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The burden and diagnostic challenges of subclinical *Plasmodium falciparum* infections in Southern Ghana

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Abstract

Background Many national malaria elimination programmes (NMEP) are intensifying campaigns for malaria control and elimination. However, these efforts are constrained by the high prevalence of subclinical infections which may sustain local disease transmission. Detection and treatment of these subclinical and low-density infection is therefore crucial in monitoring progress towards malaria control and elimination. This study sought to determine the burden of subclinical infections in three districts in Ghana, the proportion that could be detected by rapid diagnostic test (RDT), and the occurrence of *hrp2/hrp3* deletions which may impede diagnosis by HRP2-based RDTs.

Methods A community-based, cross-sectional study was conducted in the Nkwanta South, Sekyere South, and Ga South districts in Ghana. A total of 1134 whole blood samples were screened for malaria using HRP2-based rapid diagnostic test (RDT), expert microscopy, and *var*ATS qPCR. Three hundred and four (304) *P. falciparum* positive samples were typed for *hrp2/hrp3* deletions by digital PCR (dPCR).

Results Parasite prevalence was 57.1% by qPCR, 40.9% by RDT, and 8.4% by microscopy. Approximately, 33.8% (219/647) of infections were sub-patent. Compared to qPCR, the sensitivity of RDT was 65.7%, and specificity 91.9%, making it significantly more sensitive than microscopy (sensitivity 14.4%, specificity 99.4%). Parasite prevalence was highest in children aged 5–15 years (68.2%), followed by adults > 15 years (51.2%) and children < 5 years (45.3%). Prevalence also differed across the three districts, ranging from 44.0% (183/416) in Sekyere South, 55.8% (143/253) in Ga South, to 68.8% (321/466) in Nkwanta South. No *hrp2* deletions were observed, and one sample (1/304) from Nkwanta South district carried *hrp3* deletion.

Conclusion The high prevalence of subclinical malaria infections is likely to be a potential reservoir in sustaining malaria transmission. HRP2-based RDTs detected two-thirds of the subclinical infections. Given the absence of *hrp2* deletions, community testing and treatment programs using highly sensitive HRP2-based RDTs could be a valuable strategy in detecting the parasite reservoir and potentially help in ensuring a sustainable decline in disease transmission.

Keywords Subclinical, Low-density, *Plasmodium falciparum*, Malaria

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Background

Malaria is one of the most devastating parasitic diseases globally. Despite the great strides made towards controlling malaria, the disease was responsible for over 263 million clinical cases and 597,000 associated deaths in 2023, with 94% of the cases reported in WHO African Regions [1]. In Ghana, the National Malaria Elimination Programme (NMEP) has increased efforts to disrupt malaria transmission and reduce morbidity. The distribution of insecticide-treated bed nets (ITN), indoor residual spraying (IRS), larviciding, intermittent preventive treatment with sulfadoxine pyrimethamine (IPTp-SP) in pregnancy, and the use of artemisinin-based combination therapies have been effective in reducing the malaria morbidity and mortality rate by 48.7% from 2011 to 2019 [2]. This notwithstanding, malaria remains the leading cause of hospitalization and is responsible for over 30% of out-patient attendances [2]. *Plasmodium falciparum* mono-infection is the predominant malaria infection type accounting for a national average of 98% with *P. ovale* and *P. malariae* mono and mixed infections with *Plasmodium falciparum* sharing the remaining 2% [3–6].

Subclinical and low-density infections are common across all age groups, and play a critical role in sustaining a large proportion of malaria transmission [7–9]. Sustained malaria transmission in endemic areas could be attributed to a significant proportion of subclinical infections. Several studies found that 95% of transmission originates from reservoirs that do not exhibit fever or any acute clinical symptoms [10–13]. The detection and treatment of these subclinical infections is therefore crucial for malaria control [11, 14].

A large number of subclinical infections are characterized by low parasite density. Community and household-based surveillance of subclinical infections as well as point-of-care (POC) detection of malaria, to a great extent, rely on rapid diagnostic tests (RDT) and light microscopy. Due to their limited sensitivity and scarcity of trained microscopist, microscopy and RDTs underestimate the true burden of subclinical infections [15, 16]. Unlike RDT, the accuracy of light microscopy is dependent on the expertise of the microscopist examining the slides [17]. As a result, light microscopy is reported to have a varying limit of detection of about 50 to 500 parasites/μL [18–20]. The latest generation of RDTs achieves a limit of detection of < 50 parasites/μL [21].

The most sensitive RDTs for *P. falciparum* detection rely on the detection of the HRP2 and HRP3 proteins. Deletions of the *hrp2* and/or *hrp3* genes have been reported in several countries, particularly in the Horn of Africa [22, 23] and South America [24, 25], where they pose a significant obstacle to malaria control. In Ghana, deletion frequency appears to be low [4, 26].

To date, studies about the burden and prevalence of subclinical infections and the challenge associated with their detection in Ghana are sparse. As Ghana is transitioning into malaria elimination phase, a comprehensive understanding of the prevalence of subclinical malaria infections is necessary to track progress and guide national policy. In this study, we assessed the prevalence of subclinical malaria infections by qPCR and HRP- 2 based RDTs, and the occurrence of *hrp2/hrp3* gene deletions in three regions of Ghana.

Methods

Definition of terms

Subclinical malaria infection, herein, was defined as *Plasmodium falciparum* positive individual or case without fever (i.e. temperature ≤ 37.5 °C) at the time of sampling as well as the absence of any malaria related symptoms [27]. Subpatent malaria infections were defined as infections exclusively detected (positive) by *var*ATS qPCR [28].

Study areas and population

The study was conducted at three districts/regions in Ghana; Afamananso in Sekyere South District (Ashanti Region), Obom in Ga South (Greater Accra Region) and Gekrong, Pawa, Nsuogya, and Keri (Nkwanta South Municipal) in the Oti Region of Ghana (Fig. 1). Sekyere South District (latitude 6° 50'N and, longitude 1° 40'W) is one of the 43 districts in the Ashanti region of Ghana and located about 40 km away from Kumasi on the Kumasi-Mampong road. Sekyere South has a population of 120,076 distributed into 29,892 households [29] . The Ga South district lies within latitude 5°35'N and longitude 0°10' W and occupies a land area of 284.08 square kilometers with about 412 communities. Ga South has a population of 350,121 while Nkwanta South Municipal in Oti region occupies a land area of 2,473 square kilometers with a population of 135,936 [29]. The rural population together with the urban dwellers in the Nkwanta district are distributed in about 22,429 households with an average household size of 4.2 persons [29].

Study design and sample collection

Cross-sectional, community-based surveys were conducted at different time points in the aforementioned areas. Samples were obtained in Sekyere South from December 2020 to January 2021 while data were collected from Nkwanta South and Ga South in October and July, 2021 respectively. Prior to the commencement of the study, opinion leaders in each community were engaged where aims and procedures of the study were explained clearly in layman's term. Upon visiting the communities, persons of all age groups, both male and females, were

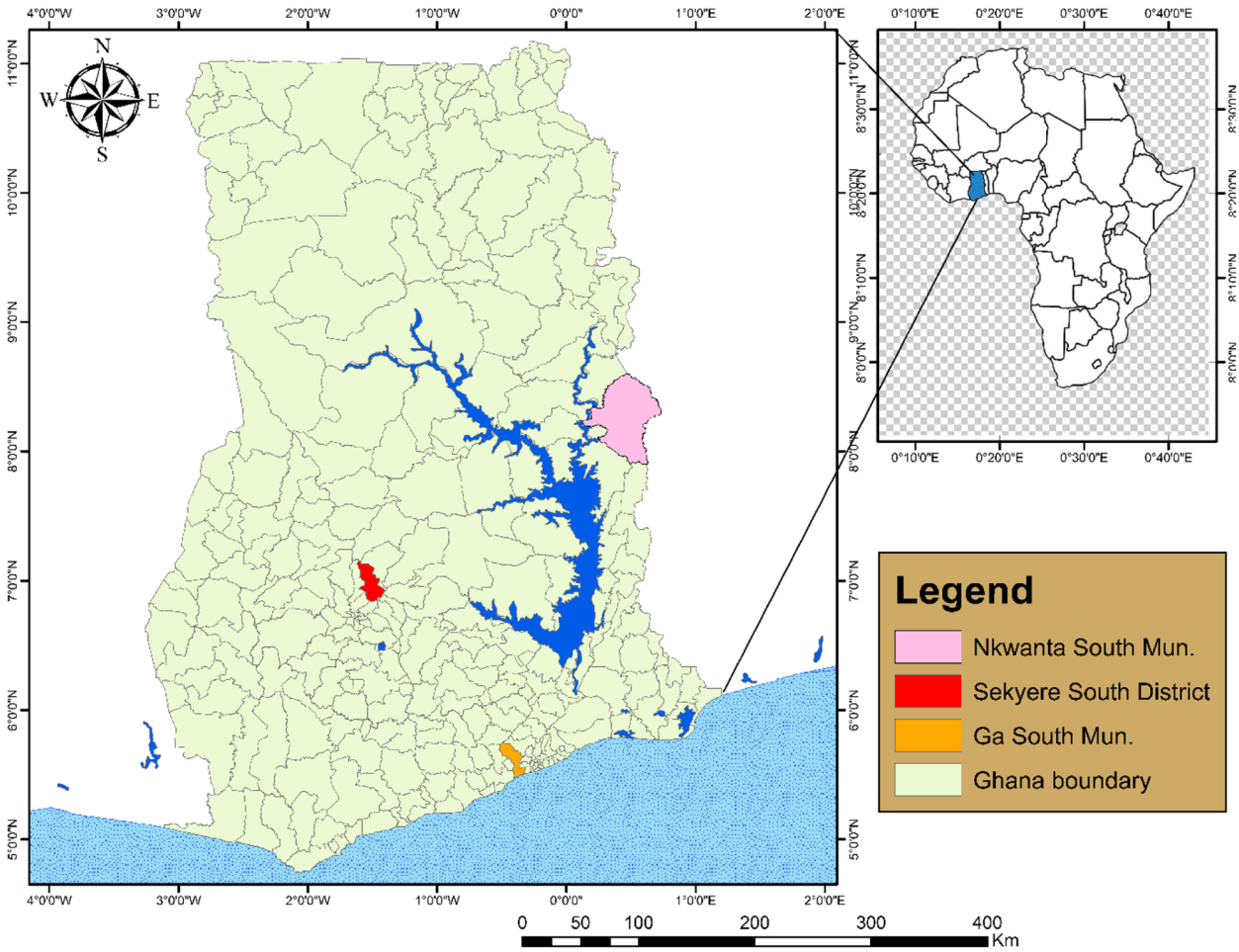

Fig. 1 Map showing the different study sites

invited to take part in the study. Written informed consent was obtained from all participants aged 18 and older. For participants younger than 18, consent was obtained from a parent or guardian. The temperature of each participant was measured using infrared thermometers. Two milliliter of venous blood was obtained from consenting participants into EDTA tubes by trained phlebotomists. Thick and thin blood smears were prepared for microscopy and RDT diagnosis performed. An aliquot of 200 µL of whole blood was pipetted into 1.5 ml Eppendorf tubes and transported on ice to the Vector-Borne Infectious Disease laboratory at Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana for storage in − 80° C freezer until they were needed for further laboratory analysis.

Sample processing and laboratory investigations

Rapid diagnostic test

The Biocredit Malaria Ag Pf (pLDH/HRPII) RDT (lot no: HOO6 C007D) manufactured by Rapigen, Inc. was used to diagnose *Plasmodium falciparum* in the study. In addition to the control band, the test kit has two bands (HRP2 and pLDH), allowing for the phenotypic detection of potential *hrp2*/*hrp3* deletions. The performance of this RDT has been evaluated before, and it was found to be more sensitive than AccessBio Carestart Malaria Pf RDT, the test routinely used by the Ghana NMCP [21]. The test kit was used according to the manufacturer's protocol and results recorded after 15–20 min. In case an RDT showed very faint bands, the test was repeated.

Microscopy

Thick and thin blood films were prepared. The thin blood film (2µL) was fixed with absolute methanol. Blood smears were stained with 10% Giemsa solution and examined under light microscope by two expert microscopists. Parasites were quantified after counting 200 or 500 White Blood Cells (WBC) [30, 31]. A slide was declared negative when no malaria parasite was seen after scanning 100 high power fields (HPFs) [32].

The parasites quantified were expressed as parasite per microliter of blood.

DNA extraction, *var*ATS qPCR, and *hrp2/3* deletion typing

DNA extraction, *var*ATS qPCR and *hrp2/3* deletion typing were performed at the University of Notre Dame, USA. Genomic DNA was extracted from 100µL of blood using the Macherey–Nagel Nucleomag extraction kits (Düren, Germany) and eluted in equal volume of elution buffer. qPCR was performed on ThermoFisher QuantStudio 3 instrument in a total reaction volume of 12 µL, including 4 µL of DNA. The multicopy *var*ATS gene was amplified. This gene is present in approximately 60 copies per parasite genome, of which approximately 20 copies are amplified [28]. To obtain absolute density estimates, dilution series of cultured NF54 *P.falciparum* parasites quantified by dPCR were run along field samples.

Samples positive for *P. falciparum* and with a Ct value of ≤28 were typed for deletion of the *hrp2* and *hrp3* genes by digital PCR [33]. In the absence of the *hrp2* gene, anti-HRP2 antibodies also recognize the HRP3 protein, a structural homolog that shares numerous epitopes with HRP2 [34]. In the dPCR assay, primers targeting *hrp2* or *hrp3* genes are multiplexed with an assay targeting serine-tRNA ligase (PF3D7_0717700) as control. The ratio of positive partitions of *hrp2*/*hrp3* to tRNA indicates the presence or absence of *hrp2* or *hrp3.* For the analysis of dPCR assay, a minimum of five droplets positive for the reference (tRNA) gene were considered. Samples were re-run if deletion were recorded but ≤5 positive droplets were observed for the reference gene.

Data analysis

Data was analyzed using GraphPad Prism 8.0 (San Diago, California), Stata 17 (Stata Corp. LLC, College Station, Texas, USA) and IBM SPSS Version 27 (Armonk, New York). Binary logistic regression was used to assess the association of potential risk factors (age, sex temperature and community) with qPCR results. Prevalence between age groups was compared using the chi-square (χ^2)-test. Five percent (5%) level of significance was used for all statistical tests.

Results

Sociodemographic characteristics of the study population

A total of 1,134 participants were recruited for the study. These included 466 (41.1%) from Nkwanta South, 416 (36.7%) from Sekyere South, and 252 (22.2%) from Ga South districts (Table 1). Among the participants, 63.6% (n= 720) were female, with a median age of 19 years (IQR= 10–43). Adults >15 years of age made up 56.8% (n= 644) of the participants, followed by 5–15 years (36.6%, n= 415). Approximately 68.7% (n= 781) of the participants reported to own an insecticide treated bed net (ITN), and among them, 40.1% (n= 313/781) reported usage of the ITN 24 h prior to the study (Table 1). The use of the ITN was significantly lower in school-aged children 5–15 years (23.8%; n= 99/415) compared to young children <5 years 30.9% (n =23/75) and adults 29.6% (n =191/644) (X^2= 6.2; P= 0.04).

Table 1 Baseline characteristics of study participants

Characteristics	N (%)	Parasite Prevalence by qPCR n(%)	*P* value
Sex			
Female	720 (63.6%)	392 (54.4%)	0.02
Male	414 (36.5%)	255 (61.6%)	
Age (years)			
< 5	75 (6.6%)	34 (45.3%)	
5–15	415 (36.6%)	283 (68.2%)	< 0.0001
> 15	644 (56.8%)	330 (51.2%)	
ITN Ownership			
Yes	781 (68.7%)	453 (58.0%)	0.22
No	353 (31.1%)	194 (54.9%)	
ITN usage			
Yes	313 (40.1%)	145 (46.3%)	< 0.0001
No	468 (59.9%)	278 (59.4%)	
Study site			
Nkwanta South	466 (41.1%)	321 (68.8%)	
Sekyere South	416 (36.7%)	183 (44.0%)	< 0.0001
Ga South	253 (22.2%)	143 (55.8%)	

P. falciparum prevalence by qPCR, microscopy, and RDT

The prevalence of *P. falciparum* in this study was 57.1% (n= 647/1134) by qPCR., 40.9% (n= 464/1134) by RDT, and 8.4% (n= 96/1134) by microscopy. Of the 464 RDT-positive cases, PfHRP2 was detected in all of them; in 48.5% (n= 225/464) of RDT-positive cases, both PfHRP2 and pLDH were detected. Prevalence by qPCR was highest in Nkwanta South at 68.8% (n= 321/466), followed by Ga South 55.8% (n= 143/252) and Sekyere South 44.0% (n= 183/416) (X^2= 55.6, P< 0.0001). The prevalence was highest in children aged 5–15 years (68.2%, n=283/415) compared to young children <5 years (45.3%, n= 34/75) and adults >15 years (51.2%, n= 330/644) (X^2= 34.1, P< 0.0001). 33.8% (n= 219/647) of infections were exclusively detected by qPCR. The proportion of sub-patent infections was highest in adults >15 years 46.7% (n= 154/330), followed by 23.5% (n= 8/34) in young children <5 years and 20.1% (n= 57/283) in children aged 5–15 years.

Compared to qPCR, the sensitivity of the HRP2-based RDT was 65.7% (95% CI 62.92%—68.45%), and sensitivity of microscopy was 14.37% (95% CI 12.33%–16.42%)

Table 2 Diagnosis accuracy of RDT and microscopy using qPCR as reference standard

	RDT	Microscopy
Sensitivity % (95% C.I)	65.7% (62.9—68.5)	14.4% (12.3–16.4)
Specificity% (95% C.I)	92.% (90.4–93,6)	99.4% (98.9–99.8)
Positive Predictive value% (95% C.I)	91.4% (90.0–93.2)	96.9% (95.8–97.9)
Negative Predictive value% (95% C.I)	66.9% (64.9–69.6)	46.6% (43.7–49.5)

RDT: rapid diagnostic test, *C.I:* confidence interval, *qPCR:* quantitative polymerase chain reaction

(Table 2). The sensitivity of RDT and microscopy also varied across the different study areas. RDT was highly sensitive in Nkwanta South (80.94%; 95%CI 70.26%—84.86%), followed by Sekyere South (57.38%; 95 CI 50.13%—64.32%) and Ga South (42.66%; 95 CI 37.06%—48.45%). In contrast, microscopy demonstrated poor sensitivity in Nkwanta south (18.07; 95 CI 14.25%—22.65%), Sekyere South (12.57%; 95 CI 8.52%—18.15%) and Ga South (15.48%; 95%CI 10.63%—22.01%). Figure 2 shows parasite densities of RDT and microscopy positive and negative samples. 91.6% ($n = 425/464$) of samples identified as positive by RDT and 96.8% ($n = 93/96$) by microscopy were also positive by qPCR.

Infections that were detected by all three tests had significantly higher geometric mean parasite density ($n = 90$, 1619 parasite/μL; 95% CI 1007—2603) as compared to samples that were detected by qPCR only (Geometric mean = 1.21 parasite/μL; 95%CI 0.78–1.87) (Fig. 2). The geometric mean parasite density of the RDT positive samples (1.31 parasites/μL; 95% CI 50.81–100.1) was almost 70-fold higher than RDT negative but qPCR positive samples (0.89 parasites/μL; 95% CI 0.61–1.30) (Fig. 2).

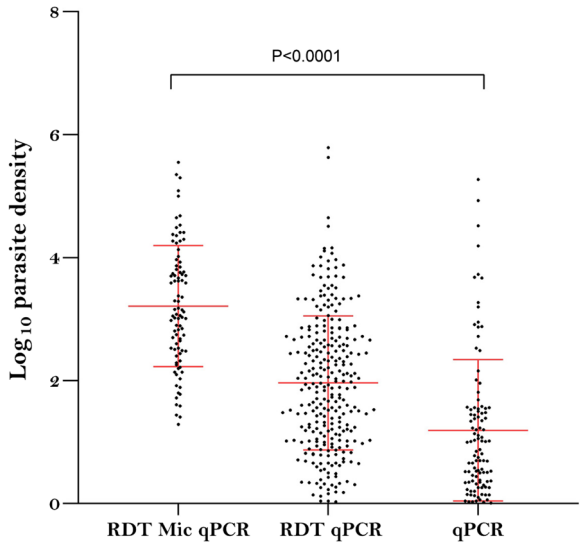

Fig. 2 Parasite density of samples determined by the combination of RDT, microscopy and *var*ATS qPCR. Y-axis shows the log10 parasite density of the samples, while the X-axis represents the different combinations of diagnostic techniques. Error bars (red lines) show the mean and standard deviation (SD)

Risk factors associated with subclinical infection

In logistic regression analysis, there was no significant association between gender and malaria infection. The temperature (*OR* 1.84 95%CI 1.07–3.19) and the district from which a participant was sampled were strong predictors of infection. The >15 years age group (*OR* 2.13 95%CI 1.60–2.83) was significantly associated with malaria infection when compared to children <5 years old (Table 3). Individuals who reported usage of insecticide treated nets 24 h prior to the study had lower odds of malaria infection (*OR* 0.54 95% CI 0.421–0.714) (Table 3).

Plasmodium falciparum hrp2/hrp3 deletion typing by dPCR

Deletion typing was conducted on 304 samples (166 from Nkwanta South, 86 from Ga South, and 52 from Sekyere South). For both *hrp2/hrp3* typing, 98.7% ($n = 300$) of the samples met the inclusion criteria of ≥5 partitions positive for tRNA. No *hrp2* deletion were observed. One of the isolates from Nkwanta South district in Oti region

Table 3 Logistic regression for potential risk factors associated with qPCR detectable malaria

	OR	P value	95% CI	
			Lower	Upper
Age (years)				
< 5	Ref			
5–15	0.763	0.232	0.490	1.189
> 15	2.127	< 0.0001	1.600	2.829
Gender				
Male	Ref			
Female	1.217	0.154	0.929	1.595
District				
Sekyere South	Ref			
Ga South	0.492	< 0.0001	0.350	0.692
Nkwanta South	0.272	< 0.0001	0.202	0.367
Temperature	1.846	0.028	1.069	3.188
ITN usage				
No	Ref			
Yes	0.548	< 0.0001	0.421	0.714

P value > 0.05 significant, *OR:* Odds ratio, *District:* various sampling areas

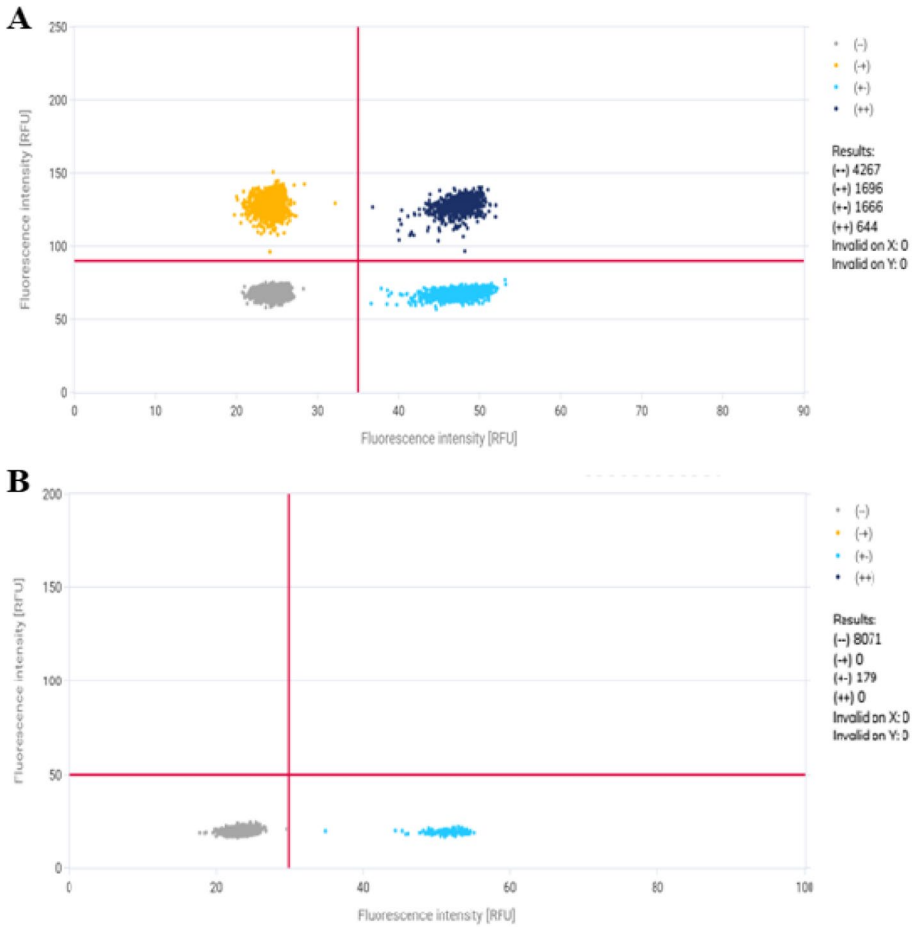

Fig. 3 Plot of *hrp3* deletion typing by dPCR**.** Bottom right: Partitions positive for tRNA (control gene) are shown in dark blue. Bottom left: Negative partitions for both target and tRNA are shown in gray. Top left: Partitions positive for the target gene are shown in yellow. Top right: Partitions positive for both target and tRNA are shown in light blue. **A** Wild type sample with no deletion. **B** *hrp3* deletion sample: Droplets positive for tRNA (control), but no droplets are positive for the target gene (*hrp3*)

examined carried a deletion of the *hrp3* gene. Figure 3 shows the plot of wild-type samples and the sample with deletion of *hrp3* gene.

Discussion

In this study, the prevalence of subclinical *P. falciparum* infection was determined using microscopy, RDTs of the latest generation, and qPCR. By qPCR, 57% of the population tested positive. Light microscopy showed very poor sensitivity (14%). This finding is consistent with earlier studies that reported considerable prevalence of the parasite reservoir in the form of subclinical infections across sub-Saharan Africa [3, 35–37]

The ultrasensitive RDT used in the present study detected about two-thirds of infections and may therefore offer possibilities for shrinking the reservoir of subclinical infections e.g. through mass testing and treatment (MTAT), reactive case detection (RCD), or focal test and treat (FTAT) programs in Ghana. Subclinical infections were shown to be the major source of transmission in several countries [10, 13]. Their ability to infect mosquitos greatly depends on their gametocyte density, which depends on parasite density [38]. In mosquito feeding experiments, very low parasite density samples did not infect mosquitos [39]. A study in Uganda found that subclinical infections were the source of >99% of infected mosquitos [10], yet, submicroscopic infections were the source of only approximately 15% of transmission. The sensitivity of the RDT (65.7%) in the current study exceeded the sensitivity of microscopy (14.37%). Thus, using this RDT in a well-designed program to screen for submicroscopic infections and administer treatment to those testing positive, presumably a large proportion of the subclinical infectious reservoir could be cleared.

The sensitivity of HRP2-based RDTs could be affected by deletions of *hrp2/3* genes. In this study, no *hrp2* deletions were observed, and one sample carried *hrp3* deletion, corroborating previous reports of low deletion

frequency in Ghana [26, 40]. Hence, HRP2-based RDTs remain an appropriate tool to detect *P. falciparum* infections in Ghana.

Consistent with previous studies, the prevalence of infection was higher in school age children (5–15 years) compared to young children <5 years and adults >15 years, and they may serve as an important reservoir for onward parasite transmission [30, 41]. These patterns can be explained by the frequent exposure of adolescents to the parasite and the gradual acquisition of immunity in older individuals [42, 43]. Beyond the partial immunity acquired, school-aged children are frequently exposed to outdoor biting vectors since they typically spend more time outdoors compared to young children.

The high prevalence of subclinical infections is likely a result of the limited utilization of preventive interventions such as insecticide treated nets (ITN) as observed in this study [44]. In Ghana, ITNs are distributed to all regions across the country especially in endemic communities as well as antenatal and Child welfare clinics [45]. This study showed that over two-thirds of the participants possessed an ITN but less than half of them reported usage prior to the study. Individuals who reported usage of ITN had reduced likelihood of being infected, corroborating the vital role it plays in preventing malaria infections. Previous studies indicated that the use of ITNs has been crucial in reducing malaria cases in Ghana by approximately 68% since the year 2000 [46, 47].

Conclusion

The current study found a high prevalence of subclinical infections by qPCR. Periodic reassessment of the burden and distribution of subclinical infections may be of crucial importance to strengthen malaria surveillance and monitor control/elimination progress. No *hrp2* deletions were detected, thus HRP2-based RDTs remain efficient for *P. falciparum* detection in Ghana. The highly sensitive RDT used in this present study detected two thirds of the infections. Screen-and-treat campaigns using these RDTs could be a vital tool in Ghana's malaria surveillance campaign and to accelerate progress towards malaria control and elimination.

Abbreviations

PCR	Polymerase chain reaction
dPCR	Digital polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
Pfhrp2/3	*Plasmodium falciparum* Histidine rich protein 2/3
ITN	Insecticide treated bed net
RDT	Rapid diagnostic test
*var*ATS	Var acidic terminal sequence
IQR	Interquartile range

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Authors' contributions

Study concept and design: KB, CK. Data generation: AHM, CK, KB, SOA, TKA, KBA, EVT and CAVA. Data analysis and statistical review: AHM, CK, KBA, CAVA and SOA. Drafting: AHM. Revision of manuscript: KB, CK, AHM, MGA, ABT. Critical appraisal and approval for submission: all authors. All authors read and approved the final manuscript.

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Data availability

All data generated or analyzed are included in this manuscript.

Declarations

Ethics approval and consent to participate

The study was approved by the Committee on Human Research, Publication and Ethics of the Kwame Nkrumah University of Science and Technology, School of Medical Sciences and Komfo Anokye Teaching Hospital (CHRPE/AP/030/20), and the ethics review board of University of Notre Dame, USA (approval no. 19–04 - 5321). This study adhered to the principles and guidelines outlined in the Declaration of Helsinki. Before sample collection, written informed consent was obtained from participants aged 18 years and above. For children between the ages of 8 to 17 years, consent of a parent or legal guardian was obtained, followed by written assent of the child.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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