PERSISTENCE OF *PLASMODIUM FALCIPARUM* HRP2 ANTIGEN AFTER EFFECTIVE ANTIMALARIAL THERAPY

O.S. Michael[1], A.E. Orimadegun[2], and C.O. Falade[1]

1. Department of Pharmacology and Therapeutics, College of Medicine, University of Ibadan, Nigeria.
2. Institute of Child Health, College of Medicine, University of Ibadan, Nigeria.

Correspondence:
Dr. O.S. Michael
Dept. of Pharm. and Therapeutics,
College of Medicine,
University of Ibadan,
Email: micobaro@gmail.com

ABSTRACT

Introduction: **Histidine Rich Protein 2 based (HRP2-based) malaria rapid diagnostic tests (mRDTs) have been shown to perform as well as routine light microscopy, however, they are limited by some factors including persistence of HRP2 antigenemia. In this paper we report the evaluation of an HRP2-based mRDT in a prospective study that enrolled children and followed them up for 28 days.**

Methods**: Children aged below five years, with acute episode of fever/pyrexia, were enrolled. The enrolled participants had expert malaria microscopy and RDT done at enrolment (Day 0), and on days 1, 2, 3, 7, 14, 21, and 28. The malaria RDT test was considered positive when the antigen and control lines were visible in their respective windows, negative when only the control band was visible and invalid when the control band was not visible. Faint test lines were considered positive. The RDT results were compared to those of expert microscopy.**

Results**: Two hundred and twenty-six children aged 29.2 ± 15.5 months were enrolled. The proportion of children positive by expert malaria microscopy and RDT was 100% and 95.6% respectively. During the 28 day follow up of the children the proportions positive by microscopy and RDT on days 3, 7, 14, and 28 were 1% and 94.6%, 0% and 93.5%, 0% and 91%, and 16.5% and 80.6% respectively. Gender and age dependent analysis of proportion of positive children were similar. Proportion of children with persistence of HRP2 antigen appeared to be lower in those with parasite density below 200/µL, however, this observation requires further evaluation in larger studies.**

Conclusions**: the study revealed a high proportion of persistence of HRP2 antigen in the children 28 days after effective antimalarial therapy. Histidine rich protein 2 based malaria rapid diagnostic tests are not recommended for monitoring of antimalarial therapies.**

Keywords: Malaria, RDT, Persistence, HRP2, Nigeria

INTRODUCTION

Parasite-based diagnosis is the cornerstone of effective antimalarial therapy.[1] Microscopy and rapid diagnostic tests are currently the most effective methods of malaria diagnosis. Microscopy, which is currently the reference standard for malaria diagnosis, is limited by scarcity of expert microscopists, poorly maintained microscopes, erratic electricity supply, and associated fatigue during the procedure.[2] However, microscopy retains certain essential advantages. Light microscopy visualizes viable parasitemia, parasites in different stages of malaria life cycle, enables the estimation of parasite density, and visualization of fragmented or isolated components of the parasite (e.g. chromatin dots). In addition, with additional equipment and/or special staining, microscopy can be automated with enhancement of parasite density estimation.[3,4] These advantages have made microscopy retain the status of gold standard for malaria diagnosis. However, despite these advantages, the use of malaria rapid diagnostic tests (mRDTs) as alternatives to microscopy has continued to increase.[5,6]

Rapid diagnostic tests are user friendly and results can be read by almost everyone after minimum training. They are instrument-free tests that provide results within minutes and can be used by community health workers. They are useful where: rapid diagnosis of malaria is needed, in communities with irregular electricity supply required for microscopes and where there is scarcity of expert microscopists. Their introduction has yielded many benefits; parasite-based malaria diagnosis is now a possibility at points of care and their use could potentially improve malaria in many settings.[7] Accurate diagnosis of malaria is an essential aspect of the preservation of our shrinking number of effective antimalarial therapies. Studies evaluating mRDTs have

also shown satisfactory accuracy compared to microscopy in diagnosis of malaria.[8,9] All these have led to the growing use of mRDT as a method of diagnosis of malaria worldwide.[10] However, there are some drawbacks with mRDT, one of which poses a significant challenge to their use in endemic countries with high rates of malaria transmission.

Majority of Malaria rapid diagnosis assays are based on three bimolecular pathways for the detection of presence of malaria parasites; parasite specific lactate dehydrogenase (pLDH), aldolase-based, and histidine rich protein2 (HRP2-based mRDTs.[11] Histidine rich protein 2-based mRDTs are the most evaluated and deployed for field diagnosis of malaria.[12] The performance of HRP2-based RDTs has been overwhelmingly shown to have adequate diagnostic accuracies. However, the challenges of using the tests need to be noted by the end user. The limitations of malaria RDTs include poor sensitivity at low parasite densities[13], susceptibility to the prozone effect[14], false-negative results due to *Plasmodium falciparum* Histidine Rich Protein 2 (Pf-HRP2) deficiency in the case of Pf-HRP2 gene deletions[15,16], cross-reactions between Plasmodium antigens and detection antibodies, false-positive results by other infections and susceptibility to heat and humidity.[17]

While false positive results have been reported in many studies evaluating HRP2-based mRDTs, persistence of HRP2 antigen has been reported in much fewer studies.[18,19] In a prospective study of Pf-HRP2 mRDT conducted at Thailand in 2001, HRP2 antigenemia was found to persist in a large proportion of the participants for over two weeks.[18] This poses a serious challenge to countries like Nigeria where malaria prevalence is high and transmission intense. Persistent HRP2 makes it challenging to identify treatment failures and may result in repeated treatment of malaria when such treatment may be unnecessary. In this paper we report the evaluation of an HRP2 mRDT in a prospective study that enrolled children and followed them up for 28 days.

METHODS

Description of Study

A prospective trial that evaluated the use of malaria rapid diagnostic test (mRDT) in the diagnosis, management, and follow-up of children below five years of age with acute febrile illnesses. Children who had severe illnesses were excluded from the study and, treated or referred to a tertiary health center by study clinicians. Diagnosis of malaria was based on clinical presentation, microscopy, and RDT. Viral and bacterial illnesses were diagnosed based on clinical and laboratory features.

Treatment: Children with acute uncomplicated malaria were treated with artesunate-amodiaquine combination therapy and followed up for 28 days. Children with bacterial infections were treated with appropriate antibacterial medicines, while those with other illnesses were appropriately managed by study clinicians. Microscopy was done alongside RDT.

Study Sites and Duration: this study is a sub- set of a main study, a trial which was conducted at two sites; a rural primary health center and an urban secondary health center, in Ibadan, Southwest Nigeria. Southwest Nigeria is hyper-endemic for malaria and malaria diagnostic services are very limited in rural areas. The trial lasted from November 2013 to December 2014. This period spanned the two (dry and rainy) seasons in Nigeria.

Clinical and Laboratory Procedures

At presentation, after voluntary, witnessed and informed consent procedures, all participants went through detailed clinical evaluation. Biodata, history of the presenting illness, and clinical examination were done and results recorded in study case record forms. The children had their weights and heights measured. Nutritional assessment was also done at enrolment and follow- up visits. Fresh blood samples were taken using capillary flow method for both the malaria RDT (mRDT) and microscopy.

Malaria Rapid Diagnosis Procedure

Malaria RDT was done using SD Bioline Malaria HRP2-Based RDT kit manufactured in Gyeonhhi-do, South Korea. Trained clinical research staff conducted the RDT procedure. All tests were performed and interpreted by research staff in accordance to manufacturers' instructions. Enrolled children had RDT and malaria microscopy done at enrolment (Day 0), and at days 1, 2, 3, 7, 14, 21, and 28. The tests were considered positive when the antigen and control lines were visible in their respective windows, negative when only the control band was visible and invalid when the control band was not visible. Faint test lines were considered positive. Presence of malaria trophozoites or schizonts on microscopy was considered positive. Blood smears were declared microscopically negative if peripheral parasites were not seen after screening of 1000 high power fields. in positive smears, parasite density was estimated assuming a white blood cell count of 8,000/uL to equal one 1uL of blood.

Persistent HRP2 Antigenemia

This referred to persistence of positive result on the evaluated HRP2-based mRDT in those whose patent peripheral parasitemia has cleared from the blood by expert microscopy after antimalarial therapy.

Determination of HRP2 Dynamics

malaria rapid diagnosis positive participants were considered to have detectable levels of HRP2 antigenemia. The proportion (%) of participants positive during the course of follow-up was used to plot graphs of persistence of HRP2 antigen over the 28-day duration of the study. The plots of microscopy determined parasite clearance were done simultaneously.

Data Analysis

Data was entered into a study database and analyzed using SPSS[IBM] software. Parasite densities obtained by microscopy were presented as geometric means. Means and Standard Deviation (SD) were calculated for continuous variables. Chi square test was used to evaluate differences in proportion and a P value <0.05 was considered significant.

Ethical Considerations

The study was approved by the University of Ibadan/ University College Hospital Institutional Ethics Review Board before commencement.

RESULTS

Study Period

The study was conducted between November 2013 and December 2014.

Baseline characteristics of participants with acute uncomplicated malaria

Two hundred and twenty-six children were positive for malaria parasites by expert microscopy. The mean age of enrolled children who were positive for malaria 29.2 ± 15.5 months. Baseline parasite density ranged from 20 – 611600/uL with a geometric mean of 7,821/uL. Sixteen of the two hundred and twenty-six children who were positive for malaria had parasite densities less than or equal to 200/uL; four of them had false negative mRDT results. Six children, of the 210 with parasite densities >200/uL, had false negative results on mRDT (P = 0.000430). Table 1 summarizes the baseline profiles of the enrolled children.

Adherence to follow up schedules

During the period of the study, the number of children that returned for follow up on days 1, 2, 3, 7, 14, 21,

Table 1: Baseline demographic profiles of children enrolled into the study.

	Male	Female	Total
Gender	140	86	226
Age (months)	28.8 ± 15.6	29.9 ± 15.4	29.2 ± 15.5
Weight (Kg)	11.7 ± 3.1	11.5 ± 2.7	11.7 ± 2.9
Height (cm)	83.7 ± 14.2	84.5 ± 13.9	84.0 ± 14.0
Mean PCV (%)	30.0 ± 4.8	29.6 ± 5.5	29.9 ± 5.1
Number with Temperature> 37.4 ^{o}C	85	55	140
Parasitemia /uL	8540	6778	7821
(GM and range)	(20 – 611600)	(101 – 320000)	(20 – 611600)

Values are Mean ± SD
SD = Standard deviation
GM = Geometric Mean

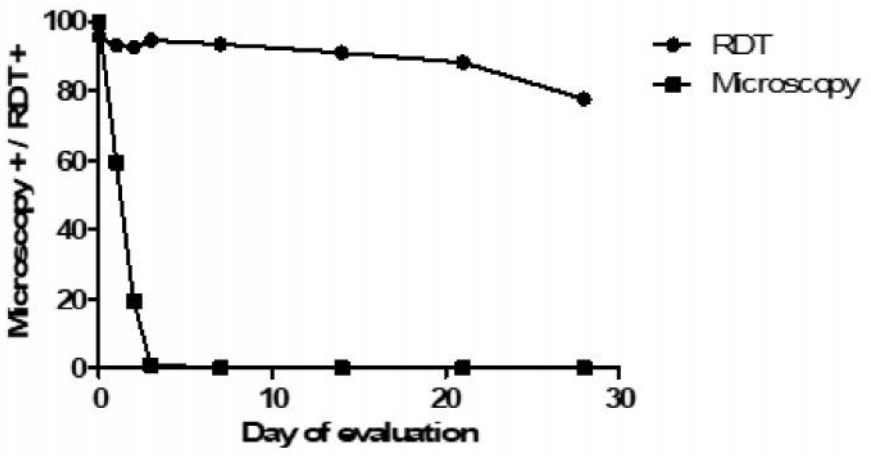

Figure 1: Proportions of study participants with HRP2 antigen on RDT (HPR2) and patent peripheral parasitemia on microscopy at enrolment and follow up for 28 days

and 28 were 220, 208, 207, 199, 189, 189 (of which 20 had patent parasitemia), and 191 (43 had patent parasitemia) respectively. Some of the children missed one or more days but were traced or they were brought during other visit days. Proportions of patients positive by microscopy and mRDT were done in children who came for the particular visit days (Table 2).

HRP2 Dynamics: Ten children had false negative results on mRDT. Parasite density (count/uL) in the ten children was 194, 2476, 229, 742, 880, 5538, 20, 410, 76, and 101/uL (Geometric mean 346/uL, range 20 – 5538/uL). Dynamics of positive results on microscopy and mRDT revealed a high proportion of children with persistence of HRP2 antigenemia,

Table 2: Proportions (%) of study participants with HRP2 antigen on RDT (HPR2) and patent peripheral parasitemia on microscopy (MP) at enrolment and follow up for 28 days.

Day of study	All study participants		Males		Females	
	RDT	MP	RDT	MP	RDT	MP
0	95.6	100	97.1	100	92.9	100
1	93.1	59.5	97.2	62.5	86.5	54.5
2	92.6	19.4	95.5	21.2	88.1	16.7
3	94.6	1.0	95.2	1.6	93.6	0
7	93.5	0	97.7	0	88.2	0
14	91	0	92.2	0	88.9	0
21	89.6	10.4	91.8	10.9	86.3	9.6
28	80.6	16.5	86.0	20.2	73	10.8

mRDT = Malaria Rapid Diagnostic Test
MP = Microscopy for Malaria Parasites

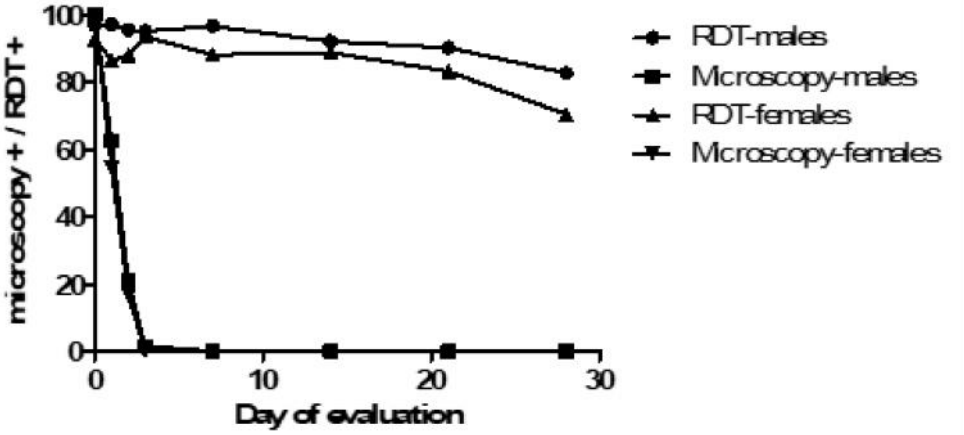

Figure 2: Parasite clearance/persistence of HPR2 Antigenemia by gender of enrolled children.

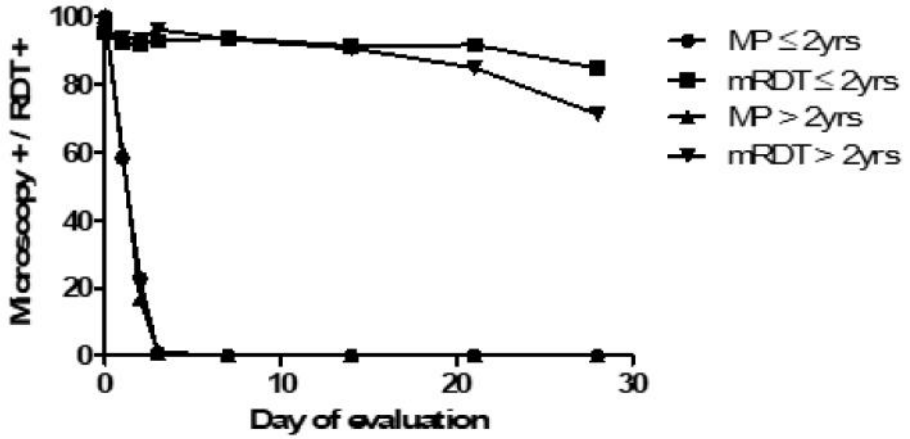

Figure 3: Parasite clearance/persistence of HPR2 Antigenemia by age (equal or below 2 years vs. age > 2 years).

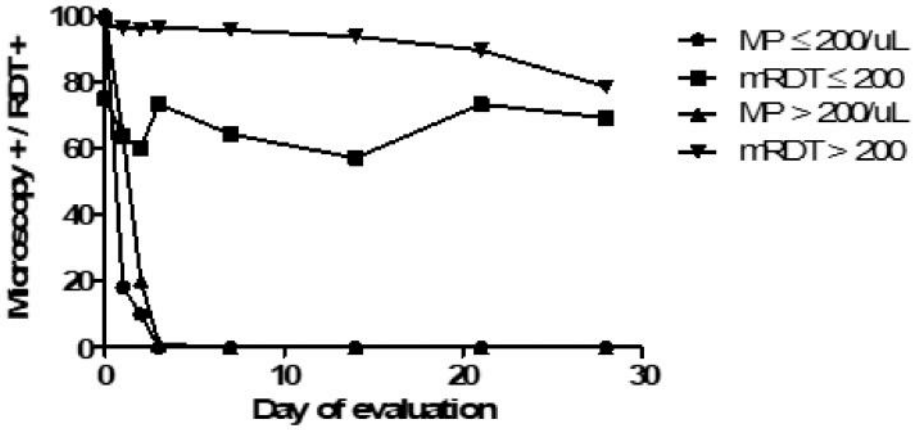

Figure 4: Parasite clearance/persistence of HPR2 Antigenemia by parasite density (equal or below 200/uL vs. Parasite density > 200/uL).

ranging from 73% to 86 % at day 28 of follow up (figures 1, 2, and 3). Proportions appeared to be lower ($p = 0.000430$) in children with parasitemia less than or equal to 200/uL on days 0, 1, 2, 3, 7 and 14 (Figure 4). The patterns of proportions were similar by gender and age.

DISCUSSION

The study showed a high proportion of persistence of HRP2 in the blood of enrolled children during the follow up period. At enrolment, the performance of mRDT compared to microscopy was comparable in terms of proportion of children diagnosed as positive. Our findings are similar to other reports of comparative good performance of mRDT compared to microscopy.[20-21] Over the years, it has become unequivocal that malaria rapid diagnostic tests have offered workers in different settings in malaria endemic countries the ability to comply with new WHO guidelines that recommend parasite-based diagnosis in every case of malaria, except in complicated malaria where prompt treatment should be commenced.

Follow-up of enrolled children revealed persistence of HRP2 antigen in a large proportion of the study participants. By day 3 when all the parasites have been cleared from the peripheral blood film, the mRDT results remained positive in 94.6% and were still positive in 80.6% by day 28 of follow-up. This finding suggests that mRDT may be less reliable for many days after episodes of malaria in children. Some previous studies have reported this persistence of HRP2 antigen in children after effective antimalarial therapy. In a study by Mayxay and others conducted in Thailand, persistence of HRP2 antigen occurred in 89% of patients with severe malaria and 61% of patients with uncomplicated malaria for greater than two weeks after successful antimalarial therapy. They also reported that persistence of HRP2 antigenemia did not predict treatment failure.[18]

In 2004, Iqbal and his colleagues reported results of a study in which 240 patients with *Plasmodium falciparum* monoinfection were tested for persistent parasite antigenemia after successful standardized antimalarial therapy by using the ICT Malaria Pf/Pv and OptiMAL-IT assays that detect the malaria antigens *Plasmodium falciparum* histidine-rich protein 2 (HRP2) and parasite lactate dehydrogenase (pLDH), respectively, as well as a panmalarial antigen (PMA).[23] The results showed that persistence of HRP2 was as high as 90.4% on day 3 with gradual decrease to 34.9% by day 14. The study also showed that false positive results were commoner with HRP2-based mRDT than parasite lactate dehydrogenase (pLDH)-based mRDT. This was attributed to the cessation of pLDH when the malaria parasite dies. They also showed that persistently positive results were not associated with gametocytemia.

In endemic countries with high malaria prevalence and transmission, persistence of HRP2 antigenemia poses a major challenge. In such settings, children are prone to repeated episodes of malaria within short intervals of time. The sensitivity of mRDT in such children will be near zero with a high proportion of false positive results once the children have been infected. This may result in a situation where children may be prescribed antimalarials repeatedly based on mRDT false positive results. It is thus recommended that expert microscopy should remain the more reliable modality of diagnosis during periods of high transmission in malaria endemic countries.

Interestingly, the baseline performance of the RDT studies was near that of microscopy. This may suggest

that the children may have been negative at the beginning of malaria transmission season and may be having their first infection at the time they were enrolled into the study. Additionally, they may have been malaria free for many weeks before presenting at the malaria clinic. There is thus a need to determine the time interval required for persistent HRP2 antigens to be cleared from the blood in different populations exposed to varying intensities of malaria transmission.

The causes of persistence of HRP2 antigen are not clear, however, some theoretical possibilities can be considered. It is highly probable that the parasites are still within the blood, but at densities below the detection limit or routine light microscopy. Prolonged screening of blood films to cover more fields than the routine may be able to detect persistence of submicroscopic malaria. Another reason may be attributed to artemisinin induced dormancy phenomenon. Several studies have observed the phenomenon of malaria parasite assuming quiescent states on exposure to artemisinin for different intervals of time.[24-26] The relationship between this phenomenon, parasite recrudescence, and treatment failure is still being evaluated. However, the phenomenon may explain the high proportion of treatment failures following artemisinin monotherapy.[27] Whether this phenomenon is related to persistence of HRP2 antigen or not remains to be determined. The study by Mayxay *et al.* did not find any association between persistence of HRP2 antigen and parasite recrudescence[18], however, the follow up duration was similar to ours (28 days); a longer follow up duration may be required. Finally, persistence of mRDT may be due to delayed clearance of the antigen from the blood. This may be linked to association between parasite density and persistence of HRP2 antigen where the higher the parasite density the longer it takes for the antigen to clear. Our study showed that the proportion of children with persistence of HRP2 antigen appeared to be lower in those with low parasite density (parasitemia equal to or below 200/uL) compared to those with higher parasitemia, however, the population with such low parasitemia was small and thus we were unable to make any strong deductions from this observation.

CONCLUSION

In conclusion, our study revealed a high proportion of persistence of HRP2 antigen in Nigerian children 28 days after effective antimalarial therapy. In regions of high malaria prevalence and transmission like Nigeria, this finding poses a challenge to the use of HRP2-based mRDTs in follow up of antimalarial therapy as they are not recommended for monitoring of antimalarial therapies in our population infected with malaria. The causes and associations of persistence of HRP2 antigenemia need to be further evaluated by workers in malaria endemic countries. Expert microscopy remains relevant in endemic countries like Nigeria, where malaria transmission is intense.

Conflict of Interest

We declare that we have no conflict of interest.

Authors Contribution

Prof Catherine Falade designed the study. All authors participated equally in conducting the study, analyzing, interpreting the data, and writing the manuscript. All authors read and approved the final draft of the manuscript.

ACKNOWLEDGEMENT

We thank the children and guardians who participated in the study for their time effort made towards adhering to the study protocol. We are very grateful to our research staff, Mrs Fatimah Abdus-Salam, Mrs Bolatito Akinyele, Ms. Grace Egunyomi, and our village health workers. We also wish to appreciate all physicians, nurses, and other medical staff that participated in the study.

REFERENCES

1. **Reyburn H.** New WHO guidelines for the treatment of malaria. Bmj 2010;340:c2637.
2. **Hanscheid T.** Diagnosis of malaria: a review of alternatives to conventional microscopy. Clin Lab Haematol 1999;21(4):235-45.
3. **Frean JA.** Reliable enumeration of malaria parasites in thick blood films using digital image analysis. Malar J 2009;8:218.
4. **Wongsrichanalai C,** Pornsilapatip J, Namsiripongpun V, *et al.* Acridine orange fluorescent microscopy and the detection of malaria in populations with low-density parasitemia. Am J Trop Med Hyg 1991;44(1):17-20.
5. World Health O. World malaria report 2015. Geneva: World Health Organization; 2015 2015.
6. **Harvey SA,** Jennings L, Chinyama M, *et al.* Improving community health worker use of malaria rapid diagnostic tests in Zambia: package instructions, job aid and job aid-plus-training. Malaria Journal. 2008;7(1):160.
7. **Skarbinski J,** Ouma PO, Causer LM, *et al.* Effect of malaria rapid diagnostic tests on the management of uncomplicated malaria with artemether-lumefantrine in Kenya: a cluster randomized trial. Am J Trop Med Hyg 2009; 80 (6):919-26.
8. **Munier A,** Diallo A, Sokhna C, Chippaux JP. [Assessment of a rapid diagnostic test for malaria

in rural health care facilities in Senegal]. Med Trop (Mars) 2009;69(5):496-500.
9. **Falade CO,** Adesina-Adewole B, Dada-Adegbola HO, *et al.* Evaluation of Paracheck-Pf(TM) rapid malaria diagnostic test for the diagnosis of malaria among HIV-positive patients in Ibadan, south-western Nigeria. Pathog Glob Health 2013;107 (2):69-77.
10. **Wilson ML.** Laboratory diagnosis of malaria: conventional and rapid diagnostic methods. Arch Pathol Lab Med 2013;137(6):805-811.
11. **Mouatcho JC,** Goldring JP. Malaria rapid diagnostic tests: challenges and prospects. J Med Microbiol 2013;62(Pt 10):1491-505.
12. **Wongsrichanalai C,** Barcus MJ, Muth S, *et al.* A review of malaria diagnostic tools: microscopy and rapid diagnostic test (RDT). Am J Trop Med Hyg 2007;77(6 Suppl):119-127.
13. **Bell DR,** Wilson DW, Martin LB. False-positive results of a Plasmodium falciparum histidine-rich protein 2-detecting malaria rapid diagnostic test due to high sensitivity in a community with fluctuating low parasite density. Am J Trop Med Hyg 2005;73(1):199-203.
14. **Gillet P,** Mori M, Van Esbroeck M, *et al.* Assessment of the prozone effect in malaria rapid diagnostic tests. Malar J 2009;8:271.
15. **Maltha J,** Gamboa D, Bendezu J, *et al.* Rapid diagnostic tests for malaria diagnosis in the Peruvian Amazon: impact of pfhrp2 gene deletions and cross-reactions. PLoS One 2012;7(8):e43094.
16. **Cheng Q,** Gatton ML, Barnwell J, *et al.* Plasmodium falciparum parasites lacking histidine-rich protein 2 and 3: a review and recommendations for accurate reporting. Malar J 2014;13:283.
17. **Maltha J,** Gillet P, Jacobs J. Malaria rapid diagnostic tests in endemic settings. Clin Microbiol Infect 2013;19(5):399-407.
18. **Mayxay M,** Pukrittayakamee S, Chotivanich K, *et al.* Persistence of Plasmodium falciparum HRP-2 in successfully treated acute falciparum malaria. Trans R Soc Trop Med Hyg 2001;95(2):179-182.
19. **Houze S,** Boly MD, Le Bras J, *et al.* PfHRP2 and PfLDH antigen detection for monitoring the efficacy of artemisinin-based combination therapy (ACT) in the treatment of uncomplicated falciparum malaria. Malar J 2009;8:211.
20. **Andreoli A,** Giorgetti PF, Pietra V, *et al.* Evaluation of a PfHRP-2 based rapid diagnostic test versus microscopy method among HIV-positive and unknown serology patients in Ouagadougou, Burkina Faso. Am J Trop Med Hyg 2015; 92(4): 834-837.
21. **Ajumobi O,** Sabitu K, Nguku P, *et al.* Performance of an HRP-2 rapid diagnostic test in Nigerian children less than 5 years of age. Am J Trop Med Hyg 2015;92(4):828-833.
22. **Mboera LE,** Fanello CI, Malima RC, *et al.* Comparison of the Paracheck-Pf test with microscopy, for the confirmation of Plasmodium falciparum malaria in Tanzania. Ann Trop Med Parasitol 2006;100(2):115-122.
23. **Iqbal J,** Siddique A, Jameel M, Hira PR. Persistent histidine-rich protein 2, parasite lactate dehydrogenase, and panmalarial antigen reactivity after clearance of Plasmodium falciparum monoinfection. J Clin Microbiol 2004;42(9):4237-4241.
24. **LaCrue AN,** Scheel M, Kennedy K, *et al.* Effects of artesunate on parasite recrudescence and dormancy in the rodent malaria model plasmodium vinckei. Plos One 2011;6(10).
25. **Teuscher F,** Gatton M, Chen N *et al.* Characterizing artemisinin induced dormancy in plasmodium falciparum and its potential impact on treatment efficacy. American Journal of Tropical Medicine and Hygiene 2009;81(5):152-152.
26. **Teuscher F,** Gatton ML, Chen N, *et al.* Artemisinin-induced dormancy in plasmodium falciparum: duration, recovery rates, and implications in treatment failure. Journal of Infectious Diseases 2010;202(9):1362-1368.
27. **Codd A,** Teuscher F, Kyle DE, *et al.* Artemisinin-induced parasite dormancy: a plausible mechanism for treatment failure. Malaria Journal 2011;10.