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ABSTRACT

***Background*: Pregnancy poses specific challenges in the diagnosis of *Plasmodium falciparum* infection due to parasite sequestration in the placenta. The diagnosis of *Plasmodium falciparum* infection in pregnant mothers therefore requires highly sensitive methods in order to detect the presence of parasites. These include those that detect the presence of antigens and those that detect and quantify the presence of the malaria parasites.**

***Objective*: The study assessed the performance of mRDT diagnostic test ((PfHRP2 -RDT) in the detection of malaria infection in blood samples from nulliparous pregnant women within the first trimester of pregnancy in Western Kenya.**

***Methods*: This was a prospective study on blood specimens collected from pregnant women in a malaria-endemic region in Kenya. m-polymerase chain reaction (mPCR) and mRDT tests were performed. The diagnostic accuracy of m-RDT was compared with mPCR as the gold standard for the purpose of this study.**

***Setting*: Twelve primary health facilities in Busia, Bungoma and Kakamega Counties in Kenya**

***Results*: Out of 264 mPCR positive samples, 130 were mRDT positive (true positives) while 134 were mRDT negative (false negative). And out of 441 mPCR**

negative samples, 41 were positive on mRDT (false positive). Thus, in comparison with mPCR, the sensitivity and specificity of mRDT to detect malaria infection in nulliparous pregnant mothers in first trimester was 49.2% and 88.9% respectively
***Conclusions*: The sensitivity of mRDT to detect *Plasmodium falciparum* infections in nulliparous pregnant mothers in the first trimester was not satisfactory compared to mPCR tests.**

INTRODUCTION

The diagnosis of malaria in malaria-endemic areas is typically based on microscopy, which is economical, widely accepted and has for a long time been regarded as the gold standard, especially in endemic areas[1,2]. However, with the advent of highly specific polymerase chain reaction (PCR) testing technologies for rapid and accurate diagnosis of malaria parasites in blood, PCR tests, where accessible, have replaced microscopy as the gold standard confirmatory test[3]. This is largely because microscopy requires significant expertise, equipment, electricity and reagents. As an alternative, rapid diagnostic tests (RDTs) have been developed for use in endemic countries where skilled microscopists are scarcely available[4].

Malaria rapid diagnostic tests (mRDTs) have to date had enormous global impact, which has influenced the World Health Organization's (WHO's) paradigm shift from empiric treatment to obtaining a parasitological diagnosis prior to treatment[5]. The RDT are less complex, which allows for utilization in austere environments while achieving similar sensitivities and specificities. Currently, there are over 200 different RDT brands that utilize three antigens: Plasmodium histidine-rich protein 2 (pfHRP-2), Plasmodium lactate dehydrogenase (pLDH) and Plasmodium aldolase (pALDO). PfHRP-2 is exclusively produced by *Plasmodium falciparum*[4] and most of the widely used RDTs are based on the detection of histidine-rich protein 2 (HRP2)[6,7]. Previous reports suggests sensitivities and specificities of over 80%, relative to the thick blood smear[8,9]. The validity of mRDTs can be determined by comparing to mPCR test, now considered the gold standard where applicable.

The current study involved the determination of the validity of mRDT test, based on its sensitivity and specificity. Sensitivity was determined by the proportion of nulliparous pregnant women in the first trimester who were truly infected with malaria and required appropriate management. Nulliparous malaria infected pregnant women who tested positive on both mRDT and mPCR tests were considered true positives, whereas those non-infected women with malaria who tested negative on both mRDT and mPCR tests were considered true negatives. The women who tested negative on mRDT but positive on mPCR were considered false negative and would have therefore missed interventional treatment which may have in turn complicated their pregnancy outcomes. Those who tested positive on mRDT, but negative on mPCR were considered false positive, and therefore underwent unnecessary treatment, which may have affected their pregnancy outcomes. False negative and false positive results therefore pose challenges in nulliparous pregnant women in their first trimester since they affect the pregnancy outcomes. This study aimed to determine the proportion of false positive and false negative mRDT test results in nulliparous pregnant women in their first trimester in western Kenya, a malaria endemic region.

The management of malaria in pregnancy is a global and national priority, yet the diagnosis and treatment still remain a challenge, especially in low- and middle-income settings. The current recommendation is to screen all pregnant women at their first antenatal visit through either microscopy or rapid diagnostic tests (mRDTs). Indeed, false negative mRDTs has been reported in sub-Saharan countries including Kenya; but the true prevalence of the false-negative results has not been fully elucidated[10]. It has been postulated that false-negative testing is due to *P. falciparum* malaria strains that lack Histidine Rich Protein 2 (HRP2) on which the m-RDT tests are based. The Kenya National Malaria Treatment Guidelines recommend artemisinin combination therapies (ACTs) such as artemether-lumefantrine or dihydroartemisinin-piperaquine (Duo-Cotecxin®) as first-line treatment for asymptomatic *P. falciparum* malaria in 2nd and 3rd trimesters of pregnancy. However, oral quinine is recommended in the 1st trimester as ACTs are contraindicated owing to insufficient safety data. In practice, antimalarial treatment on the basis of clinical suspicion should only be considered in situations where a parasitological diagnosis is not accessible[11].

MATERIALS AND METHODS

Study design

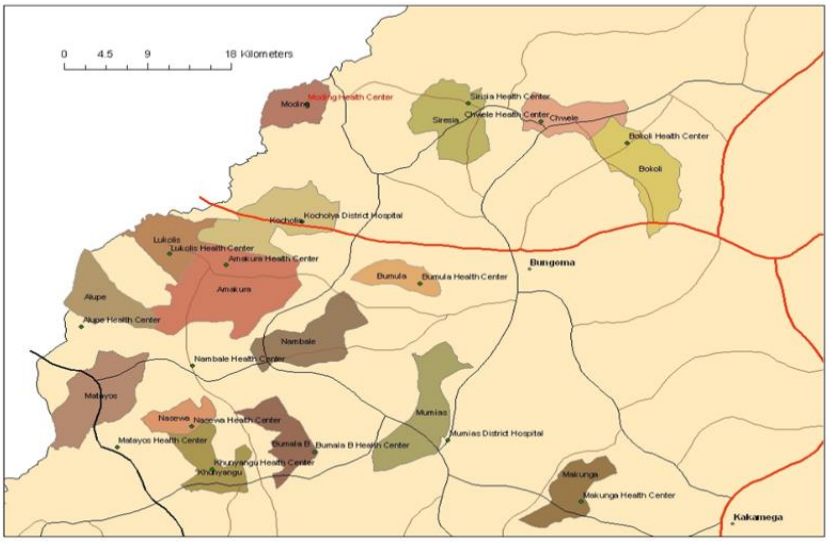
This population-based cohort study was conducted between November 2016 and May 2018. The study population consisted of nulliparous pregnant women with asymptomatic 1st trimester malaria recruited into the malaria sub-study, aged between 14 and 39 years in 12 selected clusters in Western Kenya. The sample size was 900 participants who had been enrolled into the ASPIRIN study in the study area[12].

Study Sites

This was part of the Malaria sub-study of the Global Network's ASPIRIN study conducted in Democratic Republic of Congo, Kenya and Zambia.

In Kenya, it was conducted in 12 health facilities in Busia, Bungoma and Kakamega Counties in Western Kenya[12]. The ASPIRIN study was a randomized, placebo-controlled, double-blinded multicentre clinical trial that assessed the efficacy of low dose aspirin (LDA) in the reduction of preterm birth in nulliparous women aged between 14 to 40 years. The Kenya site was classified into clusters whereby a cluster represents the catchment area of a primary healthcare centre of between 300 to 500 annual births. Currently, Kenya has a 6-tier health care system; with the basic unit being a community unit staffed by community health workers. The Malaria study was conducted in 12 community-based clusters: Sirisia, Bokoli, Chwele, Makunga, Mumias, Madende, Nasewa, Lukolis, Khunyangu, Amukura, Matayos and Bumala B.

Map of the study area



Kenya ASPIRIN study sites incorporating malaria sub-study
Source: Global Network maps

Study population

The study population consisted of 1st trimester nulliparous women with asymptomatic malaria, recruited into the ASPIRIN study. Malaria tests were conducted on 900 of these women. Community health workers were engaged and trained on how to identify pregnant nulliparous women at household level and refer them to hospital facilities for malaria screening using mRDT. Community mapping, continuous household surveys and bi-weekly household revisits were used to identify, sensitize and refer the women to the health facilities. . Participants who met enrolment criteria for the ASPIRIN study were then screened and consented to the malaria sub-study.

Inclusion Criteria

Nulliparous women aged between 14 to 40 years with asymptomatic malaria.

No more than two previous first trimester pregnancy losses

No medical contraindications to dihydroartemisinin-piperaquine (Duo-Cotecxin®)

Single live intrauterine pregnancy (IUP) between 6 (0/7) and 13 (6/7) weeks; GA corroborated by an early dating ultrasound and with heart rate greater than >110 bpm

Exclusion Criteria

Women who had been on (Duo-Cotecxin®) 3 days' prior

Multiple gestations

Foetal anomaly by ultrasound (most foetal anomalies are not detectable by ultrasounds done at this early gestation. Subsequent discovery of a foetal anomaly was not viewed as an exclusion)

Haemoglobin of < 7.0 gm/dl at screening

Presenting with malaria symptoms at time of enrolment

Any other confirmed medical conditions that may be considered a contraindication per the judgment of the site investigator (e.g., Lupus, Type 1 Diabetes, hypertension, or any other known significant disease)

Ethical Considerations

The study was approved by the Moi Teaching and Referral Hospital/Moi University School of Medicine Institutional Research and Ethics Committee (IREC) (Reference IREC/2015/81.

Approval number: FAN: IREC 1429 on 6th July 2015). Written informed consent was obtained from study participants.

Data Collection

Blood samples from 746 consented asymptomatic women were tested using two methods: A commercial mRDT (CareStart™ Malaria HRP2.Pf (Access Bio, Inc.) and mPCR designed to detect malaria species-specific markers for *P. falciparum*[13]. CareStart™ mRDT provides a rapid qualitative detection of malaria HRP2 (histidine rich protein 2) *P. falciparum* in human blood[13]. The device contains a membrane strip which is pre-coated with a monoclonal antibody as a single line across the strip which is specific to HRP2 of the *P. falciparum*. The conjugate pad is dispensed with antibodies absorbed on gold particles, which are *P. falciparum* specific to HRP2 of *P. falciparum*. The presence of two colour-lines (one-line band in the screen next to "C" and another line band in the screen next to "T") indicates a positive result for *P. falciparum*, while the presence of a line next to "C" indicates a negative result[13]. All the participants were subjected to mRDT and mPCR test upon enrolment. Samples of peripheral venous blood were collected from all the consented participants for malaria diagnosis using mRDT and mPCR tests. Results were obtained after running the samples using RDTs immunochromatography whereby a coloured detecting antibody marker binds to lysed parasite antigen and is carried by capillary action on a nitrocellulose strip and arrested by a capture antibody, resulting in a coloured band on a test strip[13]. The mPCR samples were prepared in dry blood spots of 3.5 x 3.5 Whatman's chromatography paper and safely packaged and stored at 4 degrees awaiting transportation to a reference laboratory. The samples were transported to the lab using cooler boxes as per the laid standards Operating Protocols. Quantitative PCR for malaria parasite was performed at the Steve Meshnick laboratory, University of North Carolina, USA.

Data analysis

Malaria tests using mRDT and mPCR tests were used to determine sensitivity and specificity for mRDT using mPCR as the gold standard for this study.

RESULTS

The mPCR test, was performed on 746 samples of nulliparous pregnant women in their first trimester. The test was able to amplify 705 samples. Out of these, 264 (37.4%) had positive mPCR test results whereas 441 (62.6%) had negative results. On mRDT test, 179 (25.4%) had positive results while 526 (74.6%) had negative results (Table 1). The test correctly identified 130 (49.2%) women as truly positive, and 392 (88.9%) as truly negative. However, it also showed 49 (27.4%) false positive and 134 (50.8%) false negative results (Table 1).

Table 1

Comparison of the mRDT and mPCR test results for 705 nulliparous pregnant women in their first trimester

		mPCR results		
mRDT results		Positive	Negative	Total
	Positive	130	49	179
	Negative	134	392	526
	Total	264	441	705
		Sensitivity = (130/264) *100 =49.2%	Specificity = (392/441) *100 =88.9%	

Sensitivity and specificity for the mRDT

These were calculated using the mPCR test results. Based on the mPCR test, the sensitivity and specificity of the mRDT were 49.2% and 88.9%, respectively. (Table 1). This suggests that 50.8% of the participants who required treatment (half of the positive participants) may have potentially missed treatment as they tested negative on mRDT, while 11.0% got treatment despite not having malaria.

DISCUSSION

An accurate and prompt diagnosis of malaria is the surest way to effectively treat and eventually eliminate the disease in a population at risk of fatal outcomes such as pregnant women. The study intended to determine the proportion of malaria cases missed through mRDT tests but detected through mPCR. Previous studies have reported that microscopy missed about 50% of PCR positive malaria infections, and hence the need for evaluation of the current alternatives, including the WHO preferred mRDT test. PCR is currently the most sensitive test in comparison to RDT and microscopy, especially in the detection of *P. falciparum species*[3,14]. However, it has to date not been optimized for routine diagnosis owing to among other issues cost, versatility and more importantly, the longer duration before the results are obtained It is therefore largely used as a confirmatory test or "Gold Standard" in endemic areas where resources permit[1,15]. In the context of this study, the sensitivity of mRDT test (true positive rate) was defined as the proportion of nulliparous women in the first trimester with malaria and a positive mRDT test. The test results were compared with those from the mPCR test.

From the results, the sensitivity of the mRDT test was 49.2%, suggesting that the test could only identify 49.2% (130) of the nulliparous women in their first trimester infected with the malaria, but missed 50.8% (134) of the women with the disease who may have gone untreated. Additionally, the specificity was 88.9%, implying that 11.1% (49) of the participants were false positive on mRDT, but negative on mPCR, and may have therefore been treated for malaria without having the disease. Both results could have resulted in serious pregnancy outcomes, especially the missed intervention. The specificity result (88.9%) compares well with previous studies conducted in similar settings, but the sensitivity outcome (49.2%) is way below (8, 9). However, high false negative rates of mRDT have also been reported from related studies within the region[16]. This perhaps calls for more studies before the mRDT tests are rolled out for mass screening programmes as recommended by WHO. It is important to note however that the WHO recommendation relate to clinical, but not asymptomatic malaria as in the case of

our study population which comprised of nulliparous women in first trimester who were on routine clinic visits. Indeed, false negative RDTs have been shown to be more prevalent in persons with asymptomatic infections[17].

There are many factors that may have contributed to the low mRDT sensitivity results including the quality of our mRDT kits, low parasitaemia, inadequate transportation and storage conditions and incorrect testing process including incorrect interpretation of the results[18]. Being from a malaria endemic area, the participants may also have had high antibody levels which has the potential to supress parasitaemia[19]. They may also have had high antigen levels in circulation as a result of recent malaria infections. The test techniques and interpretation methods may also not have been as robust as they were carried out by different people at different sites. It is also worth noting that our RDT tests were carried out on the field, whereas PCR tests were carried in a high precision lab in the USA. It is also worth noting that this was a sub-study within a clinical trial. A randomly controlled trial may provide a correct perspective with regards to the performance of the mRDT.

Several factors have been demonstrated to affect the sensitivity of RDTs based on detection of HRP-II, including inherent limitations of the device, low parasitic densities, mutation or deletion of the gene encoding the HRP-II, and storage conditions[20]. mRDT tests require regular training and adequate quality control. Lack of appropriate training and correct use of RDT within variety of contexts in sub-Saharan Africa has been described as one of the reasons for the varied results. False positives may also result from poor performance of specific RDT brands and low-parasite density infections[10,21]. However, as earlier noted, high false positive rates have also been reported, despite the high quality RDTs, good storage, proper handling and highly trained operators[16]. False negatives have also been attributed to the RDT brands, and *P. falciparum* type. Although *P. falciparum* isolates without the hrp2 gene are important causes of false-negative HRP2-based RDTs, pLDH-based RDT have also been linked[10,16,22]. Indeed, some researchers have even touted for the use of combined HRP2/pLDH-based RDTs in order to reduce the impact of false-negative HRP2-based RDTs in the detection of symptomatic *P. falciparum* malaria, although the combination is currently not recommended by WHO[13].

Circulating antibodies against *P. falciparum* histidine-rich protein 2 (PfHRP2) have been reported to interfere with antigen detection by RDTs[19]. This has been attributed to the potential of pre-formed host anti-PfHRP2 antibodies to block target antigen detection, thereby causing false negative test results[19]. Further, and as earlier noted, the deletion of the genes that encode for HRP2 and HRP3 (*pfhrp2 and pfhrp3*) have also been demonstrated to result in false-negative HRP2-RDT results, to the extent that some researchers have proposed that interpretation of RDT results should be supported by microscopy in conjunction with clinical observations[22,23].

CONCLUSION

The results from this study suggest that mRDT testing may not be that accurate in the detection of malaria in asymptomatic pregnant women since the sensitivity was only 49.2%, implying that 50.8% (134) of the women with the disease may have gone untreated. Additionally, the specificity was 88.9%, implying that 11.1% (49) of the participants were false positive on mRDT, but negative on

mPCR, and may have therefore been treated for malaria without having the disease.

Several factors may have been responsible for this including the test techniques and interpretation methods, inherent limitations of the device used, low parasitic densities and poor storage of the test kits. There should be further evaluation of the mRDT kits in different settings before they are rolled out for mass screening programmes.

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