Estimating the Prevalence of Asymptomatic Malaria Parasite Carriage in Southern Ghana: Utility of Molecular and Serological Diagnostic Tools

Hamza Bukari Abagna

University of Ghana Noguchi Memorial Institute for Medical Research

Eric Rodgier

CDC: Centers for Disease Control and Prevention

Aminata Colle Lo

University of Ghana Noguchi Memorial Institute for Medical Research

Zakaria Abukari

University of Ghana Noguchi Memorial Institute for Medical Research

Sophie Allen

CDC: Centers for Disease Control and Prevention

Ben Gyan

University of Ghana Noguchi Memorial Institute for Medical Research

Michael Aidoo

CDC: Centers for Disease Control and Prevention

Linda E Amoah (✉ lamoah@noguchi.ug.edu.gh)

University of Ghana <https://orcid.org/0000-0002-1652-8489>

Research

Keywords: Malaria, bead-based multiplex, HRP2, PET-PCR, asymptomatic, RDT, microscopy

Posted Date: August 18th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-805420/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background. Asymptomatic malaria infections can serve as potential reservoirs for malaria transmission. These infections range from microscopic to submicroscopic densities, making an accurate estimation of asymptomatic parasite carriage highly dependent on the sensitivity of the tool used for the diagnosis. This study sought to evaluate the sensitivities of a variety of molecular and serological diagnostic tool at determining the prevalence of asymptomatic *Plasmodium falciparum* parasite infections in two communities of varying malaria parasite prevalence.

Methods. Whole blood from 194 afebrile participants aged between 6- and 70-years old living in a high (Obom) and a low (Asutsuare) malaria transmission setting of Ghana was used in this study. Thick and thin blood smears, an HRP2-based malaria rapid diagnostic test (RDT) and filter paper dried blood spots (DBS) were prepared from each blood sample. Genomic DNA was extracted from the remaining blood and used in *Plasmodium* specific photo-induced electron transfer polymerase chain reaction (PET-PCR) and Nested PCR, whilst the HRP2 antigen content of the DBS was estimated using a bead immunoassay. Comparison of prevalence as determined by each method was performed.

Results. Parasite prevalence in the high transmission site of Obom was estimated at 71.4%, 61.9%, 60%, 37.8% and 19.1% by Nested PCR, the HRP2 bead assay, PET-PCR, HRP2-RDT and microscopy respectively. Parasite prevalence in the low transmission site of Asutsuare was estimated at 50.1%, 11.2%, 5.6%, 0% and 2.2% by Nested PCR, the HRP2 bead assay, PET-PCR, RDT and microscopy respectively.

Conclusions: Nested PCR exhibited the highest sensitivity by identifying the highest prevalence of asymptomatic *P. falciparum* in both the high and low parasite prevalence setting. However, parasite prevalence estimated by the HRP2 bead assay and PET-PCR had the highest level of inter-rater agreement relative to all the other tools tested and have the advantage of requiring fewer processing steps and producing quantitative results relative Nested PCR. These advantages make PET-PCR and the HRP2 bead assay very useful tools for estimating malaria parasite prevalence in community surveys in these settings.

Background

Asymptomatic parasite carriage in *Plasmodium falciparum* infections is a well-known phenomenon [1]. Previously, it was assumed that residents of high transmission areas were at a greater risk of harboring asymptomatic (subclinical) infections as a result of acquired immunity to clinical manifestation of the infection developed over repeated exposures [1, 2]. However, recent studies conducted in low-transmission areas of malaria endemic countries, especially in Africa have identified a high prevalence of asymptomatic *P. falciparum* carriers [3]. Asymptomatic *Plasmodium* carriage in low transmission settings has been suggested to be responsible for 20–50% of all malaria transmission in those settings [4].

Recent estimates of high asymptomatic parasite carriage in low transmission settings could be due to the sensitivity of the parasite detection tools used, where highly sensitive molecular tools increase parasite prevalence estimates [5]. Light microscopy, the gold standard for laboratory confirmation of malaria [6] has a sensitivity of detection ranging from 30–50 parasites/μl of blood according to Gilles 1993 [7] or 50–500 parasites/μl according to Moody *et al* [6]. In addition to having low sensitivity, microscopy is dependent on the quality of reagents and techniques used in preparing and staining the smear [8] as well as the expertise of the microscopist who examines the smear [9]. These limitations and the difficulty of deploying microscopy to all testing sites have led to the expansion of tools used in malaria diagnosis and detection of infection to include tools such as the rapid diagnostic tests (RDTs), with a sensitivity of ~ 100 parasites/μl [6, 9] and molecular tools such as polymerase chain reaction (PCR), with a sensitivity of about 2–5 parasites/μl of blood for Nested PCR [10] and 0.01 to 1 parasite/μl of blood for real time PCR [11].

Although the main rationale to improve malaria diagnostic tools is to ensure prompt and accurate parasite detection and treatment of clinical cases, the new diagnostic tools are frequently used by Malaria Control Programs to assess parasite carriage in population surveys [5, 12, 13].

Malaria RDTs are predominantly based on the histidine-rich protein (HRP2) and or lactate dehydrogenase (LDH) antigens, and despite RDTs having a similar sensitivity to microscopy [14–16], their ease of use and fast turnaround time have made them a preferred diagnostic tool [17, 18]. The most commonly used malaria RDTs are the HRP2-based tests, because of abundant production of the protein by the parasite and its enhanced sensitivity compared to LDH [19, 20]. A major limitation of RDTs is the fact that they are not quantitative [21]. Additional limitations of HRP2-based RDTs include a high false positive rate due to HRP2 antigen persisting in the blood for up to four weeks after the clearance of an active infection [22] and increasing reports of false negative results due to parasites not producing HRP2 as a result of *pfhrp2* gene deletions [23].

A recently developed tool for detecting parasite antigen is a sensitive HRP2 bead assay, which can simultaneously measure multiple parasite antigens including HRP2, LDH and aldolase. The HRP2 bead assay has a limit of detection of 0.24 pg/mL, 1.43 pg/mL or 71.9 pg/mL for three unique forms of HRP2 antigens (Type A, B, and C, respectively) that are captured by the beads [24]. The main disadvantage of the HRP2 bead assay is that it cannot be used as a point of care test [24–26].

Molecular diagnosis of malaria largely comprises the use of a wide variety of polymerase chain reaction (PCR) platforms to detect parasite nucleic acids. A photoelectron induced transfer PCR (PET-PCR), has a limit of detection of 3.2, 5.8, 3.5 and 5 parasites/μl for *P. falciparum*, *P. ovale*, *P. malariae* and *P. vivax* respectively, and the possibility of duplex detection of both *P. falciparum* and another human *Plasmodium* species in a single reaction is presently available [27]. PET-PCR has also been optimized for use in detecting asymptomatic malaria parasite carriers in large community surveys [24]. Although molecular tools are more sensitive than microscopy and RDTs, they are not suitable for point of care

diagnosis as they are time-consuming and require expensive specialized equipment and reagents as well as highly-skilled personnel to run them [10].

This study evaluated the sensitivities of a variety of malaria parasite detection tools; microscopy, HRP2-based malaria RDT, HRP2 bead assay, PET-PCR and Nested PCR in determining the prevalence of asymptomatic *P. falciparum* parasite carriage amongst participants from two communities with varying malaria parasite prevalence in southern Ghana.

Methods

Ethical consideration

Ethical approval for the study was obtained from the Institutional Review Board of the Noguchi Memorial Institute for Medical Research, Ghana (Study number 089/14-15). Written informed consent, assent and parental consent (for children) were obtained from all study participants.

Study site and population

This pilot study used consecutive sampling to select 194 participants from a larger cross-sectional study conducted in Obom and Asutusare during the off-peak malaria season (February 2016) [28]. Participants from the larger study were aged between 6 and 70 years old and selected based on the absence of any sign or symptom suggestive of malaria. Obom is a high malaria parasite prevalence setting in the Ga South municipality of Greater Accra Region of Ghana (Figure 1) with a microscopy estimated parasite prevalence of 35% in 2014 [5, 12] and 41.8% in 2019 [29]. Asutsuare is a low malaria parasite prevalence setting in the Shai Osudoku District of the Greater Accra Region of Ghana. Microscopy estimates of parasite prevalence in Asutsuare was 8.9% in 2009 [30] and 3.6% in 2016 [31]. According to the WHO, an annual parasites prevalence of 1-10 % is considered as low and ≥ 35 % considered as high [32].

Sample collection and processing

Prior to sample collection, the axillary temperature of each participant was measured using a digital thermometer. Venous blood (5 ml) was collected from each volunteer into EDTA vacutainer® blood collection tubes (BD, New Jersey, USA). An aliquot of the blood was used to prepare thick and thin blood smears for microscopy. The blood smears were air dried, fixed (thin film only) and stained with Giemsa following the WHO standard protocol [8, 33]. The slides were observed at 100X magnification under a light microscope by two microscopists working independently. A sample was scored as negative for malaria if no parasite was seen after observing 200 fields and scored positive if parasites were observed. Parasite density (PD/ μ l) was determined as the number of malaria parasite observed per 200 white blood cells (WBCs) X 40, with the assumption that 1 μ l of blood contains 8,000 WBCs [34].

Additionally, 5 μ l of the blood was used for *P. falciparum* diagnosis using the Malaria Pf (HRP2) Ag RDT Multi Kit (Access Bio Inc, New Jersey, USA), following the manufacturer's instructions.

Four, 50 µl drops of blood sample were spotted on Whatman #3 filter paper (GE Life sciences, USA). The filter paper blood spots were individually air dried and stored at room temperature in a sealed plastic bag containing a desiccant. The remaining blood from each volunteer was separated into plasma and packed blood cells, which were subsequently stored frozen at -20 °C until required. All samples from the field were subsequently transported to the Immunology Department of the NMIMR for further processing and analysis. An aliquot of the whole blood was sent to the CDC (USA) for analysis.

DNA extraction

DNA for the Nested PCR was extracted at the NMIMR from two 3 mm disks punched out of the DBS using the Chelex extraction method as previously described [35]. Whereas DNA for the PET-PCR was extracted at the CDC from 200 µl of packed blood cell pellets using the QIAamp DNA Mini Kits (Qiagen, USA) according to the manufacturers protocol. The DNA extracted from both procedures was either stored at 4 °C for immediate use or stored at -20 °C for later use.

Nested PCR

The Nested PCR amplification of the *P. falciparum* 18S rRNA gene was adapted from Singh *et al.* [36] with slight modification as previously reported [12]. Briefly, 200 nM dNTPs, 2 mM MgCl₂, 133 nM each of forward (rPLU6) and reverse (rPLU5) primers (Additional file Table s1) and 1 U OneTaq DNA polymerase (NEB, UK) was used to amplify the 18S rRNA gene from 5 µl (~20 ng) of DNA in the primary PCR. The secondary PCR was performed using similar concentrations of reagents as in the primary reaction mix; however, rFal1 (forward) and rFal2 (reverse) primers were used to amplify 1 µl of the primary product. The cycling parameters for the primary and secondary reactions are listed in Additional file Table s1. Genomic DNA from the 3D7 strain of *P. falciparum* (MRA 102G) was used as the positive control sample and a distilled water (no template) served as the negative control sample. Positive and negative control samples were included in each PCR reaction set up. The amplified PCR products were separated alongside a 100 bp ladder (New England Biolabs, UK) on a 2% agarose gel stained with Ethidium bromide. The gels were subsequently viewed under ultra violet light using the FUSION-FX7 advanced (Vilber Lourmat, Germany) chemiluminescence documentation system. All PCR assays were performed using the Eppendorf Mastercycler Nexus PCR Cycler (Eppendorf, UK).

PET-PCR

The multiplex PET-PCR assay was performed as previously described [27]. Briefly, the amplification of *Plasmodium* genus was performed in a 20 µl reaction containing 2 µl (~20 ng) of each DNA template, TaqMan Environmental buffer 2.0 (Applied BioSystems, USA), 125 nM each of forward and reverse primers (Additional file Table s1) except for the *P. falciparum* HEX-labeled primer which was used at a 62.5 nM. The cycling parameters used were an initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 10 sec, annealing at 60 °C for 40 sec. Genomic DNA from the 3D7 strain of *P. falciparum* (CDC, USA) was used as positive control. All assays were performed in duplicate and using the Agilent Mx3005pro thermal cycler (Agilent technologies, USA).

HRP2 bead assay

The HRP2 concentrations (pg/ml) of each sample was determined using an HRP2 bead assay previously described by Rogier *et al.* [24]. Briefly, a 6 mm disc was punched out of the dried blood spot (DBS) and incubated overnight in 200 µl of buffer (0.05% phosphate-buffered saline, Tween 20, 0.5% bovine serum albumin, 0.1% casein, 0.5% polyvinylalcohol, 0.5% polyvinylpyrrolidone, 0.05% NaN₃, and 3 µg/mL *E. coli* extract to prevent non-specific binding). The test samples, buffer blank and negative controls (O+ Packed red blood cells that tested negative by two RDT kits and microscopy) were added in duplicate on each plate. Following the assay incubation steps, 100 µl PBS was added to each well and was incubated at room temperature with shaking for 1 min. The plate was subsequently read on a Luminex-200 machine (Luminex Corporation, USA) with a target of 50 beads per region.

Data analysis

All samples that yielded visible fragments after agarose gel electrophoresis or positive CT values after real time PCR analysis were classified as positive for the particular PCR reaction. The CT cut off for the PET-PCR was set at 40. The mean threshold fluorescence intensity (MFI) positivity cut off value for the HRP2 bead assay was the log normal of the background signal (bg) + 3 SD of the MFI value for the malaria-negative population. IBM SPSS version 20 was used to generate the descriptive statistics including median and to compare median age, hemoglobin and temperature between the two sites. Graph Pad Prism version 7 was used to determine Pearson Chi-Square for sex and parasite prevalence estimated by RDT, microscopy, Nested PCR and HRP2 bead assay, Mann-Whitney test for age and Cohen's kappa test was used to determine the level of agreement between parasite prevalence estimates determined by two different tests (RDT, microscopy, Nested PCR and HRP2 bead assay). Statistical significance was set as $P \leq 0.05$, unless otherwise stated. Kappa values of < 0 are classified as no agreement (disagreement), 0.0 - 0.20 are classified as poor agreement; 0.21 – 0.40 are classified as fair agreement; 0.41 – 0.60 are classified as moderate agreement and values of 0.61 – 0.80 classified as substantial agreement and 0.81-1.0 as an almost perfect agreement [37].

Results

Demographics

Of the 194 participants, 105 (54.1%) were residents of Obom, a high parasite prevalence area and 89 (45.9%) were residents of Asutsuare, a low parasite prevalence area. There was no significant difference ($p= 0.652$) in the distribution of males between the two study sites (53% in Asutsuare and 49% in Obom) (Table 1) or in terms of age ($p= 0.109$). The median (IQR) age of participants from Obom was 14 (12 – 24.3) years and the median (IQR) age in Asutsuare was 16 (13 – 25.8).

Table 1. Demographics of the study participants

Parameters	Obom (n=105)	Asutsuare (n=89)	P-value
Sex			
Male/Female	48/50*	43/38*	0.652 ^a
Age (yrs)			
Median	14	16	0.109 ^b
Range	64	56	
Min-Max	6.0-70.0	10.0-66.0	
Temp (°C)			
Median	35.9	36.5	
Range	7.4	4.3	
Min-Max	30.1-37.5	33.2-37.5	
Parasite Positivity			
Microscopy n (%)	20 (19.1)	2 (2.2)	0.0002 ^a
HRP2 RDT n (%)	37 (37.8)	0	0.0001 ^a

yrs=year, Min = minimum, Max = maximum, Temp = temperature, and n = total number of samples tested.

^a Pearson Chi-Square, ^b Mann Whitney (Two-tailed). *a few samples had missing gender data.

Estimation of parasite prevalence and density by microscopy

A total of 19.1% (20/105) and 2.2% (2/89) of the samples were identified containing *P. falciparum* by microscopy in the high (Obom) and low (Asutsuare) transmission sites respectively (Figure 2 and Table 1). One of the samples from Obom contained a mixture of *P. falciparum* and *P. malariae* (however, this was not confirmed by PCR). A higher number of *P. falciparum* parasite carriers were detected in the high parasite prevalence setting (Obom) relative to the low parasite prevalence setting of (Asutsuare) (Pearson Chi-Square, $p = 0.0002$) (Table 1). Parasite density estimated per microlitre (PD/ μ l) blood from Obom ranged between 32 and 5080 with a median (IQR) of 180 (80-405), whilst in Asutsuare, both samples that tested positive by microscopy had a parasite density of 40 (Figure 3).

Estimation of parasite prevalence based on antigen detection

RDT positivity rates

The HRP2-RDT identified a total of 37.8% (39/101) of the samples collected from the high transmission area as positive. RDT results were not available for 4 samples from the high parasite prevalence area.

None of the samples from the low parasite prevalence setting of Asutsuare tested positive by the HRP2 RDT (Figure 2 and Table 1).

HRP2 Bead detection of Plasmodium antigen levels

Detection of the *P. falciparum* HRP2 antigen was significantly higher in Obom (61.9%) when compared to Asutsuare (11.2%), $p < 0.0001$. The *P. falciparum* HRP2 antigen levels of samples in Obom ranged from 226.0 pg/ml to 820,368 pg/ml, with a median of 4689.0 pg/ml and 49.4 pg/ml to 44,980 pg/ml with a median of 236.4 pg/ml in Asutsuare. The median HRP2 antigen levels in samples from Obom (4689.0 pg/ml) was significantly higher than samples from Asutsuare with median HRP2 antigen level of 236.4 pg/ml (Mann Whitney test, $p < 0.0001$) (Figure 3).

Estimation of parasite prevalence based on molecular tests

Nested PCR

The prevalence of *P. falciparum* by Nested PCR (nPCR) in Obom (71.4% (70/98)) was significantly higher (Fisher's exact test, $p = 0.0056$) than parasite prevalence in Asutsuare (50.6% (42/83)) (Figure 2). The nPCR could not be performed for 7 and 6 samples from Obom and Asutsuare respectively.

PET-PCR Analysis

In Obom, 60% (63/105) of the samples tested positive for *P. falciparum*, with CT values ranging from 23.8 to 36.3 and a median CT value of 30.5. The corresponding parasite density estimates ranged from 0.4 p/μl to 7,002 p/μl, with a median of 37.1 p/μl. In Asutsuare, 5.6% (5/89) of the samples tested positive for *P. falciparum*, with a CT range of 27.7 to 33.1 and a median of 31.8. The corresponding parasite density estimates range from 5.0 p/μl to 331.7 p/μl, with a median of 14.0 p/μl (Figure 3). Although a significantly higher number of parasites were detected in Obom than in Asutsuare, (Fisher's exact test, $p < 0.001$) (Figure 2), there was no significant difference between the estimated parasite densities of the two sites when their median parasite density was compared (Mann Whitney test, $p = 0.8879$).

Illustration of relationships among sensitive detection methods by areas

A total of 84.1% (53/63) of the PET-PCR positive samples and 28.5% (12/42) of the PET-PCR negative samples from Obom tested positive by the HRP2 bead assay (Figure 4A). Whilst in Asutsuare, 80% (4/5) of the PET-PCR positive samples and 7.1% (6/84) of the PET-PCR negative samples tested positive by the HRP2 bead assay (Figure 4B).

There were 8 samples from Obom that tested positive for *P. falciparum* by all the five methods tested (Figure S1), whilst 8 samples were negative by the same five methods (Figure S1). In the low transmission setting, no sample was identified as positive by all the methods (Figure S1), whilst 35 samples were identified as negative by all the five tests.

Comparison among sensitive detection methods by areas

In the high transmission setting, parasite prevalence estimated by Nested PCR was significantly higher than that estimated by PET-PCR and the HRP2 bead assay (Pearson Chi square=13.06 and 6.76 respectively, $p < 0.001$ for both), but parasite prevalence estimated by the HRP2 bead assay and PET-PCR were similar (Pearson Chi square=31.89 and $p > 0.05$) (Table S3).

In the low transmission setting, parasite prevalence estimated by the HRP2 bead assay was significantly higher than that recorded by PET-PCR (Fisher's Exact Test $p < 0.000$) (Table S3) and the difference between parasite prevalence estimated by both Nested PCR and PET-PCR on the one hand and Nested PCR and the HRP2 bead assay on the other were similar (Fisher's Exact Test $p = 1.000$ and 0.156 respectively).

Comparison between antigen detection methods by areas

The HRP2 bead assay detection of HRP2 antigen levels identified a significantly higher number of positive samples compared to the HRP2 based RDT in the high malaria transmission setting (Pearson Chi-Square=17.22, $p < 0.001$) (Figure 2 and 5B). Comparisons could not be made in the low transmission site, as no sample tested positive by HRP2 RDT (Figure 5D, Table 2 and Table S3).

Comparison between nucleic acid detection methods by areas

Nested PCR identified a significantly higher number of positive samples compared to PET-PCR in both the high transmission setting, Obom (Pearson Chi-Square=13.06, $p < 0.001$) (Figure 2, 5A and 5C) and the low transmission setting - Asutsuare. In comparing diagnostic methods that measure similar parasite features, HRP2 antigen (RDT and the HRP2 bead assay) and parasite DNA (Nested PCR and PET-PCR), fair and significant agreements were observed only for the samples collected from the high transmission setting (Obom) (Table 2). A crosstabulation analysis between PET-PCR and the HRP2 bead assay found that the two methods agreed substantially and significantly (Cohen kappa value = 67.9%, $p = 0.004$).

Table 2. Inter-rater agreement between similar detection tools

	N	Kappa value	P-value
Antigen Methods (RDT / HRP2 bead assay)			
Obom	97	0.262	0.004
Asutsuare	81	-	-
Overall	178	0.407	0.001
DNA Methods (PET-PCR / Nested PCR)			
Obom	98	0.348	0.001
Asutsuare	83	0.022	0.665
Overall	181	0.272	0.001

N, number of samples used in the analysis. The differences in the total number of samples used in the analysis is due to some samples missing results from one test or the other. No statistics could be computed for RDT vs the HRP2 bead assay in Asutsuare because no RDT positive samples were identified in Asutsuare.

Agreement between diagnostic tests

Microscopy is generally referred to as the gold standard diagnostic test for malaria. When results from the microscopy read out by the microscopists used in this study was set as the gold standard (reference test)(Table 3), the level of agreement between microscopy and the PET-PCR and the HRP2 bead assay tests in Obom was poor, with a fair agreement observed between results obtained by microscopy and RDT. In Asutsuare, the interrater agreement between microscopy and both PET-PCR and Nested PCR was poor but the agreement between microscopy and the HRP2 bead assay was fair. All the poor agreements were not significant, whilst the fair agreements were significant. There was no agreement between microscopy and nPCR in both Obom and Asutsuare (Table 3).

When Cohen’s kappa analysis (Table 3) was repeated with Nested PCR set as the gold standard (reference), there was a poor agreement between Nested PCR and RDT but fair agreement between Nested PCR and PET-PCR and the HRP2 bead assay in Obom, whilst in Asutsuare, all the agreements were poor. All the agreements in Obom were significant whilst those in Asustuare were not significant. There was no agreement between the results obtained by Nested PCR that was compared to microscopy in Obom (Table 3).

Table 3. Agreement analysis between microscopy/Nested PCR and other diagnostic tests

Parameter	Obom	Asutsuare	Overall
	Kappa (P-value)	Kappa (P-value)	Kappa (P-value)
Microscopy			
Microscopy vs RDT	0.212 (0.021)*	-	0.239 (0.001)*
Microscopy vs PET-PCR	0.055 (0.452)	0.061 (0.135)	0.161 (0.010)
Microscopy vs HRP2 bead assay	0.027 (0.698)	0.218 (0.028)*	0.156 (0.009)*
Microscopy vs Nested PCR	-0.014 (0.808)	-0.034 (0.768)	0.048 (0.262)
Nested PCR			
Nested PCR vs RDT	0.186 (0.019)*	-	0.182 (0.001)*
Nested PCR vs PET-PCR	0.348 (0.001)*	0.022 (0.665)	0.272 (0.001)*
Nested PCR vs HRP2 bead assay	0.248 (0.012)*	0.117 (0.084)	0.266 (0.001)*
Nested PCR vs Microscopy	-0.009 (0.874)	0.047 (0.157)	0.045 (0.204)

*, significant p value; vs, versus;

67.8%, however, there was also a significant discordance ($P < 0.001$) between the methods.

Discussion

This study independently utilized five different diagnostic tools, PET-PCR, a HRP2 bead assay in addition to commonly used HRP2 RDT, microscopy and Nested PCR to determine the prevalence of asymptomatic *P. falciparum* parasite carriage in two communities with varied malaria parasite prevalence in southern Ghana. Asymptomatic malaria infections are usually characterized by low and submicroscopic parasite densities [38] and depending on the transmission intensity of the area can contain lower than 100 parasites per microlitre [39, 40]. Relying solely on microscopy for detecting malaria infections containing such low parasite densities will likely result in missing many infections. Although microscopy, RDTs and nPCR are routinely used for malaria diagnosis in Ghana, PET-PCR and the HRP2 bead assay that are known to be more sensitive than microscopy at detecting low density parasitaemia [24, 41] are rarely used. The sensitivities of various combinations of commonly used malaria diagnostic tools have been compared in different malaria endemic countries, including Ghana [5], none of the studies conducted in Ghana have compared the performance of PET-PCR and an HRP2 bead assay to microscopy, an HRP2 based RDT and nPCR at determining malaria parasite prevalence in different settings of Ghana. This study was conducted to evaluate the sensitivities of especially PET-PCR and the HRP2 bead assay as effective tools to detect asymptomatic malaria parasite carriage in settings of varying parasite prevalence in Ghana.

In this study, microscopy and HRP2-based RDT, the most commonly used malaria diagnostic tests in community surveillance studies in malaria endemic countries [42] produced the lowest estimates of asymptomatic parasite carriage in both the high and low malaria parasite prevalence settings. This was not surprising as the parasite densities of infections in samples from even the high parasite prevalence setting was very low. Asymptomatic infections are noted to contain low (submicroscopic) parasite densities [43], below the limit of detection of both microscopy and RDT kits [42].

In this study, parasite prevalence detected by the RDT was higher than the microscopy in the high transmission setting, but a reverse trend was observed in the low transmission setting. One likely reason for these results could be that the HRP2 antigen concentrations measured in samples from the high parasite prevalence setting were often higher and can be detected by the RDT than in the low parasite prevalence setting where it is below the detection limit of the RDT [51, 52]. Higher levels of HRP2 antigen could also result from longer duration of antigen persistence in the high parasite prevalence setting due to more frequent infection. This would account for the higher positivity rates detected compared to microscopy in Obom but not in the low parasite prevalence setting (Asutsuare). Persistence of the HRP2 antigen after the clearance of infecting parasites is a well-known phenomenon [44, 45]. Consequently, HRP2 based malaria RDT kits may test positive for HRP2 antigens in the absence of an active infection. Additionally, as demonstrated in the study sites described here, parasite densities in low transmission settings are generally low and likely to be below the limit of detection of the RDT and microscopy especially in the off peak season [31].

Although the parasite prevalence estimated by PET-PCR, nPCR and the HRP2 bead assay were similar in the high prevalence setting, the level of agreement among the three tests was low. This observation may be due to differences in limits of detection, assay targets and other fundamental differences between the methods. Persistence of HRP2 antigen for up to four weeks following a resolved *P. falciparum* infection can result in false positive HRP2 bead assay results, whilst parasites with deletions in the *hrp2* gene (not tested in this study) can cause false negative tests [46–48]. Nested PCR protocols generally have much higher numbers of amplification cycles compared to real time PCR protocols including PET-PCR and as such are likely to detect and amplify lower template concentrations than real time PCR. Nested PCR has previously been found to be more sensitive than PET PCR [49, 50]. However, the increased number of steps involved in nPCR make it more tedious and prone to contaminations and other operator errors that can increase the number of false negative as well as false positive test results compared with real time PCR processes.

When the results obtained from PET-PCR and nPCR, both DNA-detecting, were compared to the results from the HRP2 bead assay, there was a much higher level of agreement between PET-PCR and the antigen-detecting assay. A possible explanation for this could be that PET-PCR and the HRP2 bead assay have a similar parasitaemia threshold of approximately two parasites per microliter [24], which is higher than that of nPCR. However, both the HRP2 bead assay and PET-PCR are quantitative, require fewer processing steps, and are faster processes than nPCR.

Limitations

The different diagnostic tests used in this study detect different parasite components and also have varying limits of detection. The samples used in this study were collected during the off-peak malaria season where parasite densities are generally low and thus would require diagnostic tests with low limit of detection and high sensitivity to detect.

Conclusion

Nested PCR exhibited the highest sensitivity by identifying the highest prevalence of asymptomatic *P. falciparum* in both the high and low parasite prevalence setting. However, parasite prevalence estimated by the HRP2 bead assay and PET-PCR had the highest level of inter-rater agreement relative to all the other tools tested and have the advantage of requiring fewer processing steps and producing quantitative results relative to Nested PCR. These advantages make PET-PCR and the HRP2 bead assay very useful tools for detecting and estimating malaria parasite density especially amongst asymptomatic individuals during community surveys.

Abbreviations

RDT - rapid diagnostic test

PET-PCR - photo-induced electron transfer polymerase chain reaction

WBCs - white blood cells

PD - Parasite density

Declarations

Ethics approval and consent to participate

Research Approval was obtained from the Institutional Review Board (IRB) of the Noguchi Memorial Institute for Medical Research (NMIMR). Written informed consent was obtained from study participants as well as the parents or guardians of participants who were minors before they were enrolled onto the study.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests

Funding

This project was supported in part by Bill and Melinda Gates Foundation under the Postdoctoral and Postgraduate Training in Infectious disease research at Noguchi Memorial Institute for Medical Research awarded to AL and the US Centers for Disease Control and Prevention.

Authors' contributions

LEA, MA and ER designed the study. AL collected the samples. HBA, ZA, ER and SA performed the experiments. LEA and HBA performed the statistical analysis. HBA, LEA, MA and ER contributed to writing the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors are grateful to all the study participants as well as the guardians of the minors who participated in the study for granting consent.

Disclaimer

The findings and conclusions presented in this report are those of the authors and do not necessarily reflect the official position of the Centers for Disease Control and Prevention.

References

1. Lindblade KA, Steinhardt L, Samuels A, Kachur SP, Slutsker L. The silent threat: asymptomatic parasitemia and malaria transmission. *Expert Rev Anti Infect Ther*. 2013;11:623–39.
2. Filipe JA, Riley EM, Drakeley CJ, Sutherland CJ, Ghani AC. Determination of the processes driving the acquisition of immunity to malaria using a mathematical transmission model. *PLoS Comput Biol*. 2007;3:e255.
3. Bousema T, Okell L, Felger I, Drakeley C. Asymptomatic malaria infections: detectability, transmissibility and public health relevance. *Nat Rev Microbiol*. 2014;12:833–40.
4. Okell LC, Bousema T, Griffin JT, Ouédraogo AL, Ghani AC, Drakeley CJ. Factors determining the occurrence of submicroscopic malaria infections and their relevance for control. *Nature communications*. 2012;3:1237.
5. Ayanful-Torgby R, Quashie NB, Boampong JN, Williamson KC, Amoah LE. Seasonal variations in *Plasmodium falciparum* parasite prevalence assessed by varying diagnostic tests in asymptomatic children in southern Ghana. *PLoS One*. 2018;13:e0199172.
6. Moody A. Rapid diagnostic tests for malaria parasites. *Clin Microbiol Rev*. 2002;15:66–78.

7. Hm G. Bruce-Chwatt's Essential Malariology Third ed. Diagnostic methods in malaria. London: Edward Arnold: ed. DA, W.; 1993.
8. Who, Giemsa Staining of Malaria Blood Films, in malaria microscopy standard operating procedure - MM-SOP-07A. 2016.
9. Mathison BA, Pritt BS. Update on Malaria Diagnostics and Test Utilization. *J Clin Microbiol.* 2017;55:2009–17.
10. Berzosa P, De Lucio A, Romay-Barja M, Herrador Z, Gonzalez V, Garcia L, et al. Comparison of three diagnostic methods (microscopy, RDT, and PCR) for the detection of malaria parasites in representative samples from Equatorial Guinea. *Malar J.* 2018;17:333.
11. Rubio J, Benito A, Berzosa P, Roche J, Puente S, Subirats M, et al. Usefulness of seminested multiplex PCR in surveillance of imported malaria in Spain. *J Clin Microbiol.* 1999;37:3260–4.
12. Adjah J, Fiadzoe B, Ayanful-Torgby R, Amoah LE. Seasonal variations in *Plasmodium falciparum* genetic diversity and multiplicity of infection in asymptomatic children living in southern Ghana. *BMC Infect Dis.* 2018;18:432.
13. Lamptey H, Ofori MF, Kusi KA, Adu B, Owusu-Yeboah E, Kyei-Baafour E, et al. The prevalence of submicroscopic *Plasmodium falciparum* gametocyte carriage and multiplicity of infection in children, pregnant women and adults in a low malaria transmission area in Southern Ghana. *Malar J.* 2018;17:331.
14. Palmer CJ, Lindo JF, Klaskala WI, Quesada JA, Kaminsky R, Baum MK, et al. Evaluation of the OptiMAL test for rapid diagnosis of *Plasmodium vivax* and *Plasmodium falciparum* malaria. *J Clin Microbiol.* 1998;36:203–6.
15. Beadle C, Long GW, Weiss WR, Mcelroy PD, Maret SM, Oloo AJ, et al. Diagnosis of malaria by detection of *Plasmodium falciparum* HRP-2 antigen with a rapid dipstick antigen-capture assay. *Lancet.* 1994;343:564–8.
16. Garcia M, Kirimoama S, Marlborough D, Leafasia J, Rieckmann KH. Immunochromatographic test for malaria diagnosis. *Lancet.* 1996;347:1549.
17. Bechem NN, Leke RF, Tietche F, Taylor DW. Evaluation of a rapid test for histidine rich protein 2 for diagnosis of *Plasmodium falciparum* infection in Cameroonian children. *Trans R Soc Trop Med Hyg.* 1999;93:46-.
18. Cunningham J, Jones S, Gatton ML, Barnwell JW, Cheng Q, Chiodini PL, et al. A review of the WHO malaria rapid diagnostic test product testing programme (2008–2018): performance, procurement and policy. *Malar J.* 2019;18:387.
19. Ochola L, Vounatsou P, Smith T, Mabaso M, Newton C. The reliability of diagnostic techniques in the diagnosis and management of malaria in the absence of a gold standard. *The Lancet infectious diseases.* 2006;6:582–8.
20. Gatton ML, Rees-Channer RR, Glenn J, Barnwell JW, Cheng Q, Chiodini PL, et al. Pan-*Plasmodium* band sensitivity for *Plasmodium falciparum* detection in combination malaria rapid diagnostic tests and implications for clinical management. *Malar J.* 2015;14:115.

21. Erdman LK, Kain KC. Molecular diagnostic and surveillance tools for global malaria control. *Travel Med Infect Dis.* 2008;6:82–99.
22. Humar A, Ohrt C, Harrington MA, Pillai D, Kain KC. Parasight F test compared with the polymerase chain reaction and microscopy for the diagnosis of *Plasmodium falciparum* malaria in travelers. *Am J Trop Med Hyg.* 1997;56:44–8.
23. Berhane A, Russom M, Bahta I, Hagos F, Ghirmai M, Uqubay S. Rapid diagnostic tests failing to detect *Plasmodium falciparum* infections in Eritrea: an investigation of reported false negative RDT results. *Malar J.* 2017;16:105.
24. Rogier E, Plucinski M, Lucchi N, Mace K, Chang M, Lemoine JF, et al. Bead-based immunoassay allows sub-picogram detection of histidine-rich protein 2 from *Plasmodium falciparum* and estimates reliability of malaria rapid diagnostic tests. *PLoS One.* 2017;12:e0172139.
25. Fouda GG, Leke RF, Long C, Druilhe P, Zhou A, Taylor DW, et al. Multiplex assay for simultaneous measurement of antibodies to multiple *Plasmodium falciparum* antigens. *Clin Vaccine Immunol.* 2006;13:1307–13.
26. Ambrosino E, Dumoulin C, Orlandi-Pradines E, Remoue F, Toure-Baldé A, Tall A, et al. A multiplex assay for the simultaneous detection of antibodies against 15 *Plasmodium falciparum* and *Anopheles gambiae* saliva antigens. *Malar J.* 2010;9:317.
27. Lucchi NW, Narayanan J, Karell MA, Xayavong M, Kariuki S, Dasilva AJ, et al. Molecular diagnosis of malaria by photo-induced electron transfer fluorogenic primers: PET-PCR. *PLoS One.* 2013;8:e56677.
28. Acquah FK, Lo AC, Akyea-Mensah K, Abagna HB, Faye B, Theisen M, et al. Stage-specific *Plasmodium falciparum* immune responses in afebrile adults and children living in the Greater Accra Region of Ghana. *Malar J.* 2020;19:64.
29. Acquah FK, Donu D, Bredu D, Eyia-Ampah S, Amponsah JA, Quartey J, et al. Asymptomatic carriage of *Plasmodium falciparum* by individuals with variant blood groups and haemoglobin genotypes in southern Ghana. *Malar J.* 2020;19:217.
30. Adu B, Dodoo D, Adukpo S, Hedley PL, Arthur FK, Gerds TA, et al. Fc gamma receptor IIIB (FcgammaRIIB) polymorphisms are associated with clinical malaria in Ghanaian children. *PLoS One.* 2012;7:e46197.
31. Amoah LE, Abagna HB, Akyea-Mensah K, Lo AC, Kusi KA, Gyan BA. Characterization of anti-EBA175RIII-V in asymptomatic adults and children living in communities in the Greater Accra Region of Ghana with varying malaria transmission intensities. *BMC Immunol.* 2018;19:34.
32. Organization WH. World malaria report 2015. World Health Organization; 2016.
33. Organization WH. Malaria Microscopy Quality Assurance Manual. Malaria Microscopy. Geneva: WHO; 2015.
34. Who. Malaria parasite counting, in Malaria microscopy standard operating procedure - MM-SOP-09. WHO: Geneva; 2016.
35. Amoah LE, Opong A, Ayanful-Torgby R, Abankwa J, Acquah FK. Prevalence of G6PD deficiency and *Plasmodium falciparum* parasites in asymptomatic school children living in southern Ghana. *Malar*

- J. 2016;15:388.
36. Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. *Am J Trop Med Hyg.* 1999;60:687–92.
 37. Altman DG. *Practical statistics for medical research.* London: Chapman and Hall; 1991.
 38. Slater HC, Ross A, Felger I, Hofmann NE, Robinson L, Cook J, et al. The temporal dynamics and infectiousness of subpatent *Plasmodium falciparum* infections in relation to parasite density. *Nat Commun.* 2019;10:1433.
 39. McGuinness D, Koram K, Bennett S, Wagner G, Nkrumah F, Riley E. Clinical case definitions for malaria: clinical malaria associated with very low parasite densities in African infants. *Trans R Soc Trop Med Hyg.* 1998;92:527–31.
 40. Wagner G, Koram K, McGuinness D, Bennett S, Nkrumah F, Riley E. High incidence of asymptomatic malaria infections in a birth cohort of children less than one year of age in Ghana, detected by multicopy gene polymerase chain reaction. *Am J Trop Med Hyg.* 1998;59:115–23.
 41. Talundzic E, Maganga M, Masanja IM, Peterson DS, Udhayakumar V, Lucchi NW. Field evaluation of the photo-induced electron transfer fluorogenic primers (PET) real-time PCR for the detection of *Plasmodium falciparum* in Tanzania. *Malar J.* 2014;13:31.
 42. Florey L, Measures of Malaria Parasitemia Prevalence in National Surveys: Agreement between Rapid Diagnostic Tests and Microscopy, in DHS Analytical Studies No. 43. 2014, USAID: Maryland, USA.
 43. Niang M, Thiam LG, Sane R, Diagne N, Talla C, Doucoure S, et al. Substantial asymptomatic submicroscopic *Plasmodium* carriage during dry season in low transmission areas in Senegal: Implications for malaria control and elimination. *PloS one.* 2017;12.
 44. Dalrymple U, Arambepola R, Gething PW, Cameron E. How long do rapid diagnostic tests remain positive after anti-malarial treatment? *Malar J.* 2018;17:228.
 45. Mouatcho JC, Goldring JP. Malaria rapid diagnostic tests: challenges and prospects. *J Med Microbiol.* 2013;62:1491–505.
 46. Amoah LE, Abuaku B, Bukari AH, Dickson D, Amoako EO, Asumah G, et al. Contribution of *P. falciparum* parasites with Pfhrp 2 gene deletions to false negative PfHRP 2 based malaria RDT results in Ghana: A nationwide study of symptomatic malaria patients. *PLoS One.* 2020;15:e0238749.
 47. Cunningham J, Update on *Plasmodium falciparum* hrp2/3 gene deletions. 2017.
 48. Who, False-negative RDT results and implications of new reports of *P. falciparum* histidine-rich protein 2/3 gene deletions. 2016.
 49. Akerele D, Ljolje D, Talundzic E, Udhayakumar V, Lucchi NW. Molecular diagnosis of *Plasmodium ovale* by photo-induced electron transfer fluorogenic primers: PET-PCR. *PLoS One.* 2017;12:e0179178.

50. Lucchi NW, Karell MA, Journeel I, Rogier E, Goldman I, Ljolje D, et al. PET-PCR method for the molecular detection of malaria parasites in a national malaria surveillance study in Haiti, 2011. *Malar J.* 2014;13:462.
51. Emily NR, Jen CCH, Issaka S, Sara AH, Mahamadoun HA, Merepen AG, et al. Ultra-sensitive RDT performance and antigen dynamics in a high-transmission *Plasmodium falciparum* setting in Mali. *Malar J.* 2020;19:323.
52. Matthew EC, Bachir A, Céline L, Nathan S, Iza C, Roberto, dT, et al. Clinical diagnostic evaluation of HRP2 and pLDH-based rapid diagnostic tests for malaria in an area receiving seasonal malaria chemoprevention in Niger. *Malar J.* 2019;18:443.

Figures

Figure 1

Map of Ghana highlighting the study sites. The study sites, Obom and Asutsuare are represented by green circles on the map. The map was created for this study by Awiah Dzantor Selorm, ACECoR,

Figure 2

Parasite prevalence determined by the different diagnostic tests. Microscopy and RTD detected the least number of *P. falciparum* parasite carriers in both the high and low parasite prevalence setting. Whilst parasite prevalence determined by Nested PCR, PET PCR and the HRP2 bead assay were similar in the high parasite prevalence setting, they were different in the low parasite prevalence setting

Figure 3

Parasite density determined by different tools. The median (IQR) parasite density of samples that tested positive for *P. falciparum* by microscopy (a) and PET-PCR (c) as well as the median (IQR) HRP2 antigen content of the samples estimated using the bead assay (b) from each site. Significant differences were observed in values obtained using microscopy (a) and the bead assay (c) but not by PET-PCR (b) when samples from Obom were compared to those from Asutusare.

Figure 4

Comparisons of PET-PCR, nPCR and the HRP2 bead assay detection tools. A Venn diagram illustrating the number of positive parasites by the three methods, [A] High transmission site, the three methods identified 42 samples as positive for the parasites, 7 positives between nPCR vs HRP2 bead assay, and also between PET-PCR vs HRP2 bead assay, and 6 positives between PET-PCR vs N-PCR. [B] Low transmission, the three methods identified 3 samples as positive for the parasites, 4 positives between nPCR vs HRP2 bead assay, and 1 positive between PET-PCR vs HRP2 bead assay, and no positives between PET-PCR vs nPCR

Figure 5

Comparisons between the nucleic acid and antigen detection tools. A Venn diagram illustrating the positive results obtained by the pair of nucleic acid detection tools (PET-PCR and nPCR) and Antigen detection tools (RDT and HRP2 bead assay) in the high transmission (A, B) and low (C, D) malaria transmission sites. [A & B] High transmission, the nucleic acid tools when paired identified 48 samples as positives for the parasite while the antigen detection tools when paired identified 30 samples as positives. [C & D] Low transmission, the nucleic acid tools when paired identified 2 samples as positives for the parasite while the antigen detection tools when paired identified none of samples as positive.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementalMaterial.docx](#)