Comparison of Real Time Polymerase Chain Reaction with Microscopy and Antigen Detection Assay for the Diagnosis of Malaria

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ABSTRACT

Objective: To determine the sensitivity of a real time polymerase chain reaction (PCR) for malaria diagnosis and to compare its accuracy with microscopy and an antigen based rapid diagnostic test (OptiMal).

Study Design: Cross-sectional analytical study.

Place and Duration of Study: Military Hospital, Armed Forces Institute of Transfusion and Armed Forces Institute of Pathology, Rawalpindi, from July to December 2011.

Methodology: Venous blood samples of 300 clinically suspected patients of malaria were tested for malaria parasite by microscopy and OptiMal; and malaria parasite index was calculated for the positive samples. *Plasmodium* genus specific real time PCR was performed on all specimens, targeting small subunit rRNA gene. Diagnostic accuracy of three tests was compared and cost analysis was done.

Results: Out of 300 patients, malaria parasite was detected in 110, 106 and 123 patients by microscopy, OptiMAL and PCR respectively. Real time PCR was 100% sensitive while microscopy and OptiMal had sensitivity of 89.4% and 86.2% respectively. All methods were 100% specific. The cost per test was calculated to be 0.2, 2.75 and 3.30 US$ by microscopy, OptiMal and PCR respectively, excluding the once capital cost on PCR equipment.

Conclusion: Genus specific real time PCR for the diagnosis of malaria was successfully established as a highly sensitive and affordable technology that should be incorporated in the diagnostic algorithm in this country.

Key Words: Malaria. Rapid diagnostic tests (RDTs). Polymerase chain reaction (PCR). Primers.

INTRODUCTION

Malaria remains the most prevalent and important disease worldwide. An estimated 3.3 billion people are at risk in about 106 endemic countries in Africa, Asia and Latin America. There were 216 million reported cases of malaria and estimated 655,000 deaths in 2010.[1] Pakistan is among the countries where malaria continues to be a health issue.[2] It is the second most prevalent and devastating disease, which accounts for 6% of all outpatient attendances and 18% admissions in hospitals.[3] The annual parasite incidence (API) is 0.75/1000 population, but in rural areas, 12% of the people are asymptomatic carrier of the parasite.[4] One of the key strategies for global malarial control is its prompt and effective diagnosis. This not only provides early treatment, but also helps in disease control and transmission.[5]

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Microscopy has been the mainstay of malaria diagnosis for more than 100 years. It is an inexpensive, simple and economical method for malaria diagnosis. It can identify as well as quantify the malaria parasites. However, the method is labour intensive and requires trained microscopist.[6] The role of light microscopy as gold standard has been questioned because its sensitivity fluctuates at low parasite levels (< 100 parasites/μl) and species identification in mixed infection is sometimes difficult to pick.[7]

In recent years, the need for a simple and reliable diagnostic method has led to the development of immunochromatography based rapid diagnostic tests (RDTs). These detect *Plasmodium* antigen or enzymes and the newer improved RDTs can separate *P. falciparum* and non *P. falciparum* species.[8] These are found useful in the remote endemic areas as non-skilled health workers can easily perform these rapid tests. However, the studies have shown wide variations in their sensitivity.[9] RDTs do not offer improved sensitivity over microscopy. Their sensitivity decreases as parasitic index falls below 200 parasites/μl.[10] RDTs are expensive and false positive results are not infrequent.

Since the 1980s, various molecular methods have been introduced for the diagnosis of malaria. The conventional nested and semi-nested PCR techniques have now been replaced by real time PCR. The target is

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usually genus specific small-subunit 18S ribosomal ribonucleic acid (rRNA) gene of the parasite.[11] The earlier PCR methodologies were time-consuming, technically difficult and contamination was frequent. Real time PCR is simple and quick to perform. It can quantitate the parasite and risk of contamination is minimal.[12]

PCR technique for the diagnosis of malaria is more sensitive than microscopy as it can detect < 5 parasite/µl. Species identification is also possible and mixed malarial infection is better detected by this methodology.[13] However, PCR technique is expensive, requires infrastructure support and skilled technologists.

This study is expected to form the basis of recommendation for incorporating PCR in diagnostic algorithm applicable to present healthcare structure of the country, with an aim of utilizing more accurate test for specific and earlier treatment of malaria.

The aim of this study was to compare the performance of a real time PCR with microscopy and one of the antigen detection assays for the diagnosis of malaria.

METHODOLOGY

This cross-sectional analytical study was conducted in the Military Hospital, Armed Forces Institute of Transfusion and Armed Forces Institute of Pathology, Rawalpindi, from July to December 2011.

The sample size was calculated using 18% prevalence of malaria and margin of error 5%. The sampling technique was non-probability consecutive and the inclusion criteria for the patients were fever of short duration, continuous / alternate day with rigors / chills, followed by sweating and/or palpable firm spleen and / or herpes labialis. Patients having fever with sore throat, relative bradycardia, rash, lymphadenopathy, soft spleen, diarrhea, cough and signs of meningeal irritation were excluded. Follow-up of malarial parasite positive patients, receiving anti-malarial treatment was done.

Five ml of venous blood in an EDTA tube was collected from each patient. Blood counts of each patient were done by Sysmex KX-21 haematology analyzer. Two drops of sample were used to prepare thin and thick blood smears. One drop was used for RDT (OptiMAL) and remaining sample was preserved for real time PCR malaria diagnostic test.

Thick and thin blood films were prepared using standardized blood volumes of 10 µl and 2 µl respectively and were air dried. The slides were stained with Leishman's stain and analyzed for the presence of malarial parasite (thick film). Thin blood films were used for identification of different *Plasmodium* species. The smears were considered negative if no parasite was seen after 100 x oil immersion fields.[14] Parasite density was determined by number of parasites per 200 white blood cells in a thick film expressed as parasites/µl. Baseline white blood cell count was used to calculate parasite density. Each slide was assessed by two independent microscopists. All samples showing disagreement among the methods were re-evaluated with extended time especially for microscopy, but the first result was used for calculation.

The rapid antigen malaria test OptiMAL (Flow Inc, Portland) was performed on each patient sample with a drop of blood. The test detects parasite lactate dehydrogenase (pLDH), an enzyme produced by metabolizing malaria parasites. Briefly, a drop of blood was added to a well in a micro titer plate and mixed with the drop of buffer. An OptiMAL test strip was placed in the well and the blood was wicked up by the nitro-cellulose strip. After the blood was completely wicked up, the strip was transferred to the next well, which contained a few drops of wash buffer which cleared the excess blood. The entire process took approximately 15 minutes and results were visually interpreted as per manufacturer's instructions. The presence of positive control line indicated that the strip is functional.

DNA was extracted from the venous blood sample collected in EDTA by using pure gene genomic DNA purification kit, Gentra USA, as per manufacturer's instructions.

The target gene for four species of *Plasmodium (P.)* was small sub unit (SSU) rRNA for PCR amplification in this study. The real time PCR was done using TaqMan probe according to the protocol of Lee *et al*.[15] The PCR primers for malarial species (Gen Bank accession numbers M19172 for *P. falciparum*, X13926 for *P. vivax*, M54897 for *P. malariae* and L48987 for *P. ovale*) were used. The sequence of the two amplification primers and the TaqMan probe was 5'-ACATGGCTATGACGGGTAACG-3' (forward primer), 5'-TGCCTTCCTTAGATGTGGTAGCTA-3' (reverse primer), 6 FAM 5'-TCAGGCTCCCTCTCC GGAATCGA-3'- TAMRA (TaqMan probe).

PCR was done in 25 µl reaction mixture containing 5 pM each of the forward and the reverse primer and the TaqMan probe, 0.5 units of Taq polymerase (Fermentas Life Sciences, Lithuania), 30 mM of each dNTP, 10 mM Tris HCL (pH 8.3), 500 mM KCL, 1.5 mM $MgCl_2$, 1mg/ml gelatin and 1 µl of DNA. Thermal cycling comprised of initial denaturation at 95ºC for 5 minutes, followed by 40 cycles each of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 60 seconds. The amplification was done in 7500 real time PCR system (Applied Biosystems, USA). Positive controls were prepared by pooling microscopy positive samples for *P. falciparum* and *P. vivax*. Blood samples from individuals who were known thalassaemia trait, afebrile for more than one year and microscopy negative for malaria parasite were used as negative controls. DNA free PCR grade water was used as blank in this study.

To establish the sensitivity of the PCR assay, a known sample positive for malaria with a parasite index of 2000/µl was used. This sample was serially diluted to make parasite density upto 1 – 2 parasite/µl. This dilution was tested several times and the cycle threshold value (C_t) of fluorescence was determined. On repeated testing, the latter was found to be between 34 – 36 cycles. The (C_t) value of 34 was used as the upper positive cut off value of PCR in this study. These same dilutions of known parasite index were also tested by microscopy and OptiMAL and minimum level of detection was determined.

The data was entered and analyzed in Statistical Package for Social Sciences (SPSS) version 15.0. Mean and standard deviation was calculated for quantitative variables like age (years) of patient, duration (days) of fever. Frequency and percentages were calculated for qualitative variables like positive and negative cases of malaria, sensitivity, specificity, PPV and NPV.

The study was approved by the Ethical and Research Review Committee of Armed Forces Institute of Pathology. Informed consent was obtained from all patients following good laboratory and clinical practices.

RESULTS

A total of 300 patients suspected of malaria were included in this study. All the patients were adult males with ages between 20 – 45 years with a mean age of 29.47 ± 6.414 years. The mean duration of fever at the time of presentation was 4.78 ± 2.617 days, with a minimum duration of 02 and maximum of 18 days (Table I). Malaria parasite was detected in 110 (36.7%) patients by microscopy. Out of these positive malaria cases by microscopy, 90 (81.8%) were *P. vivax*, 16 (14.6%) were *P. falciparum* and 4 (3.6%) were mixed malarial infection. A parasite density ranging from 45 to 81,560/µl was observed by microscopy. Twelve cases had parasite count less than 300/µl. OptiMAL, RDT was positive in 106 (35.3%) patients. Out of these, 14 (13.2%) had *P. falciparum* and non-*falciparum* species were found in 92 (86.8%). *Plasmodium* genus specific real time PCR was positive in 123 (41%) patients. PCR did not miss any case which was positive by microscopy or OptiMAL. All the patients having PCR positive malaria diagnosis

became afebrile within a week after starting anti-malarial treatment.

The percentage positivity, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of three methods are shown in Table II.

The lowest cycle threshold valve (C_t) detected for malaria positive case in PCR was 16 and highest (C_t) was 32.46 in this study. The fluorescence detection by real time PCR of positive malaria cases as well as negative cases is given in Figure 1. The C_t value of microscopy and OptiMAL negative and positive cases are shown in Table III.

Seven microscopically negative slides but positive by PCR were found to be positive on review with extended time (200 x oil immersion fields). Their parasite index was between 40 – 90 parasites/µl. Malaria parasite was detected in serially diluted sample till 1/16, 1/64 and 1/1024 dilutions by OptiMAL, microscopy and PCR having parasitic index equivalent to 145 /µl, 36.2/µl and 2.2/µl respectively. The cost of consumables per test by microscopy and OptiMAL was 0.2 and 2.75 US$ respectively. The real time PCR cost per test for malaria detection was 3.30 US$ excluding the capital expenditure on equipment.
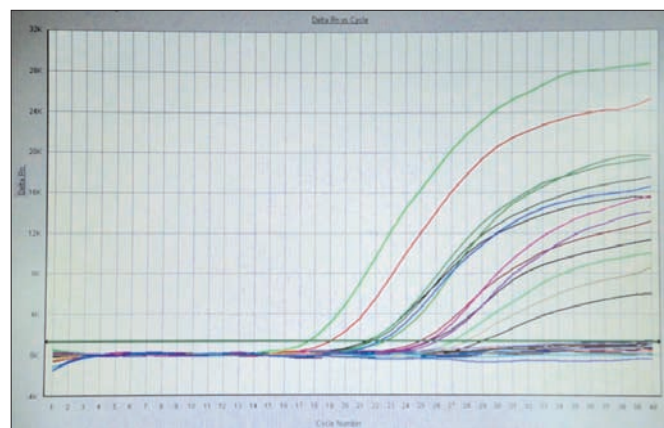

Figure 1: Real time PCR of a batch.

Table I: Descriptive statistics of age of patient and duration of fever.

	Minimum	Maximum	Mean	SD
Age (years) of patient	20	45	29.47	6.414
Duration (days) of fever	2	18	4.78	2.617

n = 300 patients

Table III: Cycle threshold value (Ct) of microscopy and OptiMAL negative and positive cases.

Diagnostic methods	Number of patients	Mean Ct value	Std. deviation (±)
Microscopy			
Negative	13	28.75	2.09
Positive	110	21.95	2.73
OptiMAL			
Negative	17	27.77	2.98
Positive	106	21.85	2.67

Table II: Frequency and percentages of PCR, microscopy and RDT (OptiMAL).

Test	Positive n (%)	Negative n (%)	Sensitivity	Specificity	PPV	NPV
PCR	123 (41%)	177 (59%)	100%	100%	100%	100%
Microscopy	110 (36.7%)	190 (63.3%)	89.4%	100%	100%	93.1%
RDT (by OptiMAL)	106 (35.3%)	194 (64.7%)	86.2%	100%	100%	91.2%

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DISCUSSION

More than one million malaria cases are registered in Pakistan annually.[4] The disease is more prevalent in rural areas and there is a human reservoir of *Plasmodium* which perpetuates malaria throughout the year. The public healthcare system in this country works at a three-tier system where the primary healthcare is supported by basic laboratory facilities and a few tertiary care laboratories have the infrastructure for molecular diagnosis. The treatment of malaria in many areas remains clinical, though the signs and symptoms of malaria have poor specificity.[16] Weak healthcare system incapable of providing quality-assured early diagnosis and prompt treatment, low coverage of preventive tools and deteriorating security situation has increased the potential threat of its spread in recent years. This situation poses threat to the health of large segments of society but especially the vulnerable population like children, pregnant ladies, immunosuppressed, thalassaemics and transfusion recipients receiving blood from malaria carriers. The management of malaria needs to be holistic and integrated within the current healthcare infrastructure and implementation of the treatment program needs utilization of all new developments on diagnostic front, both internationally and locally. Keeping in view the gaps in this approach, it was needed that a cost effective malaria diagnostic method, with high accuracy should be established, validated and made available to local health system. This study undertakes establishing a real time PCR and comparing its accuracy with other commonly used diagnostic methods i.e. microscopy and rapid diagnostic test OptiMAL.

P. vivax was the most prevalent malaria species found in this study. This correlates with the finding of data gathered by Directorate of Malaria Control Program Pakistan and published by WHO.[3] Conventional microscopy is the most commonly used methodology for malaria diagnosis especially in this part of the world. However, its sensitivity and specificity is microscopist dependent and number of tests analyzed per day. This is more important in cases of low parasitaemia where false negative results have been reported by many studies.[17,18] In this study, 13 cases were negative by microscopy and 7 out of these 13 were the cases where parasitic index was low i.e. between 40 – 95/µl found on review. The remaining 6 cases, which were positive by PCR only, were treated with antimalarials only and their time to become afebrile was similar to those who were positive in all three tests. This provided an indirect validity of PCR only positive tests.

RDTs have gained popularity worldwide since 1993, when they were first initiated by a single company. WHO has listed approximately 50 different RDTs. The newer ones can separate *P. falciparum* from other species and few had PvLDH antigen-based test.[19] RDTs are quite valuable in malaria diagnosis as they produce quick result, do not require skilled operator and can be used in remote areas. WHO sponsored malaria control programme includes provision of RDTs in endemic countries. OptiMAL is one of the immunochromato-graphic based RDT which is widely used. Its sensitivity and specificity is variable from different studies reported from Afghanistan, Turkey, Kuwait and Honduras,[20-23] in which the test showed sensitivities ranging from 79.3 to 94% but specificities ranging from 97 to 100%. This study is comparable to above quoted studies. OptiMAL missed four more cases of malaria which were positive by microscopy and PCR in this study. These were the cases which had parasite index between 75 – 150/µl. It was also observed that as the parasite index falls below 500/µl the color intensity on the strip also decreases correspondingly and the same has been reported by Rodulfo *et al*. from Venezuela.[18]

PCR based molecular detection methods for the diagnosis of malaria are being used for quite some time now. Conventional, nested and semi-nested PCR techniques were used earlier but real-time PCR is found to be superior, quick and more sensitive and specific than nested PCR.[24] Multiplex real-time PCR can identify all *Plasmodium* species, quantify the parasites and provide treatment follow-up especially in antimalarial resistant cases. These methods can detect as few as 1 parasite/µl of blood.[25] This PCR is several fold sensitive than microscopy and OptiMAL with ability to detect parasite < 5/µl. It has shown a significant gain in sensitivity over microscopy and OptiMAL while there was no difference in specificity. More or less similar advantage of real time PCR over microscopy and OptiMAL has been reported by other studies.[18,26] The yield of PCR in this study highlights that the expertise in microscopy is not upto standard and this further emphasizes the role of PCR in malaria diagnosis as gold standard. The cost per test of malaria with PCR is approximately 3.30 US Dollars excluding the capital cost on equipment as compared to OptiMAL cost per test of 2.75 US Dollars. The difference in cost is not much considering the diagnostic value of real-time PCR.

This study has few limitations. The authors could not arrange the reference samples from cultures of malaria as positive control; however, the positive control was effectively prepared from pooled sample of micro-scopically proven malaria cases. This is a preliminary study in which only genus specific real-time PCR is established. The authors intend to conduct second phase of study with multiplex real time PCR for species identification and quantification of parasite which can help monitor therapy. It is suggested that all primary care

and secondary care centres in this country be linked with at least one tertiary care centre having established real time PCR. The criterion defined for utilization of this service should be based on existing prevalence of treatment failures in malaria which resulted due to lack of laboratory proof of malaria. However, the blood samples of vulnerable patients should be referred earlier for PCR testing if microscopically negative for malaria parasite. Dried blood on filter paper for malaria PCR can be studied and this will prove to be useful for malaria PCR testing and monitoring from remote areas. Malaria transmission through blood transfusion is a real threat in this country. PCR has been reported to be more sensitive than microscopy in detecting malaria parasites in blood donors. The prevalence of transfusion transmitted malaria can be assessed by employing this sensitive technique as a first step towards policy for blood donor screening for malaria in country like Pakistan. It can be made more cost effective by testing blood donors in small pools.

CONCLUSION

Real time PCR is a very sensitive method for diagnosis of malaria than microscopy and OptiMAL especially in cases of low parasitaemia. Malaria diagnosis by real time PCR could be a valuable tool in reference laboratories to provide diagnostic help in difficult cases.

REFERENCES

1. World Health Organization. World malaria report 2011 [Internet]. Geneva: World Health Organization; 2011. [cited 2012 Nov 15]. Available from: http://www.who.int/malaria/world_malaria_report_ 2011/9789241564403_eng.pdf

2. World Health Organization. World malaria report 2005 [Internet]. Geneva: World Health Organization; 2005 [cited 2012 Dec 2]. Available from: http://whqlibdoc.who.int/publications/2005/9241593199_eng.pdf

3. World Health Organization. World malaria report 2009 [Internet]. Geneva: World Health Organization; 2009. [cited 2012 Dec 2]. Available from: http://whqlibdoc.who.int/publications/2009/9789241563901_eng.pdf

4. World Health Organization [Internet].Geneva: WHO supports malaria epidemic prevention and control in Pakistan; 2010. [cited 2012 Sep 6]. Available from: http://www.who.int/hac/crises/pak/releases/12october2010/en/index.html

5. World Health Organization. Guidelines for the treatment of malaria [Internet]. Geneva: World Health Organization; 2010. [cited 2012 Feb 15]. Available from: http://whqlibdoc.who.int/publications/2010/9789241547925_eng.pdf

6. Payne D. Use and limitations of light microscopy for diagnosing malaria at the primary health care level. *Bull World Health Organ* 1988; **66**:621-8.

7. Ochla LB, Vounatsou P, Smith T, Mabaso ML, Newton CR. The reliability of diagnostic techniques in the diagnosis and management of malaria in the absence of a gold standard. *Lancet Infect Dis* 2006; **6**:582-8.

8. Chilton D, Malik AN, Armstrong M, Kettelhut M, Parker WJ, Chiodini PI. Use of rapid diagnostic tests for diagnosis of malaria in the UK. *J Clin Pathol* 2006; **59**:862-6.

9. Tangpukdee N, Duangdee C, Wilairatana P, Srivicha K. Malaria diagnosis: a brief review. *Korean J Parasitol* 2009; **47**:93-102.

10. Singh N, Shukla M, Shukla MK, Mehra R, Sharma S, Bharti P, *et al*. Field and laboratory comparative evaluation of rapid malaria diagnostic tests versus traditional and molecular techniques in India. *Malar J* 2010; **9**:191-8.

11. Hawkes M, Kain KC. Advances in malaria diagnosis. *Expert Rev Anti Infect Ther* 2007; **5**:485-95.

12. Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Mol Biochem Parasitol* 1993; **58**:283-92.

13. Perandin F, Manca N, Calderaro A, Piccolo G, Galati L, Ricci L, *et al*. Development of a real time PCR assay for detection of *Plasmodium falciparum, Plasmodium vivax*, and *Plasmodium ovale* for routine clinical diagnosis. *J Clin Microbiol* 2004; **42**:1214-9.

14. Warhurst DC, William JE. Laboratory diagnosis of malaria: ACP broadsheet no 148. *J Clin Pathol* 1996, **47**: 4343-5

15. Lee MA, Tan CH, Aw LT, Tang CS, Singh M, Lee SH, *et al*. Real time fluorescence based PCR for detection of malaria parasites. *J Clin Microbiol* 2002; **40**:4343-5.

16. Rakotonirina H, Barmadas C, Raherijafy R, Andrianantenaina H, Ratsimbasoa A, Randrianasolo L. Accuracy and reliability of malaria diagnostic techniques for guiding febrile outpatient treatment in malaria endemic countries. *Am J Trop Hyg* 2008, **78**:217-21.

17. Zaman S, Tan L, Chan HH, Aziz L, Abdul -Samat S, Wahid R. The detection of *Plasmodium falciparum* and *P. vivax* in DNA-extracted blood samples using polymerase chain reaction. *Trans R Soc Trop Med Hyg* 2001; **95**:391-7.

18. Rodulfo H, De Donato M, Mora R, Sonzalex L, Contreras CE. Comparison of the diagnosis of malaria by microscopy, immunochromatography and PCR in endemic areas of Venezuela. *Braz J Med Biol Res* 2007; **4**:535-43.

19. Perkins MD, Bell DR. Working without a blindfold: the critical role of diagnostic in malaria control. *Malar J* 2008; **7**:55.

20. Kolaczinski J, Mohammed N, Ali I, Ali M, Khan N, Ezard N. Comparison of the OptiMAL rapid antigen test with field microscopy for the detection of *Plasmodium vivax* and *P. falciparum*: considerations for the application of the rapid test in Afghanistan. *Ann Trop Med Parasitol* 2004; **98**:15-20.

21. Palmer CJ, Lindo JF, Klaskala WI, Quesada JA, Kaminsky R, Baum MK. Evaluation of the OptiMAL test for rapid diagnosis of *Plasmodium vivax* and *Plasmodium falciparum* malaria. *J Clin Microbiol* 1998; **36**:203-6.

22. Aslan G, Ulukanligil M, Seyrek A, Erel O. Diagnostic performance characteristics of rapid dipstick test for *Plasmodium vivax* malaria. *Mem Inst Oswaldo Cruz* 2001; **96**:683-6.

23. Iqbal J, Khalid N, Hira PR. Comparison of two commercial assays with expert microscopy for confirmation of symptomatically diagnosed malaria. *J Clin Micrbiol* 2002; **40**:4675-8.

24. Peradin F, Manca N, Calderaro A, Piccolo G, Salati L, Ricci L, *et al*. Development of a real-time PCR assay for detection of *Plasmodium falciparum, Plasmodium vivax* and *Plasmodium ovale* for routine clinical diagnosis. *J Clin Microbiol* 2004; **42**: 1214-9.

25. Rougemont M, Saanen MV, Sahli R, Hinrikson HP, Bille J, Jaton K. 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**: 5636-43.

26. Alam MS, Mohan AN, Mustafa S, Khan WA, Islam N, Karim MJ, *et al*. Real-time PCR assay and rapid diagnostic tests for the diagnosis of clinically suspected malaria patients in Bangladesh. *Malar J* 2011; **10**:175-86.

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