A COMPARISON OF RAPID DIAGNOSTIC TESTING (BY PLASMODIUM LACTATE DEHYDROGENASE), AND QUANTITATIVE BUFFY COAT TECHNIQUE IN MALARIA DIAGNOSIS IN CHILDREN

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Abstract

Background: The World Health Organization (WHO) considers early and rapid diagnosis as one of the strategies to control malaria. This study compared the performance of Quantitative Buffy Coat (QBC) test and the Plasmodium lactate dehydrogenase (pLDH) rapid diagnostic test (RDT) with microscopy as the gold standard.

Materials and Methods: The study involved children ages 0-5 years who presented with a history of fever at the University College Hospital, Ibadan, Nigeria. Blood was collected from each patient and used for RDT, QBC and Giemsa-stained blood films for malaria parasites (MP). Results of QBC and RDT were compared with microscopy results for the diagnosis of malaria.

Results: A total of 370 cases (194 boys and 176 girls) were studied giving a male: female ratio of 1.1:1. Of the 370 cases tested using Giemsa-stained thick blood films for MP, 78 (21 %) were positive. For the QBC test, 78 (21%) of the cases were positive with sensitivity, specificity, positive and negative predictive values of 70.5 %, 92.1%, 70.5 % and 92.1 % respectively. Seventy-six (20%) of the cases were positive by RDT with sensitivity, specificity, positive and negative predictive values of 84.2 %, 95.2 %, 82.1 %, and 95.9 % respectively. There was no significant difference in the sensitivity of QBC compared with the RDT.

Conclusion: Both the QBC and the pfLDH (RDT) performed reasonably well in this study Malaria rapid diagnostic tests are recommended in malaria endemic clinical settings to avoid unnecessary antimalarial treatment.

Keywords: malaria, quantitative buffy coat, rapid diagnostic testing

List of Abbreviations: AO: Acridine orange, AIDS: Acquired immunodeficiency syndrome, ACT: Artemisinin-based combination therapy, CM:Cerebral malaria, BCP:Benzothiocarboxypurine, DDT:Dichloro-diphenyl-trichloroethane, DNA:DeoxyriboNucleic Acid, ELAM-1: Endothelial leukocyte adhesion molecule, G6PD: Glucose-6-Phosphate Dehydrogenase, HIV: Human immuno deficiency virus, HRP 2: Histidine Rich Protein 2, ICAM -1: Inter cellular adhesion molecule1, ICER: Incremental cost effectiveness ratio, IL-1: Interleukin -1, IFN-g: Interferon-gamma, IgG: Immunoglobulin G, MP: Malaria parasite, NADP: Oxidised Nicotinamide Adenine Dinucleotide Phosphate, NADPH: Reduced Nicotinamide Adenine Dinucleotide Phosphate, PCV: Packed Cell Volume (haematocrit), P. *falciparum:* Plasmodium *falciparum,* PLDH: Plasmodium lactate dehydrogenase, PCR: Polymerase Chain Reaction, PPV: Positive predictive value, QBC: Quantitative Buffy Coat examination, TNF: Tumour necrosis factor, NPV: Negative predictive value, RDT: Rapid diagnostic test, SP: Sulphadoxine -Pyrimethamine, SMA: Severe malarial anaemia, UM: Uncomplicated malaria, *USA: United States of America, VCAM-1: Vascular cell adhesion molecule,* WBC: White Blood Cell, WHO: World Health Organization.

Introduction

Malaria is a febrile illness caused by protozoa of the genus *Plasmodium* transmitted by female anopheles mosquitoes which feed on human blood (WHO, 2010). In spite of concerted past efforts at malaria control, it has remained a global health problem and a threat to the life of about 40% of the world population (WHO, 2010). Today, the goal of

malaria control is to prevent mortality and reduce morbidity and socioeconomic losses through the World Health Organization global Roll Back Malaria (RBM) initiative. The RBM Strategy included early case detection and prompt treatment, integrated vector management and containment of focal epidemics (Breman *et al* 2004). The World Health Organization (WHO) considers early diagnosis as the first basic element of the strategy to control the disease (WHO, 2009).

Traditionally, laboratory diagnosis had been made using the Giemsa-stained thick blood film microscopy, which continues to be the gold standard test (WHO, 2000). However, this test, in addition to being time-consuming, requires an experienced microscopist, a well-prepared film and a good quality microscope to achieve good sensitivity (Moody, 2002). Other methods have since been developed; these include methods for the detection of Plasmodia within erythrocytes (fluorescent microscopy, Quantitative Buffy Coat [QBC], dark field microscopy, nucleic acid probes and immunofluorescence), methods for the detection of plasmodial antigens in body fluids (radioimmunoassay, direct enzyme immunoassay) and methods for the detection of anti-plasmodial antibodies in serum (indirect immunofluorescence, indirect enzyme immunoassay, Western blotting) (Murray *et al*., 2008; WHO, 2009).

Giemsa microscopy and rapid diagnostic tests (RDTs) represent the diagnostics most likely to have the largest impact on malaria control today. These methods, each with characteristic strengths and limitations, together have been noted to represent the best hope for accurate diagnosis as a key component of successful malaria control (Nwuba *et al*., 2001; Wongsrichanalai *et al*. 2007; Murray *et al*. 2008; Shillcutt *et al*. 2008; Lubell *et al*. 2008; Parija *et al*., 2009; Uzochukwu *et al*., 2009; FMOH, 2011). The diagnostic performance of RDTs has been shown to vary with different locations of study to a statistically significant degree (WHO, 2000). This provides the impetus for local comparative studies of RDTs and microscopy. Furthermore, in Nigeria as a whole most studies evaluated the diagnostic performance of the Histidine-Rich Protein 2 (HRP2) based kits but not the Plasmodium Lactate Dehydrogenase (PLDH) based kits (FMOH, 2011). In addition, few studies have also been done in Nigeria on the diagnostic performance of the QBC test, which has also been shown to vary with locations of study and whose potential as a rapid and sensitive method of malaria diagnosis has been documented (Nwuba *et al*., 2001; Gaye et al 1999; Moody, 2002; Murray *et al*., 2008; Wongsrichanalai *et al*., 2007). Few studies have compared the diagnostic performances of these two methods-PLDH and QBC test- of malaria diagnosis (Nwuba *et al*. 2001; Wongsrichanalai *et al*., 2007).

This study was an experimental cross sectional design in which the QBC test was compared with a plasmodium lactate dehydrogenase (pLDH) diagnostic test (OPTIMAL) in the diagnosis of malaria, using Giemsa microscopy as gold standard. The endpoints were sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

Materials and Methods
Study design

This was an experimental cross sectional design in which the performance of QBC test and a pLDH based RDT were compared in diagnosing malaria infection using Giemsa stained microscopy as gold standard. For each diagnostic method (i.e. QBC and pLDH), the outcome measures were the proportion of cases correctly diagnosed as malaria-positive relative to Giemsa-stained MP result and cases correctly diagnosed as malaria-negative relative to the Giemsa-stained result.

Study location

The study was carried out in the children outpatient clinic and the children's emergency ward of the University College Hospital (UCH), Ibadan, Oyo State, south-western Nigeria between February 1st and May 31st, 2013. The UCH is an 850-bed tertiary hospital that serves as a referral centre for the city of Ibadan in which it is located and other parts of Nigeria depending on the diagnosis. The study population was made up of patients 0 – 5 years of age with fever at presentation or a history of fever in the three days prior to presentation, as well as other features of both uncomplicated and severe malaria. Using a malaria prevalence rate in febrile under 5 children, of 56.9% from a previous study, (Ikeh and Teclaire 2008) a minimum of 362 subjects were required.

Blood Collection Procedure

Each patient had a finger-prick using the ring finger (or big toe in infants) after thorough cleansing with 70% alcohol. Thick and thin film blood smears were made. Two millilitres of blood was then withdrawn from each patient and put into a Tri-Potassium Ethylene Di-amine Tetra acetic Acid (K_3EDTA) containing bottle and was used for Packed Cell Volume (PCV), pLDH rapid diagnostic test for malaria and QBC test.

Data Collection Procedure

All care-givers of the patients were interviewed using a structured pro-forma. History was obtained from the caregivers and children and complete physical examination was carried out on each patient by one of the investigators. All findings from physical examinations and other relevant information including the results of microscopy tests, RDTs, QBC test, PCV and parasite counts, were recorded on the standardized structured pro-forma.

Laboratory Procedures

The Giemsa stained blood films were examined by two malaria expert microscopists, one of whom has had years of experience working with an internationally-funded malaria research group (in a bid to reduce observer error) in the laboratory, without reference to the results of RDTs and clinical history of patient. Discordant results for blood film microscopy were reviewed by another expert microscopist. Every person positive for malaria by microscopy was treated with an artemisinin-based combination therapy.

OptiMAL RDTs (Diamed AG, Cressier, Switzerland) were transported refrigerated from Lagos to Ibadan and subsequently kept in a refrigerator and brought to room temperature and opened just before use to protect from humidity. The RDTs (OptiMAL-IT PLDH based test kits) were performed and interpreted according to the manufacturer's instructions by one of the researchers who was blinded to each subject's identity and to each sample's thick film result.

For the QBC technique, blood samples in acridine orange coated heparinised tubes were centrifuged at 13000 rpm for 5 minutes and the area just below the buffy coat examined in situ under a fluorescence microscope to detect the parasitized cells. The parasite nuclei fluoresced bright green, and the cytoplasm appeared yellow-orange.

Data Management and Analysis

Data was entered into a microcomputer and analysed using the SPSS 20.0 for Windows (Inc., Chicago, USA, 2001). Continuous variable estimates were expressed as mean (±SD) or median (inter-quartile range) while categorical variables were expressed in proportions and percentages. Categorical variables were compared using the chi-square test. Statistical significance level was set at $p < 0.05$. The Bayesian theory was used to estimate true positives, false positives, true negatives and false negatives and consequently the cases correctly diagnosed. The efficiency of a test corresponds to the percentage of correct test results. Statistical significance of performance differences between tests was evaluated using the McNemar's test.

Results
General Characteristics

There were 370 participants in this study, with ages ranging from 3 days to 60 months and a mean (± standard deviation) of 28 ± 19.6 months. Participants aged between 0-12 months accounted for 116 (31.4%). Of the 370 cases, 194(52.4%) were males and 176(47.6%) were females yielding a male to female ratio of 1.1:1

Malaria diagnostic test results

Of the 370 cases tested, 78(21.1%) were positive for malaria parasite by microscopy, 76(20.5%) were positive on rapid diagnostic test and 78 (21.1%) were positive on the QBC test.

Sensitivity, Specificity, Positive and Negative Predictive Values of RDT and QBC test
Comparison of Blood film for MP and Rapid diagnostic test variables

Table I: Comparison of Rapid diagnostic test results and Blood film for MP

	Blood film microscopy		Total
	+	−	
+ RDT	64	14	78
−	12	280	292
TOTAL	76	294	370

RDT: rapid diagnostic test

Based on the results in Table I, the sensitivity of RDT in detecting malaria was 84.2%, the specificity was 95.2%, the positive predictive value 82.1% and the negative predictive value 95.9%. The test efficiency was 93.0%.

Comparison of Blood film for MP and QBC test variables
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Comparison of Blood film for MP and Rapid diagnostic test variables

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Comparison of Blood film for MP and QBC test variables

Table II: Comparison of QBC test variables and Blood film for MP

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		Blood film microscopy		TOTAL
		+	−	
	+	55	23	78
QBC				
	−	23	269	292
	TOTAL	78	292	370

Based on the results in Table II, the sensitivity of QBC in detecting malaria was 70.5 %, the specificity was 92.1%, the positive predictive value 70.5 % and the negative predictive value 92.1%. The test efficiency was 87.6 %.

Table III: Paired data layout for comparison of sensitivities of RDT and QBC using blood film microscopy as reference

	QBC		
RDT	Positive (1)	Negative (0)	Total
Positive (1)	51	13	64
Negative (0)	4	10	14
Total	55	23	78

Using the McNemar test $X^2 = 4.76$; p=0.049
Comparison of the sensitivities of RDT and QBC as shown in Table III did not show any statistically significant difference.

Table IV: The paired data layout for comparison of specificities of RDT and QBC

	QBC		
RDT	Positive (1)	Negative (0)	Total
Positive (1)	4	8	12
Negative (0)	19	261	280
Total	23	269	292

$X^2 = 4.48$, p=0.052

Similarly, comparison of the specificities of RDT and QBC did not show any statistically significant difference (Table IV).

Distribution of malaria parasitaemia according   to parasite density

The Parasite densities of the patients in this study ranged from 31 to 273,831 parasites per microliter with a geometric mean parasite density of 63371.9 ± 65544.4. Forty-nine (64.4%) of the 76 positive slides for malaria parasites had parasite densities > 4,500. (Table V).

Distribution of malaria parasitaemia according   to parasite density

Table V: Sensitivities of RDT and QBC tests at various parasite densities

Parasites count (parasites μL^{-1})	Thick Blood film (MP) Positive	RDT Positive	RDT Sensitivity (%)	QBC test Positive	QBC sensitivity (%)
≤ 500	8	1	12.5	2	25.0
501 – 1,000	3	1	33.3	1	33.3
1,001 – 4,500	18	13	72.2	13	72.2
>4,500	49	49	100.0	39	79.6
Total	**78**	**64**	**82.1**	**55**	**70.5**

For both RDT and QBC tests, there was a tendency for increased sensitivity with increasing parasite densities with 100 % sensitivity for RDT and 79.6% for QBC in patients with a parasite count of >4,500 per microliter (Table V).

Discussion

Malaria is a parasitic infection of global importance and a major public health problem in Nigeria, accounting for sizeable morbidity, mortality and economic loss (NPC, 2012; Orimadegun *et al.*, 2007). The absolute necessity for rational anti-malarial therapy in the face of rampant drug resistance places importance on the accuracy of malaria diagnosis (WHO, 2009; 2010).

The 70.5 % sensitivity of the QBC test obtained from this study was low, in keeping with the findings from Adeoye and Nga (2007) in Nigeria where a sensitivity of 55.9 % was reported and the sensitivity of 78 % reported by Parija *et al.* (2009) in South India. Findings from the present study were however at variance with findings from a study by Fagbenro-Beyioku *et al.* (2007) in Lagos, Nigeria in a study involving 270 patients of various ages, where a sensitivity of 92.5 % was reported (Fagbenro-Beyioku *et al.*, 2007). Studies in other parts of the world have also yielded high sensitivities: Anthony *et al.* (1992) reported 94% sensitivity for QBC in the eastern Jayawijaya Mountains of Irian Jaya (Indonesian New Guinea), an area of low parasite transmission, and Ponsilapatip *et al.* (1990) reported 99.5% sensitivity in Thailand (Ponsilapatip *et al.*, 1990). The latter studies were conducted in areas of low malaria transmissions with the implications that observed cases were likely to be of high malaria densities thus accounting for the high sensitivities as detection of the parasites would be much easier.

Specificity of the QBC test in this test was 92.1 % which was in keeping with the finding of 95.0% reported by Wang *et al.* in a study conducted in Junlian and Mingshan counties, Sichuan, China (Wang X *et al.*, 1996).

Sensitivity and specificity, of Optimal RDT test in the present study were 84.2 and 95.2% respectively. Furthermore, the positive predictive and negative predictive values were 82.1, and 95.9 % respectively, which are consistent with findings from earlier studies (Agomo *et al.*, 2003; Chayani *et al.*, 2004). Agomo *et al.* in a study of 240 patients from South West Nigeria, reported sensitivity, specificity, positive and negative predictive values of 63.95, 92.20, 82.1 and 82.1 %, respectively for the OptiMAL RDT (Agomo *et al.*, 2003). Chayani *et al.* (2004) in their study in India, of 232 cases reported that the OPtiMAL test had sensitivity of 88.4 % and 96.8 % for *P. falciparum and P. vivax* respectively (Chayani *et al.*, 2004) Higher sensitivities have been however reported from other studies. Gbotoso *et al.* (2010) reported a sensitivity of 97.2 % for the Optimal RDT, tests performed on whole blood samples while it was 48.3 % for tests performed on the supernatant of spun saliva (Gbotoso *et al.*, 2010). In that study, patients aged 8 months to 13 years were eligible to participate in the saliva study if they had microscopically confirmed pure *P. falciparum* infection $\geq 2,000$ parasites/microlitre of blood, a temperature $\geq 37.5°C$ or recent history of fever, and absence of other concomitant illness. The selection of patients with such parasite densities apparently led to the high sensitivity obtained from the study in contrast with this present study where parasite counts were as low as 50 parasites/ microlitre. Obviously, one of the reasons for the low sensitivity of detection using Optimal-IT dipsticks may be the fact that this RDT was commercially designed to detect high levels of lactate dehydrogenase in whole blood. It could also be due to the presence of gene mutation variants of

the plasmodium species which express little or no pLDH antigen, thus resulting in a negative pLDH assay (Malik *et al*., 2004). False positive reactions may have occurred as a result of sequestration of parasites in the deep tissues and organs resulting in negative results on blood film microscopy but positive results by the RDT as the enzymes are present in blood (Makler, 1998; Murray, 2008).

Based on findings of this study, there was no statistically significant difference between the sensitivities and specificities of QBC and pLDH RDT in the diagnosis of malaria. This is in keeping with a similar study done in India by Kocharekar *et al*. where they found that the sensitivity of pLDH based RDT test was similar when compared to QBC (Kocharekar *et al*., 2014). In spite of the greater cost or more expensive equipment required (Adeoye *et al*., 2007)   our study showed no superiority of QBC over pLDH, the latter is therefore recommended in preference to QBC in a resource constrained setting like ours.

The sensitivity of the pLDH in this study was highest at parasite densities >4,500 parasites/microlitre, whereas the lowest sensitivity was documented at the parasite density less than 500 parasites/microlitre in keeping with the fact that the RDT was commercially designed to detect high levels of parasite lactate dehydrogenase in whole blood. Similar findings have been reported by different researchers. Cooke *et al*. in a study in 1999, observed similar test performances in terms of sensitivity, specificity, positive as well as negative predictive values, but also observed a marked decline in sensitivity of the test at parasitaemia less than 500 parasites/µl (Cooke *et al*., 1999). Similarly, the sensitivity of the QBC test, was related to parasite density and highest at parasite densities >4,500 parasites/ microlitre but lowest at the parasite densities less than 500/microlitre in keeping with findings by Lowe *et al*. in Kenya, where QBC had an overall sensitivity of   93.6 % but 41.7% sensitivity at parasitaemia less than 100 per microliter (Lowe *et al*., 1996). A study by Adeoye and Nga in Nigeria also showed a sensitivity of 33.3 % at low parasite densities (overall sensitivity was 55.9%) (Adeoye and Nga, 2007). The occurrence of false negative pLDH and QBC tests at low parasite densities indicates the need for a high index of suspicion in cases with clinical features in keeping with malaria. Patients that test negative to these screening tests should therefore be subjected to microscopy of stained blood films.

The WHO has recommended a minimum standard of 95% sensitivity at parasite densities of 100/µL. However, in the present study, at parasite densities of <500/µl the sensitivities were 12.5 percent. This may not be in keeping with the WHO standards, but in terms of parasite threshold associated with fever in high transmission settings, which has been noted by some authors (Smith *et al*., 1994; Mwangi *et al*., 2005; Mmbando *et al*., 2009) to be at parasite densities $\geq$ 4, 000 parasites/microliter in children aged > 1year to 15 years, the sensitivity at parasite densities >4,500 parasites/microliter was 100% for the pLDH RDT whereas it was 79.6% for the QBC test (Smith *et al*., 1994; Mwangi *et al*., 2005; Mmbando et *al*., 2009). This implies that in a high transmission setting such as in Ibadan, a negative pLDH test and QBC test in that age range would make the diagnosis of malaria unlikely. They are therefore recommended for use in settings that lack microscopists depending on the resources available after weighing the storage requirements (such as refrigeration and the initial start up high costs of the QBC equipment. Furthermore, pLDH is a very good alternative in settings where expert microscopist (microbiologist) is not available especially in endemic area of tropical African region where level of parasitaemia makes it 100 % sensitive and highly specific too.

A cost-effectiveness analysis comparing the two tests would therefore be useful in deciding which test would be suitable in any setting.

Conclusion

Malaria diagnosis has remained a useful tool in the strategy for the control of malaria. This study has revealed the usefulness of the RDT and the QBC in the diagnosis of childhood malaria in a resource constrained setting. Sensitivities of both are better at higher parasite densities. The presence of false positives at low parasite densities underscores the need to carry out microscopy in test negative cases when malaria is strongly suspected. The lack of significant difference in sensitivity between the RDT and QBC makes the RDT a better choice given its less expensive requirements. The study further underscores the importance of the use of the RDT tests to confirm diagnosis in resource constrained settings where persons are not trained in microscopy.

Ethical consideration

Ethical approval for the study was obtained from the Joint Ethics Committee of the University of Ibadan and University College Hospital, Ibadan and informed consent obtained from all caregivers.

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