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FULLENGTH

Asymptomatic low-density *Plasmodium* infection during non-transmission season: a community-based cross-sectional study in two districts of North Eastern Region, India

Hari Shankar[a,†], Sobhan Phookan[b,†], Mrigendra Pal Singh[c], Ram Suresh Bharti[a], Naseem Ahmed[a], Chander Prakash Yadav[a], Guru Prasad Sharma[a], Kuldeep Singh[b], Harpreet Kaur[d], and Neelima Mishra[a,*]

[a]*Indian Council of Medical Research–National Institute of Malaria Research, Sector-8, Dwarka, New Delhi 110077, India;* [b]*Indian Council of Medical Research–National Institute of Malaria Research Field Unit, Guwahati 781022, Assam, India;* [c]*Indian Council of Medical Research–National Institute of Malaria Research Field Unit, Jabalpur 482003, Madhya Pradesh, India;* [d]*Indian Council of Medical Research, Ansari Nagar, New Delhi 110029, India*

*Corresponding author: Tel: +91-11-25307331; E-mail: neelima1.nimr@gmail.com
†Equal authorship.

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Background: Malaria elimination requires targeting asymptomatic and low-density *Plasmodium* infections that largely remain undetected. Therefore we conducted a cross-sectional study to estimate the burden of asymptomatic and low-density *Plasmodium* infection using conventional and molecular diagnostics.

Methods: A total of 9118 participants, irrespective of age and sex, were screened for malaria using rapid diagnostic tests (RDTs), microscopy and polymerase chain reaction.

Results: Among the participants, 707 presented with symptoms and 8411 without symptoms, of which *Plasmodium* was present in 15.6% (110/707) and 8.1% (681/8411), respectively. Low-density infection was found in 5.1% (145/2818) of participants and 8327 of 9118 were Plasmodium negative. Endemicity was propotional to asymptomatic infections (high endemicity 11.1% [404/3633] vs low endemicity 5.8% [277/4778]; odds ratio [OR] 2.0 [95% confidence interval {CI} 1.7 to 2.4]) but inversely related to low-density infection (high endemicity 3.7% [57/1545] vs low endemicity 6.9% [88/1273]; OR 1.9 [95% CI 1.4 to 2.7]). The spleen rate in children 2–9 y of age was 17.9% (602/3368) and the enlarged spleen index was 1.6. Children between 8 and 14 y showed higher odds for asymptomatic (adjusted OR [aOR] 1.75 [95% CI 1.4 to 2.2]) and low-density infections (aOR 0.63 [95% CI 0.4 to 1.0)] than adults.

Conclusions: The prevalence of asymptomatic and low-density *Plasmodium* infection undermines the usefulness of standard diagnostic tools used by health agencies. This necessitates deploying molecular tools in areas where malaria microscopy/RDTs indicate a dearth of infection.

Keywords: asymptomatic malaria, epidemiology, low-density *Plasmodium* infection

Introduction

Malaria is a deadly disease and is prevalent in tropical and subtropical regions. It is presently one of the most geographically distributed and life-threatening diseases and accounted for an estimated 405 000 deaths in 2018. An estimated 228 million malaria cases occurred globally in 2018, of which 85% were reported from 19 sub-Saharan Africa countries and India.[1] India accounted for 0.34 million malaria cases in 2018 and nearly 48% of those were due to *Plasmodium falciparum* infection.[2] In this study, two northeastern districts, Udalguri and East Garo Hills, were selected due to the high malaria prevalence in these districts. The previous cross-sectional studies conducted in Udalguri district reported a high slide positivity rate (42.8%), with *P. falciparum* as the predominant species.[3] A recent systematic review of malaria in Meghalaya found a high annual parasite incidence (>10 API) in East Garo Hills district in 2016, while a dramatic reduction in the number of malaria cases was observed in this district (i.e. 20 045 cases in 2015 to 3084 in 2017) after analysing the 2015–2017 National Vector Borne Disease Control Programme

(NVBDCP) malaria epidemiology data.[4] In India, the strategy of the NVBDCP is to control malaria in high-endemic areas and eliminate it from low-endemic areas. Thus there is a need to shift from early diagnosis to active surveillance by conducting epidemiological mass surveys and detection of asymptomatic and low-density *Plasmodium* infection in the community.[5–7]

Asymptomatic *Plasmodium* infections[8] are still a major obstacle for malaria elimination programmes due to the non-treatment of asymptomatic carriers. This results in continuing malaria transmission even in dry seasons. Thus there should be methodical surveillance to identify hidden carriers of the malaria parasite and treat them to block onward transmission.[9] In addition, a significant portion of asymptomatic *Plasmodium* infections are accompanied by low-density submicroscopic infections. Such asymptomatic and low-density infections remain undetected by conventional malaria diagnostic methods but can reliably be detected using molecular methods.[10] Individuals with recurrent episodes of malaria tend to have low parasite densities and often remain asymptomatic due to the development of acquired immunity. This is one of the reasons why asymptomatic and low-density infections are observed mostly in older people,[10] although some reports suggest that school-age children may also act as a reservoir of asymptomatic malaria.[11,12] Typical submicroscopic infections show an inverse relation with transmission intensity and it has been observed that high-transmission areas (where parasite prevalence by microscopy is >75%) show nearly 20% submicroscopic infections compared with approximately 80% in low-transmission areas (where parasite prevalence by microscopy is <10%).[13] Molecular surveillance helps in identifying the sexual stage of parasites, known as gametocytes, which usually form <5% of the total parasite biomass in cases of *P. falciparum* infection. This is the only mode of transmission from humans to mosquitos. Due to the smaller proportion of gametocytes in the parasite pool, routine microscopy often remains less sensitive to detect these sexual-stage parasites. However, molecular methods such as reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR are sensitive enough to detect such hidden infectious reservoirs.[14]

Despite the fact that many malaria-endemic countries have initiated malaria elimination programmes, high prevalences of asymptomatic and submicroscopic malaria infections are persistent.[12,15,16] There are a handful of studies reported from India on the prevalence of asymptomatic and submicroscopic malaria, but these studies did not report the sampling strategy or sample size calculations, making it difficult to interpret results or generalize study findings.[9,17–19] The current study was designed to understand the situation of asymptomatic malaria and low-density *Plasmodium* infection for action by health agencies. The present study highlights asymptomatic and low-density *Plasmodium* infection prevalent in the community that remains undetected by the diagnostic tools and technologies currently being used by health agencies.

Methods

A community-based cross-sectional study was carried out from February to April 2017 during non-transmission season in two North Eastern Region districts of India. The study villages stratified as low and high malaria-endemic primary health centres (PHCs) in each district are shown in Figure 1.

Sample size

The prevalence of asymptomatic malaria was not available for either of the study districts, hence estimates from the nearest areas were used for the sample size calculation. The prevalence of asymptomatic malaria for East Garo Hills was estimated as 6.8% from a study conducted in Purulia district of West Bengal,[9] while for Udalguri district, asymptomatic malaria prevalence was estimated as 3.5% from the Assam-Arunachal border area.[20] By assuming relative precision as 15% at a 95% confidence level to estimate the prevalence of asymptomatic malaria, a total of 2340 and 4707 individuals from the districts East Garo Hills and Udalguri, respectively, were required to be screened for malaria. Further, assuming a design effect of 1.2, the desired number of samples to be collected became 2808 (rounded to 3000) and 5648 (rounded to 6000) for the districts East Garo Hills and Udalguri, respectively (total sample size was 3000+6000=9000).

Sampling methods

Two districts from two highly malaria-endemic states out of seven states in the North Eastern Region of India, namely Udalguri district from Assam and East Garo Hills district from Meghalaya, were selected based on the previous field experience of our research group working in these areas. Two PHCs from each district were selected at random (one from a high malaria-endemic PHC and one from a low-endemic PHC). Further, 10 villages from each PHC were selected using a probability proportion to size (PPS) sampling technique. In a situation where the required sample from the selected village was not available, the shortfall was covered from adjacent villages. The details of sampling from each PHC to estimate the prevalence of malaria using three diagnostic techniques are shown in Figure 2.

Field and laboratory methods

For better participation of the community, the objectives of the survey and the program schedule were communicated in the selected villages by accredited social and health activists (ASHAs) 1 d before the visit. The individuals who were willing to participate were screened for malaria, irrespective of clinical signs and symptoms related to malaria. A potential source of bias was observed to be 'volunteer bias'. Finger-prick blood was collected for on-spot diagnosis using a rapid diagnostic test (RDT) kit (SD Bioline Malaria Ag Pf/Pv, Alere Medical, Haryana, India), light microscopy using Giemsa stain and a dried blood spot on Whatman filter paper, grade 1 (Merck, Darmstadt, Germany) for molecular diagnosis and *Plasmodium* parasite species identification.

Children 2–9 y of age, irrespective of symptoms related to malaria, were examined for an enlarged spleen following the Hackett method. The spleen rate was computed by dividing the number of children with an enlarged spleen by the total number of children examined. The average enlarged spleen index was obtained as the sum of the number of children in each spleen size class multiplied by the class number (0–5) and divided by the total number of children with a palpable spleen.[21] Axillary

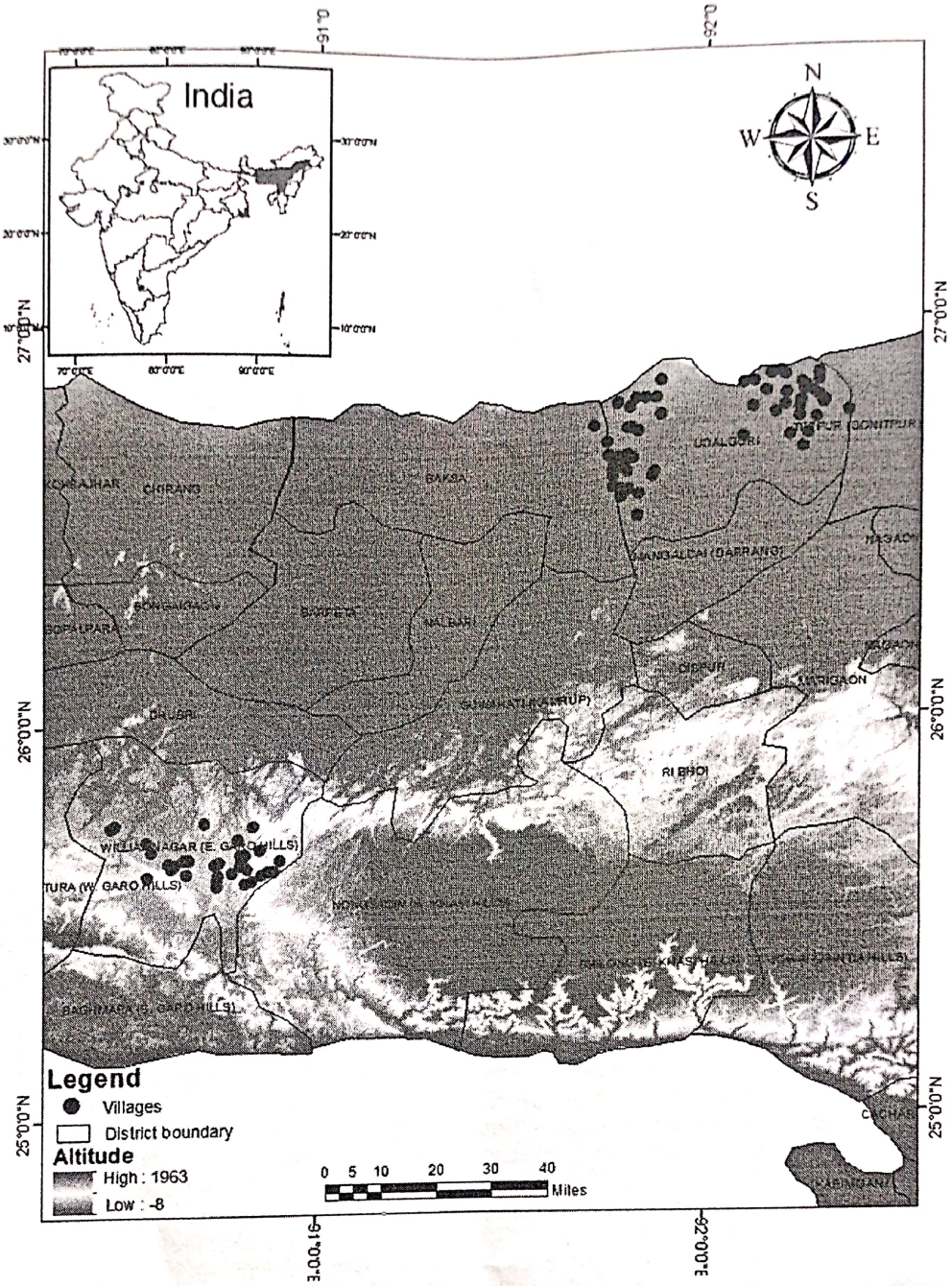

Figure 1. Study districts showing villages under investigation. The map was created with ArcGIS version 9.3 (Esri, Redlands, CA, USA).

body temperature was recorded using a digital thermometer and a history of fever and other clinical symptoms related to malaria were recorded. Fever was defined as a body temperature ≥37.5°C at the time of examination. Asymptomatic malaria was defined as participants who had neither a reported history of fever during the 2 weeks preceding the survey nor a body temperature ≥37.5°C at the time of examination but were *Plasmodium* positive by RDT, microscopy or PCR. According to a report from the World Health Organization (WHO) on low-density *Plasmodium* infections, the term 'low-density' *Plasmodium* infection may be used instead of 'submicroscopic' where the quantification of *Plasmodium* parasites is not performed. Thus low-density *Plasmodium* infections were defined as those infections detected by high-sensitivity methods, such as nucleic acid amplification–based assays (PCR in this study) but undetected using conventional diagnostics like microscopy or RDTs.[22] Since the present

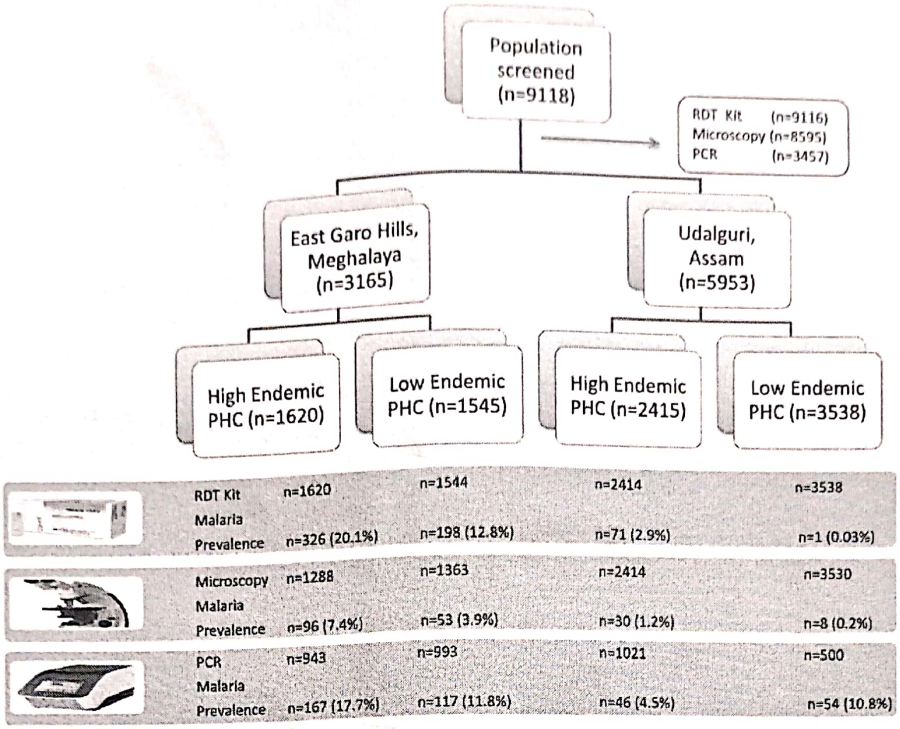

Figure 2. Flow chart showing sampling details and malaria prevalence using different diagnostic techniques. RDT results were available for 9116 individuals and 2 RDTs were invalid. Microscopy examination could be performed in 8595 slides and PCR was performed in 3457 samples due to logistics reasons explained in the Method section.

study did not perform a parasite count, the term 'low-density' *Plasmodium* infections has been used instead of submicroscopic malaria.

The outcome variable in this study was asymptomatic malaria. The exposure variables were the presence of the *Plasmodium* parasite detected by RDT, microscopy or PCR. Axillary body temperature and fever history were used as the predictors to define asymptomatic malaria.

Thick and thin blood smears were prepared at the study site and transported daily to the local laboratory, where fixation of thin blood smears was performed with methanol followed by staining of both thick and thin smears with 3% Giemsa stain solution for 30 min. All the slides were examined under oil emersion (10×100 magnification) by experienced microscopists. A blood slide was considered negative if no *Plasmodium* parasites were found after examining 100 microscopy fields. For all positive slides, malaria species identification was done. All the microscopists were kept blinded to RDT results and later the discordant results (RDT positive, microscopy negative and RDT negative, microscopy positive) were re-examined by another experienced microscopist and/or a WHO-certified microscopist who was not aware of previous microscopy/RDT results. The final microscopy results were considered as positive or negative depending upon the quality check results of the microscopy.

The *Plasmodium* species identification using PCR was done on dried blood spots prepared at ambient temperature and sealed in a zipped plastic bag containing desiccant and stored at −20°C until analysis. Due to the large sample size and logistic reasons, molecular diagnosis of malaria using PCR was performed in a subset of samples that was drawn randomly from the total samples. To perform PCR in 9000 samples is expensive and resource intensive, therefore 3500 samples were drawn randomly from the total samples and PCR diagnosis was performed on 3457 samples; 43 samples could not be amplified for PCR analysis. By doing this random selection, each sample had an equal chance of being selected for PCR diagnosis. Further, we performed power analysis by considering 90% power and an α of 5% and found that the sample size of the subset was large enough to generalize the findings.

To perform the diagnostic PCR, DNA was isolated from dried blood spots using the QIAamp Blood Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. The protocol followed in this study to identify *Plasmodium* species using PCR is described elsewhere, which was based on targeting coding sequences of the small subunit of ribosomal RNA specific to the parasite species. The limit of detection ranged from 1 to 10 parasites/μL of blood.[23] Briefly, 25-μL reaction mixtures consisted of 12.5 μL DreamTaq Green PCR mix, 0.4 μM of each primer (10 μM stock), 2 μL of DNA and sterile water up to 25 μL. All PCR amplifications were carried out in an Applied Biosystems (Waltham, MA, USA) thermocycler as follows: 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 56°C and 1 min at 72°C, with a final extension of 7 min at 72°C. PCR amplifications were analysed on 2% agarose gels prepared with 0.5× Tris-borate-ethylenediaminetetraacetic acid buffer in the presence of ethidium bromide. After obtaining the PCR results, the discordant

Table 1. Prevalence of malaria using RDT, microscopy and PCR

Variables		Malaria prevalence, n (%)	95% CI
Overall	RDT (n=9118)	596 (6.5)	6.0 to 7.1
	Microscopy (n=8595)	187 (2.2)	1.9 to 2.5
	PCR (n=3457)	384 (11.1)	10.1 to 12.2
	Irrespective of method[a] (n=9118)	791 (8.7)	8.1 to 9.2
Presented with symptoms	RDT (n=707)	93 (13.1)	10.8 to 15.9
	Microscopy (n=625)	33 (5.3)	3.8 to 7.3
	PCR (n=468)	56 (12.0)	9.3 to 15.2
	Irrespective of method[a] (n=707)	110 (15.6)	12.9 to 18.2
Presented without symptoms	RDT (n=8411)	503 (6.0)	5.5 to 6.5
	Microscopy (n=7970)	154 (1.9)	1.6 to 2.2
	PCR (n=2989)	328 (11.0)	9.9 to 12.1
	Irrespective of method[a] (n=8411)	681 (8.1)	7.5 to 8.7
Low endemicity	RDT (n=5083)	199 (3.9)	3.4 to 4.5
	Microscopy (n=4893)	61 (1.2)	1.0 to 1.6
	PCR (n=1493)	171 (11.4)	9.9 to 13.2
	Irrespective of method[a] (n=5083)	302 (5.9)	5.3 to 6.6
High endemicity	RDT (n=4035)	397 (9.8)	9.0 to 10.8
	Microscopy (n=3702)	126 (3.4)	2.9 to 4.0
	PCR (n=1964)	213 (10.8)	9.5 to 12.3
	Irrespective of method[a] (n=4035)	489 (12.1)	11.1 to 13.1

[a]Frequency used is irrespective of the diagnostic method (RDT, microscopy or PCR).

samples (RDT positive, microscopy positive, PCR negative and RDT negative, microscopy negative, PCR positive) were reconfirmed using light microscopy and PCR.

Statistical analysis and data management

The data were entered into an Excel 2007 (Microsoft, Redmond, WA, USA) worksheet and cross-checked for typographical errors. Further, quality checks were applied in order to ensure the quality of the data. Non-numerical categorical variables were coded numerically and frequency with percentage distribution was tabulated and Pearson's χ^2 or Fisher's exact test was applied for statistical comparison of 2×2 contingency tables as appropriate. Logistic regression analysis was used to calculate the odds ratio (OR) with 95% confidence interval (CI) to understand the effects of predictor variables with asymptomatic and low-density *Plasmodium* infections. Multilevel mixed effects logistic regression model analysis was performed to estimate the odds of malaria, asymptomatic infection and low-density infection among different age groups adjusted by study blocks. The critical value for statistical significance was considered at an α of 0.05. Data were analysed using R 3.4.3 for Windows (R Foundation for Statistical Computing, Vienna, Austria).

Results

A total of 9118 participants were screened by conducting mass blood surveys, of which 3165 (34.7%) participants were from East Garo Hills district and 5953 (65.3%) were from Udalguri district. Irrespective of the study districts, 5083/9118 (55.7%) individuals were from low malaria-endemic areas and 4035/9118 (44.2%) were from high malaria-endemic areas. In this survey, 57% (5197/9118) of the participants were female. The median age of the participants was 12 y (interquartile range 7–28). Spleen examination was performed in children 2–9 y of age, 1.6 as an average enlarged spleen size. The spleen rate was found to be 17.9% (602/3368).

Malaria prevalence

Individuals (n=9118), irrespective of symptoms of malaria, were screened for malaria using RDTs, of which 596 (6.5%) were found to be positive and two samples were invalid. Of the 9118 samples, 8595 were screened by traditional light microscopy, of which 187 (2.2%) were parasitaemic. A subset of samples (n=3457) from the total screened samples was randomly drawn for molecular diagnosis of malaria using PCR, which showed 384 (11.1%) positive cases. The number of *Plasmodium*-positive cases detected by RDT, microscopy and PCR was 264, 2 and 145, respectively. We found 37 that were negative by RDT but were positive for *Plasmodium* using microscopy or PCR. Such samples might have deletions of *P. falciparum* histidine rich protein-2 (HRP-2), a protein on which the RDT is based (Table 1 and Figure 3). Overall malaria prevalence estimated by RDT, microscopy or PCR was 8.7% (791/9118) and *P. falciparum* was the predominant species (95.1% [752/791]). Further, endemicity-wise malaria prevalence was found to be 5.9% (302/5083) and 12.1% (489/4035) in low- and high-endemic areas, respectively (p<0.0001). A similar trend of malaria prevalence was observed in all age groups

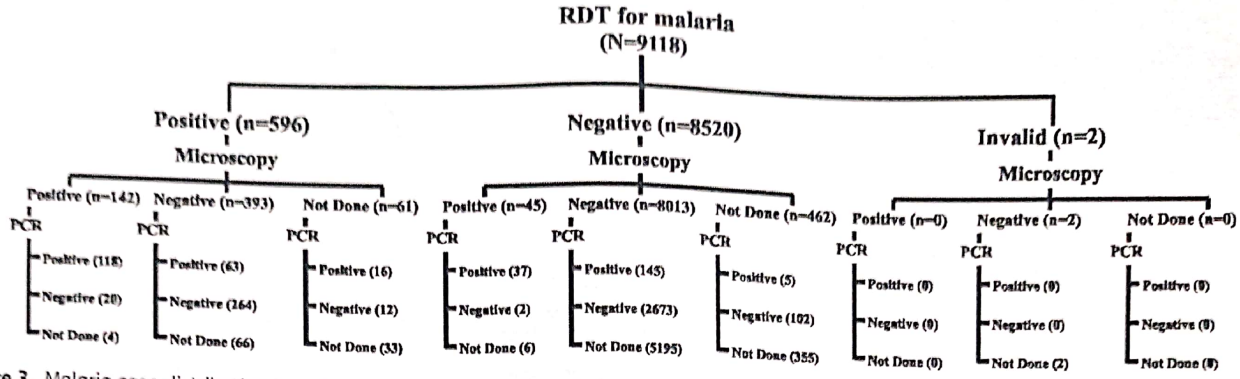

Figure 3. Malaria case distribution based on the three diagnostic techniques.

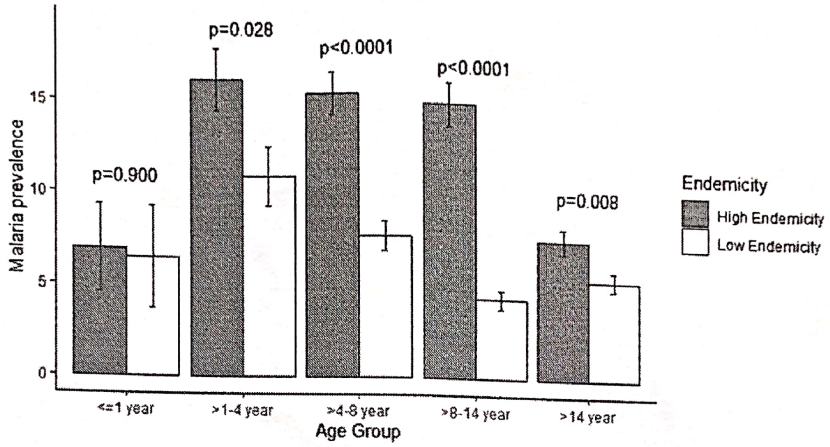

Figure 4. Malaria prevalence across the age groups in high- and low-endemic areas. Bars represent malaria prevalence (%) with standard error bars. The prevalence of malaria was calculated based on the diagnosis performed either by RDT, microscopy or PCR.

except infants, which showed a similar prevalence of malaria in low- and high-endemic areas (Figure 4). Microscopy and PCR could detect *Plasmodium* infection with nearly similar efficiency in low- and high-endemic areas, however, the prevalence calculated using the RDT varied significantly between these areas: 3.9% (199/5083) vs 9.8% (397/4035), respectively ($p<0.0001$) (Table 1). Age group analysis of malaria prevalence revealed that young children were more susceptible to infection compared with the older age group ($p<0.05$). However, the predicted difference in the odds of malaria prevalence was higher in the older age group after adjusting for study blocks, suggesting an age-dependant increase in malaria prevalence among study participants ($p<0.001$) (Table 2).

Asymptomatic malaria

Of 9118 samples, 8411 (92.2%) participants presented with no symptoms. Irrespective of diagnostic methods, 8.1% (681/8411) of participants had asymptomatic *Plasmodium* infection, where RDT could detect only 6% (503/8411) of *Plasmodium*-infected cases compared with 13.1% (93/707) of symptomatic cases (Table 1). The prevalence of asymptomatic malaria was significantly higher in high malaria-endemic areas (11.1% [404/3633]) compared with low-endemic areas (5.8% [277/4778]; OR 2.0 [95% CI 1.7 to 2.4]). Asymptomatic *Plasmodium* infection was also higher among children compared with adults ($p<0.05$) and children 1–4 y of age had 2.3 times higher odds of asymptomatic *Plasmodium* infection (OR 2.3 [95% CI 1.8 to 3.0]) compared with the >14 y age group. A mixed regression model adjusted by the study blocks revealed lower odds for asymptomatic infection in younger children compared with adults ($p=0.000$) (Table 2).

Low-density *Plasmodium* infection

Overall, 5.1% (145/2818) of cases of low-density *Plasmodium* infection were recorded from the samples that were tested by all three methods (RDT, microscopy and PCR). Low-density *Plasmodium* infection cases varied statistically between low- and high-endemic areas (6.9% [88/1273] vs 3.7% [57/1545], respectively; OR 1.9 [95% CI 1.4 to 2.7]). Also, it was observed that the prevalence of low-density infection was lowest in young children and increased proportionately with age in a non-significant manner; however, the adjusted OR was significantly higher among children 4–14 y of age compared with adults ($p<0.05$) (Table 2).

Table 2. Multilevel mixed effects logistic regression model to estimate the prevalence of malaria and asymptomatic and low-density *Plasmodium* infection among different age groups

Age groups (years)	Malaria prevalence			Asymptomatic *Plasmodium* infection			Low-density *Plasmodium* infection		
	n/N (%)	OR (95% CI)	aOR (95% CI)	n/N (%)	OR (95% CI)	aOR (95% CI)	n/N (%)	OR (95% CI)	aOR (95% CI)
0–1	13/192 (6.77)	1.33 (1.10 to 1.62)	0.50 (0.28 to 0.90)	11/152 (7.24)	1.23 (0.66 to 2.32)	0.60 (0.32 to 1.14)	2/74 (2.70)	0.41 (0.10 to 1.73)	0.37 (0.09 to 1.54)
1–4	118/853 (13.83)	1.91 (1.57 to 2.31)	1.65 (1.29 to 2.11)	96/742 (12.94)	2.35 (1.82 to 3.04)	1.66 (1.27 to 2.17)	11/310 (3.55)	0.55 (0.29 to 1.05)	0.53 (0.28 to 1.03)
4–8	227/1988 (11.42)	2.38 (1.88 to 3.01)	1.75 (1.43 to 2.14)	195/1827 (10.67)	1.89 (1.54 to 2.32)	1.72 (1.39 to 2.13)	28/640 (4.38)	0.68 (0.43 to 1.07)	0.57 (0.36 to 0.91)
8–14	205/2481 (8.26)	1.07 (0.60 to 1.92)	1.74 (1.41 to 2.14)	176/2273 (7.74)	1.33 (1.08 to 1.63)	1.75 (1.40 to 2.18)	35/695 (5.04)	0.79 (0.52 to 1.20)	0.63 (0.40 to 0.97)
>14	228/3604 (6.33)	Reference	Reference	203/3417 (5.94)	Reference	Reference	69/1099 (6.28)	Reference	Reference

aOR: adjusted OR (predicted difference in odds of infection among age groups adjusted by study blocks).

Discussion

A systematic large-scale sampling was carried out in this study to provide a representative profile of malaria in the study districts. The molecular technique used in this study detected a 2–5-fold greater malaria infection rate compared with the routinely used diagnostic tools (RDK and/or microscopy). A recent systematic review of malaria epidemiology in Meghalaya addressed the malaria situation over the past decade and mentioned that there was a significant increase ($p<0.001$) in the percentage of malaria cases among individuals ≥15 y of age (42.1% in 2015 vs 47.2% in 2017).[4] The reported malaria trend was based on the analysis of data obtained from the Meghalaya State Malaria Control Programme for 2006–2017 that involved malaria identification and confirmation using either microscopy or RDT; it did not utilize molecular tools. Our results are in agreement with a previous study[15] that also reported the inadequacy of available diagnostic tools to detect malaria in low-endemic areas during the non-transmission season. We observed that children 1–4 y of age had the highest malaria prevalence and children 4–8 y of age were highly vulnerable to malaria compared with children >14 y of age, but the mixed model regression analysis adjusted for study blocks revealed that children 4–8 and 8–14 y of age had equal odds for malaria. The spleen rate (18%) and an enlarged spleen index among children 2–9 y of age in our study population show repeated past exposure of these children to *Plasmodium* infection, which might have resulted in improved immunity in these children, thereby making them an asymptomatic reservoir of infection. Further, the spleen rate and the average enlarged spleen index in these children agree with the findings of other groups that a show positive association between the spleen rate and slide positivity rate.[21] Similar observations were reported from a yearlong study conducted in Sonitpur district of Assam that showed higher malaria prevalence in children 5–14 y of age.[20]

Higher malaria prevalence was seen in high-endemic areas due to the presence of highly forested, tribal and remote areas that are inaccessible for early malaria diagnosis and prompt treatment and due to the delay in malaria treatment-seeking behaviour of tribal populations.[24] Apart from this, socio-economic factors such as family size, type of house, use of bed nets, monthly income, distance between residence and the nearest health centre and standard of living were found to have a significant impact on malaria case management.[25,26] Previous literature suggests that the tribal population is prone to malaria due to their low socio-economic status, scattered living behaviour and cultural and regional beliefs.[4] The data presented in this study included tribal-dominant areas of the East Garo Hills district.

The perennial and persistent malaria transmission in Meghalaya[27] and Assam[20] has resulted in the development of adaptive immunity (with the progression of age) against malaria in the population residing in these areas. Because of this, adults have a much lower propensity for getting malaria[18] and more asymptomatic *Plasmodium* infections than children, similar to the findings reported in a previously published study that found a higher prevalence of asymptomatic malaria in children <15 y of age than adults.[12] There is some evidence that supports our findings on higher adjusted odds of asymptomatic infections among children 4–14 y of age than adults, as Malawi school-age children 6–15 y of age were found to be at higher risk of asymptomatic infection.[11] To support our sampling methodology, the proportion analysis on our data when compared with the census 2011 data for age and gender-wise population distribution revealed a significant difference. Although the census data are updated every 5 y, the latest available census data are from 2011. The observed significance might be because the reference data are too old and the sample size is large, thus even a slight variation can result in significant differences. In addition, it is also plausible that the population proportion might have changed during the time between the study start date and the census update year (i.e. 2011). Nevertheless, the population coverage in this study

provided representative data of the entire population according to the PPS method. Recruitment of volunteers in surveys is associated with selection bias and may result in underrepresentation of the target population. Further, the potential source of volunteer bias that may arise in any survey or clinical trial is due to the differences between recruited samples and the target population, which was nominal in our study. In addition, efforts were made while conducting this study to sample 'hard-to-reach' sections of the population so that findings could be generalized to the population residing in these regions.[28]

It is believed that both adaptive and innate immunity are the key players in asymptomatic *Plasmodium* infection,[29] however, the exact immune mechanism involved in such infection is not clearly understood. Nevertheless, a new hypothesis suggests that less virulent *Plasmodium* strains in low-endemic areas remain at a low density due to less competition with more virulent strains, thus they persist over a long period and remain asymptomatic, undetected and untreated, thereby allowing further transmission during favourable conditions.[30] Low malaria-endemic areas harbour most of the asymptomatic submicroscopic infection,[16] which was also observed in the present study, where we found a high prevalence of low-density *Plasmodium* infection in such areas, highlighting the need to tackle these silent carriers for successful implementation of malaria elimination programmes.

Further, it is evident from the data that RDT and microscopy are less sensitive than PCR for detecting malaria in low-endemic areas,[31] advocating the use of advanced molecular techniques in low-endemic settings. This study uncovered the extent of low-density *Plasmodium* infection in low- and high-endemic areas and the proportionate age-wise increase in low-density infection was contrasted by malaria prevalence observed in the respective age groups in the study population.[12] Age group distribution of malaria positivity and submicroscopic or low-density *Plasmodium* infection showed an inverse trend, which is in agreement with previous observations.[13] The dynamics of *Plasmodium* infection in different age groups have shown that despite the similar rate of new infection in school-aged children and children <5 y of age, the infection lasts for a longer period in school-aged children, as in adults.[32] This highlights the role of acquired immunity to malaria in populations residing in hyper- or holoendemic areas.[33] Importantly, low-endemic areas of our study districts contributed a greater number of cases of low-density *Plasmodium* infection that comprised the majority of asymptomatic carriers. It is assumed that submicroscopic or low-density infection not only reflects the dynamics of transmission, but also reveals the state of interventions performed by the controlling agencies. In addition, such infections have the potential to start malaria transmission under favourable conditions, as observed in Sudan, where submicroscopic infection was suggested to be the cause of a resurgence of malaria transmission in the next rainy season.[13] This is a challenging situation and it is important to understand that such carriers can act as a source of approximately 20–50% of malaria transmission even during low or non-transmission season.[13]

To conclude, our study provides exhaustive information about the prevalence and mapping of asymptomatic and low-density *Plasmodium* infection cases in the study areas. The results advocate for the use of advanced diagnostic techniques in low-endemic areas even if RDT/microscopy indicate a dearth of infection.

Authors' contributions SP was the principal investigator of the study. SP and NM conceived and designed the study. HS contributed substantially to data collection, analysis and interpretation. HS, SP, MPS and NA executed the field operations. HS, MPS and CPY performed management and analysis of data. RSB, NA and GPS performed and supervised laboratory experiments with the support of HS, MPS and NM. KS, HK and NM coordinated the field operations and logistics arrangements. HK and NM critically reviewed the manuscript for important intellectual content. HS drafted the first version of the manuscript. All authors provided intellectual input and reviewed and approved the final version.

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Competing interests None declared.

Ethics approval The study was approved by the Institutional Ethics Committee of the National Institute of Malaria Research (ECR/NIMR/EC/2015/490). All subjects or their representatives gave written informed consent prior to enrolment.

Data availability: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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