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

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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 heath 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. falciparium* 7.7% and 0.4% were of mixed infection) by microscopy. The parasite count range was detected as 320-25020 parasites/µl. Among total 132 samples (114 microscopic positive, 18 negative) were processed for nested PCR. Among microscopic positive samples with increase of the parasitaemia/µl 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/µl 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/µl 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/µl (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/µl = 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 protocol[38], and a hundred µl of DNA extracted solution was stored at -20°C until further analysis. Extracted DNA was further amplified for *Genus* specific amplification as descried by Snounou et al., 1999. Briefly, it was carried out in a 20 µl reaction mixture consisting 10× buffer, 25 mM $MgCl_2$, 10 mM dNTPs, 2.5 µM each primers (rPLU3 and rPLU4), 0.4U of Taq DNA Polymerase, 1.5 µl 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 µl 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: 95ºC for 5 minutes followed by 25 (primary reaction) and 30 (secondary reaction) cycles of annealing at 58º C for 2 minutes, extension at 72ºC for 2 minutes and denaturation at 94^0C for one minute, then the final steps was followed by extension time for 72^0C for 5 minutes. The reaction was completed by reducing the temperature to 25^0 C.[30]

The PCR amplified DNA product was well mixed with 5µl 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				
		Negative	*P. falciparum*	P. vivax	Mixed	Total
18 S rRNA Nested PCR	Negative	14	12	16	0	42
	P. falciparum	0	14	6	1	21
	P. vivax	4	3	54	1	62
	Mixed	0	2	2	0	4
	Plasmodium	0	3	0	0	3
	Total	18	34	78	2	132

The results of all the tests were noted in the register, entered in the computer and analyzed statistically with SPSS version 14.0.

RESULTS

All the collected specimens were transfer to Central Department of Microbiology (CDM), Tribhuvan University (TU) for microscopy confirmation and then subsequently for PCR at Mahidol University, Thailand. Out of the 824 Giemsa stained blood slides examined microscopically, 19.2% (157) were confirmed as infected with malarial parasite. Of those positive cases, 10.9% were infected with *P. vivax*, 7.7% with *P. falciparum* and 0.4% were of mixed infection with *P. vivax* and *P. falciparum* was detected.

The detected parasite count range was 320-25020 parasites/µl. Among the total microscopy positive cases, 55.4% had the parasite count between 1000-5000/µl, 17.2% had parasitaemia between 5000-10000/µl, 10.2% had parasitemia between 500-1000/µl, 5.7% had parasitemia between 10000-15000/µl, only 3.2% cases showed parasite count of >20000/µl, and 8.3% had parasitemia <500/µl.

A total of 114 microscopy positive and 18 microscopy negative samples were further analyzed using nested PCR. Among the microscopic positive samples with the increase of the parasitaemia/µl of the blood, the rate of detection by PCR (75.4%) was increased though the PCR failed to detect the two cases having the parasitaemia 5000-15000/µl of blood however out of 18 microscopy negative samples 4 cases were detected as *P. vivax* infection.

Out of 34 *P. falciparum* cases diagnosed by microscopy, PCR could diagnose 2 as *P. vivax* and 2 as mixed infection. Out of 78 *P. vivax* cases diagnosed by microscopy, PCR could diagnose 6 were as *P. falciparum* and 2 as mixed infection. Among, 2 mixed infection (Pf and Pv) cases diagnosed by microscopy, one was diagnosed as single infection due to *P vivax*, and next one was diagnosed as *P falciparum* by PCR The overall sensitivity, specificity, PPV and NPV of the used 18 S rRNA Nested PCR test was found to be 75.4%, 77.8%, 95.6% and 33.3%. (Table: 1 and 2).

DISCUSSION

Microscopic examination of Giemsa-stained thick and thin blood smears has been the diagnostic method of

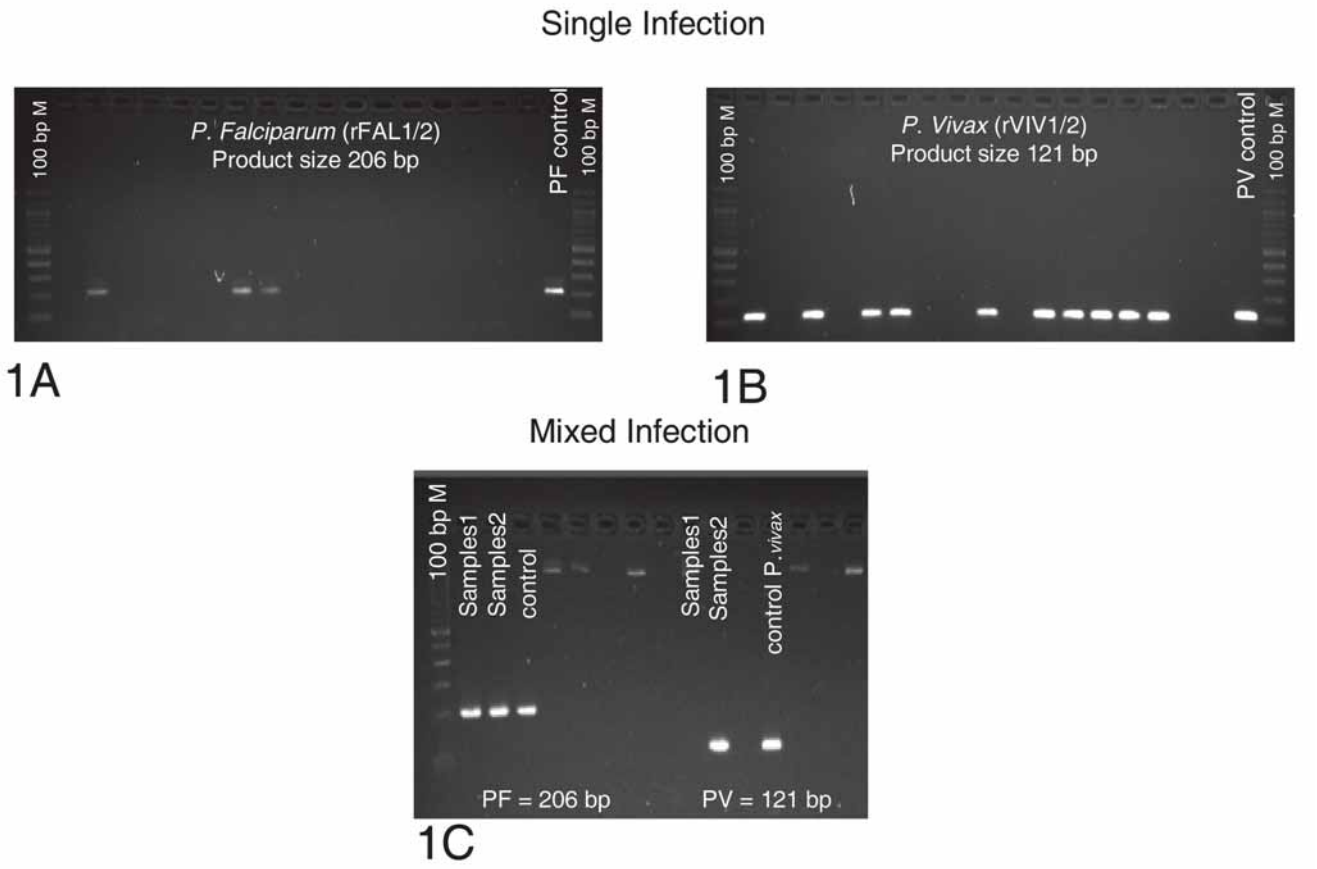

Fig.1. PCR amplification products obtained from Nested PCR; Lane 1 (M): DNA marker; 1A *Plasmodium falciparum* detected by Nested PCR; 1B *Plasmodium vivax* detected by Nested PCR; 1C Mixed Infection between *P. falciparum* and *P. vivax* detected by Nested PCR

Table - 2: Comparative Evaluation of PCR with Parasitemia

Parasitaemia	18S rRNA nested PCR findings					Total
	P. vivax (205-bp)	*P. falciparum* (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.

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