Full Length Research Paper

Evaluation of a malaria rapid diagnostic test among febrile children in Sokoto, Nigeria

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Malaria rapid diagnostic test (RDT) is an antigen capture assay that enables rapid diagnosis of malaria without the need for electricity or highly skilled technicians. Though potentially useful, its adoption needs to be guided by local test sensitivity. This study evaluated the diagnostic performance of a commercially available RDT (Malaria Pf rapid device, Biotec Laboratories Limited, United Kingdom) among 400 febrile children(aged 6months to 12 years) in Sokoto, Nigeria. It was a prospective observational study conducted at the Paediatric Outpatient Department (POPD) of UDUTH, Sokoto between March and October, 2009. Finger prick blood samples were collected from each of the patients (day 0) and immediately tested for falciparum malaria by both Giemsa microscopy and rapid diagnostic test (RDT). Patients with both positive RDT and positive microscopy results on day 0 were retested on day 7 (after antimalarial therapy) by both diagnostic methods. The prevalence of malaria among the study cohort was 40.8% by microscopy and 39.5% by RDT. The RDT had a sensitivity of 90.2% and specificity of 95.4%; with positive and negative predictive values of 93.0% and 93.4% respectively. Test accuracy was 93.3%, whereas reliability was 85.3%. Test sensitivity is reduced by low parasite density (100% at > 1600/µl Vs 69.2% at <800/µl). Of the 69 patients who were retested on day 7 after antimalarial treatment, 18 (26.1%) still had positive RDT test even though negative by microscopy and afebrile at the time of follow up. The diagnostic performance of the RDT in this study was good. Hence, it is recommended as an alternative method for diagnosis of malaria, especially when microscopy is not feasible.

Key words: Malaria, rapid diagnostic test, children, Sokoto, Nigeria.

INTRODUCTION

Malaria remains a major public health problem in sub-Saharan Africa (Oshikoya 2006, WHO 2012). Children are particularly prone to its severe manifestations and they can deteriorate rapidly if not identified early and treated appropriately. In most tropical countries, making prompt and accurate diagnosis of malaria is a formidable challenge (Hopkins, 2007). This is because malaria microscopy, which is the gold standard for routine malaria diagnosis, is not always feasible due to lack of electricity and limited supply of microscopes and reagents (Perkins 2006, Wongsrichanalai 2007, WHO 2010). It is also technically demanding and timeconsuming, often resulting in therapeutic delays. Clinical method of diagnosis, though practiced in many parts of Africa, is often unreliable due to non specificity of malaria symptoms (Murray 2008, Uzochukwu 2010, WHO 2011). This method results in over diagnosis and unnecessary treatment which increases the risk of resistance and adverse drug reactions (Perkins 2006, WHO 2010). Malaria rapid diagnostic tests (RDTs) have currently been recommended by World Health Organization (WHO) as alternative method for parasitological diagnosis of malaria (WHO 2011). These immunodiagnostic tests detect specific antigens (proteins) produced by malaria parasites (Murray 2008, WHO 2011). They employ a dipstick or test strip bearing monoclonal antibodies directed against target malaria parasite antigens, which are present in the blood of infected people. The antigens currently targeted by the RDTs are Plasmodium lactate dehydrogenase (PLDH), Plasmodium aldolase and histidine rich protein 2 (HRP2) (Wongsrichanalai 2007, Murray 2008). HRP2 is a water soluble protein produced by asexual stages and young, but not mature, gametocytes of P.falciparum (Moody 2002). The amount released in vitro increases throughout the intra erythrocytic cycle, with large quantities being released during schizoint rupture (Moody 2002). Several formats of HRP2-based RDTs are now commercially available and are increasingly being used in Africa. They are rapid, simple to operate and easy to interpret

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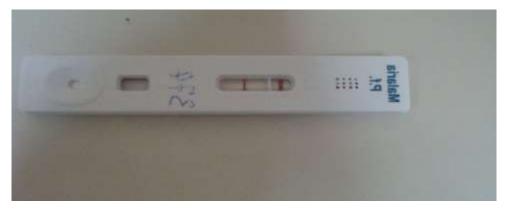


Figure 1. A positive RDT test: observe the appearance of vertical (pink) bands in both the control and test windows.



Figure 2. A negative test: note the presence of a single vertical (pink) band in the control window only. Unlike figure 1, a second vertical (pink) band is absent in the test window.

Table 1. Age distribution of 400 febrile children seen at POPD (UDUTH, Sokoto).

Age (years)	Frequency (n)	Percentage (%)	Cumulative (%)
0.5- 1.0	63.00	15.75	15.75
1.1-5.0	219.00	54.75	70.50
5.1-10.0	85.00	21.25	91.75
>10.0	33.00	8.25	100.00
Total (N)	400.00	100.00	200.00

(Wongsrichanalai 2007); hence useful in rural areas with no electricity or laboratory facilities and skill for microscopy (Nwuba et al., 2001, Ben-Edet et al., 2004). Though they are potentially useful, their adoption needs to be guided by local test sensitivity. This study therefore evaluated the diagnostic performance of one of the commercially available HRP2 based RDTs (Biotech Malaria pf), with the aim of determining its sensitivity and specificity using microscopy as gold standard. It also compared test results of the RDT with that of microscopy after anti malarial therapy.

MATERIAL AND METHODS

The study was conducted at the Pediatric Outpatient Department (POPD) of Usmanu Danfodiyo University Teaching Hospital (UDUTH), Sokoto, which is a tertiary health care facility located in Sokoto, Northwestern Nigeria (Sokoto 2008). The climate in this region is characterized by seasonal variation, with a hot dry season that spans from October to April and a rainy season which starts in April and lasts up to October. In the study area, the diagnosis of malaria is based mainly on clinical features supported by microscopy. RDTs are never routinely used; partly due to the exorbitant cost of the kits for RDT, non availability and lack of expertise in the usage of this diagnostic method. The study was a prospective observational study conducted over a period of eight months, from March to October 2009. This period coincided with rainy season which is characterized by increased malaria transmission. The study subjects were children who presented to the POPD of UDUTH, Sokoto with fever or history of fever. Those who satisfied the following criteria were included for the study: Age 6 months to 12 years; presence of fever (defined as axillary temperature of $\geq 37.5^{\circ}$ c) and informed written or thumb-printed consent by the subjects and/or their parents/guardians. Assuming a malaria prevalence of 33.6% for the north western Nigeria (FMOH 2004) and attrition rate of 10%, a minimum sample size of 377 children was determined. Over the period of study, a total of 400 children were recruited consecutively as they presented to the POPD. The study was approved by the Ethics and Research Committee of UDUTH, Sokoto.

Initial assessment and evaluation

Relevant demographic information and clinical history including age, gender, presenting symptoms, duration of fever, clinical diagnosis, and history of treatment in the preceding 2 weeks were obtained from the patients or their parents/quardians and then recorded into a predesigned study proforma data sheet. All the patients had complete physical examination. measurements of their axillary temperatures and body Prior to commencement of the study, the principal investigator had undergone six weeks training on the use of RDT and retraining on the technique of staining and microscopic examination of blood films at the School of Medical and Laboratory Sciences of

Usmanu Danfodiyo University, Sokoto (UDUS). The training was under the supervision of a qualified and experienced laboratory scientist who was also a lecturer at the school.

Rapid diagnostic test (Biotec Malaria pf Rapid Device)

The rapid diagnostic test for malaria was performed using Malaria pf Rapid device (Biotec Laboratories limited, United Kingdom). It has a shelf life of two years at the time of usage. Each kit has 25 individual pouches, with each pouch containing a blue-coloured desiccant and a 5µl sample applicator. The kit also contains a clearing buffer and an instruction manual.

Test principle

The Biotec Malaria pf Rapid Device is an in-vitro immunochromatograhic test which detects circulating *P. falciparum* Histidine-Rich Protein 2 (HRP2) antigen in whole blood. When blood is added it flows along the strip. If malaria parasite antigen (HRP2) is present, a control and a positive test bands are formed. In the absence of the antigen, only the control band is formed.

Quality Control measures

As part of quality control measures, all the RDT test kits were stored within the recommended temperature range of 4-40°C using an air conditioned room and the integrity of the kits was ascertained before commencement of the test by checking for the blue colour of the desiccant. A colour change indicates exposure to moisture which might affect RDT sensitivity. In addition, quality control (QC) testing of each of the RDT pack was done using positive and negative control samples which were prepared with the assistance of Chief Laboratory Scientist at the UDUTH Paediatric side laboratory.

Test technique

For each patient that was enrolled for the study, finger prick blood sample, which is ideal for detection of malaria parasites, was collected. The sample was used for testing by both the RDT and the Giemsa microscopy methods. The rapid diagnostic test (RDT) was performed according to manufacturer's instruction. Results were read blindly and independently by both the investigator and the laboratory scientist at the pediatrics side laboratory, within 15 minutes as recommended. RDT was considered positive for P. falciparum when pink coloured bands appeared in both the control window 'C' and test window 'T' (see Figure 1). The test was recorded as negative for p. falciparum when only one pink coloured band was seen in the control window 'C' (see Figure 2). However, it was considered invalid when no band appeared the device or when the control line was absent.

Table 2. Result of Giemsa stained microscopy against RDT.

Giemsa microscopy	Rapid diagnostic test (RDT)				
	Positive	Negative	Total		
Positive	TP =147.0	FN =16.0	163.0		
Negative	FP = 11.0	TN=226.0	237.0		
Total	158.0	242.0	400.0		

TP=True positive, TN=True negative, FP=False positive, FN=False negative

Table 3. Parasite density against RDT sensitivity.

Parasite density(/µl)	No. Positive by microscopy(N=163.0)	No. Positive by RDT(N=158)	Sensitivity (%)
None	0.0	11.0	
<200	4.0	0.0	0.0
200-400	9.0	3.0	33.3
401-800	13.0	9.0	69.2
801-1600	28.0	26.0	92.9
1601-3200	41.0	41.0	100.0
3201-6400	20.0	20.0	100.0
6401-12,800	23.0	23.0	100.0
>12,800	25.0	25.0	100.0

Giemsa stained Malaria microscopy test

Thick and thin blood films were prepared and stained according to standard guidelines as described by Cheesbrough (1998) and by Warhaust and Williams et al., (1996). Blood films were examined by the

investigator blindly, without having prior knowledge of the corresponding RDT result. The examination was done at the UDUTH paediatrics side laboratory with a light microscope, using 40x and 100x objectives. A positive test is when asexual forms of falciparum are seen. A slide is considered negative only after 100 microscopy fields were examined (Cheesbrough1998). Discordant results (between the RDT and microscopy were rechecked independently experienced laboratory scientist, at the UDUTH main Microbiology Laboratory. They were first blinded to the clinical status of the patient, the result of the RDT and that of initial microscopy. Results obtained by the two independent Laboratory scientists were regarded as final. Parasite density was determined from thick film by counting the number of parasites against 200 leucocytes and assuming that each subject has 8000 leucocytes /ul of blood as described by other workers (Warhaust and Williams et al. 1996). Therefore, parasite count = (number of parasite x 8,000)/200.

Treatment and follow up

All patients with microscopically confirmed p. falciparum parasitaemia were given artemisinine-based combination (ACT) therapy according to standard guidelines (FMOH 2005). They were told to return for follow up on day 7 after treatment or within 48 hours if there is no resolution of fever. At follow up, axillary temperature was measured and patients were clinically examined for pallor, presence of complications of malaria or other systemic illnesses. To compare test results of RDT and microscopy after anti malarial therapy, patients with both positive RDT and microscopy tests on day 0(day of first contact) were simultaneously retested on day 7(after treatment) by both diagnostic methods.

Data analysis

Data was entered and analyzed using Epi info version 332, statistical software and also with the help of Graph Pad inStat version 3.05 Software. Nominal data was summarized using proportions and frequency tables were used (X^2) illustrate quantitative data. Chi square Yates correction was used to compare proportions. But where more than 20% expected frequencies are less than 5. Fisher's used. Test exact probability test was of significance was determined at p < 0.05. The variables determined using following were microscopy as gold standard: number true positive (TP), false positive (FP), true negative (TN), and false negative (FN). Test performance including sensitivity, specificity, positive indices value (PPV), negative predictive value predictive (NPP), test accuracy and reliability (J index) were also calculated. Furthermore, sensitivity of the RDT was determined in relation to parasite densities and to other variables including gender, duration of fever and type of concomitant illnesses at presentation. Degree of agreement between two tests was measured using Kappa statistic (k) as described by Kramer and Fernstein (1981).

RESULTS

Demographic and clinical characteristics of the study population

The study population comprised of 230 males and 170 females, giving a male to female ratio of 1.4:1. Mean age of the subjects was 4.1 + 3.5 years (range 6months to 12 years). The age distribution of the patients is depicted in Table I. Children under the age of five constituted 70.5% of the patients. The mean axillary temperature and mean duration of fever of the subjects at presentation were 38.40C +0.80C (range- 37.50C to 40.80C) and 4.3 days + 3.6 days (range: 1-14 days) respectively.

Laboratory diagnosis of malaria: comparison of RDT with gold standard microscopy

The prevalence of uncomplicated malaria among febrile children was 40.8% by microscopy and 39.5% by the RDT. Overall, 147 patients had a matching positive microscopy and RDT results (true positive tests), whereas 226 patients were negative by both diagnostic test methods (true negative tests). There were 11 false positive and 16 false negative results (Table 2). The sensitivity and specificity of the RDT were 90.2% and 95.4% respectively. Both positive and negative predictive values were 93%. Test accuracy was 93.3%, whereas reliability of the test (J index) was 85.3%. Using Kappa statistics, a Kappa value (K) of +0.86 was obtained which showed good degree of agreement (concordance) between Giemsa microscopy and the RDT.

Parasite density

Parasite density ranged from 40 to 45,714 parasites / µl (Mean 4,393±7,932 Table 3 parasites μl). shows the distribution of positive **RDT** results and corresponding sensitivities the stratified bν density. When parasite parasite count was less than 200 / µl, the RDT failed to positive detect all the four slides. However. the **RDT** increased the sensitivity Ωf parasite consistently from 33% at density range of 201-400 / µl to 93% at 800-1600 / μl. With parasite count greater than 1600 / µl, sensitivity was 100%. It the is of note that 84.0% (137/163)of patients with positive had slides parasite density greater than 800/ **RDT** μl which is the level above which achieved sensitivity range of 93-100%. Of patients the 16 with false negative **RDT** results, 10 parasite than had density less 400/µl, 4 had density below 800 / μΙ while had relatively the remaining 2 patients high parasite ranging between 840/µl density and 1500 / µl.

Table 4. RDT sensitivity among patients with and without concomitant illnesses

Concomitant	TP	TN	FN	FP	Sensitivity (%)
Malaria alone	•••	114	1 11	• • •	(70)
(n=316)	132.0	164.0	9.0	11.0	93.6
Malaria + ARI					
(n=56)	11.0	40.0	5.0	0.0	68.8
Malaria + DDx					
(n=13)	2.0	10.0	1.0	0.0	66.7
Malaria + others					
(n=15)	2.0	12.0	1.0	0.0	66.7

Comparison of RDT and microscopy test results after treatment

All patients with positive microscopy results (163) and those with positive RDT test were treated with Artemisinin-based combination therapy (ACT) according to national anti malaria drug policy. The 16 patients with false negative results were excluded for post treatment retesting on day 7. Of the remaining 147 patients with concordant positive results on day 0, 75 were lost to follow up. Three patients were admitted emergency pediatric ward on account of deterioration of their clinical condition and were treated for severe malaria with intravenous quinine according to the national antimalarial treatment guidelines (FMOH 2005). They responded well to treatment, with resolution of fever by second day of therapy in two of the patients and by the 4th day in the other patient. All the three patients were negative both by microscopy and RDT tests on day 7 after treatment with quinine. The remaining 69 patients with matching positive microscopy and RDT results were followed, up to day 7 post treatments. All of them were afebrile and clinically stable by day 7. When retested on day 7 after treatment, 18 of the 69 patients (26.1%) were positive by the RDT, but negative by thick blood film microscopy. However, the remaining 51 patients (73.9%) tested negative by both diagnostic methods. The study showed that sensitivity of the RDT varies with duration of fever at presentation, being 90.5% below 3 days and falling to only 50% when the duration of fever was more than 7 days. False negative rate was significantly higher in patients presenting with fever duration less than 3 days (15/238 or 6.3%) than among those with fever beyond this period (1/162 or 0.62%). Conversely, the rate of false positive RDT tests was significantly higher when duration of fever presentation was more than 3 days (11/162 or 6.7% at > 3days vs 0% at <3days). (Fishers exact test, 2 sided p <0.0001). Similarly, there were significantly more false positive RDT results among patients who had preceding antimalarial drug therapy (8/146 or 5.5%) compared to those who had no treatment prior to presentation (3/254 or 1.2%).(Fisher exact test, 2 tailed p=0.0212). The Proportion of positive results increases with rising temperature (31.8%, 49.6%, 57.1% by microscopy and 30.9%, 46.3%, 58.5% by RDT at temperature ranges of 37.5-38.4 $^{\circ}$ c, 38.5-39.4 $^{\circ}$ c, and >39.5°c respectively). Though both Microscopy and the RDT tests showed increasing positivity with rising temperature, there was no statistically significant difference in parasite detection rate between the two methods at any given temperature. Patients who were clinically diagnosed with concomitant illnesses such as acute respiratory infection (ARI), diarrheal disease (DDx) or enteric fever in addition to malaria tend to have proportionately higher number of false negative results and hence reduced RDT sensitivity (Table 4).

DISCUSSION

The present study shows that the RDT (Biotec Malaria pf) had an overall sensitivity of 90.2% and specificity of 95% among febrile children attending our pediatric out patients' department. The result compares favorably with that of several studies (Singh et al., 1997, Beecham et al., 1999, Nwuba et al., 2000, Ben-Edet et al., 2004, Marx 2007, Oguonu 2007, Khainar 2009, Ansah 2010), which reported a variable sensitivity of 84.2-100% and specificity of 82.8%-98%. However, the sensitivity in our study was lower than that of Iran (100%) which used an RDT format (Biotec Mal pv/pf rapid device) from a similar manufacturer (Samane 2010). When compared to previous Nigerian studies, the sensitivity obtained by the present study (90.2%) was slightly lower than that of Ibadan. South-western Nigeria (93.1%) (Nwuba et al., 2001), but much higher than what was reported in Lagos (69.9%) (Ben- Edet et al., 2004). Variations in test sensitivity between these studies may be due to differences in the types of RDT formats used or due to variations in epidemiologic characteristics of the study population, level of parasitaemia, test methodology and skill (Wongsrichanalai microscopists et al., (2007).Nevertheless, the present study like earlier studies in Nigeria (Nwuba et al., 2001, Oguonu 2007), further confirms the efficacy of the RDT as alternative method for rapid diagnosis of malaria in endemic region. The sensitivity of the RDT decreases at low parasite density. This was evident by the fact that the RDT gave negative results for all the four study patients with parasite density below 200/µl; and it reached satisfactory sensitivity range of 93-100% only at density >800/µl. Fortunately, most of the parasitaemic patients (84%) in our study had parasite count above 800/µl, making the RDT still relevant for routine clinical use. Similar observation was made in Ibadan, Nigeria where sensitivity fell to 71% at parasite density less than 519/ μl(Nwuba et al., 2001) and in Lagos, Nigeria (Ben-Edet et al., 2004) where it decreased to as low as 14.3% at density below 400/ µl. Several other studies have demonstrated variation in RDT sensitivity with parasite density and have emphasized the tendency of the test to misdiagnosed patients with low parasitaemia (Singh et al., 1997, Beecham et al., 1999, Wongsrichanalai et al., 2007). Though 14 of the 16 patients with false negative results in our study had low parasitaemia with density below 800/ μl (10 <400/ μl , 4<800/ μl), the remaining two patients with negative results had relatively higher parasitaemia (840/ μ l and 1600/ μ l). Other workers (Beadle et al., 1994) have similarly observed false negative results in some patients with high parasitaemia, up to 18,000/ μ l. The explanation for this was not clear, but HRP-2 deletion/ mutation and existence of anti-HRP-2 antibodies or an inhibitor in patient's blood may be responsible (Wongsrichanalai et al., 2007). HRP-2 antigen is also known to have significant geographic diversity and some variants may escape monoclonal recognition, leading to false negative result (Murray 2008). Factors such as temperature, duration of fever and prior anti malarial drug therapy can affect the diagnostic performance of the RDT. The study showed that >50% of patients who tested positive by the RDT had temperature above

>39.5°c where as only 30.9% of the patients with temperature <38.5°c gave positive RDT result. Hence, patients with higher temperature are significantly more likely to test positive for malaria. This has been attributed to increase release of HRP2 antigens into the blood stream simultaneously with fever-inducing cytokines and other pyrogens at the time of schizont rupture (Ben-Edet et al., 2004). Beside temperature, duration of fever at presentation may have significant effect on RDT result. Our study observed that 15 of the 16 patients with false negative results had fever duration less than 3 days. The reason, as highlighted by Beecham Ngu et al., in Cameroon (1999), may be due to the fact that within the first few days of malaria infection, significant secretion of HRP2 antigens beyond the detection limit of the RDT might not have sufficiently occurred. Therefore, the RDT may be negative despite the presence of parasitaemia. On the other hand, the study showed that patients with longer duration of fever are more likely to have false positive results; as all the 11 patients with false positive tests had fever duration beyond 3days (8 of them between 3 and 7 days, the remaining 3 above 7 days). These facts should be born in mind while interpreting results of the RDT. In our study, malarial parasitaemia was detected in only 30.8% of patients who had preceding antimalarial drug treatment, compared to 46.5% in those who had no prior treatment at presentation. It is known that prior anti malarial drug therapy is associated with significant decrease in the rate of Giemsa positive test results (Warhust and Williams 1996). This is because such treatment can grossly reduce parasitaemia, making it harder to detect by microscopy (Warhust and Williams 1996). However, for the RDT, prior anti malarial drug treatment was not associated with significant reduction in parasite detection rate. Instead, an increase in the rate of false positive results (8 of 11) was observed in patients who had anti malarial treatment before presentation. This finding was similarly highlighted by Singh and Valechi et al., (1997) and by previous Nigerian studies in Ibadan (Nwuba et al., 2001) and Lagos (Ben-Edet et al., 2004). In the latter study, all the two patients with false positive results had received treatment with chloroquine and quinine before the tests were conducted. The phenomenon of persistent antigenemia after treatment was studied by several workers, with results suggesting

that HRP2 antigens can persist for a variable length of time (Beadle et al., 1994, Humar et al., 1997, Hopkins et al. 2007). Humar et al., (1997) observed antigenemia in 68% of patients by day 7 after treatment; whereas Hopkins and colleagues (2007) noted that the HRP-2 antigen persisted even beyond 28 days in some of their patients. However, Beadle et al. (1994) in Kenya found no evidence of circulating antigenaemia by day 6 post treatment. Among our study cohort, 26.1% (18/69) remained positive by the RDT on day 7 after treatment even though negative by microscopy and afebrile on clinical examination. The study is limited by the fact that follow up period could not be extended up to days 14 and 28. Another limitation is the inability to do Polymerase chain reaction and ascertain whether such positive RDT test is actually a false result or indeed a truly positive result due to re-infection. The implication of antigenemia persisting well after completion of therapy is that it limits the usefulness of HRP2-based RDT in monitoring drug resistance or treatment failure malaria (Moody 2002). This is in contrast to rapid test formats that are based on enzyme assays such as plasma parasite lactate dehydrogenase (PLDH). This enzyme, unlike HRP2 antigen, correlates directly with parasitaemia (Moody 2002, Gerstl 2010) and can therefore be used to monitor antimalarial therapy and drug resistant malaria (Hopkins 2007). Compared to Microscopy, the rapid diagnostic method is easier to learn and perform. It is also more rapid as results were ready within 15 minutes in all the study subjects compared to Giemsa microscopy which ranged between 45 and 60 minutes duration. The duration can be much more prolonged in settings with high out patients' case load and erratic power supply. At the time of the study, a single pack of 25 RDT units was procured from a sole distributor at a cost of six thousand naira (N6000.00 or \$37.5 equivalent) or two hundred and forty naira (\$1.25) per unit kit. The hospital fee for Giemsa microscopy, on the other hand, is only fifty naira (N50.00 or \$0.35) for the pediatric patients in UDUTH. This price gap between the RDT and Malaria microscopy test has also been noted in most other developing countries (U.S.\$0.55-U.S.\$1.50 per unit Vs U.S.\$0.12-\$0.40 per malaria smear) (Wongsrichanalai 2007). The apparently high cost of the RDT can discourage its widespread use in resource limited, malaria endemic countries. Hence, there is need for increase in subsidy on the RDTs to make them easily available and affordable. This will enhance rapid malaria case diagnosis and management, and play an important role in the fight against malaria.

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