Evaluation of Sensitivity, Specificity, and Cost-Effectiveness of Paper-Based Microfluidics for DNA Diagnostics of Malaria versus Nucleic Acid Test (NAT) Versus Rapid Diagnostic Tests (RDT) in Resource-Limited Settings: A Protocol

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Authors’ contributions

This work was carried out in collaboration between both authors. Author UA designed the study, wrote the protocol and wrote the first draft of the manuscript. Author TR managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Objective: The objective of the study is to compare three techniques, routinely used rapid diagnostic tests (lateral flow immune chromatography) versus nucleic acid amplification test (NAT) versus Paper-based microfluidics for DNA diagnostics of Malaria, in terms of their sensitivity and specificity as diagnostic tests in detecting malarial infection among febrile illnesses, suspected of malaria, as well as to compare their cost-effectiveness.

Methodology: Three seventy febrile cases suspected of malaria with negative results with RDT will be screened by real-time PCR and DNA microfluidics techniques, sensitivity and specificity of these as screening tests will be compared. The number of extra positive cases detected by NAT gives us the yield. Cost-effectiveness analysis will be done by calculating the incremental cost-effectiveness ratio (ICER) and average cost-effectiveness ratio (ACER) for the tests.

Statistical Analysis: Statistical analysis will be done using SPSS version 21. Sensitivity, specificity, Positive predictive values will be computed. Comparison of sensitivity and specificity of

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NAT, a paper microfluidic technique for DNA diagnostics and RDT will be carried out using McNemar’s test. Receiver operating curves will be generated separately to assess the utility of the NAT.

**Conclusion:** The Implications of this study from the patient's perspective would mean early diagnosis which forms the tenet of control of the disease by increasing the yield. Early diagnosis at the community level would translate into the application of efficient prevention mechanisms to spread the infection. The cost-effectiveness analysis would provide a scientific basis for the adoption of the best test for the diagnosis, given the economic feasibility of the study.

*Keywords:* P. vivax; RDT; NAT; DNA microfluidics.

### 1. INTRODUCTION

Malaria is a protozoan disease transmitted by the bite of infected *Anopheles* mosquitoes. It is transmitted in 106 countries containing 3 billion people and causes approximately 2000 deaths each day; mortality rates are decreasing as a result of highly effective control programs in several countries. Malaria has been eliminated from the United States, Canada, Europe, and Russia; in the late twentieth and early twenty-first centuries, however, its prevalence rose in central areas of Africa, Asia, and Latin America, where it contributes as one of the world’s greatest public health problems. Prevalence of malaria in India as per WHO report 2017, only eight percent of malaria cases were diagnosed in 2016 in India, which constituted six percent of the 216 million new cases globally. The report also suggested that India was the third on the list of 15 countries which accounted for 80 percent of all malaria cases in the world in 2016. According to the report, only 8 percent of malaria cases were detected in India due to poor surveillance mechanisms [1].

According to World Health Organisation, in South East Asia Region, 70% of malaria cases were from India [2]. The global estimate shows developing countries report a maximum number of cases of malaria with the majority of infected people living in urban India. When we consider the prevalence of malaria in Karnataka, a study reports that Dakshina Kannada, a southern coastal district of Karnataka State contributes almost 50% of the cases to the state malaria profile [3]. Due to this fact, it is justifiable to study malaria in Mangaluru, Dakshina Kannada. As screening techniques used for malarial parasite detection have their drawbacks, it is justifiable to compare their sensitivity, specificity, and cost-effectiveness.

### 1.1 Diagnostic Approaches for the Detection of Malarial Infection and Difficulties Associated

The Giemsa-stained blood slide using thin and thick smears for malaria parasites has been the gold standard method for nearly a century [4]. No alternative method still could be established to replace this universally accepted gold standard method. This technique to confirm the clinical suspicion of malaria is labor-intensive [5] and sometimes unreliable due to lack of skilled microscopists, limited supplies, inadequate maintenance of microscopes and reagents, and inadequate or absence of quality-control systems [6].

In recent times, lateral flow immunochromatographic-based rapid diagnostic tests (RDT) have been developed for the diagnosis of suspected malaria patients and are widely used in remote labor-intensive world [7]. Rapid diagnostic test (RDT) kits offer great potential for the immediate diagnosis of malaria infections. Rapid diagnosis, prompt treatment, especially in rural settings. RDTs are lateral flow immunochromatographic tests that detect *Plasmodium* parasite antigens in blood [8]. Three antigens are detected by current RDTs: histidine-rich protein 2 (HRP2), lactate dehydrogenase (LDH), and aldolase.

However, histidine- of such assays has been limited by their generation of false-positive results, which occur when nonspecific biomolecules present in the blood, such as the rheumatoid factor, for example, react with the test antigens [9]. Technical issues linked to capillary flow and to reagent stability in challenging environmental conditions, including high humidity and temperature [10], have also affected the reliability of RDT immunodiagnostics. Test sensitivities are often only 70 to 75% in the field [11], despite being
much higher in well-controlled laboratory conditions [12].

1.2 Nucleic Acid TESTING (NAT) is a Sensitive Technique for the Detection of Malarial Parasites

Nucleic acid amplification-based tests (NAATs) provide a promising approach for DNA-based malaria diagnostics. These tests both amplify and detect the genomic material of the parasite directly from a patient sample, providing a sensitive, species-specific test that will also identify whether an infection is current rather than historical [13,14]. In addition, the signal used for detection does not depend on the patients' immune responses and the technique can be both quantitative and more accurate than Immuno-RDTs [15].

Many nucleic acid testing (NAT) approaches for the diagnosis of human malaria infection have been developed in the past two decades [16-23]. Studies suggest that NAT can detect and quantify parasites more sensitively and precisely than by microscopy or rapid diagnostic tests (RDTs). NAT is valuable for controlled human malaria infection studies of investigational drug and vaccine candidates, for drug efficacy studies as well as for epidemiological surveillance [24]. Parasite load can be quantified by NAT assay 2–6 days earlier than microscopy [25-28].

In many reference laboratories, PCR-based amplification assays remain the gold-standard NAAT [29], although the requirement for trained staff and external power has limited their application in areas with reduced resources.

In recent times, a real-time PCR method has been established for the quantitative detection of malaria parasites [30-32]. Real-time PCR is reliable and yield high sensitivity and specificity when compared with microscopy or nested PCR [32,33].

In a Chinese study, Yan et al compared the sensitivities of RDTs and microscopy with that of nucleic acid testing by nested PCR. The study concluded that Compared to PCR, both microscopy and RDTs had lower sensitivities, especially for P. vivax diagnosis [34]. A study by Alam et al, in Bangladesh, compared the sensitivities of rapid diagnostic tests and nucleic acid amplification tests and the study concluded that SYBR Green-based real-time PCR assay could be used as an alternative gold standard method in a reference setting. Commercially-available RDTs used in the study are quite sensitive and specific in detecting P. falciparum, although their sensitivity in detecting P. vivax was not satisfactory compared to the real-time PCR assay [35]. Prandini et al reported better sensitivity with RT PCR in detecting malarial parasites as compared to RDT [36].

The major constraint for implementing NAT as a routine screening technique in India appears to be its high cost per test and the time duration required.

We would like to use a device, Paper-based microfluidics for DNA diagnostics that uses origami to enable multiplexed, sensitive assays that may be superior to PCR-based laboratory assays and provide high-quality, fast precision diagnostics for malaria. The paper-based microfluidic technology combines vertical flow sample-processing steps, including paper folding for whole-blood sample preparation, with isothermal amplification and lateral flow detection, incorporating a simple visualization system.

The study would aim to compare the diagnostic sensitivity and specificity of paper-based microfluidics for DNA diagnostics of malaria in resource-limited settings like India. We would like to demonstrate that advanced, low-cost DNA-based sensors can be implemented in underserved communities at the point of need cost-effectively.

1.3 Cost-Effectiveness Analysis

Cost-effectiveness analysis is an important tool to assist clinicians, scientists, and policymakers in determining the efficiency of healthcare interventions, guiding societal decision-making on the financing of healthcare services, and establishing research priorities. Diverse approaches to synthesize evidence have been considered in biomedical research, including economic evaluations of healthcare interventions [37-40]. At the same time, decision-making in health care requires an understanding of the state of economic evaluation at a national level, where the completeness of the reporting is generally less well understood but where specific priorities are often set. Cost-effectiveness analysis (CEA) compares two diagnostic tests, where the costs are identified in monetary terms and the outcomes in non-monetary terms.
Measurement of cost-effectiveness could be made in two different ways:

1. ACER – Average Cost-Effectiveness Ratio
2. ICER – Incremental Cost-Effectiveness Ratio

It helps a decision-maker to compare one treatment/diagnostic test to another thereby quantifying the opportunity cost of decisions.

1.4 Novelty / Innovation

The study could demonstrate that paper-based microfluidic devices can deliver precision diagnostics for malaria in low-resource, underserved settings with a sensitivity that is higher than that of the current malaria diagnostic tests used in the field and with performance that is similar to that of a laboratory-based real-time PCR test. These diagnostic devices could have a meaningful, positive impact on the provision of mass screening and treatment in campaigns to eliminate infectious disease. These campaigns have had limited success to date in combating malaria transmission, which has been linked to the inability of current field-based diagnostic tools to detect low-level infections. Thus, the availability of easy-to-use, highly sensitive NAATs, such as those provided by this device, could potentially detect these missed cases and reduce the opportunity for transmission. This would have a significant impact on public health in areas where malaria is highly prevalent.

1.5 Study Objectives

- To compare sensitivity, specificity, and yield of diagnostic tests, rapid diagnostic tests (RTD) and nucleic acid testing (NAT) by real-time PCR and Paper-based microfluidics for DNA diagnostics of malaria in the detection of malarial infection with \( P. \text{ vivax} \)
- To compare the cost-effectiveness of the above techniques, by calculating incremental cost-effectiveness ratio (ICER), average cost-effectiveness ratio (ACER)

2. METHODOLOGY

2.1 Study Design
Cross-sectional.

2.2 Study Centre
The study will be carried out in the Genetic division of Central research Laboratory, KS Hegde Medical Academy, located in Mangalore, Karnataka, India.

2.2.1 Sample size calculation
A sample size of 370 confirmed \( P. \text{ vivax} \) cases will be included. The sample size estimates are based on a proportion obtained from simple random sampling, with a sampling design effect \( \text{(deft)} = 1.5 \) and a probability of committing a type-1 error = 95% (1-sided test), such that the 95% confidence interval does not overlap with the threshold of 5%.

\[
 n \geq \frac{Z^2 \hat{p}(1-\hat{p})}{D^2}
\]

The sample size is based on estimating output indicators, where the upper bound of the 95% CI does not overlap with 5% for estimates in which the observed prevalence of false-negative RTD results is below 5% and where the lower bound of the 95% CI does not overlap with 5% for estimates in which the observed prevalence is above 5%.

2.2.2 Project implementation plan

2.2.2.1 Study participants
Individuals in the age group of 18-65 of either gender, seeking care for febrile illness at health facilities with negative RTD results.

Exclusion criteria: Malarial infection with ovale and malariae, any other febrile illnesses, RTD Positive cases.

2.3 Laboratory Investigations

Symptomatic individuals will be screened for malaria infection by using malaria rapid diagnostic test kit. Five microlitres of fresh whole blood will be added to the card pad, and three drops of a specific lysing agent will be added. The RTD result will read in 15–20 min according to the manufacturer’s instructions and immediately recorded.

Blood samples of individuals who will be negative for RTD will be subjected to nucleic acid amplification testing by RT-PCR and Paper-based microfluidics for DNA diagnostics.

2.4 Procedure for NAT by Real-time PCR (RT-PCR)

2 ml blood will be collected into EDTA vacutainer under strict aseptic conditions.
DNA will be extracted from EDTA preserved blood samples using the QiaAmp blood mini kit following the manufacturer's instructions at the Parasitology Laboratory of ICDDR. The DNA sample will be stored at 4°C until PCR could be completed.

Real-time PCR will be done by the primer sets described by Perandin et al with some modification to a singleplex reaction [37]. SYBR Green I dye will be used for visualizing the amplification. PCR condition will also be modified slightly to fit with Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen Corporation, USA) following the instructions of the manufacturer. Purified DNA templates will be amplified in a real-time system with a species-specific primer set. A 25-μL PCR mixture will be prepared using 1 μl of template DNA, 12.5 μl Platinum SYBR Green qPCR supermix (Platinum® Taq DNA polymerase, SYBR Green I dye, Tris-HCl, KCl, 6 mM MgCl2, 400 μM dGTP, 400μM dATP, 400μM dCTP, 800 μM dUTP, uracil DNA glycosylase, and stabilizers), 320 nM concentration of each of parasite species-specific primer set. Amplification and detection will be performed as follows: 50°C for 2 min and 95°C for 2 min. After that 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min 30 sec for a single cycle will be performed. 40 cycles will be considered for P. falciparum. The melt curve will be performed. 40 cycles will be considered for P. falciparum. The plate read will be taken after the extension at 72°C. The melt curve will be prepared from 50°C to 95°C with an increment of 0.5°C each after five seconds. To establish the minimum number of parasites detectable by the Plasmodium SYBR Green assay (detection limit), blood samples from patients infected will be collected, and parasitemia will be calculated using 200 WBC counts as reference. The infected blood samples will be diluted with uninfected erythrocytes from healthy individuals with known baseline erythrocyte counts. Ten-fold serial dilution will be made to obtain a final parasitemia of 1% (1 parasite/μl of blood) for each sample. All DNA aliquots purified from the dilutions will be treated in duplicate for real-time PCR assay. The clinical sensitivity and specificity of the modified Plasmodium real-time PCR assay for detecting and identifying malaria parasites will be calculated on 370 whole blood samples.

### 2.4.2 Cost-effective analysis of diagnostic techniques

The incremental cost-effectiveness ratio (ICER) and average cost ratio (ACER) will be calculated as follows:

**Incremental cost-effectiveness ratio:** It compares the incremental cost divided by the incremental effect. This can be described in an incremental cost-effectiveness ratio (ICER), and can be expressed in an equation:

\[
\text{ICER} = \frac{C1 - C2}{E1 - E2}
\]

where C1 and E1 are the cost and effect due to NAT/paper microfluidic device, and C2 and E2 are the cost and effects of RDT, effect(E1 & E2) being the number of cases detected.

A high ICER indicates more expenditure for better health outcomes while compared to a lower ICER. Hence an intervention with a lower ICER would be preferred.

**Average Cost-effectiveness Analysis:** The cost-effectiveness ratio of each diagnostic test will be calculated and the two ratios are compared. Specifies the cost of an intervention required to achieve each unit of effect.

**Average cost-effectiveness ratio (ACER)**

\[
\text{Cost of diagnostic test} \times \frac{\text{Cost per unit of positive cases detected}}{\text{Number of cases detected}}
\]

**Average cost-effectiveness ratio (ACER)**

\[
\text{Cost of diagnostic test} \times \frac{\text{Cost per unit of positive cases detected}}{\text{Number of cases detected}}
\]
A validated QOL questionnaire will be administered to all positive cases to calculate the QOL score. The SF-36 is a set of standard validated questions which is used to judge the quality of life in patient/subjects. This questionnaire contains 36 items spread over different domains such as physical function, physical role, general health, vitality, body pain, social well-being, emotional role, and mental health of the individual in the past 4 weeks. The score of each question when entered in the excel sheet will be converted into a number depending on the response to the question. Upon entering the response to all the questions the software will give the consolidated response of the physical and mental health domain. The final quality of health of an individual will be calculated by dividing the total SF-36 value by 100. The QOL of a participant/patient ranges from 0 to 1, where 1 refers to perfect health and 0 refers to worst health (death).

Quality-adjusted life year (QALY) gained will be calculated as QOL score multiplied by several days of early detection among positive NAT/paper microfluidic technique for DNA diagnostics results.

Cost per QALY gained by NAT/paper microfluidic technique for DNA diagnostics will be calculated as follows:

\[
\text{Cost per QALY} = \frac{\text{cost of NAT/paper microfluidic technique} + \text{Cost of RDT for NAT positive patients}}{\text{QALY gained}}
\]

A pre-tested semi-structured questionnaire containing socio-demographic details of the voluntary donors like age, gender, place of residence, will be collected after formal written consent maintaining the confidentiality of identity. The participants will be analysed for laboratory tests and cost-effective analysis. University ethics committee approval will be sought before starting the study. Informed consent will be obtained from the study subjects.

2.5 Statistical Analysis

Quantitative data like NAT, a paper microfluidic technique for DNA diagnostics and RDT results, yield, cost of the tests will be expressed in mean and standard deviation. Data thus collected will be coded, validated, and entered into Microsoft Excel version 2010 and analysed using SPSS (Statistical Package for Social Sciences) version 21. Sensitivity, specificity, Positive predictive values will be computed for NAT, paper microfluidic technique for DNA diagnostics and RDT. Receiver operating curves will be generated separately to assess the utility of the NAT, a paper microfluidic technique for DNA diagnostics and RDT and Area under the curve (AUC) will be determined. The cut-offs for the sensitivity without a significant decrease in specificity will be chosen for each of the tests. The significance level will be set as p< 0.05.

3. EXPECTED OUTCOMES

3.1 Primary Output Measures

Comparison of sensitivity and specificity of RDT versus Paper-based microfluidics for DNA diagnostics of malaria versus nucleic acid test by RT-PCR in the detection of infection by P. vivax.

3.2 Secondary Output Measure

Cost-effectiveness analysis of diagnostic tests, RDT, NAT by RT-PCR, and Paper-based microfluidics for DNA diagnostics in detecting P. vivax.

4. LIMITATIONS

Small sample size.

5. CONCLUSION AND APPLICABILITY OF THE STUDY

The study may demonstrate that paper-based microfluidic devices can deliver precision diagnostics for malaria in low-resource, underserved settings with a sensitivity that is higher than that of the current malaria diagnostic tests used in the field and with performance that is similar to that of a laboratory-based real-time PCR test. These diagnostic devices may have a meaningful, positive impact on the provision of mass screening and treatment in campaigns to eliminate infectious disease. These campaigns have had limited success to date in combating malaria transmission, which has been linked to the inability of current field-based diagnostic tools to detect low-level infections. Thus, the availability of easy-to-use, highly sensitive NAATs, such as those provided by this device, could potentially detect these missed cases and reduce the opportunity for transmission. This would have a significant impact on public health in areas where malaria is highly prevalent. It could also inform current thinking within governments and nongovernmental
organizations concerning improvements in the effectiveness and cost-effectiveness of prophylactic approaches to control diseases (where new precise diagnostic tools are required to rapidly and accurately target where treatment is needed).

Economic assessments of diagnostic tests are inherently difficult than assessments of therapeutic interventions because of uncertainty about the relation between diagnosis and result or outcomes of care. Towards the end, this study would evaluate the economic feasibility of the introduction of paper microfluidic techniques for DNA diagnostics as a diagnostic test for malaria. The economic evaluation of the cost-effectiveness of NAT using the Yield of the NAT test vis a vis the conventional RDT test would have profound implications concerning policy-making and utility of the test for diagnosing individuals with malaria. If the malarial infection detection rate by paper microfluidic technique for DNA diagnostics were to be proven to be beneficial, it would pave new roads for early diagnosis of the disease by providing scientific evidence for possibly implementing this test as a useful diagnostic test. This study would help in planning out further strategies for the effective management and treatment of individuals detected by the test. It would also dive into newer research areas to establish the subsequent decrease in the morbidity and mortality associated with malaria given appropriate facilities for early treatment after detection would be mandated at the policy level.

5.1 National Relevance

The Implications of this study from the patient’s perspective would mean early diagnosis which forms the tenet of control of the disease by increasing the yield. Early diagnosis at the community level would translate into the application of efficient prevention mechanisms to spread the infection. The cost-effectiveness analysis would provide a scientific basis for the adoption of the best test for the diagnosis, given the economic feasibility of the study. Early diagnosis will aid the clinician in providing timely treatment by reducing morbidity and mortality due to malarial infection.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. WHO Malaria report; 2017. Available:www.who.int/malaria/publications/world-malaria-report-2017/en/
2. Dash AP, Valecha N, Anvikar AR, Kumar A. Malaria in India: Challenges and opportunities. J Biosci. 2008;33:583-92.
3. Available:http://www.mangalretoday.com/main/malaria - high-risk- in-seven wards- of Mangalore-city-national program-experts.
4. Murray CK, Bell D, Gasser RA, Wongsrichanalai C. Rapid diagnostic testing for malaria. Trop Med Int Health. 2003;8:876-883.
5. Bojang KA, Obaro S, Morison LA, Greenwood BM: A prospective evaluation of a clinical algorithm for the diagnosis of malaria in Gambian children. Trop Med Int Health. 2000;5:231-236.
6. Coleman RE, Maneenchai N, Rachaphaew N, Kumpitak C, Miller RS, Soyseng V, Thimasarn K, Sattabongkot J. Comparison of field and expert laboratory microscopy for active surveillance for asymptomatic Plasmodium falciparum and Plasmodium vivax in western Thailand. Am J Trop Med Hyg. 2002;67:141-144.
7. Fogg C, Twesigye R, Batwala V, Piola P, Nbasumba C, Kiguli J, Mutebi F, Hook C, Guillerm M, Moody A, Guthmann JP. Assessment of three new parasite lactate dehydrogenase (pan-pLDH) tests for diagnosis of uncomplicated malaria. Trans R Soc Trop Med Hyg. 2008;102:25-31.
8. Howard RJ, Uni S, Aikawa M, Aley SB, Leech JH, Lew AM, et al. Secretion of a malarial histidine-rich protein (Pf HRP II) from Plasmodium falciparum-infected erythrocytes. J Cell Biol. 1986;103(4):1269–77.
9. Lee J-H, et al. False-positive results for rapid diagnostic tests for malaria in patients with rheumatoid factor. J Clin Microbiol. 2014;52:3784–3787.
10. Wongsrichanalai C, Barcus MJ, Muth S, Sutamihardja A, Wernsdorfer WH. A
review of malaria diagnostic tools: Microscopy and rapid diagnostic test (RDT). Am J Trop Med Hyg. 2007;77(6): 119–127.

11. Wanja EW, et al. Field evaluation of the diagnostic performance of malaria rapid diagnostic tests in western Kenya. Malar J. 2016;15:456.

12. Feleke DG, Tarko S, Hadush H. Performance comparison of CareStart™ HRP2/ pLDH combo rapid malaria test with light microscopy in north-western Tigray, Ethiopia: A cross-sectional study. BMC Infect Dis. 2017;17:399.

13. Rougemont M, et al. Detection of four Plasmodium species in blood from humans by 18S rRNA gene subunit-based and species-specific real-time PCR assays. J Clin Microbiol. 2004;42:5636–5643

14. Hopkins H, et al. Highly sensitive detection of malaria parasitemia in a malaria-endemic setting: Performance of a new loop-mediated isothermal amplification kit in a remote clinic in Uganda. J Infect Dis. 2013;208:645–652.

15. Wu L, et al. Comparison of diagnostics for the detection of asymptomatic Plasmodium falciparum infections to inform control and elimination strategies. Nature. 2015;528: S86–S93.

16. Coleman RE, Sattabongkot J, Promstaporn S, Maneechai N, Tippayachai B et al. Comparison of PCR and microscopy for the detection of asymptomatic malaria in a Plasmodium falciparum/vivax endemic area in Thailand. Malar J. 2006;5:121.

17. Han ET, Watanabe R, Sattabongkot J, Khuntirat B, Sirichaisinthop J et al. Detection of four Plasmodium species by genus- and species-specific loop-mediated isothermal amplification for clinical diagnosis. J Clin Microbiol. 2007;45:2521–2528.

18. Mangold KA, Manson RU, Koay ES, Stephens L, Regner M, et al. Realtime PCR for detection and identification of Plasmodium spp. J Clin Microbiol. 2005;43:2435–2440.

19. Mens PF, Schoone GJ, Kager PA, Schallig HD. Detection and identification of human Plasmodium species with real-time quantitative nucleic acid sequence-based amplification. Malar J. 2006;5:80.

20. Mixson-Hayden T, Lucchi NW, Udhayakumar V. Evaluation of three PCR-based diagnostic assays for detecting mixed Plasmodium infection. BMC Res Notes. 2010;3:88.

21. Safaeukui I, Millet P, Boucher S, Melinard L, Fregeville F, et al. Evaluation of FRET real-time PCR assay for rapid detection and differentiation of Plasmodium species in returning travelers and migrants. Malar J. 2008;7:70.

22. Alemayehu S, et al. Comparative evaluation of published real-time PCR assays for the detection of malaria following MIQE guidelines. Malar J. 2013; 12:277.

23. Tangin A, Komichi Y, Wagatsuma Y, Rashidul H, Wataya Y, et al. Detection of malaria parasites in mosquitoes from the malaria-endemic area of Chakaria, Bangladesh. Biol Pharm Bull 2008;31:703–708.

24. Vo TK, Bigot P, Gazin P, Sinou V, De Pina JJ, et al. Evaluation of a real-time PCR assay for malaria diagnosis in patients from Vietnam and returned travelers. Trans R Soc Trop Med Hyg. 2007;101:422–428.

25. Murphy SC, Shott JP, Parikh S, Etter P, Prescott WR, et al. Malaria diagnostics in clinical trials. Am J Trop Med Hyg. 2013; 89:824–839.

26. Sheehy SH, Spencer AJ, Douglas AD, Sim BK, Longley RJ, et al. Optimising Controlled Human Malaria Infection Studies Using Cryopreserved Parasites Administered by Needle and Syringe. PLoS One. 2013;8:e65960.

27. Roestenberg M, Bijkker EM, Sim BK, Billingsley PF, James ER, et al. Controlled human malaria infections by intradermal injection of cryopreserved Plasmodium falciparum sporozoites. Am J Trop Med Hyg. 2013;88:5–13.

28. Roestenberg M, O’Hara GA, Duncan CJ, Epstein JE, Edwards NJ, et al. Comparison of clinical and parasitological data from controlled human malaria infection trials. PLoS One. 2012;7:e38434.

29. Alemayehu S, et al. Comparative evaluation of published real-time PCR assays for the detection of malaria following MIQE guidelines. Malar J. 2013; 12:277.

30. Murphy SC, Prentice JL, Williamson K, Wallis CK, Fang FC, et al. Real-time quantitative reverse transcription PCR for monitoring of blood-stage Plasmodium falciparum infections in malaria human challenge trials. Am J Trop Med Hyg. 2012;86:383–394.
31. Swan H, Sloan L, Muyombwe A, Chavalitshewinkoon-Petmitr P, Krudsood S, Leowattana W, Wilairatana P, Looareesuwan S, Rosenblatt J: Evaluation of a real-time polymerase chain reaction assay for the diagnosis of malaria in patients from Thailand. Am J Trop Med Hyg. 2005;73:850-854.

32. Mangold KA, Manson RU, Koay ES, Stephens L, Regner M, Thomson RB Jr, Peterson LR, Kaul KL: Real-time PCR for detection and identification of Plasmodium spp. J Clin Microbiol 2005;43:2435-2440.

33. Shokoples SE, Ndao M, Kowalewska-Grochowska K, Yanow SK: Multiplexed real-time PCR assay for discrimination of Plasmodium species with improved sensitivity for mixed infections. J Clin Microbiol. 2009;47:975-980.

34. Khairnar K, Martin D, Lau R, Ralevski F, Pillai DR: Multiplex real-time quantitative PCR, microscopy and rapid diagnostic immunochromatographic tests for the detection of Plasmodium spp: performance, the limit of detection analysis and quality assurance. Malar J. 2009;8:284.

35. Juan Yan, Nana Li, Xu Wei, Peipei Li, Zhenjun Zhao, Lili Wang, Siying Li, Xiaomei Li, Ying Wang et al. Performance of two rapid diagnostic tests for malaria diagnosis at the China-Myanmar border area. Malaria Journal. 2013;12:73.

36. Mohammad Shafiul Alam, Abu Naser Mohon, Shariar Mustafa, Wasif Ali Khan, Nazrul Islam, Mohammad Jahirul Karim, Hamida Khanum, David J Sullivan Jr, Rashidul Haque. Real-time PCR assay and rapid diagnostic tests for the diagnosis of clinically suspected malaria patients in Bangladesh. Malaria Journal. 2011;10:175.

37. Perandin F, Manca N, Calderaro A, Piccolo G, Galati L, Ricci L, Medici MC, Arcangeletti MC, Snounou G, Dettori G, Chezzi C: Development of a realtime PCR assay for detection of Plasmodium falciparum, Plasmodium vivax, and Plasmodium ovale for routine clinical diagnosis. J Clin Microbiol. 2004;42:1214-1219.

38. Elixhauser A, Luce BR, Taylor WR, Reblando J. Health care CBA/CEA: an update on the growth and composition of the literature. Med Care. 1993;31:JS1–JS11, JS18–149.

39. Elixhauser A, Halpern M, Schmier J, Luce BR. Health care CBA and CEA from 1991 to 1996: an updated bibliography. Med Care. 1998;36:MS1–9, MS18–147.

40. Neumann PJ, Stone PW, Chapman RH, Sandberg EA, Bell CM. The quality of reporting in published cost-utility analyses, 1976–1997. Ann Intern Med. 2000;132:964–72.

41. Xu G, et al. Paper-origami-based multiplexed malaria diagnostics from whole blood. Angew Chem Int Ed Engl. 2016;55:15250–15253.