Diagnostic performance of blood film microscopy and PfHRP2-based RDT in a routine clinical setting of a secondary health facility in Ghana

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ABSTRACT

BACKGROUND

MALARIA REMAINS A MAJOR PUBLIC HEALTH THREAT CLAIMING MANY LIVES PARTICULARLY IN SUB-SAHARAN AFRICA. LIGHT MICROSCOPY AND RDTS ARE THE MAINSTAY TESTS IN THE CLINICAL SETTINGS FOR MALARIA DIAGNOSIS. MANY STUDIES REPORT VARYING LEVELS OF VALIDITY OF THESE TESTS COMPARED TO MOLECULAR METHODS LIKE PCR. DOCUMENTATION ON SUCH COMPARATIVE STUDY INVOLVING THE USE OF MOLECULAR TECHNIQUES AS REFERENCE TEST IS SCANTY IN GHANA. THIS STUDY THEREFORE ASSESSES THE DIAGNOSTIC PERFORMANCE OF THESE TESTS COMPARED TO PCR.

METHODS

BLOOD FILM MICROSCOPY (THIN AND THICK), RDT AND NESTED PCR WERE RUN ON BLOOD SAMPLES FROM A TOTAL OF 188 MALARIA SUSPECTED PATIENTS. THE ACCURACY INDICES OF THE MICROSCOPY AND RDT WERE CALCULATED USING THE RESULTS OF THE PCR AS THE REFERENCE TEST.

RESULTS

A TOTAL OF 188 PATIENTS WERE RECRUITED WITH FEMALES CONSTITUTING THE MAJORITY 128 (68%). THE PAEDIATRIC AGE GROUP 1-10 YEARS CARRIED THE LARGEST BURDEN OF MALARIA BY MEANS OF ALL THE 3 TESTS.A SENSITIVITY OF 47.37% (95% CI, 37.03 – 57.88%) WAS SHOWN BY BOTH THE MICROSCOPY AND RDT WITH SPECIFICITY OF 93.55% (95% CI, 86.48 – 97.60) AND 100% (95% CI, 96.11 – 100.00%) AND KAPPA CO – EFFICIENT OF 0.41 AND 0.47 RESPECTIVELY.

CONCLUSION

BOTH MICROSCOPY AND RDT EXHIBITED HIGH LEVEL OF SPECIFICITY BUT LOW SENSITIVITY. SIGNIFICANT NUMBER OF MALARIA PARASITAEMIC PATIENTS AS REVEALED BY THE PCR WAS MISSED BY BOTH THE RDT AND BLOOD FILM MICROSCOPY AND THUS WENT UNDIAGNOSED.

Keywords RDT, Microscopy, PCR, Malaria, Plasmodium

Background

A substantial reduction in the burden of malaria has been achieved across the globe for several decades. However, Malaria remains a major public health concern claiming many lives particularly in Sub-Saharan Africa. In 2016 alone, 445,000 deaths due to malaria occurred worldwide of which 91% were recorded in the WHO Africa Region [1]

Malaria continues to drain the economy of developing countries with impact also on the economy of developed countries as they commit resources to malaria control programmes and research.

Clinical malaria is caused mainly by 5 species and *Plasmodium falciparum* is by far the deadliest. Malaria is mainly transmitted through the bite of infected female Anopheles mosquito.

Blood film light microscopy remains the standard technique for malaria diagnosis in most clinical settings. Microscopy allows for detection and identification to the species level, it makes room for parasite quantification and it can be used for monitoring parasite clearance following therapy. Notwithstanding, the method requires trained personnel and this could be a hindrance to rapid malaria diagnosis and treatment especially in human resource constrained settings. Carrying out blood film microscopy in settings without electricity or with unstable power supply as the case is in many rural areas and even some urban settings in Sub-Saharan Africa could be very challenging. These challenges and possibly other factors like poor confidence in laboratory systems might have led to presumptive treatment of suspected malaria cases without laboratory evidence of malaria parasitaemia. In a study carried out at a regional hospital in Bamenda, Cameroon, out of 469 patients who were presumptively treated for malaria, only 30 actually had malaria parasitaemia[2]. Similarly, a study conducted in Ghana reported that of 605 feverish children who sought medical care at a hospital in Accra, only 11% were found positive for

malaria by microscopy after 80% had been diagnosed presumptively with malaria and treated with anti-malarial medication [3].

In the mid 1990s, antigen – based rapid detection test kits (RDT) were introduced to address some of the challenges associated with blood film microscopy. RDTs are simple to use with fast turnaround time and they do not require skilled personnel to perform. Accordingly, the proportion of suspected malaria cases receiving parasitological testing before treatment in the formal sector has risen from 36% in 2010 to 87% in 2016 in Africa. The rise is mainly due to the increased use of RDTs which accounted for 63% of the test in 2016 [1]. The increase use of RDTs has led to significant reduction in the over – diagnosis of malaria and over prescription of anti - malaria drugs with attendant increased clinicians' attention on non-malaria acute febrile conditions. RDTs for malaria have the potential to be highly cost-effective compared with presumptive treatment across most of Africa [4]. For instance, in a study conducted in Senegal from 2007 – 2009, an increased in parasiste – based diagnosis nationally from 3.9% of reported malaria – like febrile illness to 86% over the 3 year period led to a drop in prescription of anti – malaria drug (ACT) from 72.9% of malaria – like febrile illness to 31.5% averting a whooping 516576 courses of ACT that would have been inappropriately prescribed [5].

There have been several reports of varying sensitivity and specificity of both blood film microscopy and RDTs compared to molecular techniques like PCR. Compared to PCR, the sensitivity of blood film microscopy ranges from 16.5% - 46% whilst that of RDT ranges from 17% - 91% [6, 7, 8]. Comparative studies in which molecular techniques such as PCR are employed as the reference test are scanty in Ghana. This study therefore compares the accuracy indices of routine blood film microscopy in the clinical settings and an HRP2 – based RDT (SD

BIOLINE Malaria Ag Pf which is commonly used in health facilities in Ghana) with Polymerase Chain Reaction.

Methods

Study design, site and study population

Design: Accuracy of blood film microscopy and HRP2 – based RDT on a cross-section of patients presenting with suspected malaria was determined using Nested PCR as the Gold Standard. Recruitment and sample taking span over a period of 2 weeks in October, 2018.

Study site and Population: The study site was Kumasi South Hospital which is the second largest hospital in the Ashanti region of Ghana located at the south of the regional capital Kumasi. The hospital serves as primary hospital and also referral centre for close to one million people in the Kumasi Metropolis and its adjoining towns. The study population included all patients seeking medical care at the study site; Kumasi South Hospital (Ashanti Regional Hospital) over the 10 day period and those patients sent to the laboratory for blood investigations constituted the sample frame from which those undergoing blood investigation for suspected malaria were sampled.

A total of 200 patients with suspected malaria sent for routine blood film microscopy for malaria parasites by clinicians were approached. However, 12 did not consent to the study giving a sample size of 188.

Sample Collection

About 2mls of venous blood sample was obtained from each participant. Four (4) aliquots each of about 0.5ml in volume were made from the initially drawn blood sample and labeled as

aliquots A, B, C and D for each participant. All aliquots A were stored at -20°C for PCR detection of Plasmodium DNA. Rapid Diagnostic Test was run on all aliquots labeled B. Thin and thick films were prepared from all aliquots labeled C and D respectively for malaria parasite microscopy.

Rapid Diagnostic Test

SD BIOLINE Malaria Ag Pf which is routinely used in the health facilities in the country was employed. This Rapid Diagnostic Test (RDT) kit contains monoclonal anti-bodies designed to capture PfHRP2 antigen that may be present in patients' blood. The kit detects infections caused by *Plasmodium falciparum* species only. Blood samples from aliquots labeled B from each participant were used to run the Rapid Diagnostic Test. About 5µL of whole blood from aliquots labeled B from each participant was used to run the RDT following the manufacturer's instruction.

Blood Film Microscopy

Blood aliquots from all participants labeled D and C were used to prepare thin and thick Giemsa stained slides for microscopic examination for malaria parasites respectively. With the thick film, a drop of blood was placed on a clean grease-free specimen slide. Using the corner of a clean spreader slide, the drop of blood was spread in a circle of about 2cm in diameter. The film was left to air-dry for 30 minutes and then staining was done with Giemsa solution (7.5%, pH 7.2) for 30 minutes, after which the excess stain was washed off with buffered distilled water and then air-dried. Examination under the microscope with X 100 was done by an expert WHO certified microscopist for the presence or otherwise of *Plasmodium* parasite. A blood film was considered

negative when no malaria parasites or trophozoites were observed after 100 high power fields (hpf) had been examined on the thick film. Where parasites were seen, they were counted against 200 white blood cells (WBCs). The parasite count per microliter (µl) of blood was obtained using the formula: (Parasite count/200WBC) × Absolute WBC count.

The thin film of each participant was prepared in parallel, a similar drop of blood from aliquot C of same participant was placed on clean grease-free specimen slide and a clean spreader slide was held at 45-degree angle toward the drop of blood on the specimen slide such that the blood spread along the entire width of the spreader slide. The spreader slide was then pushed forward rapidly and smoothly still at the 45-degree angle. The blood smear was left to air dry for about 30 minutes and then fixed with 100% methanol. After complete drying, Giemsa staining was done as previously described and then subsequently observed under the microscope with X 100 magnification by an expert microscopist for Plasmodium species and stage of parasite identification.

For samples with high parasitaemia, ≥ 100 parasites per high power field, parasite density was more accurately determined on the thin film. In the thin film, parasitized RBCs were counted against 1000 RBCs. The parasite count per microliter (µl) was obtained using the formula: (parasitizedRBCs/1000 RBCs) × Absolute RBC count.

Polymerase Chain Reaction

A nested PCR adapted from a similar study [9] was used targeting small subunit ribosomal RNA (18S rRNA) gene. DNA was extracted from the whole blood stored in EDTA tubes (aliquots labeled C) using QIAamp DNA Blood Mini Kit and protocol (QIAGEN, Germany). The first PCR (Nest 1) involved the use of three primers (HUF, REV and PLF) which amplified a human

gene and the *Plasmodium* genus. The human gene complex formed was UNR-HUF with a band size of 231basepairs (bp) which was expected in all samples irrespective of the presence or otherwise of Plasmodium infection. That of the Plasmodium was UNR-PLF with band sizes between 783-821 bp depending on the species. In this PCR, a 20µL reaction of 1X OneTaq HotStart Standard Buffer, 0.075 µM each of PLF and HUF, and 0.0125 µM of HUF and 5µL of the extracted DNA and nuclease free water were used. The PCR was carried out at 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 58°C for 1 minute, 68°C for 1 minute and then 68°C for 5 minutes.

The second PCR (Nest 2) detected Plasmodium specific species and incorporated the Nest 1 PCR products and primers for the various species (Table 1). In this PCR, a 25µL reaction of 1X OneTaq HotStart Standard Buffer (from New England Biolabs), 0.15 µM each of NewPLFshort and falshort (for P. falciparum), 0.25µM Malshort, 0. 1µM Vivshort, 0.36µM OvaNew and 0.1µM NewPKrev for *P. malariae, P. vivax, P. ovale* and *P. Knowlesi* respectively and 0.5µL of a 1 in 10 dilutions of Nest 1 PCR products. The PCR was then carried out at an initial 94°C for 3 mins, followed by 35 cycles of 94°C for 30seconds, 53°C for 1 minute, 68°C for 1 minute and then 68°C for 5 minutes. The PCR was done using a 2720 Thermal Cycler from Applied Biosystems. The PCR products were then run on a 2% agarose gel. After gel electrophoresis, the samples were visualized using a Toyobo Ultra-Violet (UV) transilluminator (from Japan). The PCR was considered positive in cases in which Plasmodium DNA was amplified by NEST 2 which was 1 in 10 dilutions of NEST 1 PCR products.

Controls

For NEST 1, the positive control employed DNA from cultured 3D7 strain (*Plasmodium falciparum*). Again, the human gene complex (UNR-HUF) present in each sample further served as positive control. For NEST 2, *P. falciparum* DNA sample obtained from human participants from another study and two samples of mixed *P. falciparum* and P. *malariae* also from another study provided by the Department of Immunology, Noguchi Memorial Institute for Medical Research, Ghana together with *P. vivax* DNA sample obtained from a laboratory in France were used as positive controls. The negative controls for both NEST 1 and NEST2 consisted of only the regents without DNA sample.

Data analysis

Data were entered into Microsoft Excel (2013), processed and analyzed using Statistical Package for Social Sciences (IBM SPSS Statistics, 2015). To assess sensitivity and specificity, results of the microscopy and RDT were compared with the Nested PCR results. The sensitivity was derived as the percentage of positive test results obtained among samples scored as containing malaria parasites by the Nested PCR; the specificity was calculated as the percentage of negative test results obtained among samples whose PCR results were negative. Positive and negative predictive values were also calculated as the proportion of true-positive or true negative results among all samples scored as positive or negative by PCR, respectively. Kappa (κ) values, expressing the agreement beyond chance were calculated.

Results

Majority of the participants were females 128 (68%). The largest number of participants 50 (26.60%) fell within the age group 21 – 30. The paediatric age group 1 – 10 years carried the greatest malaria burden by means of all the 3 test methods

Majority of the participants 136 (72.35%) were afebrile (fever defined as axillary temperature of 37.5℃ or more) at the time of sampling whilst the remaining 52 recorded axillary temperature of 37.5°C or more (Table 1). Of the 188 participants, Microscopy detected 51 (27.13%) to have malaria parasitaemia and all those positive cases were *Plasmodium falciparum*; no other species were identified. The RDT tested positive for malaria in 45 (23.94%) of the participants whilst 95 (50.53%) were found to be positive for Plasmodium DNA by means of the PCR. All those PCR positive cases fell on *Plasmodium falciparum* band; like the microscopy, no other species was detected (Figures 1 and 2). Many of the febrile participants 37(71.15%) were positive for Plasmodium DNA by means of the PCR whilst 27(51.92%) and 25 (48.08%) of the febrile participants tested positive for malaria by means of blood film microscopy and RDT respectively (Table 1).

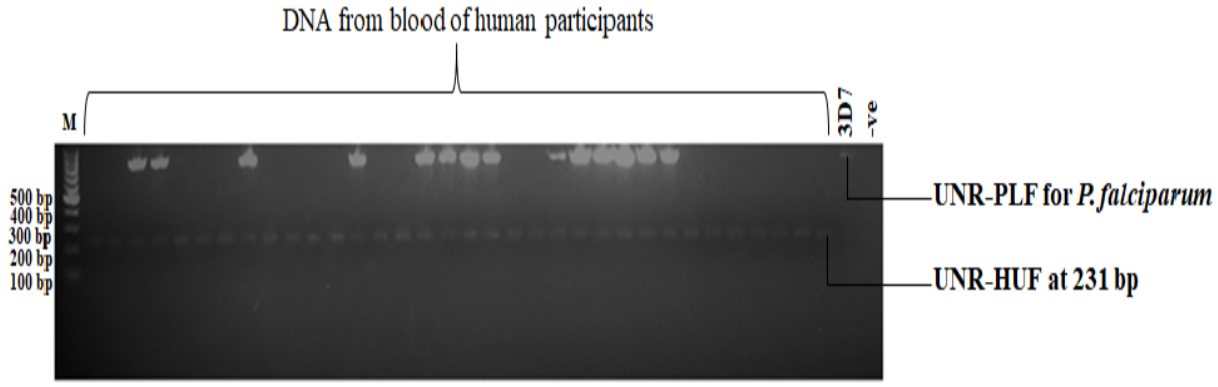

Figure 1: Gel electrogram of NEST I PCR products.
UNR-HUF at 231 bp is seen for all samples obtained from humans except 3D7 as this was obtained from cultured *P. falciparum*. A second band seen for samples that are positive for *Plasmodium* species (at 783 -821 bp depending on the specific species). M = molecular marker. 3D7 is DNA obtained from cultured P. falciparum and -ve is a no template control or negative control.

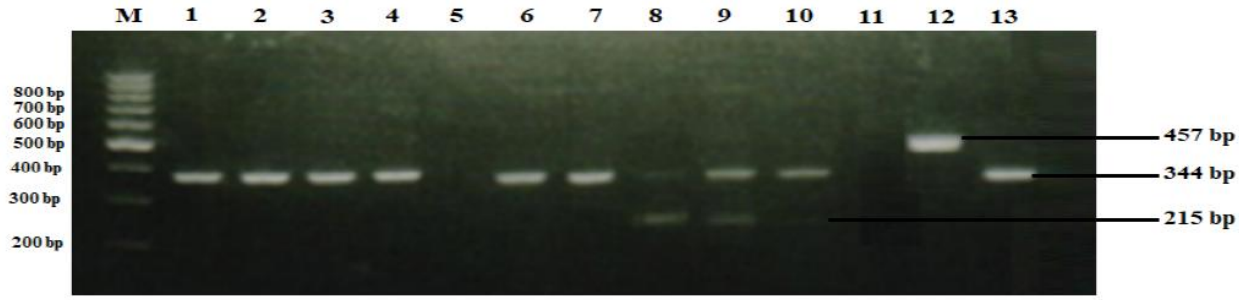

Figure 2: Gel electrogram of NEST 2 PCR products

Where, M = 100 base pair (bp) marker, 1 to 6 and 10 are samples; 7 is *P. falciparum* DNA control obtained from human participants from another study; 8 and 9 are mixed *P. falciparum* and P. *malariae* controls also from another study provided by the Department of Immunology, NMIMR; 11 is a negative control; 12 is *P. vivax* control obtained from a laboratory in France and 13 is DNA obtained from a culture 3D7 P. *falciparum* strain.

Table 1: Prevalence rate by different test methods

TEST	POSITIVE RATE		
	Febrile participants	**Afebrile participants**	**Total**
	(n = 52)	**(n = 136)**	**(n =188)**
Microscopy	27(51.92%)	24(17.65%)	51
RDT	25(48.08%)	20(14.71%)	45
PCR	37(71.15%)	58(42.65%)	95

Comparison of Microscopy and PCR results

Both the thick and thin film microscopy detected malaria parasites in the trophozoite stages in 51 out of the 188 participants. The thin film detected all 51 cases as *P. falciparum.* The PCR

detected 45 of the microscopy positive cases as positive for *P. falciparum* and the remaining 6 were false positive. With the PCR results as the reference test, the sensitivity and specificity of the microscopy test were 47.37% (95% CI, 37.03% - 57.88%) and 93.55% (95% CI, 86.48% - 97.60%) respectively. The negative predictive and positive predictive values of the Microscopy test were obtained as 65.03% (95% CI, 58.80% - 67.96%) and 88.24% (95% CI, 77.07% - 94.36%) respectively (Table 2).

Comparison of RDT and PCR results

The RDT detected 45 (47.37%) out of the 95 PCR positive cases as positive for malaria. None of the PCR negative samples was detected to be positive by RDT. With PCR as the gold standard, the sensitivity, specificity, negative predictive and positive predictive values of the RDT were 47.37% (95% CI, 37.03% - 57.88%), 100% (95% CI, 96.11-100.00%), 65.03% (95% CI, 60.58-69.24%), 100% (96.11-100.00%) respectively (Table 2).

Table 2 Accuracy indices of the microscopy and RDT compared to the nested PCR

Test Methods	Sensitivity 95% CI	Specificity 95% CI	PPV 95%CI	NPV 95%CI	Kappa(κ)
Microscopy	47.37% (37.03-57.88)	93.55% (86.48-97.60)	88.24% (77.07-94.36)	63.50% (58.80-67.96)	0.41
RDT	47.37% (37.03-57.88)	100% (96.11-100.00)	100% (96.11-100.00)	65.03% (60.58-69.24)	0.47

PPV = positive predictive value, NPV=negative predictive value, pLDH=Plasmodium Lactate Dehydrogenase, PfHRP2=*Plasmodium falciparum* Histidine-Rich Protein, RDT = Rapid Diagnostic Test, CI = Confidence Interval.

Discussion

The present study brings to attention some important observations. Firstly, the importance of parasite based testing before treatment is highlighted as more than half of patients presenting with malaria-like symptoms and signs including fever were malaria free. This observation is consistent with several studies in malaria endermic regions [2, 3, 5].Thus presumptive diagnosis and treatment of malaria without prior parasite-based testing is likely to lead to over – diagnosis of malaria and subsequent less attention to other conditions that present with signs and symptoms that are akin to malaria. WHO recommends that only in settings where parasite-based diagnostic testing is impossible should malaria treatment be started based only on clinical suspicion [1].

Secondly, both the blood film microscopy and RDT recorded a significant number of false negative rates (low sensitivity) and thus appreciable number of malaria positive cases as revealed by the Nested PCR went undiagnosed. Similar levels of low sensitivity but high specificity with blood film microscopy have been reported in studies in India [10], Kenya [11], Malawi [12] and in a study conducted concurrently in Uganda and Burkina Faso [13].

The low sensitivity of blood film microscopy may be due to inherent inability of the technique to detect parasitaemia below 10 – 50 parasites/microlitre of blood for the thick film and even higher thresholds (~500 parasites/microlitre) for the thin film [14]. However, there is the need for continuous in – service training for laboratory personnel to improve the efficiency of detection as sensitivities as high as 90.44% for blood film microscopy have been reported in some centres [15]

This RDT employed in this study was SD BIOLINE Malaria Ag Pf which contains monoclonal anti-bodies against *Plasmodium falciparum* Histidine-Rich Protein 2 which is specific only for

falciparum species. The accuracy indices obtained in this study were lower than those obtained in a similar study in Indonesia where the sensitivity and specificity of PfHRP-2 based RDT were compared with PCR [16].

In Ghana, a study conducted at KATH [17] and Techiman [18] obtained sensitivities of 97% and 100% and specificities of 91% and 73% respectively when RDT was compared with Microscopy. However, both studies did not utilize a molecular technique like PCR as the reference test.

The levels of sensitivity and specificity obtained in this study for the PfHRP2 – based RDT are comparable to those reported in similar studies in India [14], Ethiopia [6], Eastern Sudan [19], Malawi [12], Burkina Faso [13].

In a low prevalence setting in Zambia however, the sensitivity of a PfHRP2-based RDT was reported as 17% which is much lower than the 47.37% obtained in this study [7]. The specificity obtained in the Zambian study was however comparable to that obtained in this study.

The stability of the impregnated anti-bodies, product design and quality, transport, storage and handling conditions as well as the evolving HRP2 gene deletions as being continually reported [20, 21] could explain the apparent difference in the performance of the HRP2-based RDTs. Many studies have also reported inherent inability of the RDTs to detect malaria positive cases at low parasite densities [1, 22].

Conclusion

Both the RDT and blood film microscopy showed similar levels of sensitivity and specificity with both exhibiting moderate agreement with the PCR results. Significant number of malaria

parasitaemic patients as revealed by the PCR was missed by both the RDT and blood film microscopy and thus went undiagnosed.

Recommendation

It is recommended that further multi-centred study with greater sample size to ascertain the true performance of RDTs and Microscopy across the country. The quality assurance systems in the laboratories should be strengthened.

Abbreviations

Histidine-Rich Protein 2 (HRP2), Polymerase Chain Reaction (PCR), Rapid Diagnostic Test (RDT), Positive Predictive Value (PPV) Negative Predictive Value (NPP), Confidence Interval (CI)

DECLARATIONS

Ethical Approval

The authors declare that all aspects of the study were examined and approved by the Committee on Human Research, Publication and Ethics (CHRPE) School of Medical Sciences of the Kwame Nkrumah University of Science and Technology (KNUST) and KATH in Kumasi, Ghana with reference number CHRPE/AP/520/17

Competing Interest: The authors declare that they have no competing interest.

Authors' Contribution: FOA helped with study conception, design, field work, data analysis and writing of the manuscript. DA, RMP and AOO helped with study supervision and critical revision of the manuscript for intellectual content. DA and RMP provided logistics for the study.

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REFERENCES

1. WHO, World Malaria Report. Geneva: World Health Organization, 2017

2. Parsel S.M, Gustafson S.A., Friedlander E., Shnyra A.A., Adegbulu A.A., Liu Y., Parrish N.M., Jamal S.A., Lofthus E., Ayuk L., Awason C., Henry C.J. & McArthur C.P. Malaria over-diagnosis in Cameroon: diagnostic accuracy of Fluorescence and Staining Technologies (FAST) Malaria Stain and LED microscopy versus Giemsa and bright field microscopy validated by polymerase chain reaction. *Infect Dis Poverty*. 2017, **6**(1):32

3.Malm K.B.P.C., Armah G., Gyapong J., Owusu-Adjei S., Koram K. & Binka F. Malaria as a cause of acute febrile illness in an urban paediatric population in Ghana. Proceedings of the Annual Meeting of the American Society of Tropical Medicine and Hygiene, Atlanta, GA, November 11–15, 2012

4.Shillcutt S., Morel C., Goodman C., Coleman P., Bell D., Whitty C.J.M. & Mills A. Cost-effectiveness of malaria diagnostic methods in sub-Saharan Africa in an era of combination therapy. *Bull World Health Organ* 2008**, 86**:101–110.

5.Thiam S., Thior M., Faye B., Ndiop M., Diouf M.L., Diouf M.B., Diallo I., Fall F.B., Ndiaye J.L., Albertini A., Lee E., Jorgensen P., Gaye O. & Bell D. Major Reduction in Anti-Malarial

Drug Consumption in Senegal after Nation-Wide Introduction of Malaria Rapid Diagnostic Tests. *PLoS One*. 2011, **6**(4)

6. Golassa L., Enweji N., Erko B., Assefa A. & Swedberg G. Detection of a substantial number of sub-microscopic Plasmodium falciparum infections by Polymerase chain reaction: a potential threat to malaria control and diagnosis in Ethiopia. *Malar. J* 2013, **12**:352

7. Laban M.N., Kobayashi T., Hamapumbu H., Sullivan D., Mharajurwa S., Thuma P.E., Shiff C.J. & Moss W.J. Comparison of a PfHRP2-based rapid diagnostic test and PCR for malaria in a low prevalence setting in rural southern Zambia: implications for elimination. *Malar. J* 2015, **14**:25

8. Batwala V., Magnussen P. & Nuwaha F. Are rapid diagnostic tests more accurate in diagnosis of Plasmodium falciparum malaria compared to microscopy at rural health centres? *Malar. J* 2010, **9**:349

9.Miguel-Oteo M., Jiram A.I., Ta-Tang T.H., Lanza M., Hisam S. & Rubio J.M. Nested Multiplex PCR for identification and detection of human Plasmodium species including Plasmodium knowlesi. *Asian Pac J Trop Med* 2017, **10**(3): 299-304

10. Haanshuus C.G., Chandy S., Manoharan A., Vivek R. & Mathai D. Correction: A High Malaria Prevalence Identified by PCR among Patients with Acute Undifferentiated Fever in India. *PLoS One*. 2018, **13**(2) 23

11. Macedo de Oliveira A., Skarbinski J., Ouma P.O., Kariuki S., Barnwell J.W., Otieno K., Onyona P., Causer L.M., Laserson K.F., Akhwale W.S., Slutsker L. & Hamel M. Performance of Malaria Rapid Diagnostic Tests as Part of Routine Malaria Case Management in Kenya. Am J Trop Med Hyg 2009, **80**(3)470–474

12. Parr J.B., Belson C., Patel J.C., Hoffman I.F., Kamthunzi P., Martinson F., Tegha G., Thengolese I., Drakeley C., Meshnick R.S., Escamillia V., Emch M. & Juliano J.J. Estimation of *Plasmodium falciparum* Transmission Intensity in Lilongwe, Malawi, by Microscopy, Rapid Diagnostic Testing, and Nucleic Acid Detection Am J Trop Med Hyg 2016, **95**(2), 373–377

13. Kyabayinze D.J., Zongo I., Cunningham J., Gatton M., Angutoko P. & Ategeka J. HRP2 and pLDH-Based Rapid Diagnostic Tests, Expert Microscopy, and PCR for Detection of Malaria Infection during Pregnancy and at Delivery in Areas of Varied Transmission: A Prospective Cohort Study in Burkina Faso and Uganda. *PLoS One*. 2016, **11**(7) E0156954-E0156954

14. Ochola L.B., Vounatsou P., Smith T., Mabaso M.L. & Newton C.R. The reliability of diagnostic techniques in the diagnosis and management of malaria in the absence of a gold standard. *Lancet Infect Dis* 2006, **6**(9):582–588.

15. Pakalapati D., Garg S., Middha S., Kochar A., Subudhi A.K., Arunachalam B.P., Kochar D.K. & Das A. Comparative evaluation of microscopy, OptiMALand 18S rRNA gene based multiplex PCR for detection of Plasmodium falciparum &Plasmodium vivax from field isolates of Bikaner, India. Asian Pac J Trop Med 2013, **6**(5) 346-351

16. Francisca L., Kusnanto J.H., Sabatoto T.B.T., Sebayang B., Supriyanto, Andriyan E. & Bangs M.J. Comparison of rapid diagnostic test Plasmotec Malaria-3, microscopy and quantitative real-time PCR for diagnoses of Plasmodium falciparum and Plasmodium vivax infections in Mimika Regency, Papua, Indonesia. *Malar. J* 2015, **14**:103

17. Ameyaw B., Mutocheluh M., Owusu M., Khalifah A.A., Duneeh V.R. & Narkwa W. Performance of First Response® and CareStart™ Malaria Rapid Diagnostic Tests for the Detection of *Plasmodium falciparum* in a Tertiary Hospital in Ghana. *BJMMR* 2017, **19**(8): 1-7

18. Baiden F., Webster J., Tivura M., Delimini R., Berko Y., Amenga-Etego S., Agyeman-Budu A., Karikari A.B., Bruce J., Owusu-Agyei S. & Chandramohan D. Accuracy of Rapid Tests for Malaria and Treatment Outcomes for Malaria and Non-Malaria Cases among Under-Five Children in Rural Ghana. *PLoS One* 2012, **7**(4) e34073

19. Osman M.M.M., Nour Y.M.B., Sedig M.F., De Bes L., Babkir A.M., Mohamedani A.A. & Mens P.F. Informed decision making before changing to RDT: a comparison of microscopy, Rapid Diagnostic Test and molecular techniques for the diagnosis and identification of Malaria parasites in Kassala Eastern Sudan. *Malar. J* 2010 **9**(Suppl 2):32

20. Amoah L.E., Abankwa J. & Oppong A. Plasmodium falciparum histidine rich protein 2 diversity and the implications for PfHRP 2-based malaria diagnostic tests in Ghana. *Malar. J* 2016, **15**:101

21.WHO: False-negative RDT results and implications of new reports of *P. falciparum* histidine-rich protein 2/3 gene deletions. World Health Organization, Geneva, Switzerland, 2016.

22. Mathison B.A. & Pritt B.S. Update on malaria diagnostics and test utilization. *J Clin Microbiol* 2017, **55**(7):2009-2017