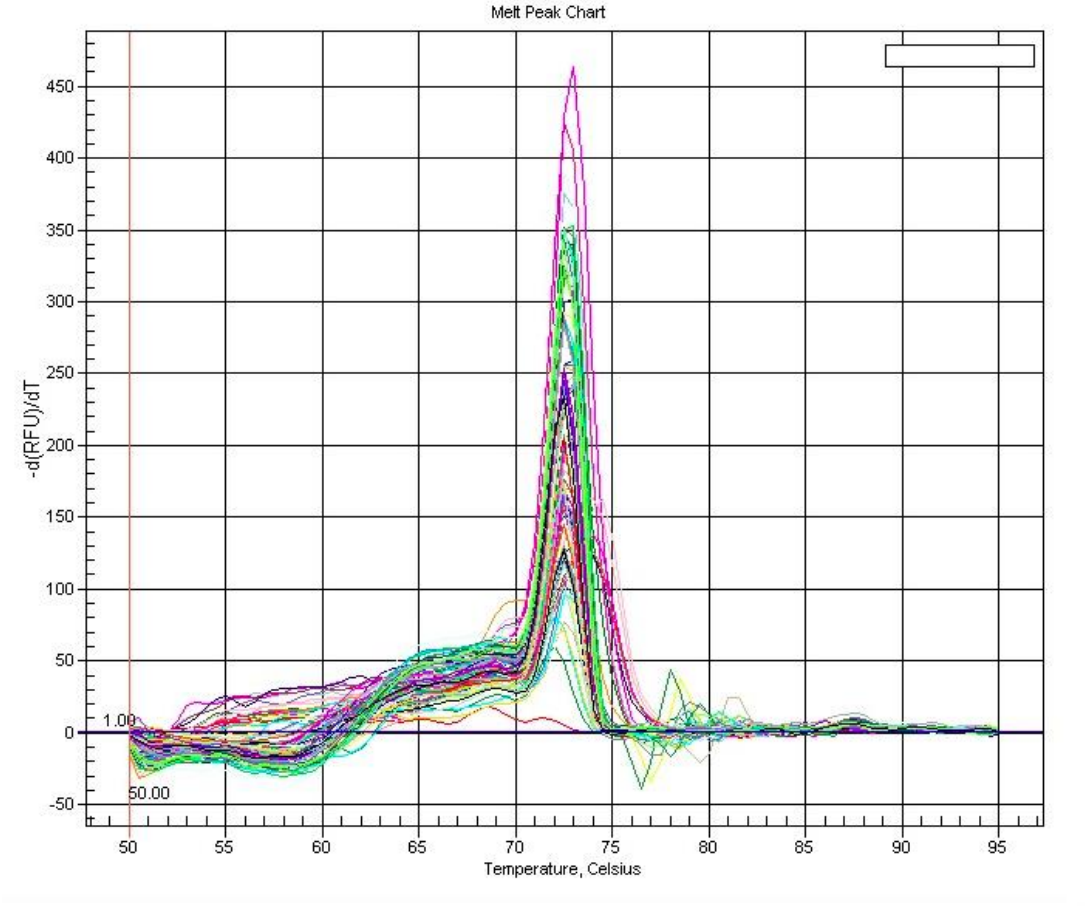
*Evolutionary analyses were conducted in MEGA7[48].*




## Real Time PCR Sample Output

*Amplification and melt peak for a subset of samples during the* P. falciparum *species-specific amplification reaction*


Amplification Chart
PCR Base Line Subtracted Curve Fit RFU
Cycle
3.85


Melt Peak Chart
-d(RFU)/dT
Temperature, Celsius
1.00
50.00