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Performance of rapid diagnostic test, light microscopy, and polymerase chain reaction in pregnant women with asymptomatic malaria in Nigeria

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ABSTRACT

Objectives: Rapid diagnostic tests (RDTs) offer an attractive tool for diagnosing malaria in pregnancy. This study assessed the effectiveness of a *Plasmodium falciparum*–specific RDT compared with microscopy and polymerase chain reaction (PCR) in diagnosing asymptomatic malaria in pregnant women in southwest Nigeria.
Methods: The study included 406 asymptomatic pregnant women seeking antenatal care. Blood samples were collected and tested using RDT (SD Bioline, Standard Diagnostics Inc. Korea) and light microscopy and confirmed using nested PCR.
Results: The study revealed that the malaria parasite positivity rate was 8.9% by RDT, 21% by microscopy, and 32% by nested PCR. RDT had a sensitivity of 51.4% and specificity of 69.5%, whereas microscopy had a sensitivity of 65.3% and specificity of 98.2%. The combined testing of microscopy and RDT had a sensitivity and specificity of 100%. The study also showed a high prevalence of mild anemia among participants.
Conclusions: Despite the RDT's low sensitivity, its high negative predictive value suggests it could be useful in combination with microscopy in ruling out asymptomatic malaria in pregnancy. Further study will help identify more suitable RDTs for routine malaria diagnosis in Nigeria and strengthen malaria prevention programs in pregnant women.

Introduction

Malaria is one of the leading causes of morbidity and mortality in sub-Saharan Africa [1]. In 2022, an estimated 249 million malaria cases were reported in 85 endemic countries worldwide. About 96% of malaria deaths worldwide were in 29 countries. Nearly half of all malaria deaths worldwide in 2022 occurred in four countries: Nigeria (31%), the Democratic Republic of the Congo (12%), Niger (6%), and the United Republic of Tanzania (4%) [2].

Pregnant women are especially vulnerable to malaria infection caused mainly by *Plasmodium falciparum* due to immune system changes, which increases their risk of serious complications such as severe anemia, placental malaria, premature delivery, and low birth weight in mothers residing in high-transmission areas. The manifestation of malaria in pregnancy (MiP) depends on the malaria transmission level. In high malaria transmission areas, such as Nigeria, the prevalence of malaria infection among pregnant would range from 25% to 50% [3].

MiP is mostly asymptomatic in malaria-endemic areas, probably due to acquired semi-immunity, and acts as a malaria transmission reservoir [3]. Asymptomatic parasitemia occurs when a person carries *Plasmodium* parasites in their bloodstream; however, due to partial immunity, the parasites are incapable of causing symptoms in the affected individual [4]. Asymptomatic individuals have low health-seeking behavior due to the absence of disease symptoms and, hence, are not treated. This

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may worsen malaria's effects on the mother and fetus, as well as cause maternal and neonatal mortality.

The World Health Organization (WHO) recommends intermittent preventive treatment in pregnancy (IPTp) with sulfadoxine/pyrimethamine (SP) given during antenatal visits at least twice during pregnancy, once during the second trimester, and once at least one month after the first treatment to protect women from malaria during pregnancy [5]. In 2005, Nigeria implemented IPTp-SP as a national strategy per the 2002 WHO strategic framework recommendation for preventing malaria in Africa during pregnancy [6]. This strategy is expected to reduce the incidence of asymptomatic MiP; however, the escalating SP resistance means that there is a need for proper diagnosis so that malaria treatment may be initiated in those with SP-resistant parasites [7,8]. Timely and accurate diagnosis is needed to make decisions for any therapeutic intervention. Although rapid diagnostic tests (RDTs) and expert microscopy (gold standard) are considered adequate for malarial diagnosis in endemic populations, unfortunately, such methodologies are unable to detect low-density infections, need expertise, and are relatively expensive with reduced sensitivity.

Intermitted screening and treatment of pregnancy (ISTp) with artemisinin combined therapy is an alternative strategy for controlling MiP. The concept of ISTp is to provide scheduled screening of pregnant women, ideally using RDT, and treat positive cases with artemisinin-based combination therapy (ACT) whether symptoms are present or not [9]. RDT has been shown to be as effective as routine microscopy in diagnosing malaria [10]. Thus, RDT testing is recommended in uncomplicated malaria case management. Other factors supporting routine RDT use over microscopy include ease of use, low cost, and test results obtained within 15-20 minutes. These attributes make RDTs attractive for inclusion into an ISTp program. Molecular tests could also be used; however, high skill and infrastructure requirements pose a significant impediment at the point of care, especially in rural settings where most MiP occur [11].

To date, RDT diagnostic performance in the context of ISTp has not been adequately evaluated in pregnant women from Nigeria. This is particularly important because *P. falciparum* infections in MiP are mostly sub-microscopic. Precise diagnosis of *Plasmodium* species is critical in malaria control during pregnancy. Therefore, this study evaluated RDTs (index test) with microscopy and nested polymerase chain reaction (PCR) as the reference in detecting asymptomatic *P. falciparum* in pregnant women attending antenatal care in Ogun State, Nigeria. The study will contribute to the drive toward malaria elimination and Sustainable Development Goal 3, which seeks to ensure healthy lives and promote well-being for all ages.

Materials and methods

Study area, design, and population

This study was conducted in Ogun State, Southwest Nigeria, where malaria transmission is perennial. In Nigeria, pregnant women are given a supervised curative dose of SP at least twice during the second and third trimesters of pregnancy, during routinely scheduled antenatal clinic visits, irrespective of whether the woman is infected or not, according to WHO guidelines for malaria control in pregnancy [12]. Afebrile pregnant women (body temperature >37.5°C) seeking antenatal care in four hospitals (State Hospital Ijebu-Ode, State Hospital Abeokuta, General Hospital Ijebu-Igbo, and General Hospital Ifo) in Ogun State were recruited after a signed informed consent form was provided. Details of the participant's characteristics and sampling techniques have been previously described [7].

Ethical consideration

Ethical approval was obtained from the ethical review committees of the Ogun State Ministry of Health Hospital Management Board, Abeokuta (SHB/2427/45). After approval, participants who signed a written informed consent form were enrolled after being adequately informed about the study objectives, risks, and potential benefits. Assent was also obtained from pregnant women under 16 years of age. To ensure confidentiality, the names of the participants were not requested and recorded.

Study design, sample collection, and processing

Pregnant women who were eligible for the study were recruited through convenience sampling. The study's minimum sample size was determined using the sample size formula for a single proportion [13], with an 8.7% prevalence of malaria parasitemia [14], a precision of 5%, and a standard normal deviation of 1.96 at 95% confidence intervals. Given a 10% non-response rate, the study required a minimum sample size of 298 participants.

Peripheral blood samples obtained from a finger prick were used to diagnose malaria using the malaria RDT (RDT; SD Bioline, Standard Diagnostics Inc. Korea; Lot; 05EDHOO5A) and microscopic examination of Giemsa-stained blood smears. Hemoglobin (Hb) level was estimated using a hematology analyzer (Automated hemoglobin analyzer, Sysmex, USA). Approximately 100 µl of blood was also spotted on the Whatman 3 MM filter paper for molecular analysis. Anemia was defined as an Hb level <11 g/dL and classified as previously described. [15]; severe anemia (Hb <7 g/dL), moderate anemia (Hb: 7-9.9 g/dL), and mild anemia (Hb: 10-10.9 g/dL). Insecticide-treated net use and pregnancy outcomes were also recorded.

DNA extraction and molecular analysis

QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany) was used to extract total genomic DNA from dried blood spots according to the manufacturer's instructions. The 18S ribosomal RNA–specific nested PCR was used to detect *Plasmodium* species using previously published primers [16]. The PCR master mix was prepared by adding 50 ng of total genomic DNA into a master mix containing 1× PCR buffer, 0.2 mM dNTPs, 100 nM of each primer, and 1 unit of Taq DNA polymerase in a total volume of 20 µL. Speciation of the *Plasmodium* genus was conducted using 1 µL of outer PCR amplicons as a template for the nested PCR. Thermocycling conditions of nested PCR are the same as those of the outer PCR, except for the annealing (58°C) temperature. PCR amplicons were separated using 2% agarose gel electrophoresis.

Statistical analysis

The diagnostic performance of microscopy and RDT was determined by calculating sensitivity, specificity, positive predictive value, and negative predictive value (NPV). The Cohen kappa statistic was used to estimate the level of agreement between the diagnostic tests. The Pearson chi-square test was applied to determine the association between independent categorical variables. Statistical significance was set at $P < 0.05$. All statistical analyses were performed in SPSS (Version 21.0), and PCR served as the reference method in this study.

Results

Demographic characteristics of study participants

A total of 406 afebrile pregnant women at different gestational ages were recruited in this study (Table 1). The median age was 29 years (interquartile range: 25-35 years), and most participants (63%) were between the ages of 25 and 34 years. A total of 39% of the participants were primigravida. The remaining participants were multigravida with ≥2 pregnancies. Most of this study's pregnant women (76%) slept under an insecticide-treated net and 67 (16.5%) of the participants were found to be anemic.

Table 1
Characteristics of the study population.

Characteristics	N = 406 (%)
Age (years)	
Median age (interquartile range)	29 (25-33)
Age groups	
13-24	93 (22.9)
25-34	254 (62.7)
>35	59 (14.5)
Gravidity	
Primigravida	160 (39.4)
Multigravida	246 (60.6)
Gestational Trimester	
First	24 (5.9)
Second	286 (70.4)
Third	96 (23.6)
Bed-net use	
Yes	309 (76.1)
No	97 (23.9)
Anemia	67 (16.5)

Anemia defined as hemoglobin <11 g/dL.

Malaria prevalence by microscopy, rapid diagnostic test, and nested polymerase chain reaction

The study reveals a malaria parasite detection rate of 21.2% (86 of 406) by microscopy, 8.6% by RDT (35 of 406), and 32% (130 of 406) by nested PCR. Speciation by nested PCR shows 107 samples were *P. falciparum*, whereas five were non-*falciparum: Plasmodium ovale* (n = 3) and *Plasmodium malariae* (n = 2). Co-infection was observed in 18 samples with *P. falciparum/P. ovale* (n = 13), *P. falciparum/P. malariae* (n = 3), and *P. ovale/P. malariae* (n = 2).

Distribution of malaria diagnosis test results based on participant age, gravidity, gestational age, and insecticide-treated net

The distribution of malaria test results for microscopy, RDT, and nested PCR were similar among different age groups, gravidity, and gestational age. No significant difference was observed between malaria diagnosis test results and participant age, gravidity, gestational age, and insecticide-treated net use. However, a significant difference was observed between pregnant women who were positive for malaria by microscopy ($P = 0.011$) and RDT ($P = 0.0001$) and those who were sleeping under an insecticide-treated net (Table 2).

Association between maternal anemia among pregnant women and malaria diagnosis tests

A total of 67 (16.5%) of 406 pregnant women were anemic (Hb <11 g/dL). Of these, 53 pregnant women had mild anemia, 11 had moderate anemia, and three had severe anemia (Table 3). Most maternal (mild and moderate) anemic cases were not infected with malaria parasites. RDT detected malaria parasites in all three pregnant women with severe anemia, whereas microscopy and PCR detected the presence of malaria parasites in only two.

Malaria parasite detection in neonates of different pregnancy outcomes

A poor pregnancy outcome was observed in 56 (14%) neonates (Table 4). Anemia and low birth weight were observed in 33 and 17 neonates, respectively, with three premature and stillbirths each. No significant difference was observed between positive malaria parasites by microscopy and PCR among neonates and pregnancy outcomes, except for RDT ($P = 0.0001$).

Table 5 shows the diagnostic performance of microscopy and RDTs using PCR as the reference method. Microscopy gave a moderate sensitivity (65.3%) but a very high specificity (98.2%), whereas RDT has a low sensitivity (51.4%) and moderate specificity (69.5%). The combination of microscopy and RDT has a very high sensitivity and specificity (100%) compared with microscopy and RDT only. The positive predictive values and negative predictive values (95% confidence intervals) for microscopy were 94.2% (89.4-100.0) and 86.6% (76.6-98.7), respectively, RDT has 13.7% (9.14-22.4) and 93.8% (89.9-96.1), respectively, and microscopy and RDT has 37.5% (21.3-40.5) and 97.5% (93.6-100.0), respectively.

Discussion

This study evaluated the diagnostic performance of RDT and microscopy for detecting malaria parasites in pregnant women in Ogun State, southwest Nigeria. Our study shows low RDT sensitivity (51.4%) but moderate sensitivity of microscopy (65.3%). RDT sensitivity doubled when combined with microscopy. Other findings include a high asymptomatic *Plasmodium* infection rate (32%) by PCR, high insecticide-treated net use (76%), and relatively low maternal anemia (17%), as well as a low occurrence of poor pregnancy outcome (14%).

Due to the endemicity of *P. falciparum* in sub-Saharan Africa, MiP characterized by sub-microscopic infection predominantly occurs. MiP is associated with asymptomatic *P. falciparum* infection, a parasite

Table 2
Distribution of malaria diagnosis test results based on participant age, gravidity, gestational age, and bed net.

	Microscopy			Rapid diagnostic test			Polymerase chain reaction		
	Positive n (%)	Negative n (%)	*P*-value	Positive n (%)	Negative n (%)	*P*-value	Positive n (%)	Negative n (%)	*P*-value
Age (years)									
15-24	27 (31.4)	66 (20.6)	ns	12 (34.3)	79 (21.3)	ns	34 (26.2)	59 (21.4)	ns
25-34	48 (55.8)	206 (64.4)		20 (55.6)	234 (63.1)		76 (58.5)	172 (62.3)	
>35	11 (12.8)	48 (15.0)		3 (8.3)	56 (15.1)		20 (15.4)	45 (16.3)	
Gravidity									
Primigravida	33 (38.4)	127 (39.7)	ns	15 (41.7)	145 (39.1)	ns	48 (36.9)	113 (40.9)	ns
Multigravida	53 (61.6)	193 (60.3)		20 (57.1)	224 (60.4)		82 (63.1)	163 (59.1)	
Gestational trimester									
First	2 (2.3)	22 (6.9)	ns	2 (5.6)	22 (5.9)	ns	12 (9.2)	19 (7.2)	ns
Second	61 (70.9)	225 (70.3)		20 (55.6)	265 (71.4)		87 (66.9)	192 (71.9)	
Third	23 (26.7)	73 (22.8)		13 (36.1)	83 (22.4)		31 (23.9)	65 (23.6)	
Bed net									
Yes	56 (18.1)	253 (81.9)	0.011	8 (22.9)	300 (80.7)	0.0001	90 (69.2)	213 (77.2)	ns
No	30 (30.9)	67 (69.1)		27 (27.8)	69 (18.6)		40 (30.8)	63 (22.8)	

Sample size (n) = 406; statistical significance set at $P < 0.05$, ns: not significant.

Table 3
Association between maternal anemia in pregnant women and malaria diagnosis tests.

Malaria Tests		Maternal anemia				*P*-value
		Mild n (%)	Moderate n (%)	Severe n (%)	Negative n (%)	
Rapid diagnostic test	Neg	44 (83.0)	7 (63.6)	0	319 (94.1)	0.0001
	Pos	9 (17.0)	4 (36.4)	3 (100.0)	20 (5.9)	
Microscopy	Neg	37 (69.8)	7 (63.6)	1 (33.3)	275 (81.1)	0.031
	Pos	16 (30.2)	4 (36.4)	2 (66.7)	64 (18.9)	
Polymerase chain reaction	Neg	32 (60.4)	9 (81.8)	1 (33.3)	241 (71.1)	0.161
	Pos	21 (39.6)	2 (18.2)	2 (66.7)	98 (28.9)	
Total		53 (100.0)	11 (100.0)	3 (100.0)	339 (100.0)	

Statistical significance set at $P < 0.05$.
mild anemia = Hb between 10 and 10.9 g/dL; moderate anemia = Hb between 7 and 9.9 g/dL; negative anaemia ≥11 g/dL; severe anemia = Hb level <7 g/dL.
Hb, hemoglobin; Neg, negative; Pos, positive.

Table 4
Malaria parasite detection among neonates of different pregnancy outcomes.

Malaria diagnosis		Pregnancy outcome					*P*-value
		Anemia	Low birth weight	Premature	Stillbirth	Normal	
Rapid diagnostic test	Neg	28 (84.8)	9 (52.9)	2 (66.7)	1 (33.3)	330 (94.3)	0.0001
	Pos	5 (15.2)	8 (47.1)	1 (33.3)	2 (66.7)	20 (5.7)	
Microscopy	Neg	24 (72.7)	11 (64.7)	2 (66.7)	2 (66.7)	281 (80.3)	0.442
	Pos	9 (27.3)	6 (35.3)	1 (33.3)	1 (33.3)	69 (19.7)	
Polymerase chain reaction	Neg	23 (69.7)	10 (58.8)	2 (66.7)	1 (33.3)	247 (70.6)	0.562
	Pos	10 (30.3)	7 (41.2)	1 (33.3)	2 (66.7)	103 (29.4)	
Total		*33 (100.0)*	*17 (100.0)*	*3 (100.0)*	*3 (100.0)*	*350 (100.0)*	

Neg, negative; Pos, positive.
Statistical significance at $P < 0.05$.

Table 5
Diagnostic performance of microscopy and RDTs using PCR as the gold standard.

		PCR			Test performance				
		Pos	Neg	Total	Sensitivity (95% CI)	Specificity (95% CI)	Positive predictive value (95% CI)	Negative predictive value (95% CI)	κ value
Microscopy	Pos	81	5	86	65.3% (56.4-89.4)	98.2% (87.3-100.0)	94.2% (89.4-100.0)	86.6% (76.6-98.7)	0.73
	Neg	43	277	321					
RDT	Pos	18	17	35	51.4% (33.2-66.8)	69.5% (62.7-76.1)	13.7% (9.14-22.4)	93.8% (89.9-96.1)	0.103
	Neg	113	258	371					
Microscopy-RDT	Pos[a]	6	10	16	100% (78.1-100.0)	100% (72.4-100.0)	37.5% (21.3-40.5)	97.5% (93.6-100.0)	0.179
	Neg	0	390	390					

Microscopy-RDT = combined test.
CI, confidence interval; Neg, negative; PCR, polymerase chain reaction; Pos = positive; RDT, rapid diagnostic test; κ, kappa.
[a] Any positive by either microscopy or RDT was positive. PCR served as the reference method.

known to sequester at the placenta. This phenomenon consequently causes maternal anemia and adverse pregnancy outcomes viz, anemia, abortion, low birth weight, premature birth, and stillbirth [17]. Presently, MiP control relies on IPT-sp, which is composed of at least three doses of IPTp-SP once a month, given during antenatal visits from the second gestational trimester [5]. Despite being effective, increasing *P. falciparum* resistance against IPTp-SP and plausible treatment failure call for an improved IPTp-SP program. For instance, the integration of malaria testing and subsequent treatment during antenatal care in the ISTp approach.

In this study, RDT targeting *P. falciparum* histidine-rich protein 2 (PfHRP2) had a low sensitivity (51.4%) and low positive predictive value (13.7%), as reported previously in Tanzania [16]. Several factors have been linked to low RDT sensitivity, including low parasitemia, PfHRP2 gene deletions, operator error in disease prevalence, poor storage conditions, and variations in RDT brand and lot performance [18]. Generally, the parasitemia of the study participants was low and mostly sub-microscopic, which may, in turn, explain the low sensitivity of the RDT in the study. Further investigation, including the PfHRP2 gene deletion status in the study area, will be needed to accurately dissect other factors contributing to the low RDT sensitivity.

Another significant factor worth considering is the inhibition of parasite replication by IPTp-SP, which could potentially lower parasite density below the RDT limit of detection. A multicenter prospective study conducted in Burkina Faso and Uganda showed that increasing gravidity and IPTp-SP treatment decrease RDT sensitivity [19]. In other studies, however, RDT targeting PfHRP2 has been reported to be more sensitive than what is reported in this study [20,21]. Molecular tests are sensitive and specific under similar circumstances and have a low parasite density. However, the implementation of molecular diagnosis of malaria is highly underutilized because of the high initial and overhead costs required for a point-of-care test, particularly, in resource-limited settings.

This study's relatively low (69.5%) specificity of RDT is congruent with studies conducted in malaria-endemic settings. This is often attributed to prolonged PfHRP2 persistence after parasite clearance. A previous longitudinal study revealed that PfHRP2 could persist in pregnant women for >28 days after treatment with ACT [22]. PfHRP2 further compounds this from frequent *P. falciparum* infections prevailing in many malaria-endemic settings in sub-Saharan Africa, including Nige-

ria. Furthermore, PfHRP2 persistence could be extended further by increased antigen production by gametocytes, particularly, when gametocyte production is elevated after induction by IPTp-SP [23]. This consequently causes RDT false positivity and may lead to falciparum malaria overdiagnosis and increased antimalaria used and may mask or delay the diagnosis of other illnesses in pregnancy.

Using RDT, targeting other *Plasmodium* antigens, such as lactate dehydrogenase could improve the poor diagnostic performance or combine RDT with other tests [24]. In this study, the RDT-microscopy combination attained double the sensitivity of RDT, specificity and positive predictive value declined marginally, NPV improved from 93.8% to 97.5%, and sensitivity was doubled. Similar findings were reported by a study conducted in Uganda [25]. This infers that microscopy could supplement RDT testing of malaria in this setting. However, routine use of microscopy in resource-limited settings is still a significant challenge owing to its dependence on electricity supply, skilled labor, and access to good quality microscopes and reagents, whereas RDT targeting lactate dehydrogenase are less sensitive than PfHRP2-based RDTs [26]. Despite these, a high NPV (93.8%) indicates that the RDT evaluated in the present study can be used confidently to rule out MiP [27].

This study detected *P. ovale and P. malariae* less frequently, as reported previously in other West African countries [28]. Previous studies have also shown that *P. ovale* and *P. malariae* rarely affect pregnancy and its outcome [28]. Failure to detect *P. vivax* in this study was not surprising because Africa bears a lower *P. vivax* burden than other parts of the world, and it is rarely reported among pregnant women from sub-Saharan Africa. *P. vivax* can adversely affect pregnancy, especially primigravidae, but the impact is less prominent than in *P. falciparum*–infected pregnant women [29]. The high prevalence of *P. falciparum* in pregnant women in this study underscores the deployment of effective MiP control strategies in southwest Nigeria.

Taken together, this study demonstrates that the use of RDT to diagnose malaria in pregnant women in the current setting has poor sensitivity unless combined with microscopy. However, the underlying limitation of malaria diagnosis by microscopy makes it unattractive for combination with RDT, especially in resource-limited regions. This underscores the urgency to either improve the sensitivity of existing RDTs or develop low-cost, easy-to-use, and ultra-sensitive tests for malaria diagnosis in pregnancy. Tests with improved diagnostic performance will help avert poor pregnancy outcomes and improve the estimation of the true prevalence of *Plasmodium* infection in pregnant women. This is paramount, especially with the increasing *P. falciparum* resistance against IPTp-SP and ACTs.

Declaration of competing interest

The authors have no competing interests to declare.

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Ethical approval

Ethical approval was obtained from the ethical review committee, Ogun State Ministry of Health Hospital Management Board, Abeokuta, Nigeria (SHB/2427/45). The research followed the code of ethics of the World Medical Association (Declaration of Helsinki). Before being recruited into the study, every participant gave written or verbal informed consent.

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Author contributions

SAA recruited patients, obtained informed consent, and collected samples; ABO, KAF, and DN carried out molecular genotyping and analysis; KAF, DN, ABO, and SAA drafted the manuscript; BNT, TPV, and OO reviewed the manuscript and contributed to the discussion and the overall scientific content; AOJA, TPV, and OO conceived, designed, and provided oversight and leadership responsibility. All authors read and approved the final version of the manuscript.

Data statement

The data supporting this study’s findings are available from the corresponding author (OO) upon reasonable request.

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