JMID/
Journal of Microbiology and Infectious Diseases

2018; 8 (2):55-60
doi: 10.5799/jmid.434593

RESEARCH ARTICLE

Efficacy of Histochemical Staining Techniques in the Detection of *Plasmodium falciparum* Histidine-Rich Proteins in Blood of Children with Malaria

Mfoniso Udonkang[1], Mokutima Eluwa[2], Paul Inyang-Etoh[3], Imeobong Inyang[1], Amabe Akpantah[2], Theresa Ekanem[2]

[1]*Histopathology Unit, Department of Medical Laboratory Science, University of Calabar, Calabar, Cross River State, Nigeria*
[2]*Department of Human Anatomy, University of Calabar, Calabar; Cross River State, Nigeria*
[3]*Microbiology and Parasitology unit, Department of Medical Laboratory Science, University of Calabar, Calabar, Cross River State, Nigeria*

ABSTRACT

Objective: *Falciparum malaria* predominates in sub-Saharan Africa and children below five years are the most vulnerable. Giemsa-stained microscopy is the gold standard in malaria diagnosis. Diagnosis with rapid diagnostic test (RDT) kit is also common and over 80% of available malaria RDT kits is *Plasmodium falciparum* histidine-rich protein 2-based (Pfhrp2). However, these histidine-rich protein 2-based kits have been observed to give false positive and negative results due to persistent antigenemia and low parasitaemia respectively. Thus, the methods of Pauly, Perls, and Means & Feeney were adopted to explore the advantage of using microscopy for specific detection of these histidine-rich proteins and their usefulness in detecting low parasitemia in children.

Methods: Children aged 0-5 years (n=200) visiting three hospitals and private laboratories in Calabar were recruited. Whole blood samples were tested with CareStart Malaria HRP2-based kit, and blood films were made and stained with Giemsa, Pauly, Perls and Means & Feeney for microscopy.

Results: The sensitivity and specificity were Giemsa (56.4%, 79.8%), Means & Feeney (52.5%, 77.8%), Perls (47.5%, 85.9), Pauly (45.5%, 86.9%), and RDT (23.8%, 96%). Pauly method had the highest area under the curve of 0.830 while RDT method had the lowest of 0.661. Among the positive cases, low parasitemia detected by the histochemical methods was Perls 36 (75%), Pauly 32 (69.6%), and Means & Feeney 34 (64.2%), and for Giemsa method 40 (70.2%).

Conclusion: Pauly method was the most accurate. All three methods were sensitive in detecting low parasitemia. These diagnostic methods are useful in malaria diagnosis in this endemic population. *J Microbiol Infect Dis 2018; 8(2):55-60*

Keywords: Histochemical stains, Histidine-rich proteins, Malaria, *Plasmodium falciparum*, Efficacy

INTRODUCTION

In Nigeria, malaria accounts for about 100 million hospital cases, over 300,000 deaths, about 60% of all outpatient visits to hospitals, and death of children under the age of 5 years [1]. Children under the age of 5 years are mostly affected because of their low immunity and variable signs and symptoms especially at low parasitemia where diagnosis is difficult [1,2].

The routine diagnostic methods for malaria in Nigeria have been microscopy and rapid diagnostic tests (RDT). Giemsa-based microscopy has been used for several years as a gold standard method [3,4]. It has the advantages of detecting low parasitemia (as low as 10 parasites per µl of blood), quantification, and species identification. This method has contributed in the diagnosis and elimination of malaria in most part of the world. However, it needs technical expertise for accurate diagnosis. Also, the incorporation of HRPs in RDT was recommended by World Health Organization (WHO) for the diagnosis of malaria

Correspondence: Dr. Paul Columba Inyang-Etoh, Medical Laboratory Science Department Faculty of Allied Medical Sciences, College of Medical Sciences, University of Calabar, P. M. B. 1115, Calabar, Cross River State, Nigeria
E-mail: inyangetoh@yahoo.com
Received: 19 August 2017 Accepted: 22 March 2018

in whole blood [1]. Histidine-rich protein (HRP) is one of the biomarkers of *Plasmodium falciparum* which aids in its specific detection for effective management and control of malaria [5,6]. Histidine rich proteins are different antigens synthesized and expressed by asexual forms of *Plasmodium falciparum* in infected erythrocytes which are responsible for its virulence [6]. The HRP1 or knob-associated histidine-rich protein (KAHRP) is responsible for rosetting, cytoadhesion, increased parasitemia and anemia [7-9]. While HRP2 is a soluble protein found in plasma and on the membrane and cytoplasm of infected erythrocytes responsible for haemozoin formation, anemia, fever, organ dysfunction including cerebral malaria, and other associated life-threatening complications [2,7,10,11]. It has been reported that the use of *Plasmodium falciparum* histidine-rich protein-based RDT kits comes with false positive and false negative results [6,12]. False negative results occur due to low parasitemia (<200 p/µl) or mutant genes [12], and false positive due to circulating antigenemia within two weeks of treatment [4]. This has lead to misdiagnosis of malaria in most settings. Thus, all RDT results are subject to confirmation by microscopy.

Among these histochemical staining techniques, Perls method gives specific staining of ferric ion of hemozoin, the brown-black malaria pigment [13]. The methylene blue method of [14] has been reported to be specific in the detection of histidine, the amino acid which polymerizes to more than 20 histidine repeats units called histidine-rich proteins. While the Pauly stain detects the HRPs of the malaria parasite by chemodetection of the imidazole group of histidine using diazonium salt [15]. In this study, these three histochemical methods for microscopic detection of these Pfhrps where adopted with the aim of improving on the Giemsa and RDT methods especially for malaria diagnosis in low parasitemia and in asymptomatic cases frequently encountered among children.

METHODS

Study area

The study was carried out in Calabar metropolis the capital of Cross River State, located in South-South of Nigeria with coordinates $4^{o}57'0"N$ $8^{o}19'30"E$ by $4.95000^{o}N$ $8.32500^{o}E$. Two Local Government Areas, Calabar South and Calabar Muncipal make up the metropolis. Calabar boost of a population of 371,022 million persons and an area of 406 km^2. Calabar is located in the rain forest belt of Nigeria with rainfall recorded throughout the year with about 80% of the rainfall occurring between April and October [16].

Ethical approval

Ethical approval was obtained from the Research Ethics Committee of the Cross River State Ministry of Health (Rec. No. 2015/315). Verbal or written informed consent was sought from the parents/guardians of participants before commencement of the study.

Study subjects and sample collection

A total of 200 children between 0 and 5 years of both genders were recruited. Test subjects (n=101) visiting the General hospital, Calabar, Immanuel Infirmary, Calabar, University of Calabar Medical Centre, and Incopa Medical Laboratory were used. Control subjects without symptoms of malaria (n=99) were recruited from the general population. Each subject personal and demographic data was obtained with a structured questionnaire. Blood samples were collected by finger prick.

Inclusion criteria: The test group comprised subjects with clinical signs of *Falciparum malaria*. While the control group comprised subjects with no clinical signs of malaria.

Processing of samples

Rapid diagnostic test

The RDT was carried out according to the manufacturer's instructions with capillary whole blood samples using CareStart PfHRP2 RDT test kits manufactured by Access Bio, USA.

Giemsa staining technique

The Giemsa microscopy method was carried out with thick and thin blood films stained with 10% Giemsa solution for 10 minutes. Results were reported according to WHO standard reporting format [17].

Histochemical staining techniques

The thick and thin blood films of the subjects were stained for HRPs with three histochemical stains of [14,18,19].

Means & Feeney method

In the methylene blue method of [14], the blood films were flooded with 0.01% methylene blue for 30 minutes. All stained slides were viewed with the x100 objective of Kyowa light microscope and photomicrographs were taken using a LEICA 750DM photomicroscope.

Pauly method

In the Pauly Diazo method [18] the stain was prepared according to method of [15] and blood films were stained for 15 minutes.

Perl method

In the Perl Prussian blue method [19], the blood films were flooded for 60 minutes with freshly prepared Perl stain.

Parasite density estimation

The positive slides were reported quantitatively as parasite density counts in the thick blood films according to the standard by [1] reference of parasite count/µl of blood. The number of parasites/200 high power fields was recorded. The parasite density were classified as mild (<200 parasite count/µl), moderate (>200 parasite count/µl), and as severe malaria (>1000 parasite count/µl).

Statistical analysis

Statistical Package for Social Sciences (SPSS) version 20 (Armonk, New York: IBM Corporation) was used to analyze the results. The results were expressed as mean ± standard deviation and percentages. Prevalence was calculated using a 2 x 2 contingency table and receiver's operator characteristics (ROC) curve was used to determine the area under the curve. Probability level of less than 0.05 ($p<0.05$) was statistically significant.

RESULTS

The demographic data of human subjects in Table 1 showed that of the 200 participants 101 (34%) were symptomatic (test) while 99 (66%) were asymptomatic (control). Males were 98(48.7%) and females were 102 (51.3%). The highest number of participants were found among children aged 13-25 months 36 (24%) and the lowest number were aged 0-12 months 26 (17.3%).

The diagnostic tests results of *P. falciparum* are as shown in Table 2. Among the test subjects, Giemsa stain detected malaria in 57 (56.4%) and the RDT method was positive for HRP2 in 24 (23.8%). Histidine-rich proteins were detected with Means & Feeney method in 53 (52.5%), Perls method in 48 (47.5%) and Pauly method in 46 (45.5%). In the test group, the most intense infection was observed in the Perls method (530.00 ± 1617.616) as compared with Giemsa method (5227.37 ± 24992.33). Among the controls, Means & Feeney method detected more HRPs in 22 (22.2%) and had the most intense infection (243.08 ± 144.65) when compared with Giemsa in 20 (20.2%) and (70.00 ± 53.311) respectively.

The performance characteristics of the diagnostic methods are shown in Table 3. The sensitivity, specificity, PPV, and NPV for each method were as follows: Giemsa (56.4%, 79.8%, 26.0%, 35.8%); Means & Feeney (52.5%, 77.8%, 29.3%, 38.4%); Perls (47.5%, 85.9%, 22.6%, 37.85); Pauly (45.5%, 86.9%, 22.0%, 39.0%); and RDT (23.8%, 96.0%, 14.3%, 44.0%). The Pauly method had the highest AUC of 0.830 while the RDT method had the lowest AUC of 0.661 and was statistically significant ($p=0.001$).

Table 1. Demographic and clinical data of symptomatic and asymptomatic subjects.

Descriptive (n=200)	No. of Subjects (%)
Asymptomatic	99 (49.5)
Symptomatic	101 (50.5)
Symptoms	
Cough/catarrh	16(8.0)
Fever	67(33.5)
Gastroenteritis	2(1.0)
Cough/catarrh/Fever/Gastroenteritis	14(7.0)
Convulsion	2(1.0)
Gender	
Male	98 (49.0)
Female	102(51.0)
Age (months)	
0-12	34(17.0)
13-25	48(24.0)
26-38	40(20.0)
39-51	39(19.5)
52-65	39(19.5)

Table 4 shows the classification of malaria severity based on the parasitemia levels. Among the positive test subjects, low parasitemia was detected by all the methods as Giemsa method 40 (70.2%), Perls 36 (75%), Means & Feeney 34 (64.2%), and Pauly 32 (69.6%). The most intense infection was observed with the Means & Feeney 95.29 p/µl when compared with

Giemsa 64.0 P/µl. At moderate parasitemia, Means & Feeney method had the highest cases of 11 (20.7%) and most intense infection of 345.45 p/µl when compared with Giemsa which had 9 (15.8%) and intensity of 355.56 p/µl. At high parasitemia, Means & Feeney method had similar prevalence of 7.9% with Giemsa (7.9%) but it was the Perls method that had the most intense infection of 4112.00P/µl when compared with MPD of Giemsa of 36525.00 p/µl.

Table 2. Prevalence and intensity of malaria infection according to the different diagnostic methods.

Test subjects (n=101)			Control subjects (n=99)	
Methods	**No. of positive (%)**	**MPD ± SD (P/µl)**	**No. (%) positive**	**MPD ± SD (P/µl)**
Giemsa	57 (56.4)	5227.37 ± 24992.325	20 (20.2)	70.00 ± 53.311
Means and Feeney	53 (52.5)	455.85 ± 1148.279	22 (22.2)	243.08 ± 144.648
Perl's	48 (47.5)	530.00 ± 1617.616	14 (14.1)	65.71 ± 33.676
Pauly	46 (45.5)	476.52 ± 1769.124	13 (13.1)	67.27 ± 43.445
RDT	24 (23.8)	-	4 (4.0)	-

N=Total number, MPD=mean parasite density, SD=standard deviation, P/µl=parasite per microliter of blood

Table 3: Performance characteristics of the different diagnostic methods

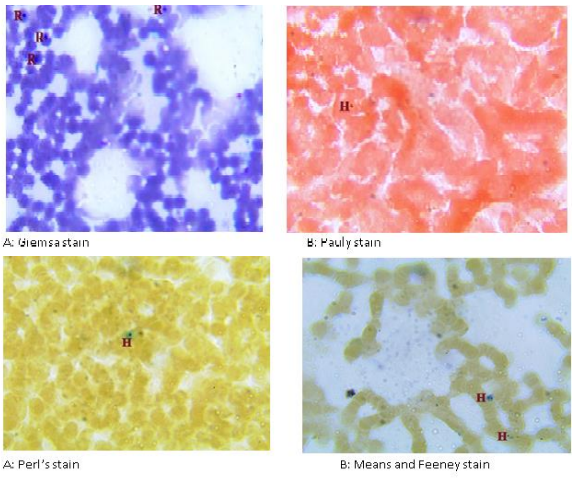
Methods	Sensitivity	Specificity	PPV	NPV	AUC	P value
Giemsa	56.4	79.8	26.0	35.8	1.000	
Pauly	45.5	86.9	22.0	39.0	0.830	0.001
Perl's	47.5	85.9	22.6	37.8	0.829	0.001
Means & Feeney	52.5	77.8	29.3	38.4	0.826	0.001
RDT	23.8	96.0	14.3	44.0	0.661	0.001

PPV=positive predictive value, NPV=negative predictive value, AUC=area under the curve

Table 4: Classification of malaria severity based on parasitaemia levels with the different diagnostic methods.

Methods	No. of LP (%)	MPD ± SD (P/µl)	No. of MP (%)	MPD ± SD (P/µl)	No. of HP (%)	MPD±SD (P/µl)
Giemsa (n=57)	40 (70.2)	64.00±29.77	9(15.8)	355.56±90.43	8(14.03)	36525.00±60783.05
Pauly (n=46)	32 (69.6)	76.25±37.14	8(17.4)	335.00±90.55	6(13.04)	2800.00±4540.10
Perl's (n=48)	36 (75)	68.89±33.96	7(14.6)	314.29±87.72	5(10.4)	4112.00±3568.57
Means & Feeney (n=53)	34 (64.2)	95.29±49.25	11(20.8)	345.45±88.13	8(15.1)	2140.00±2424.92

LP=low parasitaemia (<200P/µl), MP=moderate parasitaemia (200-500P/µl), HP=high parasitaemia (>500P/µl), MPD=mean parasite density, SD=standard deviation



Plates 1 and 2 show the photomicrographs of malaria parasite stained with these methods.

DISCUSSION

On account of the wide use of HRPs as biomarkers in the diagnosis of *P. falciparum* with RDTs, its potential microscopic detection was explored. In this study, three histochemical methods of Pauly, Perls, and Means & Feeney were used to diagnose malaria by microscopic detection of HRPs in human *P. falciparum* species.

The demographic data showed that children below the age of five years are susceptible to malaria. It also revealed that malaria may be symptomatic or asymptomatic among the children examined. The ability of the diagnostic tests to diagnose malaria in both the symptomatic and asymptomatic groups is in confirmation that malaria may be symptomatic or asymptomatic in presentation [1].

The histochemical methods detected HRPs in varying specificities when compared with the Giemsa and RDT methods. This may be due to the staining mechanism of the different methods. The Perls method stained the iron component of the hemozoin pigment formed from HRP2 [13].

Pauly stain has been shown to react with the imidazole group of the histidine amino acid of HRP 2 and 3 [15] while the methylene blue method of [14] reacts with the histidine amino acid to produce staining reactions. This confirms the reports of other researches that HRPs are produced by *P. falciparum* [6,20,21].

All the methods including the Giemsa gold standard had sensitivities of 26-56% among the subjects. This shows that the subjects within this group are more susceptible to malaria due to their low immunity [22-24]. Thus, these stains help in early diagnosis because these groups become symptomatic earlier [25].

The sensitivities of Giemsa (56.4%), Means & Feeney (52.5%), Perls (47.5%), and Pauly (45.5%) were better than that of RDT (23.8%) for all the subjects tested in this study. This finding is supported by researches that HRP-based RDTs are less accurate than microscopy [4]. Baker et al. [6] had stated that antigenic variability and low HRP2 antigenemia at low parasitemia are responsible for the low performance of RDTs. This questions the reliability of RDT as a routine method for diagnosis especially at low parasitemia, although its merit of high specificity can be explored in ruling out false positive results as it meet the recommended specificity limit of WHO [26]. A recent report by [1] has recommended that the use of RDT should only be restricted to areas where microscopy is not available and its result must be confirmed by microscopy. Another advantage these methods have over RDTs is that they can quantify the amount of HRPs present in blood similar to the Giemsa method. Thus, they can be adopted in the management and monitoring of malaria treatment similar to the Giemsa method [4].

Among the three histochemical methods, Pauly stain proved to be the most accurate method by having the highest area under the ROC curve (0.830). This is in line with a study that the area under the ROC curve shows the accuracy of a diagnostic test [27]. These findings also confirm the report of [15] that "Pauly method is sensitive and specific in the quantitative detection of HRPs in *P. falciparum*". Its accuracy is comparable to the ELISA and western blot methods. This is explained by the specific staining of the imidazole group of the histidine amino acid by diazonium salts [15].

The histochemical methods were valuable in measuring the level of prevalence and intensity of malaria based on low parasitemia, moderate parasitemia, and high parasitemia almost similar to Giemsa method. This important finding further emphasizes the relevance of these histochemical methods in detecting HRPs and as a useful marker in predicting malaria severity. Thus, correlates with other previously reported findings [3,28,29].

During microscopy, it was observed that the HRPs were detected both in thin and thick films. Speciation with thin film was possible as the morphology of the red cells were intact, a characteristic finding of falciparum infection. Thus, the histochemical methods can be used in species identification similar to the Giemsa stain [4].

As conclusion, this study reveals that the three histochemical methods of Pauly, Perls, and Means & Feeney can be employed in the routine diagnosis of *Falciparum malaria*. The quantitative staining of the HRPs is an indication of the ability of these methods to aid in the determination of the level of parasite density thus aid in better management and treatment of malaria. On the whole, these histochemical methods are capable of decreasing Falciparum malaria-related morbidity and mortality among children below five years in endemic countries including Nigeria.

ACKNOWLEDGMENTS

Thanking: The authors are grateful to the management and staff of Incopa Medical Laboratories for their support in terms of equipment and manpower support during the period of research work.

Conflict of interest: The authors declare no personal or financial conflict of interest.

Financial Support: The authors declared no financial support.

REFERENCES

1. WHO. Guidelines for the treatment of malaria, 3rd edn. Geneva: WHO, 2015: 1-7. http://www.who.int/malaria/publications/atoz/9789241549127/en/

2. Roberts LS, Janovy JJ. Gerald D Schmidt, Roberts LS Foundations of Parasitology. 8th edn. Boston: McGraw Hill Educational Foundation, 2009; p:147-170.

3. Kifude CM, Rajasekariah HG, Sullivan Jr DJ, et al. Enzyme-linked immunosorbent assay for detection of

Plasmodium falciparum histidine-rich protein 2 in blood, plasma, and serum. Clin Vac Immuno 2008; 15(6):1012-1018.

4. Mouatcho JC, Goldring JPD. Malaria rapid dignostic tests: challenges and prospects. J Med Microbiol 2013; 62(10):1491-505.

5. Marquart L, Butterworth A, McCarthy JS, Gatton ML. Modelling the dynamics of *Plasmodium falciparum* histidine-rich protein 2 in human malaria to better understand malaria rapid diagnostic test performance. Malaria J 2012; 11:74.

6. Baker J, Gatton ML, Peters J, Ho M-F, McCarthy JS. Transcription and expression of *Plasmodium falciparum* histidine-rich proteins in different stages and strains: implications for rapid diagnostic tests. PLoS ONE 2011; 6(7):e22593.

7. Kakkilaya BS. Malaria Pathogenesis. 2011; 1-5. Retrieved online on 20th February, 2015.

8. Lamikanra AA, Theron M, Kooij T WA, Roberts DJ. Hemozoin (malarial pigment) directly promotes apoptosis of erythroid precursors. PLoS ONE 2009; 4(12):e8446.

9. Wiser MF. Cellular and molecular biology of Plasmodium. Tulane University. 1999. Retrieved online on 20th June, 2015. http://www.tulane.edu/CellularandMolecular BiologyofPlasmodium

10. Francis U, Issac Z, Yakubu A, Enosakhare A, Felix E. Haematological parameters of malaria infected patients in the University of Calabar Teaching Hospital, Calabar, Nigeria. J Hematol Thromboembo Dis 2014; 2:171.

11. Miller LH, Baruch DI, Marsh K, Doumbo OK. The pathogenic basis of malaria. Nature 2002; 415: 673-9.

12. Mwakalinga SB. Variant surface antigen expression by sexual and asexual stages of *Plasmodium falciparum*. PhD thesis, Faculty of Health and Medical Sciences, University of Copenhagen, United Kingdom. 2012; 1-12 http://cmp.ku.dk/degreenews/phd/mwakalingasteven/thesiscover/thesis_mwakalinga.pdf

13. Rupani, AB, Amarapurkar, AD. Hepatic changes in fatal malaria: an emergency problem, Ann Trop Med Parasitol 2009; 103(2):119-127.

14. Means GE, Feeney RE. Chemical modification of proteins. San Francisco: Holden-Day Incorporated 1971; 226-227.

15. Sahal D, Kannan R, Sinha A, et al. Specific and instantaneous one-step chemodetection of histidine-rich proteins by Pauly's stain. Anal Biochem 2014; 308(2):1663-70.

16. Eze BE, Effiong J. Morphometric parameters of the Calaber River Basin:Implication for hydrologic processes. J. Geog Geol 2010; 2(1):18-26.

17. WHO. Basic malaria microscopy. Part 1, Learner's guide, 2nd edn. Geneva: WHO, 2010; 1-83. http://www.who.int/malaria/publications/atoz/9241547820/en/

18. Pauly H. Über die Konstitution des Histidins. Hoppe-Seyler's Zeitschrift fur Physiolo Chemie 1904; 42(5-6):508-18.

19. Perls M. Nachweis von Eisonoxyde in Pigmenten. Vir Archeo Patholo Anat 1867; 39:42.

20. Rock EP, Marsh K, Saul AJ, et al. Comparative analysis of the *Plasmodium falciparum* histidine-rich proteins HRP-I, -II and HRP-III in malaria parasites of diverse origin. Parasitolo 1987; 95(2):209-27.

21. Hawkes M, Conroy AL, Opoka RO, et al. Use of a three-band HRP2/pLDH combination rapid diagnostic test increases diagnostic specificity for *Falciparum malaria* in Ugandan children. Malaria J 2014; 13:43.

22. Prugnolle F, Durand P, Ollomo B, et al. A fresh look at the origin of *Plasmodium falciparum*, the most malignant malaria agent. PLoS Path 2011; 7(2):e1001283.

23. Sahar T, Reddy KS, Bharadwaj M, et al. *Plasmodium falciparum* reticulocyte binding-like homologue protein 2 (PfRH2) is a key adhesive molecule involved in erythrocyte invasion. PLoS ONE 2011; 6(2):e17102.

24. Gunalan K, Gao X, Yap SSL, Huang X, Preiser PR. The role of the reticulocyte-binding-like protein homologues of Plasmodium in erythrocyte sensing and invasion. Cell Microbiol 2013; 15:35-44.

25. Ilombe G, Maketa V, Mavoko HM, et al. Performance of HRP2-based rapid test in children attending the health centre compared to asymptomatic children in the community. Malaria J 2014; 13:308.

26. WHO: World Health Organization. Malaria Rapid Diagnostic Test Performance: summary results of WHO malaria RDT product testing: Rounds 1–3(2008–2011). Geneva: WHO, 2011. http://www2.wpro.who.int/NR/rdonlyres/005E574B-FFCD-484C-BEDEC580324AC2CF/0/RDTMalariaRd3_Summary_FINAL11Oct.pdf

27. Hajian-Talaki K. Receiver operating characteristic (ROC) curve analysis for medical diagnostic test evaluation. Caspian J Intern Med 2013; 4(2):627-33.

28. Fox LL, Taylor TE, Pensulo P, et al. Histidine-rich protein 2 plasma levels predict progression to cerebral malaria in Malawian children with *Plasmodium falciparum* infection. J Infect Dis 2013; 208:500-503.

29. Rubach MP, Mukemba J, Florence S, et al. Plasma *Plasmodium falciparum* histidine-rich protein-2 concentrations are associated with malaria severity and mortality in Tanzanian children. PLoS ONE 2012; 7(5):e35985.