Accuracy of diagnosis among clinical malaria patients: comparing microscopy, RDT, and a highly sensitive quantitative PCR and the implication of submicroscopic infections

Stephen Opoku Afriyie
Kwame Nkrumah University of Science and Technology (TAB-KNUST)
Thomas Kwame Addison
Kwame Nkrumah University of Science and Technology (TAB-KNUST)
Yilekal Gebre
University of Notre Dame
Abdul-Hakim Mutala
Kwame Nkrumah University of Science and Technology (TAB-KNUST)
Kwasi Baako Antwi
Kwame Nkrumah University of Science and Technology (TAB-KNUST)
Dawood Ackom Abbas
Kwame Nkrumah University of Science and Technology (TAB-KNUST)
Kofi Agyapong Addo
Kwame Nkrumah University of Science and Technology (TAB-KNUST)
Austine Tweneboah
Kwame Nkrumah University of Science and Technology (TAB-KNUST)
Cristian Koepfli
University of Notre Dame
Kingsley Badu (kingsbadu@gmail.com)
Kwame Nkrumah University of Science and Technology (TAB-KNUST)

Research Article

Keywords: Malaria, microscopy, rapid diagnostic test, varATS qPCR, sensitivity

Posted Date: March 31st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1510630/v1

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Malaria Journal on March 4th, 2023. See the published version at https://doi.org/10.1186/s12936-023-04506-5.

Abstract

Background: The World Health Organization recommends parasitological confirmation of all suspected malaria cases by microscopy or rapid diagnostic tests (RDTs) before treatment. These conventional tools are widely used for point-of-care diagnosis in spite of their poor sensitivity at low parasite density. Several studies have compared the diagnostic accuracy of microscopy and RDT using standard 18S rRNA PCR as reference with varying outcomes. Here, we present for the first time in Ghana, the accuracy of diagnosis of microscopy and RDT using highly sensitive *var*ATS qPCR as reference in a clinical setting.

Methods: 1,040 febrile patients were recruited from two primary health care centers in the Ashanti Region of Ghana and tested for malaria by microscopy, RDT, and *var*ATS qPCR. The sensitivity, specificity, and predictive values were assessed using qPCR as gold standard.

Results: Parasite prevalence was 17.5%, 24.5%, and 42.1% by microscopy, RDT, and *var*ATS qPCR respectively. Using qPCR as the standard, RDT was more sensitive (55.7% vs 39.3%), marginally less specific (98.2% vs 98.3%), and had higher positive (95.7% vs 94.5%) and negative predictive values (75.3% vs 69.0%) than microscopy. Parasite prevalence and density was higher among the 5-14 age group than the <5, 15-30, and >30 age groups across all the tests.

Conclusions: RDT outperformed microscopy for the diagnosis of *P. falciparum* malaria in the study area. However, both diagnostic methods missed over 40% of infections that were detected by *var*ATS qPCR. Novel tools are needed to ensure prompt diagnosis of all clinical malaria cases.

Introduction

Malaria remains a major public health concern, particularly in sub–Saharan Africa where >93% of global malaria cases and deaths occur annually. Disruptions in malaria services by the COVID-19 pandemic reportedly led to an estimated 14 million cases rise in global malaria burden and 47,000 additional deaths between 2019 and 2020. Even before the pandemic, malaria elimination progress had stalled since 2018, hence important milestones set for 2020 were missed (1). Prompt diagnosis and treatment have been identified as a critical component of the 3 pillars established by the Global Technical Strategy 2016-2030 to eliminate malaria (2). Consequently, the WHO recommends parasitological confirmation of all suspected malaria cases by either microscopy or RDT before treatment (3). The advantages of confirmed diagnosis include prevention of unnecessary use of antimalarial chemotherapy which in turn will slow down parasite resistance, and enable better disease management of non-malarial fevers (4). Accurate malaria diagnosis is also beneficial to interrupt disease transmission patterns to reduce malaria morbidity and mortality (5).

Malaria is endemic across all sixteen (16) administrative regions in Ghana with the entire population at risk. Transmission is year-round and heterogeneous among different ecological zones within the country – ranging from hyperendemicity in the Upper West Region, hypo-endemicity in Greater Accra, and meso-endemicity in the forest and southern coastal areas (6,7). Ghana has seen dramatic progress in its fight against malaria, evidenced by over 50% and 65% decline in morbidity and mortality respectively between 2005 and 2015 (8). Despite the efforts made to overcome malaria in Ghana, the disease remains the leading cause of

hospitalizations, accounting for over 30% and 23% of all outpatient and inpatient admissions respectively across the country (8).

The National Malaria Control Program in Ghana promotes the usage of 'gold' standard microscopy or RDTs for clinical malaria diagnosis (6). Microscopy is generally inexpensive to operate and is capable to distinguish between the various *Plasmodium* species. (9). Additionally, microscopy is useful to estimate parasite density, monitor drug efficacy and is capable of diagnosing other infections. However, the sensitivity of microscopy is variable and largely dependent on the expertise of the microscopist, good equipment, and the quality of staining reagents (3). The limit of detection of microscopy is generally estimated as 50-500 parasites/μL of blood (10) though the expert microscopist may detect 10 parasites/μl of blood (11). Microscopy is also known to perform poorly at low parasitemia, mixed infections and is too laborious for population-level surveillance (12).

The advent of RDTs and its adoption in Ghana has contributed to a significant reduction in presumptive diagnosis across the country (13). RDTs are lateral flow immuno-chromatographic assays that detect malaria-specific antigens using dye-labelled mono- or polyclonal antibodies which are immobilized on a nitrocellulose membrane (14). The resulting antigen-antibody complex produces a colorimetric reaction which appears as a visible line on the membrane (11). Most commercially produced RDTs target histidine-rich protein-2 (HRP-2), an antigen specific for *Plasmodium falciparum*, the predominant malaria parasite in sub-Saharan Africa and the deadliest of all the malaria parasites (12). RDTs provide quicker results (~15-20mins) than microscopy, are easy to perform, and do not require electricity, thus offering a good alternative in resource-limited settings (15). Nevertheless, RDTs are only qualitative and reportedly yield more false positives (than microscopy) due to the persistent circulation of antigens (HRP-2) even after parasite clearance by antimalarials (16). Studies have shown most RDTs to be less sensitive at low parasite densities (<200 parasites/μL), thus missing chronic latent infections in asymptomatic populations, particularly in low-transmission settings (17,18). The accuracy of RDT may also be limited by mutant or *hrp2* gene deletions, thus leading to false negatives (19,20).

Nucleic acid amplification tests (NAATs) are molecular-based diagnostic techniques that provide by far the highest sensitivity and specificity for malaria diagnosis. Polymerase Chain Reaction (PCR) and Loop-mediated Isothermal Amplification (LAMP) are the commonly used NAATs to detect parasite DNA in blood (21). The limit of detection of these molecular tests range between <1-5 parasites/μl, hence their ability to detect low-density infections (22,23). NAATs are however not used for routine diagnosis due to their expensive and sophisticated process. Use is restricted to identify latent infections and drug resistance studies during epidemiological surveys. They may also serve as reference standards to compare existing and/or new diagnostic tests (12).

Several studies have been conducted across Africa and Asia to compare the accuracy of microscopy and RDT using conventional or nested 18S rRNA PCR as reference (19,22,24–27). Recently, a novel qPCR assay that targets the *var* gene family of *P. falciparum* has been developed and proven to be 10 times more sensitive than the conventional 18S rRNA PCR methods used for comparing microscopy and RDT (28). Whiles most studies in Ghana have compared microscopy and RDTs using conventional PCR methods (13,29,30), there is currently no information on the performance of these traditional malaria diagnostic tools against the highly sensitive *var*ATS qPCR assay. This study provides baseline information on how the commonly used malaria tests in Ghana perform against *var*ATS qPCR.

Methods

Study Areas

The study was conducted at two primary health centers in the Ashanti Region of Ghana (Figure 1), namely Agona Government Hospital (AGH) and Mankranso Government Hospital (MGH). AGH is situated at Agona, the administrative capital of the Sekyere South District in Ghana, where it serves as the main referral health facility for surrounding villages and towns (latitude 6^0 50'N and 70 10'N and longitude 10 40'W and 10 25'W). About 47% of the inhabitants live in rural areas and 67% are involved in agriculture. The average rainfall pattern falls between 855mm and 1,500mm with a daily warm to hot temperature at about 27^0C (31).

Mankranso Government Hospital is located at Mankranso, the capital of the Ahafo Ano South West District (formerly Ahafo Ano South District) in the Ashanti Region of Ghana. According to the 2010 Population and Housing Census, about 90% of the total population live in rural areas. About 81.7% percent of indigenous households in Mankranso are engaged in crop farming whiles others are actively involved in poultry farming. The district is located at latitude $6^0$42' N and longitude $1^0$45'N and $2^0$20'W. Mankranso has a wet semi-equatorial climate with a mean monthly temperature between 26^{o}C – 28^{o}C with two major rainfall patterns in the district (32).

Study design

This was a cross-sectional hospital-based study conducted between January and June 2021. Sampling was done three days per week between the hours of 9 a.m. and 3 p.m. each day. Inclusion criteria for the study were suspected malaria patients (*i.e.* those referred to the laboratory for malaria diagnosis by clinicians) of all ages and gender. Written informed consent was obtained from all participants who were ≥18 years old. Parental or guardian consent was obtained for all those who were below 18 years old. Unsuspected malaria patients and participants who could not provide informed consent were excluded from the study.

Sample size

The sample size was pre-calculated as the minimum number of samples required to attain 95% sensitivity and specificity assuming a 5% margin of error at 95% confidence level (α = 0.05, power 1 – β = 0.20*)* as previously described (33–35). To calculate the final sample size, a previous prevalence of 25.96% (36) was used. After substituting in the variables, a minimum sample size of 379 was required to attain 95% sensitivity and 95% specificity respectively. The present study recruited a total of 1,040 participants – 458 and 582 from MGH and AGH respectively.

Study procedures

A well-structured questionnaire was administered to participants in layman's terms to capture socio-demographic, household characteristics, and clinical data. Afterwards, 2ml of venous blood was collected from participants into EDTA tubes. RDT diagnosis was immediately performed for all participants and blood smears (thick and thin) were prepared for microscopic diagnosis. Aliquots were also taken for diagnosis by qPCR and transported in cold boxes to the Vector-Borne Infectious Diseases laboratory at TAB-KNUST for storage and further analysis.

Laboratory investigations

Rapid diagnostic testing

The CareStart™ Malaria Pf (HRP2) Ag RDT (AccessBio, USA) kit was chosen for the present study since it passed WHO-recommended criteria for routine diagnosis (37). Briefly, the RDT was labelled with a unique participant code and date before 5µL of whole blood was pipetted unto the sample well, “S”, on the RDT cassette. Afterwards, two drops (60µL) of buffer solution provided by the manufacturer was added to the well labelled “A” on the cassette. Test results were recorded after twenty (20) minutes according to the manufacturer’s instructions.

Diagnosis by ‘gold’ standard microscopy

Duplicate thick and thin blood films were prepared for each participant on clean, well-labelled, frosted glass slides. Briefly, 2µL and 6µL of whole blood were pipetted to prepare thin and thick films respectively on the slide as recommended (38). The thin smears were fixed in absolute methanol after which the slides were arranged in slide boxes for further analysis. All slides were stained using 10% Giemsa working solution. Microscopy diagnosis was done by two independent WHO-certified experts. Both microscopists were blinded to the results of RDT and each other’s results. The Giemsa-stained blood films were imaged at the ×100 objective. Parasite detection was done by examining at least 100 high power fields. Parasite quantification was estimated based on the total number of malaria parasites counted in 200 or 500 white blood cells and then multiplied by 8,000 white blood cells (WBCs) as previously described (39). Slides were adjudged “positive” if positive by either or both of the microscopists. Final parasite density estimates were obtained by taking the average of parasitemia by both microscopists.

Molecular diagnosis by *var*ATS qPCR

DNA was extracted from 200 µL whole blood using the Macherey-Nagel NucleoMag extraction kit and eluted in 100 µL buffer. qPCR was done on a ThermoFisher QuantStudio 3 instrument in a total volume of 12 µL, including 4µL eluted DNA, corresponding to 8 µL blood using the highly sensitive, *P. falciparum var* acidic terminal sequence (*var*ATS) multi-copy gene assay. The reaction mixture was made of 1.28µL of PCR water, 6µL of 1× Perfect tough PCR MasterMix, 0.48µL of 10uM forward and reverse primers, 0.24µL of 10uM probe, and 4µL eluted DNA. The forward, reverse, and probe sequence used has been previously described (28). Cycling conditions of the qPCR consisted of pre-incubation at 50^0C for 2 minutes, initial denaturation at 95^0C for 2 minutes, final denaturation at 95^0C for 10 seconds, annealing, and elongation at 55^0C for 30 seconds. Amplification of the target gene was done at 45 cycles. Parasite density of the samples was estimated using a standard curve of purified parasite genomic DNA (gDNA) quantified by droplet digital PCR of a 10-fold dilution of 3D7 parasite culture (10^3, 10^2, 10^1, 10^0, and 10^{-1} copies/µl in double-distilled water). The limit of detection of qPCR was <0.1 parasites/µL blood.

Data Analysis

Data from the questionnaire and laboratory results were coded and entered using Microsoft Excel 2016. The data was analyzed using GraphPad Prism 9.0 (San Diego, California) at 95% confidence level and a significance at $p<0.05$. 2×2 contingency tables were drawn after which sensitivity, specificity, positive predictive value, and

negative predictive values were calculated. Descriptive statistics were performed for socio-demographic features which were represented as frequencies and percentages. Mean or median was used to summarize quantitative data. Parasite density across the age groups was computed using ANOVA (geometric mean) and post-hoc analysis using Tukey's multiple comparison test. The agreement between different diagnostic tests was calculated using the Kappa (κ) measure of inter-rater agreement. Briefly, κ < 0.20 indicated poor agreement, 0.21-0.40 fair, 0.41-0.60 moderate, 0.61-0.80 good, 0.81-0.99 very good and 1.00 indicate perfect agreement (40).

Results

Comparing diagnostic accuracy of RDT and Microscopy using qPCR as reference

Using highly sensitive *var*ATS qPCR as the reference standard, 438/1,040 patients were positive for *P. falciparum*. Out of the 438 patients positive by qPCR, RDT identified 244 (55.7%) infections whereas microscopy could detect only 172 (39.3%) true positives. Similar false positive rates were recorded in RDT, 11 (1.8%) and microscopy, 10 (1.7%) (Figure 3). Microscopy reported a higher false negative rate (60.7%), missing out on 266 qPCR-positive samples than RDT which had a false negative rate of 44.3%, failing to identify 194 qPCR-positive samples.

The sensitivity of microscopy and RDT was 39.3% and 55.7% respectively. Both tests had comparable specificity (98.2% vs 98.3%), whiles RDT reported higher PPV (95.7% vs 94.5%) and NPV (75.3% vs 69.0%) than microscopy. Ultimately, RDT showed "moderate" agreement with qPCR (κ=0.571), whereas microscopy showed "fair" (κ=0.409) agreement (Table 1). The better sensitivity of RDT was particularly pronounced at low parasite density (qPCR <200mps/µl) where RDT was found to be 4-fold more sensitive than microscopy (27.9% vs 6.6%) and once again showed better agreement (κ=0.326) with qPCR than microscopy (κ=0.067) (Table 2).

Table 1: Diagnostic accuracy of microscopy and RDT using qPCR as reference

Performance metric	Test	
	RDT	Microscopy
TP (qPCR = 438)	244	172
FP (qPCR negative)	11	10
TN (qPCR = 602)	591	592
FN (qPCR positive)	194	266
Sensitivity % (95% C.I.)	55.7 (51.0-60.3)	39.3 (34.8-43.9)
Specificity % (95% C.I.)	98.2 (96.8-99.0)	98.3 (97.0-99.1)
PPV % (95% C.I.)	95.7 (92.4-97.6)	94.5 (90.2-97.0)
NPV % (95% C.I.)	75.3 (72.2-78.2)	69.0 (65.8-72.0)
Accuracy %	80.3	73.5
kappa value (95% C.I.)	0.571 (0.523-0.620)	0.409 (0.359-0.458)

TP=True Positive; FP=False Positive; FN=False Negative; TN=True Negative; PPV=Positive Predictive Value; NPV=Negative Predictive Value

Table 2: Diagnostic accuracy of microscopy and RDT at low parasite density (qPCR<200p/µL)

Performance metric	Test	
	RDT	Microscopy
TP (qPCR = 244)	68	16
FP (qPCR negative)	11	10
TN (qPCR = 602)	591	592
FN (qPCR positive)	176	228
Sensitivity % (95% C.I.)	27.9 (22.6-33.8)	6.6 (4.1-10.4)
Specificity % (95% C.I.)	98.2 (96.7-99.0)	98.3 (97.0-99.1)
PPV % (95% C.I.)	86.1 (76.8-92.0)	61.5 (42.5-77.6)
NPV % (95% C.I.)	77.1 (74.0-79.9)	72.2 (69.0-75.2)
Accuracy %	77.9	71.9
kappa value (95% C.I.)	0.326 (0.260-0.392)	0.067 (0.023-0.111)

TP=True Positive; FP=False Positive; FN=False Negative; TN=True Negative; PPV=Positive Predictive Value; NPV=Negative Predictive Value

Accuracy of RDT and microscopy across different age groups using qPCR as reference

Across the different age groups, similar sensitivity and specificity trends were observed by both RDT and microscopy. In both tests, sensitivity and specificity were highest in the 5-14 age group followed by the <5, 15-30, and >30 age group in decreasing order. Both tests recorded >94% specificity across the different age groups (Table 3).

Table 3: Diagnostic accuracy of microscopy and RDT across the age groups

Age group (years)	Sensitivity %	Specificity %	PPV %	NPV %
RDT				
<5	67.0 (57.2-75.6)	99.1 (95.2-100.0)	98.5 (91.9-100.0)	77.9 (70.5-83.4)
5-14	87.8 (78.5-93.5)	94.6 (85.2-98.5)	95.6 (87.8-98.8)	85.3 (74.3-92.0)
15-30	48.7 (40.9-56.5)	98.1 (95.2-99.3)	94.9 (87.7-98.0)	72.2 (66.7-77.1)
>30	34.5 (26.4-43.7)	98.7 (96.1-99.6)	92.9 (81.0-97.5)	74.9 (69.7-79.5)
Microscopy				
<5	44.3 (34.9-54.2)	99.1 (95.2-100.0)	97.7 (88.2-100.0)	67.7 (60.2-74.3)
5-14	60.8 (49.4-71.1)	98.2 (90.4-100.0)	97.8 (88.7-99.9)	65.1 (54.3-74.4)
15-30	37.0 (29.8-44.9)	99.0 (96.6-99.8)	96.6 (88.5-99.4)	68.1 (62.7-73.1)
>30	23.9 (17.0-32.5)	97.3 (94.3-98.8)	81.8 (65.6-91.4)	71.7 (66.4-76.5)

Values in the bracket indicate the 95% confidence interval

Parasite prevalence by microscopy, RDT, and *var*ATS qPCR

All 1,040 samples were tested for malaria by RDT, microscopy, and qPCR. Unsurprisingly, higher parasite prevalence was detected by qPCR (42.1%) than RDT (24.5%) and microscopy (17.5%). By qPCR, higher parasite prevalence was recorded at MGH than AGH (Table 4).

Table 4: Malaria prevalence by RDT, microscopy, and *var*ATS qPCR

Test	Study area		Total (n=1040)
	Mankranso (n=458)	Agona (n=582)	
	MP+ (%)	MP+ (%)	MP+ (%)
RDT	104 (22.7)	151 (25.9)	255 (24.5)
Microscopy	66 (14.4)	116 (19.9)	182 (17.5)
qPCR	195 (42.6)	243 (41.8)	438 (42.1)

MP+ = Malaria positive

Parasite prevalence across the age groups

All three different tests detected high positivity rate among the 5-14 age group, with values that were distinctly higher than the other age groups (Figure 4). The lowest prevalence was recorded in the >30 age group by all three tests.

Parasite density by *var*ATS qPCR across the age groups

A significant difference ($p<0.0001$) in parasite density estimation was observed across the age groups. The geometric mean parasite density in the 5-14 age group (1246p/µl) was 7-fold higher than in children <5years

(166.8p/µl), and 100-fold higher than that of adults >30 years (12.4p/µl). The 15-30 age group had a geometric mean parasite density of 84.3p/µl. Figure 5 illustrates $\log_{10}$ transformation of parasite density by qPCR across the age groups.

Socio-demographic characteristics of the study population

Of the 1,040 participants recruited for the study, 458 (44.0%) were obtained from Mankranso Government Hospital (MGH) with the remaining 582 (56.0%) from the Agona Government Hospital (AGH). All data is presented for both sites combined. Participants' age ranged between 3 weeks and 96 years with a median age of 22 years (IQR=7 – 36 years). The majority of the study populace fell within the 15-30 age group (34.9%), followed closely by the >30 (32.4%), <5 (20.3%), and 5-14 (12.4%) age groups in descending order. Females were more than twice (70.7%) the number of males involved in the study (29.3%). Studentship/apprenticeship was the predominant occupation (33.0%) of the participants whereas civil servants (6.1%) were the least represented in the study. The demographics of the participants are summarized in Table 5 below:

Table 5: Socio-demographic features of the study population

Baseline characteristics	Study area		Both sites, n (%)
	Mankranso, n (%)	Agona, n (%)	
Gender			
Male	114 (24.9)	191 (32.8)	305 (29.3)
Female	344 (75.1)	391 (67.2)	735 (70.7)
Age group (years)			
<5	74 (16.2)	137 (23.5)	211 (20.3)
5-14	28 (6.1)	101 (17.4)	129 (12.4)
15-30	209 (45.6)	154 (26.5)	363 (34.9)
>30	147 (32.1)	190 (32.6)	337 (32.4)
Occupation			
Farming	57 (12.6)	64 (11.0)	121 (11.7)
Trading	52 (11.5)	85 (14.6)	137 (13.2)
Civil servant	46 (10.2)	17 (2.9)	63 (6.1)
Student	114 (25.2)	227 (39.0)	341 (33.0)
Unemployed	83 (18.4)	44 (7.6)	127 (12.3)
Other	100 (22.1)	145 (24.9)	245 (23.7)
ITN usage			
Yes	275 (61.0)	215 (37.0)	490 (47.5)
No	176 (39.0)	366 (63.0)	542 (52.5)
History of fever			
Yes	307 (67.0)	386 (66.3)	693 (66.6)
No	151 (33.0)	196 (33.7)	347 (33.4)

Discussion

Prompt and accurate diagnosis of malaria is the fundamental first step to effectively identify, treat and interrupt disease transmission (5). An excellent modelling analysis predicted that >100,000 malaria-associated deaths would be averted by a 95% sensitive and 95% specific diagnostic tool that require minimal infrastructure. According to the model, >300,000 malaria-related deaths and ~450 million unnecessary treatments may be prevented by a 90% sensitive and 90% specific diagnostic test that require no infrastructure (41).

In Ghana, like most African countries, gold standard microscopy and RDTs are commonly used for routine malaria diagnosis in healthcare centers. Whiles extensive studies have compared microscopy and RDT based on standard 18S rRNA PCR results, this study is the first in Ghana to make comparisons using *var*ATS qPCR as

reference in a clinical setting. Most *P. falciparum* strains possess relatively fewer 18S ribosomal subunits (5-8 copies/genome) (42) than the multi-copy *var* gene family which has approximately 59-60 copies/genome (43,44). *var*ATS qPCR therefore has a very low limit of detection (~0.03 – 0.15 parasites/μL) and has been proven to be 10-fold more sensitive than traditional 18S rRNA PCR methods (28).

Comparisons between microscopy, RDT, and PCR need to consider vastly different limits of detection of qPCR. Extraction from whole blood results in better sensitivity compared to dried blood spots (45). The corresponding volume of blood screened by qPCR ranges from <0.1 μL (46) to ≥200 μL (47) when DNA is concentrated during extraction. Multi-copy genes such as *var*ATS offer superior sensitivity compared to single copy genes (48). As a result, the limit of detection differs across several orders of magnitude. A more sensitive PCR will result in more low-density infections being detected, and thus a lower sensitivity of microscopy or RDT compared to PCR. For the current study, we have extracted DNA from whole blood, screened 8 μL of whole blood, and amplified a multi-copy target, resulting in a very low limit of detection.

The present study revealed that microscopy missed almost two-thirds (60.7%) of infections, whereas RDT missed nearly half (44.3%) of qPCR-positive cases. This corroborates a previous study where microscopy missed 169 (57%) *var*ATS qPCR-positive infections (28). A similar study in Nigeria which compared microscopy and CareStart RDT used in this study revealed microscopy missed a higher proportion (30%) of *var*ATS qPCR-positive infections than RDT (12%) (49).

False negatives are of huge concern since failure to treat diseased persons may lead to continued disease transmission, increased morbidity, and possibly mortality, especially in children under five and pregnant women (19). The high false negative rate observed in our study may be explained by the relatively low detection limit of *var*ATS qPCR, hence its ability to detect low-density infections that were missed by microscopy and RDT. The false negatives observed in RDT may also be attributed to possible *hrp2* gene deletions which distort the expression of target antigens detected by RDT (11,14). Whiles *hrp2* gene deletions have been reported in a nationwide survey in Ghana (51), a recent study found no *hrp2* deletions at the two sites where the present study was held (20). Alternatively, the false negatives in microscopy may be attributed to the quality of the smearing and/or staining process as well as the level of expertise of the microscopists (11). Though WHO-certified experts read the slides, there is still the possibility of missing infections below the detection limit of microscopy.

Fewer false positives were reported in microscopy (10) than RDT (11) in our study. False positive HRP2-based RDT results have been associated with the persistent circulation of hrp2 antigen (up to two weeks) even after parasite clearance, a phenomenon that may explain our findings (12,14,52). Other potential causes of false-positive RDT results include persistence of clinically irrelevant gametocytes and cross-reactions with other non-*P. f*alciparum species (14). The false positives in microscopy may be due to misidentification of artifacts as parasites (53). Another plausible explanation is the inherent inability of our qPCR assay to identify non-*P. falciparum* infections that may have been detected by microscopy. In the present study, there was a single case of *P. ovale* microscopy-positive infection that was missed by our qPCR assay and RDT (Supplementary data).

From our study, RDT was more sensitive, marginally less specific, and was a better predictor of malaria than microscopy (Table 1&2). The relatively higher sensitivity of RDT could be attributed to the fact that RDT targets antigens (hrp2/hrp3) expressed by parasites and not actual parasites as in the case of microscopy, hence may

be able to pick up low density infections which may have been missed by microscopy (52). RDT showed overall higher accuracy and relatively better agreement (κ=0.571) with *var*ATS qPCR than microscopy (κ=0.409). Our findings were consistent with a community-based study in Nigeria where RDT was more sensitive (73.9% vs 63.0%) and strongly agreed (κ=0.74 vs κ=0.67) with *var*ATS qPCR than microscopy. The study in Nigeria, also reported microscopy to be more specific than RDT (99.5% vs 97%), similar to our findings, albeit a marginal difference was observed in the present study (49).

The present study also shed light on parasite prevalence at Mankranso and Agona in the Ashanti Region of Ghana. There was marginally higher parasite prevalence by qPCR at Mankranso than Agona (Table 4) despite the majority of the inhabitants in the former sleeping under ITNs (Table 5). Other socio-demographic factors such as the presence of stagnant water and/or household characteristics may have accounted for the occurrence. Our study found high parasite prevalence and corresponding high parasite density in the 5-14 age group followed in descending orders by the <5, 15-30, and >30 age groups (Figure 4&5). This finding corroborates earlier findings in Nigeria (49) where children within the 6-14 age groups presented with high parasite prevalence and density. The 5-14 age group are generally categorized as school-age children and may be exposed to more infectious bites due to their over-engagement in outdoor activities and little compliance to sleeping under ITNs as compared to the <5 age group who are likely to sleep in ITN with their parents (54), and to some extent protected by maternal antibodies in the first few months of life (55,56). Again, it is worth noting from the present study that parasite prevalence and density reduced with increasing age (Figure 4&5). Lower parasite prevalence and parasite density was observed in the older >30 age group as compared to the younger <5 and 5-14 age groups. A plausible interpretation for this occurrence is the development of acquired immunity in the older >30 age group due to frequent exposure, hence giving them the ability to control parasite multiplication to levels that may be undetectable by microscopy and RDT (57). It was also observed that the highest sensitivity and specificity values were observed in the 5-14 age group who presented with the highest parasite prevalence and parasite density (Table 3). Conversely, sensitivity decreased with decreasing parasite density and increasing age group. The decrease in sensitivity with increasing age group has been reported in previous studies (19,57). On the other hand, specificity and PPV did not appear to be affected by age group, a finding which contradicts earlier studies (19,57).

In the present study, the generally high PPV (>93%) but low NPV (<76%) indicate a positive RDT or microscopy test is a good predictor of malaria however, a negative test may not necessarily indicate no infection (58,59). This suggests clinicians should be cautious in completely ruling out malaria solely based on a negative RDT or microscopy result, especially in cases where there is high clinical suspicion. To effectively test, treat and interrupt malaria transmission, RDTs should be further optimized and rigorous refresher training given to microscopists to facilitate prompt and accurate detection of all malaria infections.

The focus of the present study was restricted to *P. falciparum,* thus the RDT and qPCR used are designed to detect *Plasmodium falciparum* infections which may have had an impact on parasite prevalence and density estimates. However, in Ghana, like most African countries, *P. falciparum* is responsible for over 95% of all malaria infections.

Conclusion

Even though RDT and microscopy are routinely used for point-of-care diagnosis, both of them miss a substantial number of infections at low parasitemia. This may not be problematic in very high transmission areas, since most adults would be carrying some parasites with no fever. But in children under five years and low transmission settings, this could have dire consequences. There is thus a critical need for improved diagnosis. It is recommended that refresher training be held for laboratory personnel to improve malaria microscopy, and for manufacturers to provide even highly sensitive rapid diagnostic tests to facilitate the quick diagnosis and treatment of malaria.

Abbreviations

RDT: Rapid diagnostic test; qPCR: Quantitative polymerase chain reaction; varATS: *var* gene acidic terminal sequence; HRP-2: Histidine Rich Protein-2; AGH: Agona Government Hospital; MGH: Mankranso Government Hospital; TAB-KNUST: Department of Theoretical and Applied Biology-Kwame Nkrumah University of Science and Technology; ITN: Insecticide-Treated Net; PPV: Positive Predictive Value; NPV: Negative Predictive Value

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the Committee on Human Research, Publications, and Ethics (CHRPE/AP/030/20) of the School of Medical Sciences, KNUST. Permission for the study was sought and obtained from the management of Agona and Mankranso Government Hospital. Informed consent was obtained for all participants involved in the study.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during the current study are included in this published article [and its supplementary information files]. Additional data may be found at Havard Dataverse https://dataverse.harvard.edu/dataverse/harvard

Competing interests

No competing interest

Funding

This project (TMA2016CDF 1605). is part of the EDCTP2 programme supported by the European Union".

Authors' contributions

KB and CK conceived and designed the study. SOA analyzed the data, interpreted the findings, and wrote the paper. YG performed the molecular tests. KBA and A-HM were involved in data collection, entry, and

review/editing of the manuscript. TKA, KAA, DAA, and AT played a role in data collection and laboratory processing of samples. All authors read and approved the final manuscript.

Acknowledgements

We appreciate the health directorate of Agona and Mankranso Government hospitals for cooperation as well as all study participants.

References

1. World Health Organization. World Malaria Report 2021. 2021.

2. World Health Organization. Global Technical Strategy for Malaria 2016–2030. Global Malaria Programme; 2015. 9–14 p.

3. World Health Organization. Malaria microscopy quality assurance manual – Ver. 2. 2nd ed. 2016. 140 p.

4. Ngasala B, Bushukatale S. Evaluation of malaria microscopy diagnostic performance at private health facilities in Tanzania. Malar J [Internet]. 2019;18(1):1–7. Available from: https://doi.org/10.1186/s12936-019-2998-1

5. Landier J, Parker DM, Thu AM, Carrara VI, Lwin KM, Bonnington CA, et al. The role of early detection and treatment in malaria elimination. Malar J. 2016;15(363):1–8.

6. NMCP. National Malaria Control Programme Ghana Malaria Programme Review. 2013;(June):24. Available from: https://www.ghanahealthservice.org/downloads/ghana_malaria_programme_review_final_report_june_2013.pdf

7. Awine T, Malm K, Bart-Plange C, Silal SP. Towards malaria control and elimination in Ghana: Challenges and decision making tools to guide planning. Glob Health Action [Internet]. 2017;10(1). Available from: https://doi.org/10.1080/16549716.2017.1381471

8. Shretta R, Silal SP, Malm K, Mohammed W, Narh J, Piccinini D, et al. Estimating the risk of declining funding for malaria in Ghana : the case for continued investment in the malaria response. Malar J [Internet]. 2020;19(196):1–15. Available from: https://doi.org/10.1186/s12936-020-03267-9

9. Anchimane VT, Shedge RT. A Review of Malaria Diagnostic Tools: Microscopy and Rapid Diagnostic Test. Asian J Med Sci. 2010;1:75–9.

10. Moody A. Rapid Diagnostic Tests for Malaria Parasites. Clin Microbiol Rev. 2002;15(1):66–78.

11. Varo R, Balanza N, Mayor A, Bassat Q. Diagnosis of clinical malaria in endemic settings. Expert Rev Anti Infect Ther [Internet]. 2020;8–20. Available from: https://doi.org/10.1080/14787210.2020.1807940

12. Mathison BA, Pritt BS. Update on Malaria Diagnostics and Test Utilization. J Clin Microbiol. 2017;55(7):1–9.

13. Quakyi IA, Adjei GO, Sullivan DJ, Laar A, Stephens JK, Owusu R, et al. Diagnostic capacity, and predictive values of rapid diagnostic tests for accurate diagnosis of Plasmodium falciparum in febrile children in Asante-Akim, Ghana. Malar J [Internet]. 2018;17(1):1–9. Available from: https://doi.org/10.1186/s12936-018-2613-x

14. Mukkala AN, Kwan J, Lau R, Harris D, Kain D, Boggild AK. An Update on Malaria Rapid Diagnostic Tests. Curr Infect Dis Rep. 2018;20(12):1–8.

15. Nijhuis RHT, van Lieshout L, Verweij JJ, Claas ECJ, Wessels E. Multiplex real-time PCR for diagnosing malaria in a non-endemic setting: a prospective comparison to conventional methods. Eur J Clin Microbiol Infect Dis. 2018;37(12):2323–9.

16. Osman MMM, Nour BYM, Sedig MF, De Bes L, Babikir AM, Mohamedani AA, et al. Informed decision-making before changing to RDT: a comparison of microscopy, rapid diagnostic test and molecular techniques for the diagnosis and identification of malaria parasites in Kassala, eastern Sudan. Trop Med Int Heal. 2010;15(12):1442–8.

17. Tambo M, Mwinga M, Mumbengegwi DR. Loop-mediated isothermal amplification (LAMP) and Polymerase Chain Reaction (PCR) as quality assurance tools for Rapid Diagnostic Test (RDT) malaria diagnosis in Northern Namibia. PLoS One. 2018;13(12):1–8.

18. Doctor SM, Liu Y, Whitesell A, Thwai KL, Taylor SM, Janko M, et al. Malaria surveillance in the Democratic Republic of the Congo: Comparison of microscopy, PCR, and rapid diagnostic test. Diagn Microbiol Infect Dis [Internet]. 2016;85(1):16–8. Available from: http://dx.doi.org/10.1016/j.diagmicrobio.2016.01.004

19. Berzosa P, De Lucio A, Romay-Barja M, Herrador Z, González V, García L, et al. Comparison of three diagnostic methods (microscopy, RDT, and PCR) for the detection of malaria parasites in representative samples from Equatorial Guinea 11 Medical and Health Sciences 1108 Medical Microbiology. Malar J [Internet]. 2018;17(1):1–12. Available from: https://doi.org/10.1186/s12936-018-2481-4

20. Vera-Arias CA, Holzschuh A, Oduma CO, Badu K, Abdul-Hakim M, Yukich J, et al. Plasmodium falciparum hrp2 and hrp3 gene deletion status in Africa and South America by highly sensitive and specific digital PCR. 2021;

21. Bell D, Fleurent AE, Hegg MC, Boomgard JD, McConnico CC. Development of new malaria diagnostics: matching performance and need. Malar J. 2016;15(1):1–12.

22. Ita OI, Otu AA, Onyedibe K, Iwuafor AA, Banwat E, Egah DZ. A diagnostic performance evaluation of rapid diagnostic tests and microscopy for malaria diagnosis using nested polymerase chain reaction as reference standard in a tertiary hospital in Jos, Nigeria. Trans R Soc Trop Med Hyg. 2018;112(10):436–42.

23. Thongdee P, Chaijaroenkul W, Kuesap J, Na-Bangchang K. Nested-PCR and a new ELISA-based NovaLisa test kit for malaria diagnosis in an endemic area of Thailand. Korean J Parasitol. 2014;52(4):377–82.

24. Faye B, Nath-Chowdhury M, Clément Tine R, Louis Ndiaye J, Sylla K, Wasquez Camargo F, et al. Accuracy of HRP2 RDT (Malaria Antigen P.f®) compared to microscopy and PCR for malaria diagnosis in Senegal. Pathog Glob Health. 2013;107(5):273–8.

25. Haanshuus CG, Chandy S, Manoharan A, Vivek R, Mathai D, Xena D, et al. A high malaria prevalence identified by PCR among patients with acute undifferentiated fever in India. PLoS One. 2016;11(7):1–13.

26. Abdalla ZA, Rahma NEA, Hassan EE, Abdallah TM, Hamad HE, Omer SA, et al. The diagnostic performance of rapid diagnostic tests and microscopy for malaria diagnosis in eastern Sudan using a nested polymerase chain reaction assay as a reference standard. Trans R Soc Trop Med Hyg. 2019;113(11):701–5.

27. Mahende C, Ngasala B, Lusingu J, Yong TS, Lushino P, Lemnge M, et al. Performance of rapid diagnostic test, blood-film microscopy and PCR for the diagnosis of malaria infection among febrile children from Korogwe District, Tanzania. Malar J. 2016;15(1):1–7.

28. Hofmann N, Mwingira F, Shekalaghe S, Robinson LJ. Ultra-Sensitive Detection of Plasmodium falciparum by Amplification of Multi-Copy Subtelomeric Targets. 2015;1–21.

29. Acquah FK, Donu D, Obboh EK, Bredu D, Mawuli B, Amponsah JA, et al. Diagnostic performance of an ultrasensitive HRP2 - based malaria rapid diagnostic test kit used in surveys of afebrile people living in Southern Ghana. Malar J [Internet]. 2021;1–11. Available from: https://doi.org/10.1186/s12936-021-03665-7

30. Osei-yeboah J, Norgbe GK, Lokpo SY, Kinansua MK, Nettey L, Allotey EA. Comparative Performance Evaluation of Routine Malaria Diagnosis at Ho Municipal Hospital. 2016;2016.

31. Ghana Statistical Service. District Analytical Report: Sekyere South District. 2014.

32. Ghana Statistical Service. District Analytical Report: Ahafo Ano South District. 2014.

33. Negida A, Fahim NK, Negida Y. Sample Size Calculation Guide - Part 4 : How to Calculate the Sample Size for a Diagnostic Test Accuracy Study based on Sensitivity, Specificity , and the Area Under the ROC Curve. Adv J Emerg Med. 2019;3(3):3–5.

34. Hajian-Tilaki K. Sample size estimation in diagnostic test studies of biomedical informatics. J Biomed Inform [Internet]. 2014;48:193–204. Available from: http://dx.doi.org/10.1016/j.jbi.2014.02.013

35. Tay SC, Badu K, Mensah AA, Gbedema SY. The prevalence of malaria among HIV seropositive individuals andd the impact of the co-infection on their hemoglobin levels. Ann Clin Microbiol Antimicrob. 2015;14(10).

36. Paintsil EK, Omari-sasu AY, Addo MG, Boateng MA. Analysis of Haematological Parameters as Predictors of Malaria Infection Using a Logistic Regression Model : A Case Study of a Hospital in the Ashanti Region of Ghana. 2019;2019.

37. World Health Organization. Recommended selection criteria for procurement of malaria rapid diagnostic tests. 2018.

38. World Health Organization. Collection of Finger-Prick Blood and Preparation of Thick and Thin Blood Films. 2016. p. 1–4.

39. World Health Organization. Malaria Parasite Counting. In: Malaria Microscopy Standard Operating Procedure. 2016. p. 1–5.

40. McHugh ML. Interrater reliability: the kappa statistic. J Croat Soc Med Biochem Lab Med. 2012;22(3):276–82.

41. Rafael AME, Taylor T, Magill A, Lim Y, Girosi F, Allan R. Reducing the burden of childhood malaria in Africa : the role of improved diagnostics. Nature [Internet]. 2006;39–48. Available from: www.nature.com/diagnostics

42. Grabias B, Essuman E, Quakyi IA, Kumar S. Sensitive real - time PCR detection of Plasmodium falciparum parasites in whole blood by erythrocyte membrane protein 1 gene amplification. Malar J [Internet]. 2019;18(116):1–9. Available from: https://doi.org/10.1186/s12936-019-2743-9

43. Dimonte S, Bruske EI, Enderes C, Otto TD, Turner L, Kremsner P, et al. Identification of a conserved var gene in different Plasmodium falciparum strains. Malar J [Internet]. 2020;19(194):1–15. Available from: https://doi.org/10.1186/s12936-020-03257-x

44. Claessens A, Hamilton WL, Kekre M, Otto TD, Faizullabhoy A, Rayner JC, et al. Generation of Antigenic Diversity in Plasmodium falciparum by Structured Rearrangement of Var Genes During Mitosis. PLoS Genet. 2014;10(12).

45. Strøm GEA, Tellevik MG, Hanevik K, Langeland N, Blomberg B. Comparison of four methods for extracting DNA from dried blood on filter paper for PCR targeting the mitochondrial Plasmodium genome. Malar J. 2014;13(137):488–94.

46. Russell TL, Grignard L, Apairamo A, Kama N, Bobogare A, Drakeley C, et al. Getting to zero : micro - foci of malaria in the Solomon Islands requires stratified control. Malar J [Internet]. 2021;20(248):1–9. Available from: https://doi.org/10.1186/s12936-021-03779-y

47. Imwong M, Hanchana S, Malleret B, Rénia L, Day NPJ, Dondorp A, et al. High-Throughput Ultrasensitive Molecular Techniques for Quantifying Low-Density Malaria Parasitemias. J Clin Microbiol. 2014;52(9):3303–9.

48. Oyedeji SI, Awobode HO, Monday GC, Kendjo E, Kremsner PG, Kun JF. Comparison of PCR-based detection of Plasmodium falciparum infections based on single and multicopy genes. Malar J. 2007;6(112):1–6.

49. Umunnakwe FA, Idowu ET, Ajibaye O, Etoketim B, Akindele S, Shokunbi AO, et al. High cases of submicroscopic Plasmodium falciparum infections in a suburban population of Lagos , Nigeria. Malar J [Internet]. 2019;1–8. Available from: https://doi.org/10.1186/s12936-019-3073-7

50. Dinko B, Djanie RA, Abugri J, Agboli E, Duodu GK, Tagboto S, et al. Comparison of malaria diagnostic methods in four hospitals in the Volta region of Ghana. 2016;7(5):1–7.

51. Eva L, Id A, Abuaku B, Bukari AH, Dickson D, Amoako EO, 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. 2020;134:1–16. Available from: http://dx.doi.org/10.1371/journal.pone.0238749

52. Mfuh KO, Achonduh-Atijegbe OA, Bekindaka ON, Esemu LF, Mbakop CD, Gandhi K, et al. A comparison of thick-film microscopy, rapid diagnostic test, and polymerase chain reaction for accurate diagnosis of Plasmodium falciparum malaria. Malar J [Internet]. 2019;18(1):1–8. Available from: https://doi.org/10.1186/s12936-019-2711-4

53. Ohrt C, Purnomo M, Sutamihardja A, Tang D, Kain KC. Impact of microscopy error on protective efficacy estimates in malaria prevention trials. Clin Pharmacol Ther. 2002;65(2):134.

54. Walldorf JA, Cohee LM, Coalson JE, Bauleni A, Nkanaunena K, Kapito-Tembo A. School-age children are a reservoir of malaria infection in Malawi. PLoS One. 2015;1–13.

55. Kangoye DT, Nebie I, Yaro JB, Debe S, Traore S, Ouedraogo O, et al. Plasmodium falciparum malaria in children aged 0-2 Years: The role of foetal haemoglobin and maternal antibodies to two asexual malaria vaccine candidates (MSP3 and GLURP). PLoS One. 2014;9(9).

56. Dobbs KR, Dent AE. Plasmodium malaria and antimalarial antibodies in the first year of life. Parasitology. 2016;143(2):129–38.

57. Siahaan L, Panggabean M, Panggabean YC. RDT accuracy based on age group in hypoendemic malaria. In: IOP Conference Series Earth and Environmental Science. 2018.

58. Safari S, Baratloo A, Elfil M, Negida A. Evidence Based Emergency Medicine Part 3: Positive and Negative Likelihood Ratios of Diagnostic Tests. Emergency [Internet]. 2015;3(3):87–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26495411%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4608341

59. Trevethan R. Sensitivity, Specificity, and Predictive Values: Foundations, Pliabilities, and Pitfalls in Research and Practice. Front Public Heal. 2017;5(November):1–7.

Supplementary data

Supplementary Data is not available with this version.

Figures

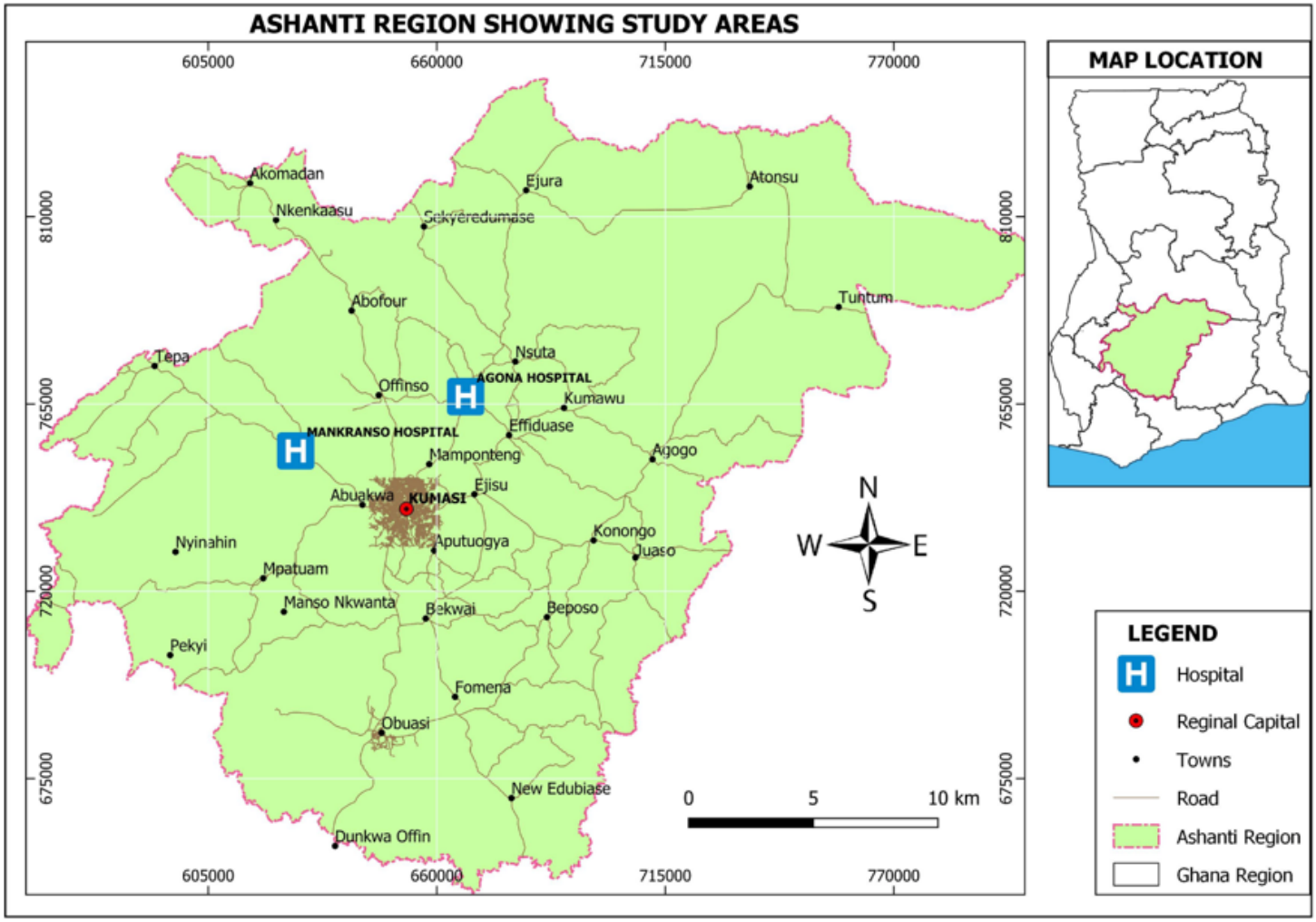

Figure 1

A map showing the location of the study areas in the Ashanti Region of Ghana. [The map was created by Mr. Ema Dari of the Department of Geography and Rural Development, KNUST using ArcGIS Desktop 10.6.1 software]

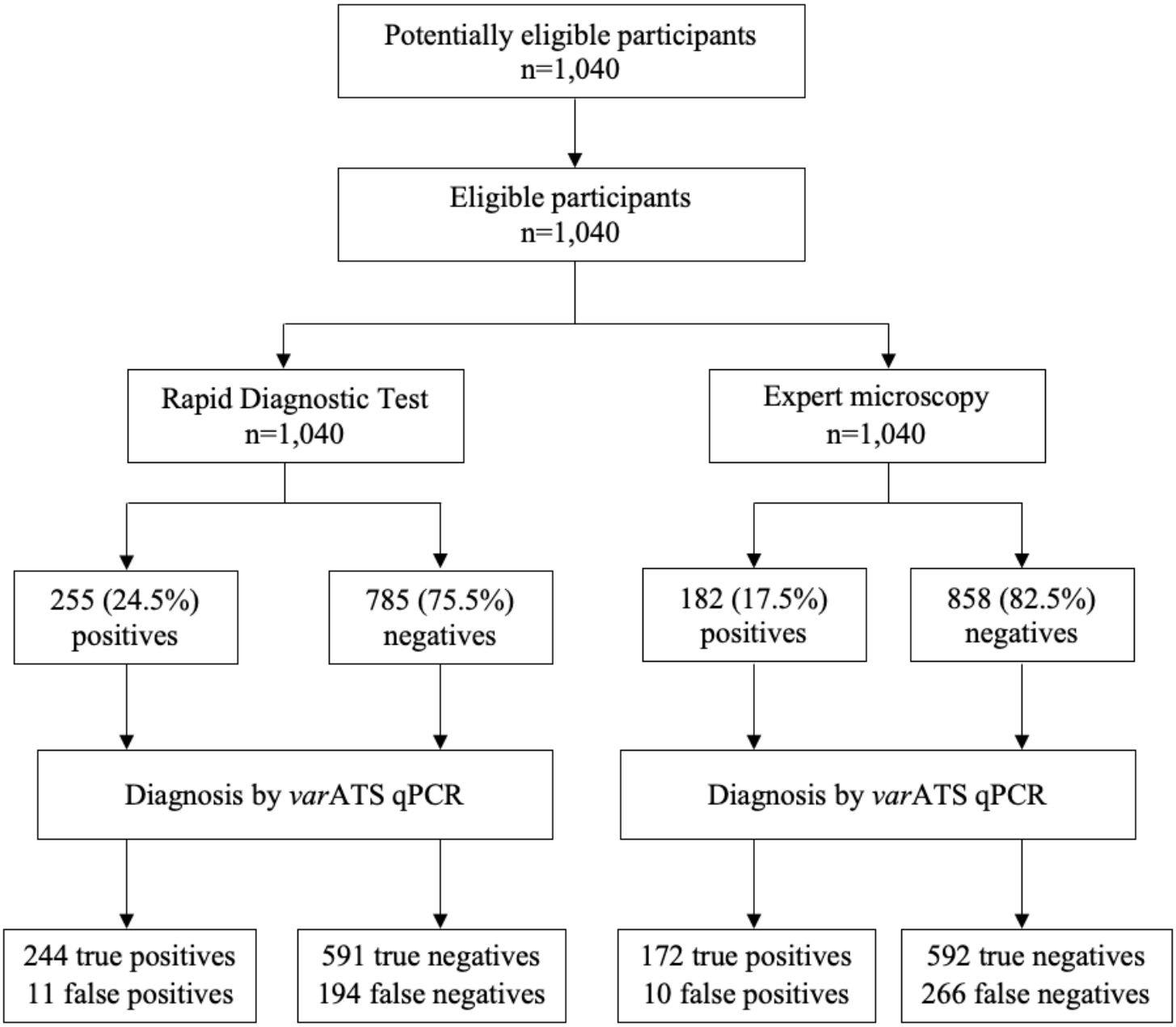

Figure 2

Flow chart describing participant recruitment and diagnostic tests performed

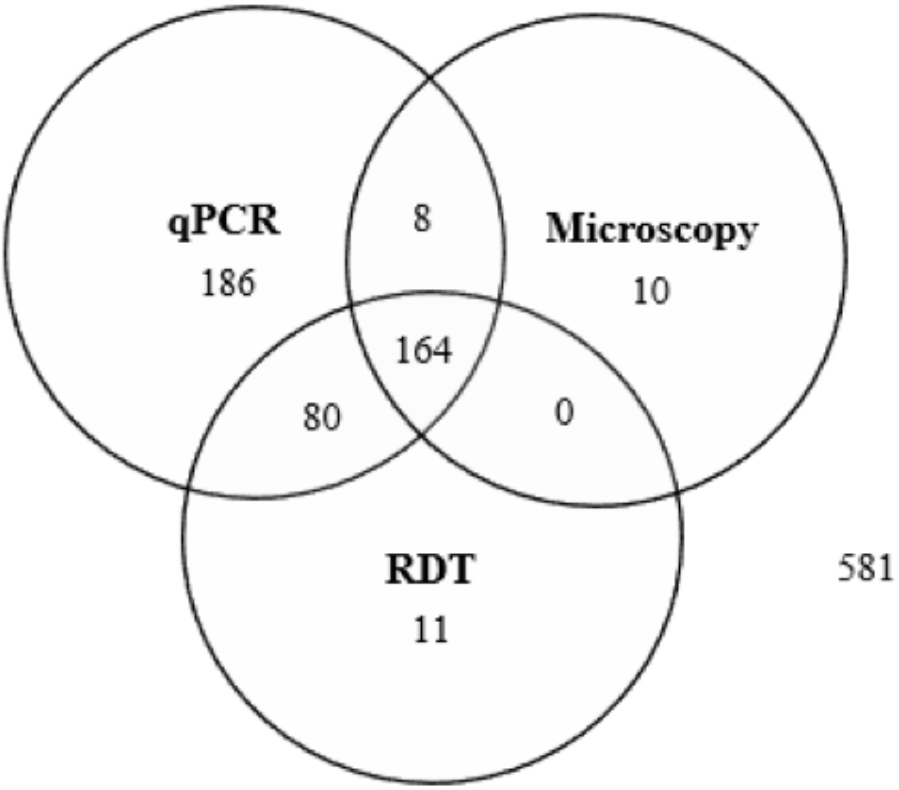

Figure 3

Detection of *P. falciparum* by microscopy, RDT, and *var*ATS qPCR

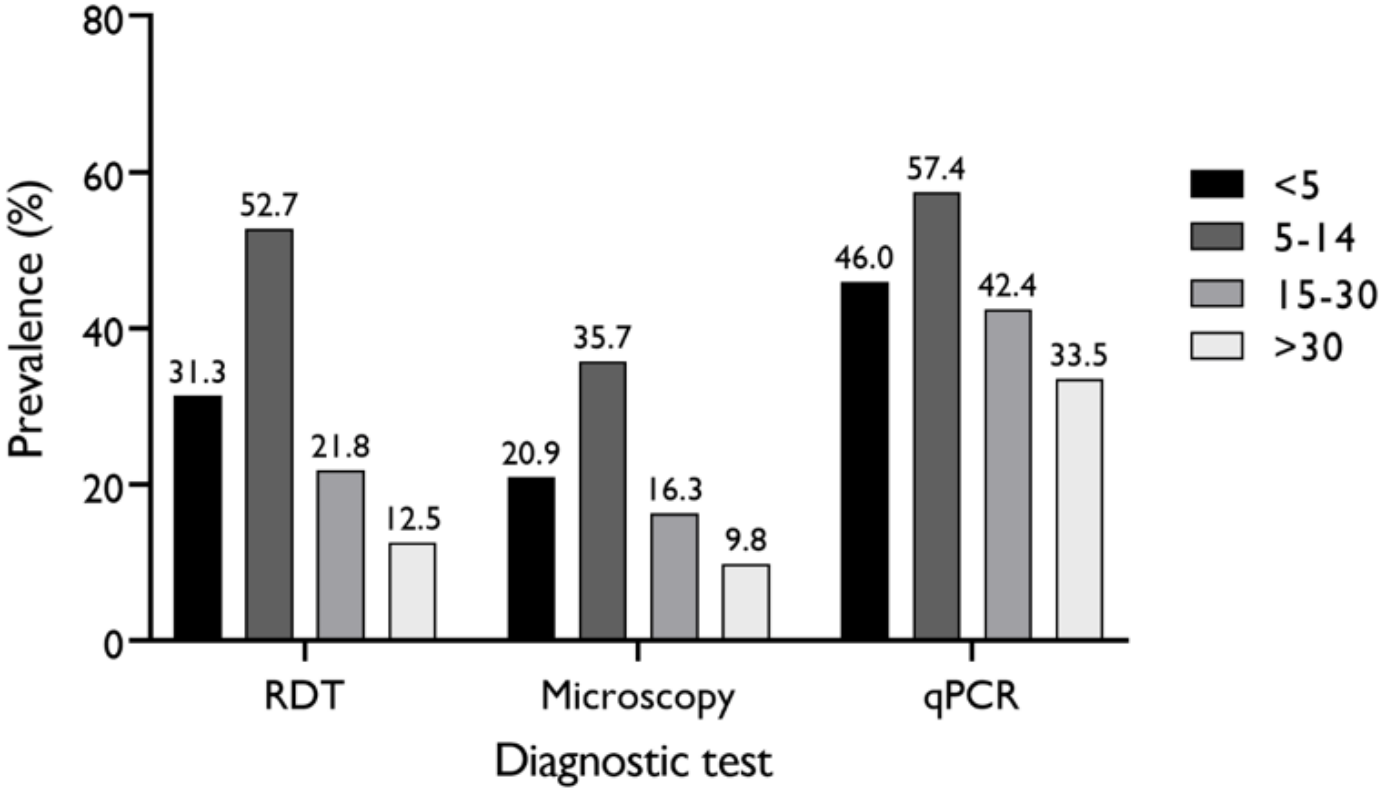

Figure 4

Parasite prevalence across different age groups

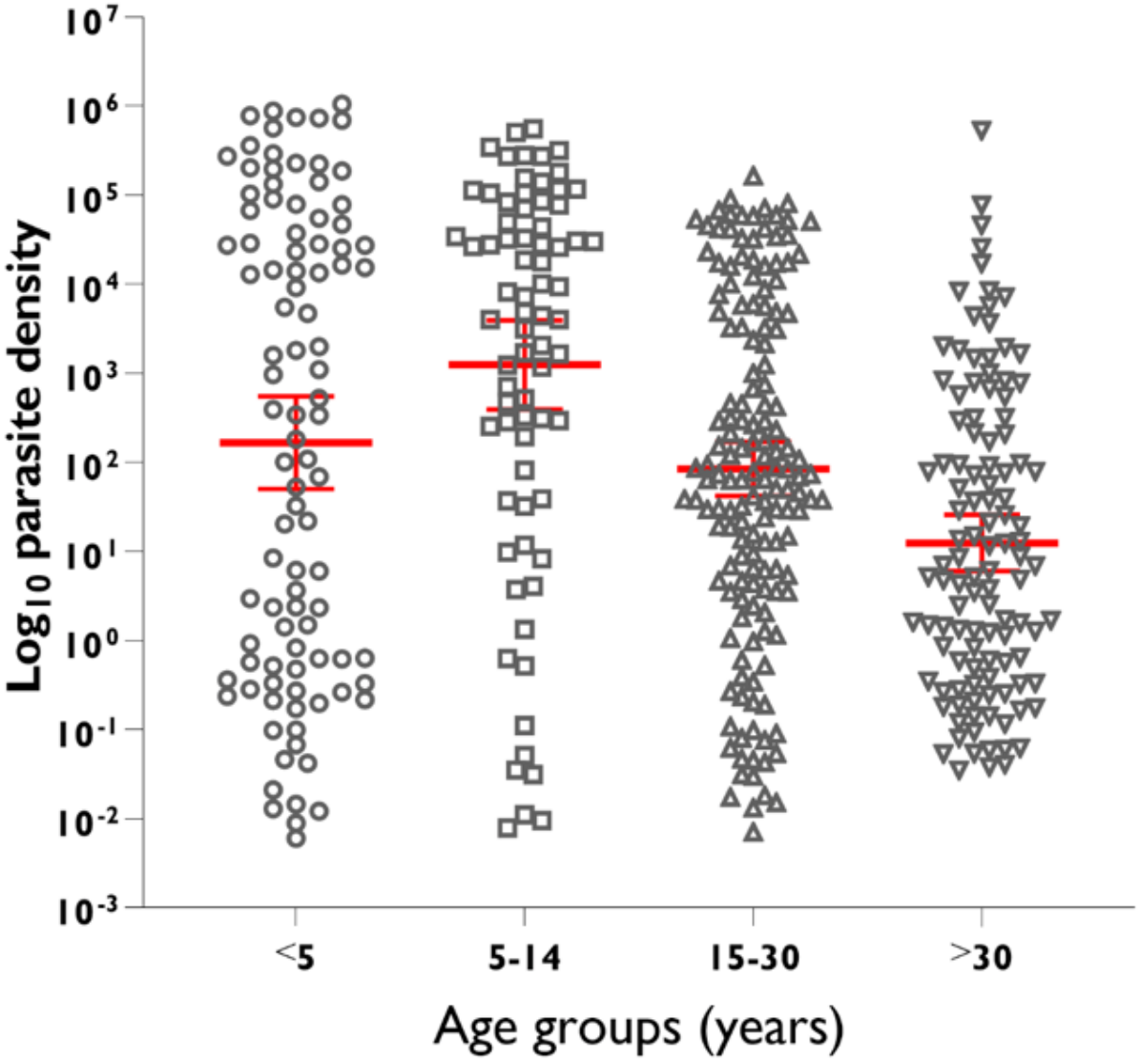

Figure 5

Parasite density across different age groups (error bars showing geometric mean with 95% CI)