

Comparative evaluation of microscopy and polymerase chain reaction (PCR) for the diagnosis in suspected malaria patients of Nepal

K Parajuli,^{1,2} S Hanchana,³ M Imwong,⁴ S Pukrittayakamee⁴ and P Ghimire¹

¹Central Department of Microbiology, Tribhuvan University (TU), ²Department of Microbiology, Institute of Medicine, TU, Kathmandu, Nepal; ³Mahidol Oxford Tropical Research Unit, Faculty of Tropical Medicine,

⁴Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

Corresponding author: Keshab Parajuli, Department of Microbiology, Institute of Medicine Maharajgunj Campus, Tribhuvan University, Kathmandu, Nepal. e-mail: pkeshab@yahoo.com; keshabparajuli@hotmail.com

ABSTRACT

Microscopy has been the most common technique for the diagnosis of malaria in Nepal. This study was undertaken at CDM, TU, Kathmandu, and Department of Clinical Tropical Medicine, Mahidol University, Bangkok to compare the efficiency of microscopy and PCR for malaria diagnosis in Nepalese context. During July-August 2007, blood samples were collected in glass slides and on filter papers from suspected malaria cases of Kanchanpur, Jhapa and Morang Districts. Sample transportation and storage was done using standard protocol. Microscopy was done at the health posts in the district in Nepal while Nested PCR using previously standardized primers was carried out at Mahidol University. Among 824 malaria suspected cases, 19.2% (157) were laboratory confirmed as malaria cases (*P. vivax* 10.9%, *P. falciparum* 7.7% and 0.4% were of mixed infection) by microscopy. The parasite count range was detected as 320-25020 parasites/ μ l. Among total 132 samples (114 microscopic positive, 18 negative) were processed for nested PCR. Among microscopic positive samples with increase of the parasitaemia/ μ l of the blood, the rate of detection by PCR (75.4%) was increased though the PCR failed to detect 2 cases having the parasitaemia 5000-15000/ μ l of blood however 4 microscopic negative cases were detected as *P. vivax* infection. Among the microscopy positive samples for *P. falciparum*, 3 were found *P. vivax* and 2 were found as mixed infection of Pv and Pf and 6 *P. vivax* were found positive for *P. falciparum* by PCR. Two microscopy positive samples for mixed infection were found be positive for one Pv and one Pf by PCR. PCR could be good tool in confirming the clinically strongly suspected but microscopically negative malaria cases and advanced molecular epidemiological studies, although its use in routine diagnosis may not be feasible.

Keywords: PCR, Microscopy, Malaria, Diagnosis, Nepal.

INTRODUCTION

There was an estimated 247 million malaria cases among 3.3 billion people at risk in 2006, causing nearly a million deaths, mostly of children under 5 years. 109 countries were endemic for malaria in 2008, 45 within the WHO African Region.^{1,2}

About 70 percent of Nepal's populations live in areas with unstable malaria transmission and reported malaria cases ranging 25,000 to 30,000 annually have now dropped to 7,000 to 9,000 with annual malaria related mortality rate of 8/100,000 in all age groups and 11/100,000 in children 0-4 years.³

Microscopic examination of Giemsa-stained thick and thin blood smears has been identified as the most common technique to diagnose malaria since last 100 years.^{4,5} Microscopy continues to be the gold standard for identification of *Plasmodium* spp. in the laboratory setting.^{6,7} Although easy to apply and cost-effective, this technique assumes that laboratories have certain infrastructure in place with highly skilled professionals

and lowest detecting level is 10 to 50 parasites/ μ l so the sensitivity may fluctuate depending upon the skill of technician.⁸

WHO has recognized the urgent need for simple and cost-effective diagnostic tests for malaria to overcome the deficiencies of both light microscopy and clinical diagnosis. To overcome such limitations, several methods have been in use including the staining of parasite DNA and RNA with acridine orange: the quantitative buffy coat method (QBC); methods based on the detection of the enzyme lactate dehydrogenase (pLDH), rapid antigen capture assay that detects circulating *P. falciparum* histidine rich protein-2 (PfHRP-II),⁹ used mainly in diagnosing malaria in non-immune individuals or in epidemiologic studies.

Polymerase chain reaction (PCR) based assays have been used mainly for the assessment of the sensitivity and specificity of microscopy and rapid diagnostic tests (RDTs) and may be of clinical value in some selected situations.¹⁰⁻¹⁴ PCR-based methods have been shown to

be powerful tool for malaria diagnosis.¹⁵⁻¹⁸ PCR based tests have shown remarkable capacity to detect malarial parasites in mixed infections and low parasite count and are also sensitive when compared to microscopic examination.¹⁹⁻²¹ It has been estimated that PCR can detect malaria infections with parasitemia as low as 5 parasites/ μ l (0.0001% parasitemia).²² Giemsa-stained or unstained thick blood smear and, particularly, blood conserved on filter-papers have been used as a source of DNA in molecular and epidemiological studies.²³⁻²⁷

Very little is known on the sensitivity and specificity of PCR based tests in Nepal for malaria diagnosis as microscopy and RDTs are only used routinely. This study is planned to understand the efficiency of PCR in comparison to conventional Giemsa stained microscopy of thick/thin smears in Nepalese context.

MATERIALS AND METHODS

During study period (2005 to 2007, malaria peak season in Nepal), blood samples were collected from a total of 824 malaria suspected patients from Kanchanpur, Jhapa and Morang districts lying in plain region in Nepal. The suspicion of the cases were made by the local health care providers based on clinical signs and symptoms like fever, chills, headache, etc. Peripheral blood smears (thick and thin) were prepared from the finger prick samples collected aseptically. Thin smears were fixed with methanol at the field condition, while thick blood smears were air dried. Three drops of blood from each suspected cases were also collected in standardized filter paper strips (Whatman No 1) for molecular analysis, with label and stored in a plastic bag with silica gel after drying the spots at room temperature. Informed consent was taken from each patient, prior to sample collection.

Blood smears were stained with Giemsa staining technique and examined for presence of malarial parasites following standard methodology.²⁸ Parasites were counted as Parasite count/ μ l = Total parasite count / WBC count X 8000, where the total leukocyte count was assumed as 8000.²⁹ The slides were reported only

negative upon not detecting any parasites when 200 fields of the slides were examined.

Filter paper strips with labels were stored at room temperature till further processing at the molecular laboratory of Department of Clinical Tropical Medicine, Mahidol University, Thailand during November-December, 2007 following previously standardized protocols. Nested PCR was carried out following the methodology previously described, which was used for the detection of any malaria parasites of the four *Plasmodium* which infect human.³⁰

DNA was extracted from the dried blood spots on filter paper using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol³⁸, and a hundred μ l of DNA extracted solution was stored at -20°C until further analysis. Extracted DNA was further amplified for *Genus* specific amplification as described by Snounou et al., 1999. Briefly, it was carried out in a 20 μ l reaction mixture consisting 10 \times buffer, 25 mM MgCl₂, 10 mM dNTPs, 2.5 μ M each primers (rPLU3 and rPLU4), 0.4U of Taq DNA Polymerase, 1.5 μ l sample (DNA template). The second round (secondary reaction) of nested PCR for *species* specific amplification was carried out in four separate tubes each containing a single primer pair (rFLA1/rFAL2, rVIV1/VIV2, rMAL1/rMAL2, and rOVA1/rPLU2). 1.5 μ l of PCR product from primary reaction and same concentration and volume was used as in first round amplification for reaction mixture. Amplification was performed under the following conditions: 95°C for 5 minutes followed by 25 (primary reaction) and 30 (secondary reaction) cycles of annealing at 58° C for 2 minutes, extension at 72°C for 2 minutes and denaturation at 94°C for one minute, then the final steps was followed by extension time for 72°C for 5 minutes. The reaction was completed by reducing the temperature to 25° C.³⁰

The PCR amplified DNA product was well mixed with 5 μ l of Orange G and the expanded DNA fragment were further analyzed running the products in Agarose gel

electrophoresis. The amplified product was visualized using 3.0% ethidium bromide staining and gel documentation under the UV light.³⁰ The bands obtained in the gels were correlated with the standards and molecular weight markers for analysis. A positive reaction was noted when primers specific for *Plasmodium falciparum* (Pf) and *P. vivax* (Pv) produce amplification products of 206-bp and 121-bp respectively.

Table - 1: Comparative Evaluation of PCR with Microscopy

Particulars		Microscopy				Total
		Negative	<i>P. falciparum</i>	<i>P. vivax</i>	Mixed	
18 S rRNA Nested PCR	Negative	14	12	16	0	42
	<i>P. falciparum</i>	0	14	6	1	21
	<i>P. vivax</i>	4	3	54	1	62
	Mixed	0	2	2	0	4
	<i>Plasmodium</i>	0	3	0	0	3
	Total	18	34	78	2	132

Table - 2: Comparative Evaluation of PCR with Parasitemia

Parasitaemia	18S rRNA nested PCR findings					Total
	<i>P. vivax</i> (205-bp)	<i>P. falciparum</i> (106—bp)	Mixed	Unidentified	Negative	
≤ 500	4	0	0	0	6	10
501-1000	4	1	0	0	8	13
1001-5000	32	13	2	3	12	62
5001-10000	12	6	2	0	1	21
10001-15000	1	1	0	0	1	3
15001-20000	3	0	0	0	0	3
20001-25000	1	0	0	0	0	1
≥ 25001	1	0	0	0	0	1
Negative	4	0	00	0	14	18
Total	62	21	4	3	42	132

choice for species identification in epidemiologic studies and medical diagnosis.³¹ The method is simple, does not require highly equipped facilities, and in most cases enables differentiation among the four species causing malaria in humans when performed by a technician. However, microscopy is often time-consuming and laborious, and it is estimated that even a skilled person can evaluate only 60 to 80 specimens per day under field conditions.³² This method can sometimes be misleading in identifying parasite species, especially in the case of low level of parasitemia and a mixed parasite infection^{33,34} or modification by drug treatment. PCR-based methods have been consistently shown to be powerful tool for malaria diagnosis.^{15,18}

PCR, in particular nested PCR, has proven to be a more sensitive diagnostic method for malaria than microscopy, particularly in cases of low parasitemia and mixed infections.^{12,30} The PCR analysis for *Plasmodium* spp. done in this study showed a sensitivity of 75.4%. In the present study, Nested PCR failed to detect parasite DNA in microscopically positive samples, both with low levels of parasitaemia, however PCR-positive results were obtained for other samples with similarly low levels of parasitaemia. Similar discordant result was also reported by Singh *et al.* (1996).²⁵ Degradation of the target DNA after extraction with repeated freezing and thawing or the presence of *P. falciparum* sequences that lack sequences recognized by used primers or technical faults while DNA storage may give false negative results.³⁵ The samples collected during this study has been collected in filter paper strips, however silica gel crystals to absorb the moisture was lacking in the packs.

The repeated freezing and thawing of the samples due to electricity cuts/load shedding might have also contributed for the degradation of the DNA in the samples. A study from an endemic area of Brazil has reported a sensitivity of PCR to be 73.0% using blood stained in filter paper stripes in condition of low level parasitemia.²¹ However, another study has reported an overall sensitivity of 97.4% for PCR compared to microscopy.²⁵

In this study specificity of PCR for *Plasmodium* spp. was 77.8. . Other reports obtained a specificity of 69% to 100%.^{35,36} However in this study 3 *P. vivax* cases were detected by PCR prior to the light microscopy becoming positive for *P. falciparum*,

and 6 cases *P. falciparum* were detected when light microscopy becoming positive for *P. vivax*. The entire 2 microscopy positive samples for mixed infection (Pf and Pv) were found be positive for one *P. vivax* and one *P. falciparum* by PCR. A study was reported by Speers et al in which, *P. vivax* was detected by PCR prior to the light microscopy becoming positive in one case, and in the second case *P. malariae* was detected when light microscopy was unable to speciate the causative *Plasmodium* species.³⁶

In present study, 4 cases which were not detected by microscopy could be detected by PCR. This might be due to the presence of low level of parasitemia. Other studies have also reported the ability of PCR to diagnose low level of parasitemia undetected by conventional microscopy.^{25,37,38} In addition, chances of cross contamination was strictly monitored and prevented following stringent quality control procedure.

Nested PCR could detect parasite DNA even in very low parasite count where microscopy could not detect, and PCR could also distinguish the species which was misdiagnosed by microscopy. In practice, PCR-based assays may not replace microscopy for routine diagnosis in developing country Nepal. However, nested PCR would be useful in discriminating the low parasitic infection cases strongly suspected by the clinicians but not supported by microscopy.

ACKNOWLEDGEMENTS

The authors would like to acknowledge CDM TU, NPHL, EDCD Teku, DPHO Kanchanpur, Jhapa and Morang Nepal, and Mahidol University, Thailand and Wellcome Trust (Grant 080867/Z/06/Z).

REFERENCES

1. WHO. World Malaria Report 2008, Geneva, Switzerland.
2. Winstanley P, Ward S, Snow R *et al.* Therapy of falciparum malaria in Sub-Saharan Africa: from molecule to policy. *Clin Microbiol Rev* 2004; 17: 612-37.
3. DoHS (Department of Health Services). Annual Report 2002/2003. Ministry of Health & Population, Nepal.
4. WHO/CDS/RBM. New perspectives malaria diagnosis. Report of a joint WHO/ USAID informal consultation, WHO, Geneva, 2000.
5. Rougemont M, Saanen MV, Sahli R *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: 5636-43.
6. Ndao M, Bandyayera E, Kokoskin E *et al.* Comparison of blood smear, antigen detection, and nested-PCR methods for screening refugees from regions where malaria is endemic after a malaria outbreak in Quebec, Canada. *J Clin Microbiol* 2004; 42: 2694-700.
7. Makler MT, Palmer CJ, Ager AL. A review of practical techniques for the diagnosis of malaria. *Ann Trop Med Parasitol* 1998; 92: 419-33.
8. Payne D. Use and limitations of light microscopy for diagnosing malaria at the primary health care level. *Bull WHO* 1998; 66: 621-6.
9. Ghimire P, Samantaray JC, Mirdha BR. Comparative evaluation of four different techniques used in the diagnosis of malaria. *J Inst Med (Nepal)* 2001; 23: 50-4.
10. Zhong KJ, Kain KC. Evaluation of a colorimetric PCR-based assay to diagnose *Plasmodium falciparum* malaria in travelers. *J Clin Microbiol* 1999; 37: 339-41.
11. Rubio JM, Buhigas I, Subirats M *et al.* Limited level of accuracy provided by available rapid diagnosis tests for malaria enhances the need for PCR-based reference laboratories. *J Clin Microbiol* 2001; 39: 2736-7.
12. Hanscheid T, Grobusch MP. How useful is PCR in the diagnosis of malaria? *Trends Parasitol* 2002; 18: 395-8.
13. Singer LM, Newman RD, Diarra A *et al.* Evaluation of a malaria rapid diagnostic test for assessing the burden of malaria during pregnancy. *Amer J Trop Med Hyg* 2004; 70: 481-5.
14. Bell D, Peeling RW. Evaluation of rapid diagnostic tests: malaria. *Nat Rev Microbiol* 2006; 4: 34-8.
15. Coleman RE, Sattabongkot J, Promstaporm S *et al.* Comparison of PCR and microscopy for the detection of asymptomatic malaria in a *Plasmodium falciparum/vivax* endemic area in Thailand. *Malaria J* 2006; 5: 121-5.
16. Berry A, Fabre R, Benoit-Vical F *et al.* Contribution of PCR-based methods to diagnosis and management of imported malaria. *Med Trop (Mars)* 2005; 65: 176-83.
17. Cox-Singh J, Mahayet S, Abdullah MS *et al.* Increased sensitivity of malaria detection by nested polymerase chain reaction using simple sampling and DNA extraction. *Int J Parasitol* 1997; 27: 1575-7.
18. Di Santi SM, Kirchgatter K, Brunialti KCS *et al.* PCR-based diagnosis to evaluate the performance of malaria reference centers. *Rev Inst Med Trop* 2004; 46: 183-7.
19. Brown AE, Kain CK, Pipithkul J *et al.* Demonstration by the polymerase chain reaction of mixed *Plasmodium falciparum* and *Plasmodium vivax* undetected by conventional microscopy. *Trans Roy Soc Trop Med Hyg* 1992; 86: 609-12.
20. Zakeri S, Najafabadi ST, Zare A *et al.* Detection of malaria parasites by nested PCR in south-eastern, Iran: evidence of highly mixed infections in Ghahbahar District. *Malaria J* 2002; 1: 2-8.
21. Scopel KKG, Fontes CJF, Nunes AC *et al.* Low sensitivity of nested PCR using Plasmodium DNA extracted from stained thick blood smears: an epidemiological retrospective study among subjects with low parasitemia in an endemic area of the Brazilian Amazon region. *Malaria J* 2004; 3: 8-12.
22. Zalis MG, Ferreira-da-Cruz MF, Balthazar-Guedes HC *et al.* Malaria diagnosis: standardization of a polymerase chain reaction for the detection of *Plasmodium falciparum* parasites in individuals with low-grade parasitemia. *Parasitol Res* 1996; 82: 612-6.
23. Kimura M, Kaneko O, Inoue A *et al.* Amplification by polymerase chain reaction of *Plasmodium falciparum* DNA from Giemsa stained thin blood smears. *Mol Biochem Parasitol* 1995; 70: 193-7.
24. Long GW, Fries L, Watt GH *et al.* Polymerase chain reaction amplification from *Plasmodium falciparum* on dried blood spots. *Amer J Trop Med Hyg* 1995; 52: 344-6.
25. Singh B, Cox-Singh J, Miller AO, Abdullah MS, Snounou G, Rahman HA. Detection of malaria in Malaysia by nested polymerase chain reaction amplification of dried blood spots on filter papers. *Trans Roy Soc Trop Med Hyg* 1996; 90: 519-21.
26. Edoh D, Steiger S, Genton B *et al.* PCR amplification of DNA from malaria parasites on fixed and stained thick and thin blood films. *Trans Roy Soc Trop Med Hyg* 1997; 91: 361-3.
27. Ivanova NV, Morozov EH, Kurkina IV *et al.* ITS1, 5.8S and A-type ITS2 rDNA sequences from *Plasmodium vivax* and development of a method for retrospective PCR diagnosis of malaria by stained thick blood smears. *Mol Biol* 2001; 35: 515-25.
28. Cheesbrough M, District Laboratory Practice in Tropical Countries, Part 1, Cambridge University Press, UK. 1999, 239-259.
29. Moody A, Rapid Diagnostic Tests for Malaria Parasites, *Rev Clin Microbiol* 2002; 15:166-78.
30. Snounou G, Singh B. Malaria Methods and Protocols, Nested PCR Analysis of Plasmodium Parasites (PCR Detection and Identification of Plasmodium Parasites), 2002; 72: 189-203.
31. Warhurst DC, Williams JE. Laboratory diagnosis of malaria. *J Clin Pathol* 1996; 49: 533-8.
32. Gardner MJ, Hall N, Fung E *et al.* Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* 2002; 419: 498-511.
33. Knowles R, White RS. Studies in the parasitology of malaria. Calcutta, India: *Indian Med Res Memoirs No* 1930; 18: 436.
34. Richie TL. Interactions between malaria parasites infecting the same vertebrate host. *Parasitol* 1988; 96: 607-39.
35. Barker RHJ, Banchongaksorn T, Courval JM *et al.* A simple method to detect *Plasmodium falciparum* directly from blood samples using the polymerase chain reaction. *Amer J Trop Med Hyg* 1992; 46: 416-26.
36. Speers DJ, Ryan S, Harnett G and Chidlow G; Diagnosis of malaria aided by polymerase chain reaction in two cases with low-level parasitaemia. *Internal Med J* 2003, 33:12, 613-15.
37. Nandwani S, Mathur M, Rawat S. Evaluation of the PCR analysis for diagnosis of falciparum malaria in Delhi, India. *Indian J Med Microbiol* 2005; 23: 176
38. Oyediji SI, Awobode HO, Monday GC, *et al.* Comparison of PCR-based detection of *Plasmodium falciparum* infections based on single and multicopy genes, *Malaria J* 2007, 6: 112.