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Comparison of Conventional and PCR based detection of Plasmodium falciparum and Plasmodium vivax infections in human blood

Afshan Iqbal[1], Rabia Mushtaq[2], Ferhana Hussain [3], Mahjabeen Saleem[4], Mamoona Naz[5], Beenish Maqsood[6]

Afshan Iqbal[1], syeda_iqbal@hotmail.com, Cell no.; 92-322-6070474
Rabia Mushtaq[2], mushtaq71@yahoo.com
Ferhana Hussain[3,] everlasting_fh@yahoo.com
Mahjabeen Saleem[4], mahjabeensaleem1@hotmail.com
Mamoona Naz[5], mamoona_nibge@yahoo.com
Beenish Maqsood[6], beenish.ibb@gmail.com
1; Corresponding author
1,2,3,4,5,6; Institute of Biochemistry and Biotechnology, University of the Punjab, Lahore, Pakistan, PO Box 54590.

ABSTRACT

Blood samples from the 100 malarial parasite (MP) negative suspected individuals and 50 malarial patients were collected in EDTA vacutainers. Genomic DNA from each individual was amplified using nested PCR assay for 18S smaller ribosomal subunit gene to detect malarial parasites in low grade parasitemia. Data was statistically analyzed afterwards. MP test diagnosed only 1% malarial infection in collected blood samples whereas it increased up to 4% using molecular methods. MP test was unable to detect mixed infection whereas the nested PCR assay detected 3% *P. falciparum* and 4% *P. vivax* mixed infections in blood samples. Sensitivity (P value) of nested PCR was statistically 0.00 which showed that PCR technique is highly significant for detection. From the present data it is concluded that PCR is more sensitive and specific technique to be used as a diagnostic tool for malarial parasite as compared to the traditional microscopical analysis as practiced in all hospitals. It should be applied in biochemical labs (pathological labs) of hospitals so that it helps in reduction of false negativity of malarial diagnosis.

Indexing terms/Keywords

Malarial infection, *Plasmodium falciparum*, *Plasmodium vivax*, Nested PCR

Academic Discipline And Sub-Disciplines

Biochemistry

SUBJECT CLASSIFICATION

Diagnostic methods

TYPE (METHOD/APPROACH)

Cross-sectional study

INTRODUCTION

Malaria is global health problem that can be transmitted through the bite of infected mosquito anophelese. *Plasmodium vivax* and *Plasmodium falciparum* being the main causative agents accounting 130-145 million infections per annum world wide [1, 2]. Clinical symptoms of malaria are due to destruction of erythrocytes. Presentation of malaria often resembles with common viral infection [3], i.e. fever, chills, headaches and diaphoresis and sometimes severe complications [4].

Rapid and timely diagnosis is very critical for malarial diagnosis. Despite being exhausting, time-consuming, and less sensitive with decrease of parasitemia, Giemsa-stained blood smear method is in regular practice because of being inexpensive [5]. Other Rapid diagnostic tests (RDTs) include fluorescent microscopy, dipstick immunoassay, polymerase chain reaction (PCR) and nested PCR (nPCR) [4]. These tests identify parasitic antigen (histidine-rich protein-2 of parasite or a parasite-specific enzyme), metabolic products or antiplasmodial antibodies [6]. Although dipstick test is rapid but is insensitive for high parasitemia levels as well as below 100 parasites/µl whereas PCR for species-specific Plasmodium genome is more sensitive detecting 10 parasites/µl of blood [5, 7, 8]. It can be helpful in determining co-infection in HIV seropositive patients [9, 10]. nPCR, which amplifies 18S rRNA, has been used to measure the frequency of mixed infections in Middle East [11]. In 2010, duplex quantitative real time PCR assay was developed, the assay proved to be less laborious and cost effective with reduced risk of contamination [12].

In this study, MP negative, suspected malarial blood samples from Pakistani patients were rescreened using nested PCR for the presence of *P. vivax* and *P. falciparum* DNA.

MATERIALS AND METHODS

Peripheral blood samples were collected during the high transmission season from 100 suspected individuals and 50 blood samples of MP positive malarial patients in EDTA vacutainers from Allama Iqbal Medical College, Lahore, Pakistan. Informed consent was taken from each individual. All samples were stored at -20°C. This study was approved by the local departmental ethical committee and was carried out in accordance with the second Helsinki Declaration.

DNA extraction from blood was performed according to the method and analyzed according to the method [13] and analyzed [14]. Genus-specific primer set corresponding to the 18S ribosomal RNA [15] (synthesized by Fermentas) was used to amplify the target sequence. Amplification of genus and species specific Plasmodium was done by using the primers: rPLU6 **(**5′-TTAAAATTGTTGCAGTTAAAACG-3′**),** rPLU5 (5′ CCTGTTGTTGCCTTAAACTT 3′), rFAL1 (5′ TTAAACTCGTTTGGGAAAACCAAATATATT3´), rFAL2 (5′ACACAATGAACTCAATCATGACTACCCGTC3´), rVIV1

(5′CGCTTCTAGCTTAATCCACATAACTGATAC3′) and rVIV2 (5′ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA3′). The PCR products obtained after amplification were 1200, 205 and 120bp in size respectively.

The primary PCR assay was carried out using the external primer pair (rPLU6+rPLU5) while the secondary PCR assay was carried out using all 4 internal primers together (rVIV1, rVIV2, rFAL1, rFAL2). For the secondary PCR assay, 1µL of amplified product from the primary PCR assay was used. Both the primary and secondary PCR cycling parameters were identical. Amplification was carried out on i-cycler (Bio-Rad). PCR was performed using 5µl of 10X PCR amplification buffer with ammonium sulphate, 5µl of 25mM $MgCl_2$, 0.4µL of 25mM dNTPs, 5µL of 1µM sense and antisense primer solutions, 5µl of template DNA, 0.2µl of 0.1u/µl Taq DNA polymerase and made the final volume to 50µl with sterile distilled water. After an initial denaturation step (94°C, 5 min), the following amplification conditions were carried out for 30 cycles: denatured at 94°C for 60 sec, annealed at 58°C for 2 min; extension was carried out at 72°C for 1.5 min and final extension at 72°C for 4 min. PCR samples were analyzed on a 2% agarose gel.

Statistical analysis was performed using SPSS version 13. For sensitivity and specificity (P value), KAPPA statistics was used. Results were considered significant at p value of $\leq$ 5%.

RESULTS AND DISCUSSION

Malaria is a rapidly growing epidemic in Pakistan like other part of the world creating big challenge to the Ministry of Health. The Government is trying its level best to eradicate this disease using available resources and is also fighting against this menace with the help of various international organizations. Government has accorded a high priority for control of malaria along with other 6 communicable diseases as embodied in its National Health Policy. Malaria is predominantly disease of the rural areas where the incidence of poverty is higher (38.65%) than in urban areas (22.39%) [16]. According to another study, a rural family loose more than 40% of its crop yields due to consequences of malaria disease. Though the malaria program presently has low coverage of attending patients even then program has significant contribution in the reduction of disease incidence and further strengthening of program highly justify the public sector investment in the malaria control (Malaria Economic Survey 2002/03). According to survey if there is no malaria control program in the country the counterfactual would be that the incidence of malaria 0.69 per 1,000 populations would rise to 1.39 per 1,000 populations without program adding at least 1 million new cases every year. Based on these assumptions, it is estimated that with the programme, the total number of malaria cases averted would be 481,356 (Economic analysis of National Malaria Control Program, 2004).

Two rounds of nested PCR were performed. Primary assay was performed to identify the genus while second round was performed to identify the infecting species of *Plasmodium*. From primary PCR, a product of 1200 bp was obtained from DNA extracted blood samples for the presence of genus *Plasmodium.* Figure 1 show the amplified products on 1% agarose gel using gene ruler™ 100bp DNA ladder (Fermentas, EU) as standard marker.

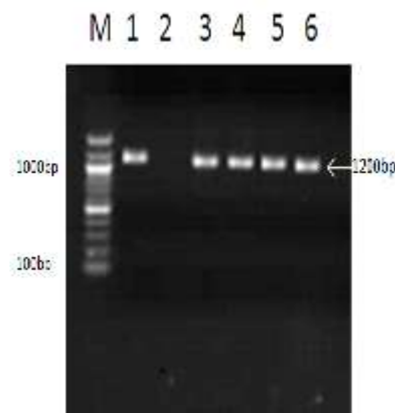

Figure 1: PCR amplification analysis of ssrRNA gene on 1% agarose gel electrophoresis. Size of PCR product obtained was 1200bp. Lane M: 100 bp DNA ladder, Lane 1: Positive control, Lane 2: Negative control, Lane 3-6: PCR product of 1200 bp.

Secondary PCR, performed for species identification, gave PCR products of 120bp for *P. vivax* and 205bp for *P. falciparum* respectively. Figure 2 indicate the obtained PCR product from *P. vivax* and *P. falciparum*, respectively.

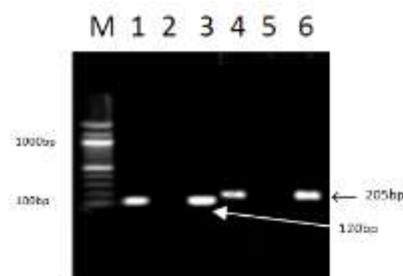

Figure 2: PCR amplification analysis of ssrRNA gene on 1% agarose gel electrophoresis. Lane M: 100 bp molecular weight DNA marker, Lane 1: Positive control, Lane 2: Negative control, Lane 3: 120bp PCR product, Lane 4: Positive control, Lane 5: Negative control, Lane 6: 205bp PCR product.

Statistical Analysis

Frequency of effected gender among patients was determined. Males were more prone to malaria with frequency of 58% among all patients as compared to females (42%). Frequency of positive malarial parasite (MP) test among collected data was determined which was then evaluated using nested PCR. Using the conventional MP test, only 1% of samples were detected to be malaria positive and it was also unable to detect mixed infections as given in Table 1.

Table 1: Frequency of MP Test and nPCR assay for Genus Identification

Result	Frequency (%)
MP +ve	1
MP -ve	99
PCR +ve	4
PCR -ve	96

Table 2 show frequency of *P. vivax* and *P. falciparum* infection. As detected by MP test, the level of mixed infection was zero whereas nested PCR assay detected 4 % of mixed infection. The P value for sensitivity and specificity of nested PCR was 0.000, proving PCR to be highly sensitive.

Table 2: Frequency of PCR for *P. vivax and P. falciparum* Identification

		Valid	Frequency (%)
Species identification	***P. vivax***	**PCR+ve**	1
		PCR -ve	96
	P. falciparum	**PCR +ve**	3
		PCR -ve	97

PCR-based assays are more specific and sensitive than microscopy for detecting and identifying malarial parasites. It is vital to distinguish between the different *Plasmodium* species in order to begin the adequate anti-malarial treatment. For instance, widespread resistance of *P. falciparum* to first-line and second-line anti-malarial drugs such as chloroquine and pyrimethamine-sulfadoxine in some countries necessitates the use of quinine or artemisin derivatives for rapid resolution of the parasitemia in vulnerable non-immune patients. Several PCR assays based on the 18S rRNA genes have thus been developed for the diagnosis of malaria. These assays are, however, not adequate for use in a clinical setting, since the turnaround time from admission to diagnosis is incompatible with the urgency to initiate treatment. RDT provide the necessary speed but perform poorly when parasite loads are low and are inadequate in cases where identification of the parasite species is desirable [17].

In this research, nested PCR technique is used to detect the *P. falciparum* and *P. vivax* in the human blood.100 microscopy negative and some microscopy positive malaria blood samples were taken from patient in metropolitan of Lahore, Pakistan. These were re-screened using PCR for the presence of *P. falciparum* and *P. vivax* DNA. We used 3 primer pairs as reported by Zaman et al; 2001 [18], for PCR screening of 100 microscopy negative and 50 microscopy positive blood samples. All blood samples were preceded through PCR by using the 3 pairs of primers rPLU5 + rPLU6 / rFAL1 + rFAL2 + rVIVI1 + rVIVI2. Four blood samples were found to be positive for malaria when PCR was performed by using rPLU5+ rPLU6 nested multiplex primer set. All of these four samples were found *P. vivax* positive by PCR using rVIVI1+rVIVI2 set of primers and 3 samples were found *P. falciparum* positive by PCR using rFAL1+rFAL2 set of primers.

In conclusion, the nested multiplex primer set rPLU5+rPLU6, rVIVI1+rVIVI2 and rFAL1+rFAL2 specific for the *P. vivax* the *P. falciparum* SSU-rRNA region, was found to be the most sensitive primer set to use when tested on DNA from malaria suspected blood samples in metropolitan of Lahore.

Prevalence of malaria in gender was determined. Frequency of males was 58 and females were 42 out of 100. Frequency of *Plasmodium* was observed to be 1% as a result of MP test whereas it was actually 4% when re-confirmed by PCR assay. MP test was unable to detect any mixed infection while the nested PCR assay showed that 4 % infection was due to *P. vivax* and 3% was due to *P. falciparum* co-infection [19].

Sensitivity and Specificity was determined by KAPPA statistics and also to determine the significant value. Significant value was calculated 0.000. Lower the P value, higher the significance. Sensitivity and specificity of PCR was calculated as 0.000 so it was found to be highly significant. Same results have been reported by Snounou, et al., in 1993, they also concluded that nested PCR is highly sensitive technique for detection of malarial parasites.

It is concluded that nested PCR is more sensitive and specific technique as a diagnostic tool for malarial parasite detection as compared to the MP test as practiced in hospitals and would be useful to replace conventional MP test, particularly in case of low parasitemia and mixed infections. It should be applied in biochemical labs (pathological labs) of hospitals so that it helps in reduction of false negativity of malarial diagnosis in Pakistan.

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Title and Authors

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Authors: Afshan Iqbal[1], Rabia Mushtaq[2], Ferhana Hussain[3], Mahjabeen Saleem[4], Mamoona Naz[5], Beenish Maqsood[6]

1; Corresponding author

1,2,3,4,5,6; Institute of Biochemistry and Biotechnology, University of the Punjab, Lahore, Pakistan, PO Box 54590.

Afshan Iqbal[1], syeda_iqbal@hotmail.com, phone no.; 92-322-6070474

Rabia Mushtaq[2], mushtaq71@yahoo.com

Ferhana Hussain[3], everlasting_fh@yahoo.com

Mahjabeen Saleem[4], mahjabeensaleem1@hotmail.com

Mamoona Naz[5], mamoona_nibge@yahoo.com

Beenish Maqsood[6], beenish.ibb@gmail.com