International Journal of Infectious Diseases 49 (2016) 161–169

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Subclinical *Plasmodium falciparum* infections act as year-round reservoir for malaria in the hypoendemic Chittagong Hill districts of Bangladesh

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ARTICLE INFO

Article history:
Received 12 April 2016
Received in revised form 16 June 2016
Accepted 20 June 2016

Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Malaria
Subclinical
Asymptomatic
Epidemiology
Rapid diagnostic test

SUMMARY

Objectives: An analysis of the risk factors and seasonal and spatial distribution of individuals with subclinical malaria in hypoendemic Bangladesh was performed.
Methods: From 2009 to 2012, active malaria surveillance without regard to symptoms was conducted on a random sample ($n = 3971$) and pregnant women ($n = 589$) during a cohort malaria study in a population of 24 000.
Results: The overall subclinical *Plasmodium falciparum* malaria point prevalence was 1.0% ($n = 35$), but was 3.2% ($n = 18$) for pregnant women. The estimated incidence was 39.9 per 1000 person-years for the overall population. Unlike symptomatic malaria, with a marked seasonal pattern, subclinical infections did not show a seasonal increase during the rainy season. Sixty-nine percent of those with subclinical *P. falciparum* infections reported symptoms commonly associated with malaria compared to 18% without infection. Males, pregnant women, jhum cultivators, and those living closer to forests and at higher elevations had a higher prevalence of subclinical infection.
Conclusions: Hypoendemic subclinical malaria infections were associated with a number of household and demographic factors, similar to symptomatic cases. Unlike clinical symptomatic malaria, which is highly seasonal, these actively detected infections were present year-round, made up the vast majority of infections at any given time, and likely acted as reservoirs for continued transmission.

1. Introduction

In Bangladesh, malaria remains endemic in 13 of 64 districts, with over 11 million people at risk of infection. The highest rates are found in the Chittagong Hill districts, where *Plasmodium falciparum* is the predominant species and *Plasmodium vivax* occurs to a lesser extent.[1,2] A diverse array of mosquito species transmit malaria in the region.[2–5] Clinical infections tend to peak from June to August during the rainy season.[5–7] Beginning in 2007, the Ministry of Health and the non-profit group BRAC began implementing the National Malaria Control Program, which provides community-based testing and treatment with artemisinin combination drugs and long-lasting insecticide treated bed nets, and has strengthened the overall malaria surveillance and control programs. During the study period, the use of long-lasting insecticide-treated bed nets was high (89.3%).[6] The BRAC provides malaria diagnostics and chemotherapy for free. Shasta shabikas are village health care workers who provide diagnosis for tuberculosis and malaria diagnostics and chemotherapy with artemether/lumefantrine , as well as prenatal care. The mean cost of a diagnosis is USD 0.39 and cost of treatment is USD 0.51.[2]

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http://dx.doi.org/10.1016/j.ijid.2016.06.019

A 65% reduction in malaria prevalence and a 91% decrease in malaria mortality occurred between 2008 and 2012, likely as a result of this focused effort.[2] Without a consistent effort at elimination, these gains are tenuous.

A cohort study performed by the Johns Hopkins Malaria Research Institute in collaboration with the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) started in 2009, aimed to better understand the epidemiology of malaria in the Hill districts. Previous papers have described seasonal patterns and risk factors for hypoendemic clinical malaria.[6,8] By contrast, this paper explores the active surveillance data to characterize subclinical infections as diagnosed by rapid diagnostic tests (RDT) and microscopy.

2. Methods

The study area included two unions in the Chittagong Hill districts of Bangladesh, an area now known as hypoendemic for *P. falciparum* and *P. vivax*, with more than a dozen resident ethnic groups and an overall population of 24 000 people in 4500 households over an area of 179 km^2.[6,9] The population was monitored year-round with demographic surveys, active and passive surveillance, entomological sampling, and mapping. Informed consent was obtained from all adult participants and from the guardians of children. Details of the surveillance methods have been described previously.[9] There were four aspects to this analysis: (1) estimating the prevalence from a randomized active surveillance of subclinical *P. falciparum* and *P. vivax* malaria and associated seasonal and geographic factors in all age groups and specifically in pregnant women year-round; (2) determining the extent of symptoms among those with subclinical *P. falciparum* infections identified in this active screening; (3) associating subclinical *P. falciparum* infections with demographic and behavioral risk factors; and (4) estimating the incidence of subclinical *P. falciparum* infections in the population and especially in pregnant women. Analysis was conducted on the randomly tested individuals selected for active surveillance, with separate results for pregnant women added when relevant.

Data were analyzed using R statistical software (Vienna, Austria), including the packages gmodels, stats, geoR, zoo, spatstat, maptools, maps, and lubridate.[10–16]

2.1. Estimation of the prevalence of subclinical P. falciparum and P. vivax infections and associated geographic and seasonal factors

The active surveillance system started in mid-October 2009 in Kuhalong Union, and was expanded to Rajbila Union in May 2010, with both being followed until mid-October 2012. One person in each of 24 geographic clusters was selected randomly for active sampling per week using a random number generator (http://www.randomizer.org), with eight people from each of three age groups (<5, 5–14, and ≥15 years). If a selected person was not available, the person four numbers away within the selected age group and cluster was sampled. All pregnant women in the study area were included in the active surveillance system for research and clinical follow-up. People who were selected multiple times made up 7.2% of the randomly selected population and 2.2% of the pregnancy selected population.

Those selected for sampling answered a survey related to symptoms and underwent an RDT for *P. falciparum* and *P. vivax* and provided blood for microscopic analysis. The prevalence by age group (<5, 5–14, 15–39, and ≥40 years) and sex was calculated. An overall age and sex-adjusted prevalence was calculated using direct adjustment, as well as sex-adjusted prevalence for 2–10-year-olds.

2.2. Determining the extent of symptoms present in those with subclinical P. falciparum infections

Active surveillance included a detailed questionnaire on physical symptoms experienced in the prior 2 weeks. The survey was answered directly by the participant, or by the caretaker for children, with input from the child when possible . Surveyors took the temperature of the participants, who were considered febrile at ≥38.3 °C for oral temperatures and at ≥37.5 °C for axillary temperatures. Both univariate and multivariate regression with common symptoms of malaria was conducted to assess associations of physical symptoms and actively detected subclinical *P. falciparum* malaria. This analysis was presented for the randomly selected population as well as for all those pregnant at the time of survey.

2.3. Determining the association of subclinical P. falciparum infection with demographic and behavioral risk factors

The cohort study included data from several other surveys including information on demographics, repeated every 3–4 months, and another on malaria control practices, household behaviors, and household proximity to environmental features, conducted yearly.[9] The elevation was determined from remotely sensed data (SRTM files). GIS (ArcGIS 10.1; ESRI, Redlands, CA, USA) was used to determine the elevation value at each house location. GIS was used to generate the hydrology layer, which is modeled on SRTM data. Then GIS was used to determine the distance from house to stream. Univariate analyses compared these socio-demographic and household factors with the prevalence of subclinical *P. falciparum* infection. These subclinical results were compared with previously published data for clinical symptomatic *P. falciparum* malaria in this cohort study.[6] Multivariate logistic regression was conducted using normalized covariates for all continuous factors. A stepwise logistic regression using forward and backward selection was then used to select the final model. Only those people with no missing data on the covariates considered were used for stepwise regression. This process was repeated with a larger dataset once several covariates with larger numbers of missing values were eliminated from the model.

2.4. Estimation of subclinical P. falciparum infection incidence

Of the 24 people selected for active surveillance each week, four were selected for longitudinal follow-up. All pregnant women were also entered into the longitudinal study. If a randomly selected woman was pregnant, another woman from that cluster was added to the study.

This population was tested by RDT and microscopy, as per the active surveillance protocol, on the initial visit and then at approximately 3, 6, and 9 months thereafter. The person-time under the nested longitudinal study and associated incident infections (not including those cases found on the initial visit) were documented and used to calculate the incidence rate. One and a half months of person-time were deducted for all incident cases with the assumption of infection at halfway through the follow-up interval. If the interval between visits exceeded 4 months, the person-time and associated cases for this interval were eliminated from the analysis.

Incidence was compared by age, sex, and pregnancy status. The assigned age group of a person for analysis was that at the time of blood draw for that interval. The overall incidence rate was adjusted by age and sex using direct standardization and the overall population age/sex distribution.

To explore the changes in incidence in pregnancy, all 15- to 39-year-old women in both the random and pregnancy follow-up

categories were divided into three groups: pregnant women, recently pregnant women, and not pregnant women. These states were defined on the basis of the pregnancy status at the specific follow-up visit. Those who were 'recently pregnant' had been pregnant during earlier follow-up visits. 'Not pregnant' women, were not pregnant on the current or any of the prior follow-up visits for this selection.

Those testing positive for malaria by either RDT and/or microscopy were treated according to the National Malaria Treatment Guidelines of Bangladesh and then followed (repeat RDT/microscopy) on days 2, 7, and 28 following diagnosis to ensure clearing of infection. Standard *P. falciparum* malaria cases were treated with artemether/lumefantrine. Pregnant women in their first trimester were treated with quinine dihydrochloride for *P. falciparum* and chloroquine for *P. vivax* infections. One person had a documented infection two times during follow-up, but as there was documented clearance of parasites between these two visits, it was treated as a second incident case.

3. Results

3.1. Demographics

Of the 3978 people surveyed for active screening, laboratory information was available for 3971. Of these, 3382 were selected through population random sampling; 664 were then entered into the longitudinal study and 589 were selected due to pregnancy. The demographic characteristics of these three groups are summarized in Table 1.

3.2. Estimation of the prevalence of subclinical P. falciparum and P. vivax infections and associated geographic and seasonal factors

Fifty-three people (1.33%; $n = 3971$) had documented subclinical *P. falciparum* infections (35 from the random sample and 18 from the pregnancy sample), with 51 positive by RDT and 44 positive by microscopy for *P. falciparum*. There was one positive blood smear for *P. vivax*. The geometric mean parasite density of the 44 positive *P. falciparum* infections was 902 parasites/μl (range 40–14 600). The nine RDT-positive/microscopy-negative cases may have occurred secondary to the low parasite density, as well as higher sensitivity of histidine-rich protein 2 (HRP2) antigen detection. The proportion of infections that were positive by sex and age group are shown in Table 2. Based on the random sample, the age and sex-adjusted point prevalence of *P. falciparum* malaria was 10.4/1000 population (95% confidence interval (CI) 7.0–13.9). Among the 1638 subjects aged 2–10 years tested, 16 had *P. falciparum* malaria, with a sex-adjusted point prevalence of 9.8/1000 population (95% CI 5.0–14.6). Among the 631 pregnant women tested, there were 20 infections of *P. falciparum* detected,

Table 1
Basic demographics of the active surveillance study population

Demographic and household factors		Active malaria survey, *n* (%) (random selection)	Nested longitudinal study, *n* (%) (random selection)	Pregnancy selected longitudinal study, *n* (%)
Union	Rajbila	1509 (44.6)	275 (41.4)	294 (49.9)
	Kuhalong	1873 (55.4)	389 (58.6)	295 (50.1)
	Total	3382 (100.0)	664 (100.0)	589 (100.0)
Sex/pregnancy	Male	1615 (47.8)	301 (45.3)	0 (0)
(On first visit)	Female	1767 (52.2)	363 (54.7)	589 (100.0)
	Pregnant	42 (2.4)	32 (8.8)	589 (100.0)
	Not pregnant	1725 (97.6)	331 (91.2)	0 (0)
	Total	3382 (100.0)	664 (100.0)	589 (100.0)
Age	6 months–<5 years	862 (25.5)	161 (24.2)	0 (0)
	5–14 years	1277 (37.8)	249 (37.5)	5 (0.8)
	15–39 years	761 (22.5)	161 (24.2)	579 (98.3)
	≥40 years	482 (14.3)	93 (14.0)	5 (0.8)
	Total	3382 (100.0)	664 (100.0)	589 (100.0)
Ethnicity	Bengali	707 (20.9)	143 (21.5)	111 (18.8)
	Tribal	2675 (79.1)	521 (78.5)	478 (81.2)
	Marma	1995 (74.6)	379 (72.7)	370 (77.4)
	Tanchangya	308 (11.5)	62 (11.9)	48 (10.0)
	Khyang	177 (6.6)	37 (7.1)	31 (6.5)
	Chakma	115 (4.3)	28 (5.4)	18 (3.8)
	Tripura	57 (2.1)	8 (1.5)	7 (1.5)
	Bawn	20 (0.7)	7 (1.3)	3 (0.6)
	Mro	2 (0.1)	0 (0)	0 (0)
	Rkhaine	1 (0.0)	0 (0)	1 (0.2)
	Total	3382 (100.0)	664 (100.0)	589 (100.0)
Religion	Buddhist	2636 (77.9)	515 (77.6)	475 (80.6)
	Muslim	607 (17.9)	125 (18.8)	94 (16.0)
	Christian	74 (2.2)	14 (2.1)	10 (1.7)
	Hindu	65 (1.9)	10 (1.5)	10 (1.7)
	Total	3382 (100.0)	664 (100.0)	589 (100.0)
Education level (age ≥15 years)	0–2 years	743 (59.8)	155 (61.0)	305 (52.2)
	3–5 years	233 (18.7)	55 (21.7)	121 (20.7)
	≥6 years	267 (21.5)	44 (17.3)	158 (27.1)
	Total	1243 (100.0)	254 (100.0)	584 (100.0)
Occupation (age ≥15 years)	Farmer	465 (37.4)	85 (33.5)	128 (21.9)
	Housewife	173 (13.9)	51 (20.1)	308 (52.7)
	Day laborer	173 (13.9)	41 (16.1)	56 (9.6)
	Student	130 (10.5)	19 (7.5)	19 (3.3)
	Jhum cultivator	95 (7.6)	17 (6.7)	22 (3.8)
	Unemployed	92 (7.4)	21 (8.3)	26 (4.5)
	Other	115 (9.3)	20 (7.9)	25 (4.3)
	Total	1243 (100.0)	254 (100.0)	584 (100.0)

Table 2
Subclinical *Plasmodium falciparum* infections by sex and age group

Sex/age category		Total (%)	Total without *P. falciparum* malaria (%)	Total with *P. falciparum* malaria (%)	OR (95% CI)	*p*-Value
Female	<5 years	426 (12.6)	424 (12.7)	2 (5.7)	1.0	-
Female	5–14 years	664 (19.6)	660 (19.7)	4 (11.4)	1.28 (0.23–7.05)	0.773
Female	15–39 years	440 (13.0)	435 (13.0)	5 (14.3)	2.43 (0.47–12.63)	0.289
Female	≥40 years	237 (7.0)	236 (7.1)	1 (2.9)	0.90 (0.08–9.97)	0.930
Male	<5 years	436 (12.9)	433 (12.9)	3 (8.6)	1.47 (0.24–8.84)	0.675
Male	5–14 years	613 (18.1)	599 (17.9)	14 (40.0)	4.95 (1.12–21.93)	0.035[a]
Male	15–39 years	321 (9.5)	317 (9.5)	4 (11.4)	2.68 (0.49–14.71)	0.258
Male	≥40 years	245 (7.2)	243 (7.3)	2 (5.7)	1.74 (0.24–12.47)	0.579
Total		3382 (100)	3347 (100)	35 (100)		

OR, odds ratio; CI, confidence interval.
[a] Statistically significant.

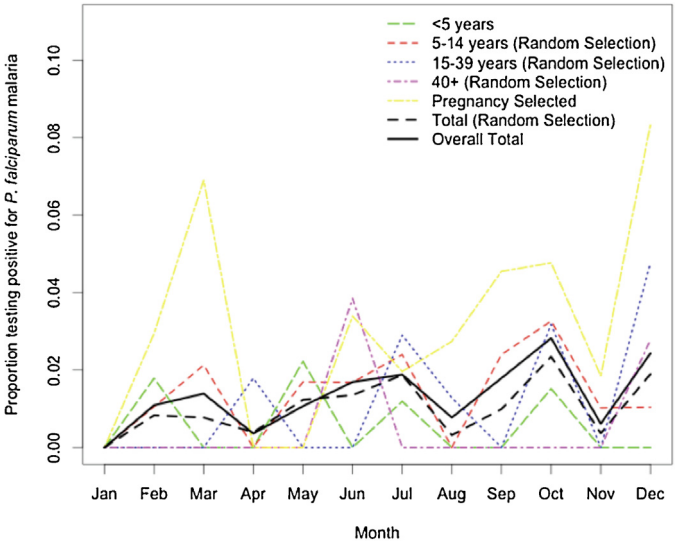

Figure 1. Proportion testing positive for *Plasmodium falciparum* infection by RDT and/or microscopy during active surveillance by month, age, and study type.

equating to a point prevalence of 31.7/1000 population (95% CI 18.0–45.4).

The proportion testing positive for subclinical *P. falciparum* infection by calendar month showed no significant difference by season (Figure 1; May–October vs. November–May, odds ratio (OR) 1.9, 95% CI 0.9–4.3; *p*-value = 0.09). The spatial intensity of all active survey participant household locations and those that tested positive for *P. falciparum* infections can be seen in Figure 2. Subclinical *P. falciparum* infections were not simply a function of population density, as areas with lower population densities had higher infection densities than would be expected if there was no spatial variation in subclinical *P. falciparum* incidence.

3.3. Determining symptoms present in persons with subclinical P. falciparum *infections*

The proportions of participants with and without *P. falciparum* infections who had specific symptoms in the prior 2 weeks (Table 3 for those in the random study and Table 4 for all pregnant women) showed that most of the symptoms surveyed were reported at higher rates among those who had subclinical *P. falciparum*. These differences in symptoms with and without *P. falciparum* malaria were also present among pregnant women, although a higher proportion of pregnant women were experiencing constitutional symptoms in both groups (Table 4).

When specifically examining patients with fever, muscle aches, fatigue, and headache, 69% of those with *P. falciparum* malaria had at least one of these symptoms compared to 18% of those without *P. falciparum* malaria (OR 9.9, 95% CI 5.2–19.8). A multiple covariate regression analysis among the randomly detected population, including fever, muscle aches, fatigue, and headache in the prior 2 weeks and actively sampled *P. falciparum* malaria, showed that when controlling for the other symptoms, fever (OR 10.3, 95% CI 4.2–24.9; $p < 0.001$) and headaches (OR 3.8, 95% CI 1.5–9.4; $p = 0.004$) were positively associated with subclinical infection, while no association was found with fatigue (OR 0.6, 95% CI 0.2–1.5; $p = 0.274$) or muscle aches (OR 1.0, 95% CI 0.4–2.6; $p = 0.944$).

When analyzing measured fever at the time of survey for the randomly selected population, two (5.7%) of the 35 positive *P. falciparum* infections and 20 (0.6%) of the 3347 without infection had a fever (OR 10.1, 95% CI 1.1–44.3; $p = 0.02$).

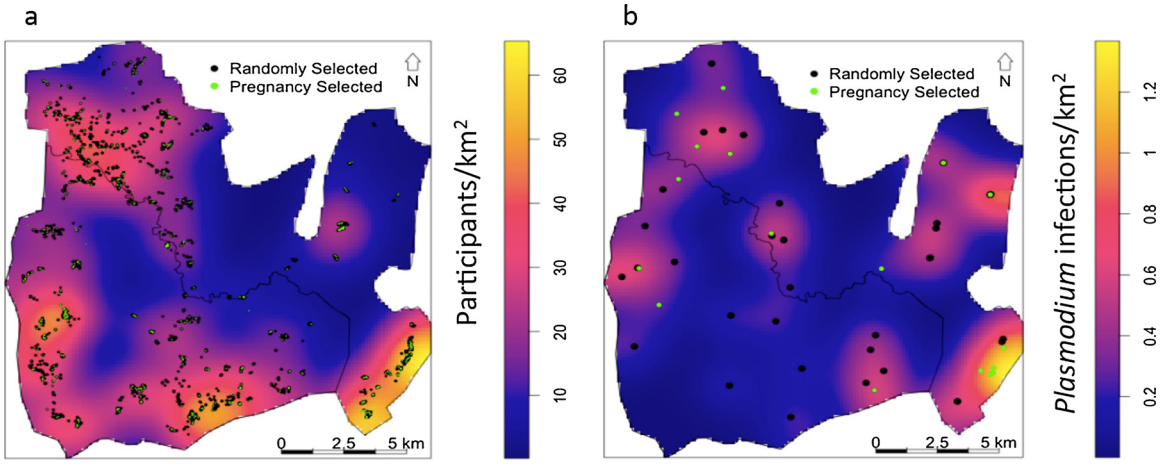

Figure 2. Spatial intensity of household locations by active surveillance study type: (a) all active survey study participants, (b) subclinical *Plasmodium falciparum* infections.

Table 3
Association of subclinical *Plasmodium falciparum* infections with reported symptoms in the prior 2 weeks—randomly selected active survey participants

Symptoms		Negative for *P. falciparum* malaria *n* (%)	Positive for *P. falciparum* malaria *n* (%)	OR (95% CI)	Fisher's exact test *p*-value
Single symptom associations					
Fever	No	3137 (93.8)	16 (45.7)	17.9 (8.6–37.7)	<0.001
	Yes	208 (6.2)	19 (54.3)		
Headache	No	3093 (92.5)	18 (51.4)	11.5 (5.5–24.1)	<0.001
	Yes	252 (7.5)	17 (48.6)		
Chills	No	3241 (96.9)	24 (68.6)	14.2 (6.1–31.2)	<0.001
	Yes	104 (3.1)	11 (31.4)		
Nausea	No	3265 (97.6)	26 (74.3)	14.1 (5.6–32.3)	<0.001
	Yes	80 (2.4)	9 (25.7)		
Vomiting	No	3293 (98.4)	29 (82.9)	13.1 (4.3–33.9)	<0.001
	Yes	52 (1.6)	6 (17.1)		
Diarrhea	No	3295 (98.5)	32 (91.4)	6.2 (1.2–20.8)	0.017
	Yes	50 (1.5)	3 (8.6)		
Cough	No	2859 (85.5)	25 (71.4)	2.4 (1.0–5.1)	0.029
	Yes	486 (14.5)	10 (28.6)		
Fatigue	No	3147 (94.1)	28 (80.0)	4.0 (1.4–9.5)	0.004
	Yes	198 (5.9)	7 (20.0)		
Muscle ache	No	3085 (92.2)	28 (80.0)	3.0 (1.1–7.0)	0.017
	Yes	260 (7.8)	7 (20.0)		
Muscle weakness	No	3048 (91.1)	27 (77.1)	3.0 (1.2–7.0)	<0.001
	Yes	297 (8.9)	8 (22.9)		
Convulsions/seizures	No	3340 (99.9)	35 (100.0)	0 (0–107.3)	1
	Yes	5 (0.1)	0 (0)		
Summary measures					
Any of the symptoms listed above	No	2492 (74.5)	9 (25.7)	8.4 (3.8–20.53)	<0.001
	Yes	853 (25.5)	26 (74.3)		
Fever, headache, fatigue, muscle aches	No	2760 (82.5)	11 (31.4)	10.3 (4.8–23.4)	<0.001
	Yes	585 (17.5)	24 (68.6)		
Total (*n*)		3345	35		

OR, odds ratio; CI, confidence interval.

Table 4
Association of subclinical *Plasmodium falciparum* infections with reported symptoms in the prior 2 weeks—all pregnant women from the active survey

Symptoms		Negative for *P. falciparum* malaria *n* (%)	Positive for *P. falciparum* malaria *n* (%)	OR (95% CI)	Fisher's exact test *p*-value
Single symptom associations					
Fever	No	543 (88.9)	8 (40.0)	11.9 (4.3–34.8)	<0.001
	Yes	68 (11.1)	12 (60.0)		
Headache	No	420 (68.7)	8 (40.0)	3.3 (1.2–9.4)	0.013
	Yes	191 (31.3)	12 (60.0)		
Chills	No	575 (94.1)	14 (7.0)	6.8 (2.0–20.3)	0.001
	Yes	36 (5.9)	6 (30.0)		
Nausea	No	518 (84.8)	11 (55.0)	4.5 (1.6–12.4)	0.002
	Yes	93 (15.2)	9 (45.0)		
Vomiting	No	536 (87.7)	15 (75.0)	2.4 (0.7–7.1)	0.160
	Yes	75 (12.3)	5 (25.0)		
Diarrhea	No	604 (98.9)	20 (100.0)	0 (0.0–22.4)	1
	Yes	7 (1.1)	0 (0.0)		
Cough	No	538 (88.1)	15 (75.0)	2.5 (0.7–7.4)	0.015
	Yes	73 (11.9)	5 (25.0)		
Fatigue	No	509 (83.3)	16 (80.0)	1.3 (0.30–4.0)	0.760
	Yes	102 (16.7)	4 (20.0)		
Muscle ache	No	485 (79.4)	9 (45.0)	4.7 (1.7–13.1)	<0.001
	Yes	126 (20.6)	11 (55.0)		
Muscle weakness	No	444 (72.7)	10 (50.0)	2.7 (0.97–7.2)	0.040
	Yes	167 (27.3)	10 (50.0)		
Convulsions/seizures	No	604 (98.9)	18 (90.0)	9.5 (0.90–54.9)	0.030
	Yes	7 (1.1)	2 (10.0)		
Summary measures					
Any of the symptoms listed above	No	286 (46.8)	3 (15.0)	5.0 (1.4–26.8)	0.005
	Yes	325 (53.2)	17 (85.0)		
Fever, headache, fatigue, muscle aches	No	313 (51.2)	3 (15.0)	5.9 (1.7–31.9)	0.001
	Yes	298 (48.8)	17 (85.0)		
Total (*n*)		611	20		

OR, odds ratio; CI, confidence interval.

3.4. Determining the association of subclinical P. falciparum *malaria with demographic and behavioral risk factors*

Regarding demographics, the relationships between subclinical *P. falciparum* infections and various demographic factors, as well as clinical incidence, were numerous (Table 5). Factors that were significantly associated with subclinical malaria included union, ethnicity, and occupation; age group was marginally associated. The farming practice of jhum cultivation was an occupational risk factor for infection.

Table 5
Comparison of socio-demographic risk factors for actively detected subclinical *Plasmodium falciparum* infections with passive incident clinical malaria infections during high- and low-transmission seasons

	Actively sampled mild/asymptomatic infections				Passive symptomatic infections[a]						
	Year-round					High-transmission season			Low-transmission season		
Household factors	*n* (%) Negative	*n* (%) Positive	OR (95% CI)	*p*-Value	Total population	Cases	Incidence per 1000/ month	*p*-Value	Cases	Incidence per 1000/ month	*p*-Value
Union											
Rajbila	1489 (98.7)	20 (1.3)	1	-	10 498	234	2.05	<0.001	34	0.30	0.4
Kuhalong	1858 (99.2)	15 (0.8)	0.60 (0.3–1.2)	0.1	12 874	171	1.3		33	0.24	
Sex											
Male	1592 (98.6)	23 (1.4)	1		11 456	217	1.8	0.053	35	0.3	0.60
Female	1755 (99.3)	12 (0.7)	0.47 (0.2–0.9)	0.037[b]	11 916	188	1.47		32	0.25	
Age											
<6 months	0	0			985	0	0	<0.001	0	0	<0.001
6–59 months	857 (99.4)	5 (0.6)	1	-	2621	43	1.5		16	0.6	
5–14 years	1259 (98.6)	18 (1.4)	2.5 (0.91–6.6)	0.077	5200	149	2.8		23	0.4	
≥15 years	1231 (99.0)	12 (1.0)	1.7 (0.6–4.8)	0.336	14 563	213	1.3		28	0.2	
15–39 years	752 (98.8)	9 (1.2)	2.1 (0.7–6.1)	0.199							
≥40 years	479 (99.4)	3 (0.6)	1.1 (0.3–4.5)	0.923							
Ethnicity											
Bengali	704 (99.6)	3 (0.4)	1	-	4821	33	0.6	<0.001	6	0.1	<0.001
Total tribal	2643 (98.8)	32 (1.2)	2.8 (0.9–9.3)	0.085	18 551	372	1.9		61	0.3	
Marma	1978 (99.1)	17 (0.9)	2.0 (0.59–6.9)	0.264	14 035	275	1.84		34	0.23	
Tanchangya	300 (97.4)	8 (2.6)	6.3 (1.6–23.8)	0.007[b]	2085	39	1.74		9	0.40	
Khyang	174 (98.3)	3 (1.7)	4.0 (0.81–20.2)	0.089	1134	3	0.24		5	0.40	
Chakma	114 (99.1)	1 (0.9)	2.1 (0.2–20.0)	0.533	802	15	1.74		7	0.81	
Tripura	54 (94.7)	3 (5.3)	13.0 (2.6–66.2)	0.002[b]	391	20	5.42		4	1.08	
Other tribal	2	0	0 (0–Inf)	0.997	104	20	20.70		2	2.07	
Education level, (age ≥15 years, *n* = 1243)											
0–2 years	735 (98.9)	8 (1.1)	1	-	8598	136	1.40	0.604	16	0.17	0.338
3–5 years	229 (98.3)	4 (1.7)	1.6 (0.48–5.38)	0.443	2668	38	1.32		8	0.28	
≥6 years	267 (100.0)	0 (0.0)	0 (0–Inf)	0.988	3297	39	1.18		4	0.12	
Occupation (age ≥15 years, *n* = 1243)											
Agricultural	461 (99.1)	4 (0.9)	1		4609	48	0.93	<0.001	11	0.21	0.643
Day labor	170 (98.3)	3 (1.7)	2.0 (0.45–9.2)	0.356	1996	42	1.91		5	0.23	
Jhum cultivation	91 (95.8)	4 (4.2)	5.1 (1.2–20.7)	0.024[b]	2544	71	2.42		5	0.17	
Other	509 (99.8)	1 (0.2)	0.23 (0.03–2.0)	0.185	5414	52	0.92		7	0.12	
Unemployed	92 (100.0)	0 (0)	0 (0–Inf)	0.996							
Housewife	173 (100.0)	0 (0)	0 (0–Inf)	0.994							
Student	149 (100.0)	0 (0)	0 (0–Inf)	0.995							
Other	114 (99.1)	1 (0.9)	1.0 (0.11–9.2)	0.992							
Total					23 372	405	1.62		67	0.27	

OR, odds ratio; CI, confidence interval.
[a] Passive data taken from prior survey in this cohort.[6]
[b] Statistically significant.

Education level was not significantly associated with subclinical infection.

There was too little power to define distinctions by age or sex categories (Table 2), but trends suggested overall higher prevalence among males than females and higher prevalence in those 5–39 years of age than among young children or older adults. The highest prevalence age group category for women was 15–39 years and for men was 5–14 years.

In terms of household risk factors, when subclinical and clinical malaria were compared (Table 6),[5] there was no association for sleeping under a bed net the night before or owning animals. There was a trend of increasing distance from ponds being a risk factor,

Table 6
Comparison of household risk factors for actively detected subclinical *Plasmodium falciparum* infections with passive incident clinical malaria cases during high- and low-transmission seasons

	Active mild/asymptomatic infections				Passive symptomatic infections						
	Year-round					High-transmission season			Low-transmission season		
Household factors	*n* (%) Negative	*n* (%) Positive	OR (95% CI)	*p*-Value	Total population	Cases	Incidence per 1000/ month	*p*-Value	Cases	Incidence per 1000/ month	*p*-Value
Bed net use (slept under net night before survey), *n* = 3257											
Yes	2950 (99.0)	31 (1.0)	1.0	-	18 869	306	1.48	<0.001	54	0.26	0.611
No	272 (98.6)	4 (1.4)	1.4 (0.49–4.0)	0.530	2255	58	2.66		7	0.32	
Own animals, *n* = 3382											
Yes	2817 (99.0)	28 (1.0)	1.0	-	19 087	337	1.65	0.407	126	0.26	0.923
No	530 (98.7)	7 (1.3)	1.3 (0.58–3.1)	0.504	4285	68	1.48		30	0.27	
Distance from house to pond (meters), *n* = 3381 (Two 'don't knows' were excluded)											
0–50	654 (99.7)	2 (0.3)	1.0	-	4312	31	0.68	<0.001	7	0.015	0.015
51–100	262 (99.2)	2 (0.8)	2.5 (0.40–17.8)	0.362	1897	13	0.64		1	0.05	
>100	167 (98.8)	2 (1.2)	3.9 (0.55–28.0)	0.174	1022	13	1.19		5	0.46	
No pond	2263 (98.7)	29 (1.3)	4.2 (1.0–17.6)	0.050	16 141	345	1.99		54	0.31	
Distance from house to forest (meters), *n* = 3382											
0–25	1134 (98.6)	16 (1.4)	1.0	-	7869	183	2.16	<0.001	34	0.40	0.013
26–50	1343 (98.8)	17 (1.3)	0.90 (0.45–1.78)	0.756	9187	126	1.29		22	0.23	
>50	870 (99.8)	2 (0.2)	0.16 (0.04–0.72)	0.016[a]	6316	96	1.43		11	0.16	
Distance from river or stream (meters), *n* = 3971											
0–50	569 (99.5)	3 (0.5)	1.0	-							
51–100	587 (98.8)	7 (1.2)	2.3 (0.58–8.8)	0.239							
100–250	1137 (99.0)	11 (1.0)	1.8 (0.51–6.6)	0.353							
250+	1054 (98.7)	14 (1.3)	2.5 (0.72–8.8)	0.148							
Altitude (meters above sea level), *n* = 3968											
7–25	734 (99.9)	1 (0.1)	1.0	-							
25–50	1545 (99.1)	14 (0.9)	6.7 (0.87–50.7)	0.067							
50–75	688 (98.7)	9 (1.3)	9.6 (1.2–76.0)	0.032[a]							
75–125	353 (10.5)	8 (2.2)	16.6 (2.1–133.6)	0.008[a]							
125–147	27 (90.0)	3 (10.0)	81.6 (8.2–810.5)	<0.001[a]							
Total	3347	35			23 372	405	1.62		67	0.27	

OR, odds ratio; CI, confidence interval.
[a] Statistically significant.

although not statistically significant for the subclinical analysis. Living closer to forests was associated with a higher risk of both subclinical infection and symptomatic malaria. There was no association of distance to streams for subclinical infections.

Elevation in the study area ranged from 7 m to 147 m above sea level. A strong association between altitude and malaria was present, with those in the highest elevation range (125–147 m above sea level) having 81.6 (95% CI 8.2–810.5) times the odds of subclinical malaria compared to those in the lowest elevation range (7–25 m above sea level).

3.5. Multivariate regression

In this analysis a selection of the above individual, household, and other demographic factors and their associations with malaria were examined. Individual characteristics included union, sex, age group, race, education, occupation, and current pregnancy status. Household factors included whether a bed net was located over the bed, whether animals were owned by members of that household, distance to the forests, distance to the closest river/stream, distance to the nearest pond, and altitude. The stepwise logistic regression was conducted with 3948 participants for whom no covariates were missing (99.4% of those surveyed). Once the use of bed nets was eliminated as a factor, a second stepwise logistic regression was conducted with a larger dataset, as much of the missing data was in this factor.

The final model included sex, age, occupation, distance to forest, elevation, and pregnancy (Table 7). Higher risk groups included pregnant women, males, jhum cultivators, those living at higher elevations and closer to forests, and marginally those aged 5–14 years and day laborers.

Ethnicity remained an important explanatory variable for subclinical infections until elevation was added to the model. The Tripura, in particular, were higher risk and tended to live on hilltops, while the Bengalis, a lower risk group, lived mostly at lower elevations.

Table 7
Regression model of subclinical *Plasmodium falciparum* infection with household and demographic factors

Demographic and household factors		Unadjusted (n = 4010)		Adjusted (n = 3986)	
		OR (95% CI)	p-Value	OR (95% CI)	p-Value
Sex	Male	1.0	-	1.0	-
	Female	0.5 (0.2–0.9)	0.037[a]	0.43 (0.2–0.9)	0.030[a]
Age	<5 years	1.0	-	1.0	-
	5–14 years	2.5 (0.91–6.6)	0.077	2.4 (0.88–6.5)	0.087
	15–39 years	2.1 (0.68–6.1)	0.199	0.59 (0.11–3.0)	0.521
	≥40 years	1.1 (0.26–4.5)	0.923	0.34 (0.05–2.2)	0.258
Occupation (all age groups)	Other	1.0	-	1.0	-
	Agricultural	0.93 (0.32–2.7)	0.896	3.0 (0.6–14.5)	0.170
	Day labor	1.8 (0.55–6.2)	0.322	4.1 (0.9–19.9)	0.075
	Jhum cultivation	4.7 (1.6–13.9)	0.005[a]	8.9 (1.7–45.9)	0.009[a]
Distance from house to forest	Per increase in Z-score	0.28 (0.1–0.8)	0.012[a]	0.42 (0.2–1.1)	0.074
Elevation	Per increase in Z-score	1.8 (1.4–2.3)	<0.001[a]	1.6 (1.2–2.0)	<0.001[a]
Pregnancy	No	1.0	-	1.0	-
	Yes	5.0 (1.2–21.6)	<0.031[a]	13.3 (2.3–77.2)	0.004[a]

OR, odds ratio; CI, confidence interval.
[a] Statistically significant.

Table 8
Incidence rate of subclinical *Plasmodium falciparum* infections by age and sex among randomly selected longitudinal survey participants

Age	Sex	Incident infections	People followed	Person years	Incidence rate (per 1000 person-years)	95% CI
<5 years	Male	2	77	50.4	39.7	10.2–154.3
<5 years	Female	1	64	40.7	24.6	3.6–170.5
5–14 years	Male	1	127	82.2	12.2	1.7–85.4
5–14 years	Female	3	133	86.2	34.8	11.4–105.8
15–39 years	Male	2	53	34.8	57.5	15.0–220.9
15–39 years	Female	0	109	76.5	0	0–Inf
≥40 years	Male	3	45	29.7	101.2	34.6–295.8
≥40 years	Female	0	46	29.7	0	0–Inf
Total	male (crude)	8	285	197.0	40.6	20.6–80.0
Total	male (adjusted)	10.9[a]	285	197.0	55.2	31.0–98.4
Total	female (crude)	4	335	233.0	17.2	6.5–45.4
Total	female (adjusted)	2.6[a]	335	233.0	11.04	3.3–37.2
Total (crude)		12	620	430.0	27.9	16.0–48.7
Total (adjusted)		14.1[a]	620	430.0	32.9	19.7–54.9

CI, confidence interval.
[a] Using direct adjustment, applying rates to age/sex structure of overall population.

3.6. *Estimation of subclinical* P. falciparum *infection incidence*

The randomly selected, nested, longitudinal study used 664 people, 620 of whom had at least one follow-up visit that was no greater than 4 months after the first. The total duration of follow-up time for these 620 individuals was 431.55 years (430.5 when adjusted for the assumption that people became infected half way through the follow-up interval).

Although not significantly different, adult males (15–39 and ≥40 years) had the highest incidence of *P. falciparum* infection, while adult females were estimated to have the lowest, with an incidence of 0 infections/1000 person-years (Table 8). The overall age and sex-adjusted incidence rate was estimated to be 39.9/1000 person-years (95% CI 19.7–54.9). The estimated rates for both currently pregnant women and recently pregnant women were both about 19.5 incident infections/1000 person-years.

4. Discussion

This study demonstrates that subclinical malaria infections make up the majority of infections in the Hill districts of Bangladesh. Extrapolating rates to the population of 24 000, the estimated incident subclinical infections per year was 790 (95% CI 473–1318), with an average of 250 (95% CI 168–332) prevalent subclinical infections at any one time. Using a formula based on microscopy prevalence from Okell et al.,[17] a PCR *P. falciparum* prevalence of about 4% was estimated, or just under 1000 people at a time.

There was an average of 189 clinical symptomatic incident infections per year detected, which makes up only 19% of estimated microscopy/RDT-positive incident infections. These clinical cases were treated promptly and thus had short durations (on the order of days to weeks). If a 2-week duration is assumed, there was an average of eight symptomatic infections at a time.

The extent to which sub-microscopic infections transmit malaria is still being explored. However, it has been estimated that in very low transmission settings, sub-microscopic infections account for 70–80% of all those infected, and 20–50% of all human-to-mosquito transmissions come from sub-microscopic carriers of infection.[17,18] It has been shown that almost all people with infections have mature gametocytes in their blood, including asymptomatic and sub-microscopic infections, and modeling experiments show that transmission to mosquitoes depends on a number of factors including gametocyte density, a relationship that is non-linear and varies by setting.[18–22]

Understanding the specifics of spatial and temporal distribution and risk factors for these subclinical infections is critical for malaria control and elimination programs.

The most prominent risk factors for subclinical *P. falciparum* infection appear to be pregnancy, living at high elevation and close to forests, being involved in jhum cultivation or other high risk occupations, being an older child or younger adult, and being male. When examining malaria incidence, the highest risk age group was adult males, whose point estimate for incidence was more than double any other age/sex group. Much of the adult male population

is involved in activities such as jhum cultivation (a type of hillside farming) and rubber plantation farming, among other activities that may lead to increased risk.

The comparison of the pregnant to non-pregnant women showed an increase in risk of infection for pregnant women and recently pregnant women in this area, reinforcing the results from previous studies.[23] This increased risk, combined with the potential complications of malaria during pregnancy, emphasizes the need for continued testing and follow-up of all pregnant individuals.

The limitations of this analysis include a low number of subclinical infections documented over the period, resulting in a lack of power for many of the subgroup analyses and multivariate regression. Furthermore, in a few cases the respondents learned their RDT results prior to finishing the symptom survey, which could have introduced a recall bias, possibly leading to over-reporting of symptoms of those with positive RDTs. Also, the inclusion of PCR would have helped to clarify the proportion of infections that were sub-microscopic. Lastly, due to the treatment of all detected infections, it was not possible to establish the natural history of the subclinical infections; thus the average duration of infections and the proportion of infections that would have developed into symptomatic cases are not known.

In conclusion, the Hill districts of Bangladesh are hypoendemic for malaria, with subclinical infections making up the majority of infections. Unlike symptomatic cases that lead people to seek treatment, and which peak in the rainy season, the subclinical infections occurred year-round. Attempts at elimination of malaria in this region will require more than finding and treating symptomatic patients, as subclinical infections likely act as the major reservoir for continued transmission.

Contributions

KS, WK, DAS, MR, GG, MN, and DJS made contributions to the concept and design of the study in the field. KS, WK, MSA, SA, CP, JK, MZH, and JA collected data in the field. KS, WK, DAS, MSA, SA, CP, JK, MR, MAH, JA, TS, and DJS were involved with data management. KS, WK, DAS, SA, MR, TS, and DJS made substantial contributions to data analysis. KS, WK, MSA, DAS, and DJS coordinated manuscript drafts. All authors (KS, WK, DAS, MSA, SA, CP, JK, MR, MZH, JA, GG, TS, SG, MN, and DJS) critically reviewed and approved the paper for submission.

Acknowledgements

This research activity was carried out with funding from the Johns Hopkins Malaria Research Institute (JHMRI) with support from The Bloomberg Family Foundation to cover costs at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), as well as partial support for personnel costs of faculty at Johns Hopkins. The research was carried out through this joint project between the two institutions. Individual graduate support for KLS was provided by the Johns Hopkins Medical Scientist Training Program and the Johns Hopkins Bloomberg School of Public Health, Department of International Health. After the JHMRI approved the general scope of work for the overall research activity (“Mapping Malaria in Bangladesh”), the JHMRI had no role in the study design, in the collection, analysis, and interpretation of the data, in the writing of the manuscript, or in the decision to submit the manuscript for publication. icddr,b is grateful to the governments of Bangladesh, Canada, Sweden, and the UK for providing core/unrestricted support.

Ethical approval: This study was approved by the Institutional Review Board of the Johns Hopkins Bloomberg School of Public Health and the Ethical Review Committee of icddr,b.

Conflict of interest: DJS receives royalties from Alere for the provision of positive control histidine-rich protein 2 included in some Binax test kits. The other authors have no additional financial interests.

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