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Comparative analysis of four malaria diagnostic tools and implications for malaria treatment in southwestern Nigeria

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ABSTRACT

Objectives: One of the problems encountered in malaria control and elimination is inaccurate diagnosis, resulting from the degree of sensitivity of the different malaria diagnostic tools. Even though microscopy remains the gold standard for malaria diagnosis, more sensitive and robust diagnostic tools such as polymerase chain reactions (PCR) are used in research settings to monitor interventions and track sub-microscopic infections due to some of the drawbacks of microscopy. Since diagnosis is a critical determinant for rational malaria treatment, it is imperative that accurate diagnosis must be assured for an effective treatment plan. Therefore, this study compared two routinely used point of care malaria diagnostic tools with two molecular tools and discussed their implication for malaria treatment.

Design: In this study, 436 individuals with suspected malaria were sampled and systematically tested using four methods, namely rapid diagnostic test (henceforth referred to as malaria RDT- mRDT), microscopy, nested PCR (nPCR), and quantitative PCR (qPCR). Test sensitivities and specificities were compared, and their level of concordance was determined.

Results: With nPCR as the gold standard, a false positivity rate of 42.2%, 8.9%, and 57.8% was obtained for mRDT, microscopy, and qPCR. Similarly, false negativity rates of 12.5%, 62.5%, and 0.8% were obtained for each of the methods mentioned above, respectively. Of all the tools assessed, qPCR gave the highest sensitivity (99.2%) and moderate specificity (42.2%), followed by the mRDT kit used (87.5%).

Conclusions: With the detection of a high false positivity rate based on mRDT and a substantial proportion of sub-microscopic carriers in this study area by nested/quantitative PCR, we recommend that these molecular tools should be in specialized laboratories within the region to (i) track and treat sub-microscopic carriers to prevent their contribution to malaria transmission; (ii) provide reliable epidemiological data using high throughput testing tools for evaluating malaria interventions.

Introduction

The tremendous gains made in cases and mortality reduction globally (from 585 000 to 405 000) between 2010–2018 result from deliberate intervention strategies (World Health Organization, 2019). This achievement is also seen in reducing the number of endemic countries from 108 to 91, with ten certified malaria-free and 29 preventing re-introduction of malaria infections during the same period (World Health Organization, 2019). Nevertheless, the practical benefits have seen a plateau in the last two years, principally in Africa, where a significant disease burden is mainly observed. In Nigeria, malaria remains a significant public health problem and accounts for 27% of the total disease burden in Africa (World Health Organization, 2019) (2020 WMR). Diagnosis remains a considerable challenge in malaria control and elimination, and the performance of tools differs in various epidemiological settings (Mahende et al., 2016a, 2016b).

Microscopy is an essential tool for malaria diagnosis in high transmission areas (Abeku et al., 2008). However, some of the drawbacks of microscopy include subjective parasite identification and counting by microscopists, inability to detect mixed species infection as well as distinguish *P. ovale* from *P. vivax* (Alves et al., 2002; Bisoffi et al., 2010), the lower detection limit of between 4–20 parasites/μl even for expert microscopists (Endeshaw et al., 2008) and very uniquely in Nigeria, an unstable power supply (FMOH, 2015). Despite these shortcomings, microscopy remains

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the gold standard for diagnosis as it can identify individuals with active malaria and provide information on the parasite density, which is helpful for monitoring the efficacy of treatment.

Current guidelines for malaria treatment by the World Health Organization (WHO) require parasitological confirmation by malaria RDT (mRDT) and/or microscopy (WHO, 2015). Malaria RDT kits are being used in many endemic countries due to the rapid turn-around time, cost-effectiveness, and ease of result interpretation requiring little training (Mayxay et al., 2004). Malaria RDTs are more cost-effective and profitable in reducing the treatment of febrile illnesses not due to malaria. The diagnostic accuracy of mRDT differs by quality and storage conditions (Maltha et al., 2013; Harvey et al., 2017). Also, the most widely recommended histidine-rich protein 2 (HRP2) mRDT is unable to distinguish between past and current infections due to antigenemia (the persistence of the HRP2 antigen in peripheral blood after effective treatment) (WHO, 2004; Swarthout et al., 2007; Shillcutt et al., 2008) which has been suggested to be the source of over-treatment with antimalarial chemotherapy (Sansom, 2009).

Polymerase chain reaction (PCR) has been used as a more sensitive diagnostic tool for both detection and quantification of parasite DNA. Several PCR techniques have been described for malaria parasite detection, including the nested or conventional PCR (nPCR/PCR) with amplification of specific parasite targets and quantitative real-time PCR (qPCR), which requires the use of labeled probes for increased specificity (Mixson-Hayden et al., 2010; Miura et al., 2013; Lau et al., 2015). Deployment of such molecular techniques has helped detect “hidden non-falciparum species,” as reported by various authors (Mekonnen et al., 2014; Ehtesham et al., 2015; Ogbu et al., 2015; Oboh et al., 2018). These tools have been very effective in estimating current malaria infection. However, the availability of such tools in resource-limited areas is lacking, and the requirement for a trained technician, quality control, and equipment maintenance might be “far-reached” in endemic populations (Hawkes and Kain, 2007; Krampa et al., 2017).

This study was therefore designed to evaluate if treatment based on mRDT-status (as it is primarily used in the study area) is indicative of under-, optimum- or over-treatment by subjecting malaria suspected samples collected (between January to March 2017) from health facilities in southwestern Nigeria, systematically to four malaria diagnostic tools.

Materials and methods

Study areas

Samples were collected from two States in Nigeria (Lagos and Edo) described in our previous study (Oboh et al. 2018). In Lagos State, samples were collected from four different Local Government Areas (LGAs), namely, Eti-osa (06°26′ N 003°29′ E), Ibeju (06°26′ N 003°56′ E), Kosofe (06°28′ N 003°22′ E) and Ikorodu (06°33′ N 003°35′ E) LGA, while in Edo State, samples were collected from two LGAs: Oredo (06°19l′ N 5°34′ E) and Ikpoba Okha (06°16′ N 5°68′ E).

In Lagos, malaria is mainly hypo-endemic, with a 1.9% prevalence rate in children aged 6–59 months. On the other hand, malaria is meso-endemic in Edo State, with a prevalence rate of 35% in children aged 6–59 months (Ebomwonyi et al., 2015; Odugbemi et al., 2016)

In both states, annual rainfall is 1400–1800 mm, and there is a short break called “August break’ with two prevailing climatic conditions; the dry season (lasting from November to March) and the wet season (from April to October), with a temperature gradient of 30–38 °C (Aigbodion and Osariyekemwen, 2013).

Study design and collection of samples

The study was a passive case detection of patients presenting clinical symptoms of malaria and visiting hospitals in any of the two locations between January to March 2017. Patients age ≥ two years with clinical symptoms of malaria detected by a febrile condition of ≥37.5 °C were included in the study. A total of 436 patients were recruited into this study after obtaining informed consent and/or assent where applicable. As it is the national policy of Nigeria, *P. falciparum*-specific HRP2 RDT kits (Care Start®, Access Bio Inc) were rapidly used on all patients' samples, and irrespective of the study outcome, those found positive were treated with artemisinin-based combination therapy as per national guideline. Blood smears and dried blood spots were made on all samples irrespective of their mRDT status following the process described in our earlier article (Oboh et al., 2018). Dried blood spots were preserved for further molecular and serological assays.

Sample processing following molecular methods

Nested PCR

Genomic DNA of all samples was extracted from three punches of 3 mm dried blood spot using the QIAamp DNA Blood Mini Kit (Qiagen®, Hilden, Germany), eluted in a 100 μl final volume and stored at −20 °C until use. For the nested PCR (nPCR), already designed primers targeting the 18S rRNA of *Plasmodium* species according to Snounou et al. (Snounou et al., 2002) were used with modification in the cycling conditions. PCR amplification was carried out with 1 μl extracted gDNA and 2 μl of primary amplicon for the primary and nested PCR, respectively, using the Gotaq Green Mater mix (Promega) as detailed in Oboh et al. (Oboh et al., 2018). 3D7 culture isolates and sterile water were used as positive and negative controls for all runs, respectively.

Real-time quantitative PCR

Regarding the real-time quantitative PCR (qPCR), the *var* gene multi-copy acidic terminal sequence (Hofmann et al., 2015) was further used to validate outcomes from RDT, microscopy, and nPCR. Primer probe sequence and cycling conditions for qPCR validation of *P. falciparum* have been detailed elsewhere (Umunnakwe et al., 2019). In brief, 5 μL of template gDNA was added to a reaction mix containing 1 μL of nuclease-free water, 10 μL of 2x Taqman Universal PCR Mastermix (Applied Biosystems, New Jersey, USA), 1.6 μL of 10 μM forward and reverse primer each and 0.8 μL of 10 μM probe. The reaction mix was run on a CFX 96 real-time system thermocycler (BioRad). Duplicates of serially diluted DNA of laboratory strain of *P. falciparum* 3D7 were used as standard with a starting concentration of 1.24 × 10^6 parasites per microliter of blood.

Statistical analysis

Data were entered in excel and exported to Statistical Package for Social Sciences (SPSS) version 21.0 (SPSS, Inc. Chicago IL, USA) for analyses. To evaluate the performance of each diagnostic tool with regard to sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), each tool was compared against the highly sensitive nPCR as the gold standard. Agreement between pairs of evaluation tools was tested based on Cohen Kappa’s statistics where ≤0 indicates no agreement, 0.01–0.40 – slight to an average agreement, 0.41–0.80 – moderate-stable agreement, 0.81–1.00 perfect agreement.

Table 1
Background characteristics of study participants.

	Lagos	Edo	Total
Number	254	182	436
Percentage	58.3	41.7	100
Age (years)			
Mean (S.E)	18.53 (13.7)	23.26 (15.40)	
Range	1–68	1–72	
Sex			
Male	121	76	197
Female	133	106	239

Results

A total of 436 participants were recruited for this cross-sectional hospital-based survey, although more than half of the subjects were from Lagos (58.3%: 254/436), the mean age of individuals from both areas was not different (18.53 years in Lagos and 23.26 years in Edo), while the sex ratio was approximately 1:1 in both study locations (Table1).

Figure 1 shows a summary of the results obtained from the comparison between the different diagnostic methods. Of the 436 symptomatic samples collected (outermost sphere), quantitative PCR (peach circle) and nested PCR (light green circle) identified 358 and 256 as positive, while mRDT (brown circle) and microscopy (dark-green circle) identified 300 and 112 as positive. Of the 256 *Plasmodium* infections detected by nested PCR, 85.5% were *P. falciparum*, 4. 7% were *P. malariae*, and 6.3% were *P. falciparum* and *P. malariae* mixed infection, and the remaining were different multiple mixed infections (see legend). Also, it is important to state that mRDT and qPCR targeted only *P. falciparum* species while the microscopy detected one (1) *P. ovale* species in addition to the *P. falciparum* (111) isolates detected.

Using nested PCR as the gold standard, a false positivity rate of 42.2%, 8.9%, and 57.8% was obtained for RDT, microscopy, and qPCR. Consequently, a false negativity rate of 12.5%, 62.5%, and 0.8% was also observed by RDT, microscopy, and qPCR, respectively.

The two molecular detection tests (nPCR and qPCR) employed in this study gave interesting sensitivity and specificity results (Table 2), highlighting their increased power of detection relative to microscopy and/or RDT. Next to these is the mRDT; however, because of its inability to differentiate passive from active infection, findings observed in this study should be interpreted cautiously relative to the gold standard (nPCR) used for comparison.

As an added advantage, the nested PCR could detect rich mixes of *Plasmodium* species from *P. malariae/ovale*, *P. falciparum/malariae P. falciparum/vivax*, and *P. falciparum/malariae/vivax*, as

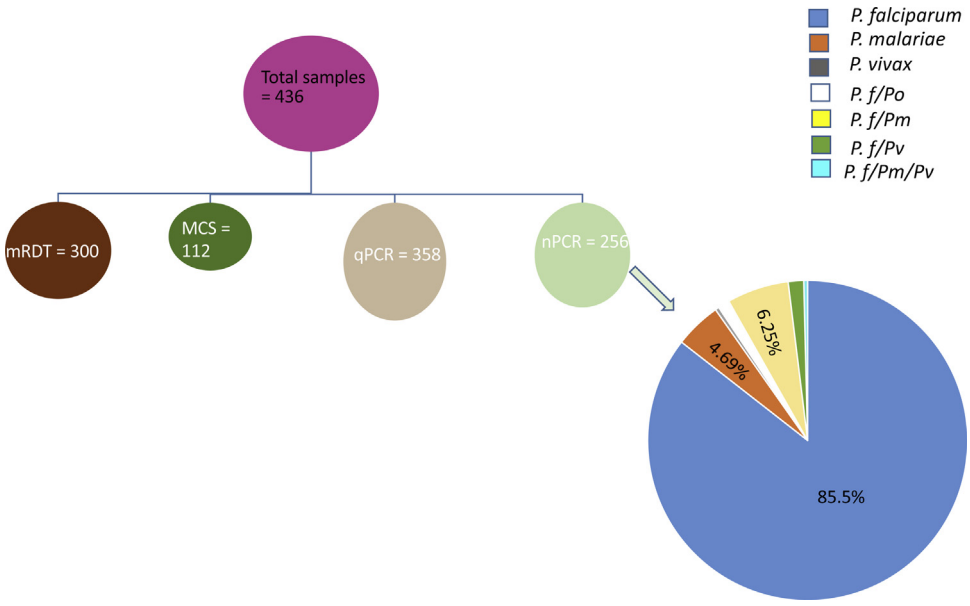

Figure 1. Visual description of the comparative evaluation (prevalence) of the performance of the different malaria diagnostic tools (quantitative PCR, nested PCR, mRDT, and microscopy).

Table 2
Test performance of the different diagnostic assays used for evaluating malaria positivity from samples collected from the study areas.

	nPCR[a]		Sensitivity % (95%CI)	Specificity % (95% CI)	PPV % % (95% CI)	NPV % % (95% CI)	Kappa's test	P-value
	Positive	Negative						
mRDT								
Positive	224	76	87.5 (82.8–91.3)	57.8 (50.21–65.1)	74.7 (71.2–77.9)	76.5 (69.7–82.1)	0.47	0.000*
Negative	32	104						
MCS[b]								
Positive	96	16	37.5 (31.6–43.7)	91.1 (85.9–94.8)	85.7 (78.6–90.8)	50.6 (47.9–53.2)	0.26	0.000*
Negative	160	164						
qPCR								
Positive	254	104	99.2 (97.2–99.9)	42.2 (34.9–49.8)	70.9 (68.3–73.5)	97.4 (90.4–99.4)	0.45	0.08
Negative	2	76						

nPCR- nested PCR; mRDT- rapid diagnostic test; MCS- microscopy; qPCR- real-time quantitative PCR. Superscript a and b refer to the use of only *P. falciparum* results from the three diagnostic tools since the mRDT and qPCR use in the study are specific for *P. falciparum*. *- statistically significant association.

depicted in Figure 1. Although the mRDT used (PfHRP2) and the real-time PCR mainly targeted *P. falciparum*, results from microscopy showed no more difference from these focused diagnostic tools, as only *P. ovale* (1) was the additional species detected apart from falciparum isolates.

Discussion and conclusion

This study provides a comparative analysis of four commonly used malaria diagnostic tools in routine and research settings to detect malaria infections from two endemic areas of southern Nigeria. Analyses from this study provide the rate of false positives and negatives, both of which have implications for malaria control and intervention programs.

The observation of a high false positivity rate with mRDT (~43%) and qPCR (≥50%) is worrisome as treatment in the study area is almost entirely based on mRDT result; hence, overtreatment would be operational. The rate of false positivity (by any of the tools) obtained in this study is higher than that witnessed in Ethiopia (Golassa et al., 2013) and Zanzibar (Msellem et al., 2009) with almost similar malaria transmission but lower than that in a low transmission setting (Reyburn et al., 2007). It has been established that *P. falciparum* HRP-2 antigen circulates in the blood long after treatment and parasite clearance, and as such, mRDT cannot distinguish passive from active infection (Bisoffi et al., 2010).

The failure of microscopy to detect a substantial proportion of sub-microscopic individuals (~63%) could be due to low parasite density or operational shortcomings by the laboratory technician. Nevertheless, this group of untreated individuals (false negative) poses a considerable challenge to the malaria control effort as they subsequently act as reservoirs of infection for continuous transmission (Starzengruber et al., 2014). Even with mass screening, especially when a sensitive technique is not being employed, this group will still go undetected, thus necessitating the use of highly sensitive and specific techniques such as PCR that could identify very low densities of parasites missed by microscopy or even mRDT as has been shown by various studies (May et al., 1999; Fru-Cho et al., 2014; Starzengruber et al., 2014; Mahende et al., 2016a; Doctor et al., 2016). Contrary to this study, the sub-microscopic prevalence of *P. falciparum* in similar studies was relatively lower than what was obtained here (May et al., 1999; Golassa et al., 2013). However, these differences could be due to variation in the transmission intensity and intervention implementation, which could influence parasite carriage's burden in various epidemiological settings.

The substantial number of sub-microscopic carriers in Nigeria, as detected by nested/quantitative PCR assays, shows how less sensitive microscopy is in identifying low-grade parasitemia. Moreover, the high false positivity rate (mainly from mRDT as it is the diagnostic tool that determines treatment) demonstrates the reduced specificity of mRDT in distinguishing a true positive from a non-positive sample, resulting in over-prescription of ACTs, and therefore calls for more sensitive detection tools such as PCR. Should elimination of malaria in Nigeria be a target, this set of sub-microscopic carriers must be systematically tracked and treated as they could serve as reservoirs of infection and propagate malaria transmission.

In addition to the routine mRDT and microscopy diagnostic tools used in the country, PCR or tools such as loop-mediated isothermal amplification (LAMP) should be added as a referral option, especially in specialist hospitals. Although they are relatively expensive compared to the standard diagnostic tool, their future benefit in malaria elimination cannot be understated. Moreover, *a Plasmodium*-RDT combo kit that detects pan-specific antigens such as lactate dehydrogenase or other brands that could detect *vivax* malaria can be used in place of the falciparum specific PfHRP2 currently in use in Nigeria. Taken together, effective implementation of these will be valuable in tracking and treating individuals with low-grade parasites and also provide reliable epidemiological data necessary for evaluating malaria interventions.

Conflict of interest

All authors declare that they have no conflict of interest.

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Ethical consideration

Ethical approval for this study was obtained from the Nigerian Institute of Medical Research, Yaba, Lagos, Lagos State Health Service Commission, and the Edo State Hospital Management Board, Benin City.

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