

Note

Molecular detection of *Plasmodium falciparum* and *Plasmodium vivax* prevalence in North Indian population

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Currently, malaria ranks as the 5th major cause of death in developing countries. There are six human malarial parasites species, with *Plasmodium falciparum* (Pf) and *P. vivax* (Pv) being the most predominant. This study enrolled 165 outpatients of a tertiary care hospital of the northeast district of Delhi. Blood samples were taken and were examined for malaria by slide microscopy and rapid malarial antigen detection test. DNA extraction was done and PCR was performed for genus as well as species identification. A total of 60 (36.36%) malaria positive samples were identified among a total of 165 samples collected. *P. vivax* and *P. falciparum* were found to be present in 51.66 and 13.33% samples, respectively and 18.33% samples were positive for both the species on the basis of PCR analysis. The present study suggests that while the traditional methods for diagnosis of malarial infection are gold standard they will not be able to detect mixed infections with low parasite count.

Keywords: Malaria, PCR

India, a major contributor of malaria with 65% incidence of malaria occurring among 2.4 million cases observed in Southeast Asia^{1,2}. The human malarial parasite species have now increased to six which includes *Plasmodium vivax*, *P. malariae*, *P. falciparum*, *P. knowlesi* and newly identified *P. ovale curtisi* (classic type) and *P. ovale wallikeri* (variant type)^{3,4}. Globally, *P. falciparum* (Pf) and *P. vivax* (Pv) have higher level of morbidity and mortality in comparison to other known species⁵⁻⁷. In India, 60-65 % of the infections are due to *P. vivax* and 35-40% are due to *P. falciparum*. Considering the severity of Pf malaria in humans, and the emerging severe form of malaria in *Plasmodium vivax* (Pv) a need to identify these parasites early and at different stages of the infection is necessary to start an early treatment.

Advanced technology such as PCR has revolutionized diagnosis of pathogenic infections with the ease of detection, accuracy and reproducibility.

Moreover, due to the emergence of drug resistance in malarial parasites and limited choice of antimalarial drugs, it becomes essential to make an early and definitive diagnosis^{8,9}.

Molecular tools have been utilized for malaria diagnosis and have improved the capacity to diagnose sub microscopic infections^{10,11}. The limitations of microscopy and rapid diagnostics tests (RDTs) in species differentiation can be overcome with molecular diagnostic tools. Here, we studied the potential of PCR in detection of malarial parasites with pan malarial primers and species specific primers (Pf and Pv) in humans.

Materials and Methods

This study enrolled 165 patients, who visited outpatient department of a tertiary care hospital of the Northeast district of Delhi, India, during the monsoon and post monsoon months of (June-August) 2015. Peripheral blood smears were giemsa stained and examined for malarial parasite by slide microscopy, and the collected blood in EDTA vial was subjected to commercially available rapid malarial antigen detection test, sure test malaria PF/PV HRP2/pLDH Combo (Access Bio Inc, NJ, USA) This immune chromatographic test has a sensitivity of 96 and 98% for *P. vivax* and *P. falciparum*, respectively with specificity of 97.5% and precision 100% (as per the manufacturers of the kit). For molecular detection, 2 mL of whole blood was taken into EDTA tubes for DNA extraction using commercial kit (Real Biotech Corporation). Primers were selected from previous published literature as shown in Table 1¹²⁻¹⁴.

Table 1 — Primer sequences, annealing temperatures and size of amplified products

Target gene	Primer sequence(5'-3')	Amplicon size (bp)	Temp. (°C)
<i>Plasmodium vivax</i> F	CGCTTCTAGCTTAATCC	120 bp	55 ¹²
	ACATAACTGATAC		
<i>Plasmodium vivax</i> R	ACTTCCAAGCCGAAGC	422 bp	47 ¹³
	AAAGAAAGTCCTTA		
<i>Plasmodium falciparum</i> F	GGA ATG TTA TTG CTA	107 bp	60 ¹⁴
	ACA C		
<i>Plasmodium falciparum</i> R	AAT GAA GAG CTG TGT	107 bp	60 ¹⁴
	ATC		
<i>Plasmodium</i> genus F	GCTCTTTCT	107 bp	60 ¹⁴
	TGATTTCTTGGATG		
<i>Plasmodium</i> genus R	AGCAGGTT	107 bp	60 ¹⁴
	AAGATCTCGTTCCG		

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Plasmodium infection was confirmed with genus specific primer (18s-rRNA region) and *Plasmodium falciparum* and *Plasmodium vivax* were differentiated by PCR.

Statistical analysis was done using Statistical Package for Social Sciences (SPSS) package (SPSS; Version 20.0). The chi square test and Fisher's exact test were used to determine statistical significance of data. P value <0.05 was considered significant. Diagnostic performances of PCR, microscopy and RDT were compared. It included true positive (A), false positive (B), false negative (C) and true negative (D). Sensitivity: $A / (A+C) \times 100$, specificity: $D / (D+B) \times 100$, positive predictive value: $A / (A+B) \times 100$, negative predictive value: $D / (D+C) \times 100$ ¹⁵.

Results and Discussion

During the three months study period from June to August 2015, a total of 60/165 (36.36%) malaria positive samples were identified by light microscopy. Fig. 1 shows the monthly distribution of samples received for testing. Table 2 depicts the evaluation of conventional and molecular malarial detection methods. Microscopy and RDT detected 47/60

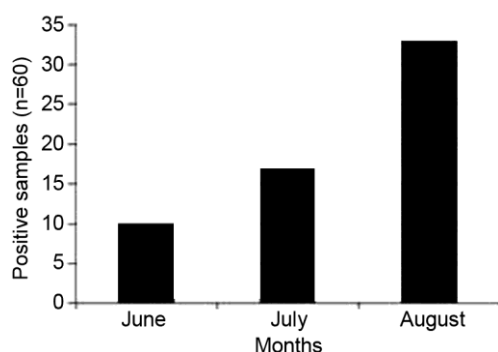


Fig. 1 —Monthly distribution of malarial positive samples

samples as positive and 13/60 samples as negative for malaria infection in comparison to PCR which showed 50/60 samples as positive and 10/60 samples as negative. Table 3 depicts the age and gender distribution of positive malarial cases showing a male preponderance with 34 (56.66%) versus 26 (43.33%) females with median age of 11.97 years. PCR detected *Plasmodium vivax* (51.6) % as the predominant species causing malaria followed by *P. falciparum* (13.33%) and 18.33% samples were positive for both the species. PCR showed a sensitivity of (98%) and specificity (100%) higher than that of microscopy and Rapid Diagnostics kit. Fig. 2 (A & B) depicts the agarose gel picture for plasmodium genus, *P. falciparum* (422 bp) and *P. vivax* (121 bp).

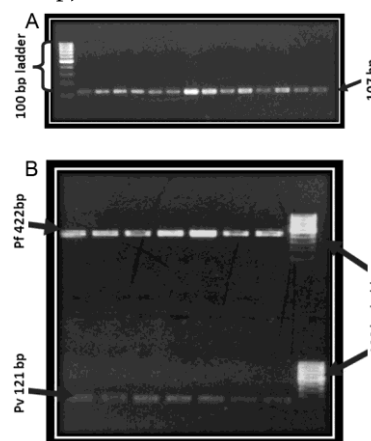


Fig. 2 — (A) PCR for detection of Plasmodium genus and species on 1.5 % agarose gel. Lane 1: molecular weight marker (100 bp), lane (2-15) plasmodium genus (107 bp); (B) Multiplex PCR for detection of Plasmodium species (*P. falciparum* and *P. vivax*) on 1.5 % agarose gel. Upper lane 1-7: *Plasmodium falciparum* (422bp), lower lane 1-7: *Plasmodium vivax* (121bp) and lane 8: molecular weight marker (100 bp)

Table 2 — Comparative evaluation of malaria positive samples by PCR assays, microscopy and malaria Antigen detection kit. (n=60)

Cause of infection	<i>P. falciparum</i>	<i>P. vivax</i>	(<i>P. falciparum</i> and <i>P. vivax</i>)	Total <i>Plasmodium</i> spp. detected	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Methods								
Microscopy (%)	8 (13.3)	30 (50)	9 (15)	47 (78.3)	94.1	92.8	97.9	81.2
PCR (%)	8 (13.3)	31 (51.6)	11 (18.3)	50 (83.3)	98	100	100	90.9
Malaria antigen (%)	7 (11.6)	30 (50)	10 (16.6)	47 (78.3)	90.3	81.2	94	72.2

Table 3 — Age and gender wise distribution of malaria positive samples by PCR

Age group in years	Male/female	<i>Plasmodium falciparum</i> (%)	<i>Plasmodium vivax</i> (%)	(Pf + Pv) (%)	Negative (%)	N=60 (%)
<5 years	14/8	3	14	2	3	22 (36.67)
5-15 years	5/4	2	1	4	2	9 (15)
16-25years	5/6	2	3	3	3	11 (18.33)
>26years	10/8	1	13	2	2	18 (30)
Total (m+f)	34/26	6+2=8 (13.33)	18+13=31 (51.66)	5+6=11 (18.33)	5+5=10 (16.66)	-

Malaria diagnosis remains a challenge, not only in the resource limited settings but also in research and laboratory environments. Our study observed that the patients were uniformly distributed in all age groups with highest number of patients in age group of less than five (<5) years. *P. vivax* and *P. falciparum* were the most dominating species as seen worldwide. A meta-analysis of 38 studies, demonstrated an average of 69 and 56% of *P. vivax* and *P. falciparum* infections, respectively¹⁶. The pathogenicity of *P. falciparum* is due to the cyto adherence properties of infected red blood cells (RBCs) to the host epithelium and sequestration into the peripheral vasculature¹⁷. Our study showed the presence of Pv and Pf co infection (18.33%) which is also similar to the other studies reported in India^{18,19}. *P. falciparum* alone was found in 13.33% samples which are in contrast to other reported study¹⁶. Previously, *P. falciparum* was known to be the dominant species but recently, *P. vivax* has emerged as a dominant *Plasmodium* species²⁰. The seasonal fluctuation, rapid urbanization and vector density, may be a possible assumption of the changing trend of malaria. Whether low-density parasitaemias are transmissible or not, it remains debatable²¹, but several studies have confirmed that sub microscopic *P. falciparum* as well as *P. vivax* infections are capable of slow transmission from hypnozoite reservoir, which are stated to be resistant to current control measures²². The pathophysiology of falciparum is unique and severe as it infects all stages of erythrocytes, which causes heavy parasitemia and severe breakdown of the RBCs provoke a hemodynamic, immunologic and metabolic effect compared to vivax species which infects only young RBCs²³. Loe *et al.*²⁴ have already associated clinical severity in *P. vivax* malaria due to cytoadhesion of infected erythrocytes (Pv-iEs) to the endothelial cells of the host partly mediated by variant interspersed repeats (VIR) proteins, encoded by the vir genes signifying their role in *P. vivax* infections.

Conclusion

PCR was found to be most accurate as compared to microscopy and RDT results, when repeated. Evident by the positive predictive value and negative predictive value as 100% and 90.9% respectively, PCR can be a valuable tool for surveillance. Therefore, a continuous molecular and clinical surveillance of malaria in symptomatic as well as in asymptomatic individual may be an advantageous tool in National surveillance programme.

References

- Centers for Disease Control and Prevention. 2011. Malaria facts. Centers for Disease Control and Prevention, Atlanta, GA. <http://www.cdc.gov/malaria/about/facts.html>.
- Brock PM, Fornace KM, Parmiter M, Cox J, Drakeley CJ, Ferguson HM & Kao RR, *Plasmodium knowlesi* transmission: integrating quantitative approaches from epidemiology and ecology to understand malaria as a zoonosis. *Parasitology*, 143 (2016) 389. doi: 10.1017/S0031182015001821.
- Mueller I, Galinski MR, Baird JK, Carlton JM, Kochar DK, Alonso PL & Del Portillo HA, Key gaps in the knowledge of *Plasmodium vivax*, a neglected human malaria parasite. *Lancet Infect Dis*, 9 (2009) 555.
- Price RN, Douglas NM & Anstey NM, New developments in *Plasmodium vivax* malaria: severe disease and the rise of chloroquine resistance. *Curr Opin Infect Dis*, 22 (2009) 430.
- Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the Global Burden of Disease Study 2015. GBD 2015 Mortality and Causes of Death Collaborators. *Lancet*, 388 (2016) 1459. doi: 10.1016/S0140-6736(16)31012-1.
- Pradhan A, Anasuya A, Pradhan MM, Ak K, Kar P, Sahoo KC, Panigrahi P & Dutta A, Trends in Malaria in Odisha, India-An Analysis of the 2003-2013 Time-Series Data from the National Vector Borne Disease Control Program. *PLoS One*, 11 (2016) e0149126. doi: 10.1371/journal.pone.0149126.
- Hay SI, Guerra CA, Gething PW, Patil AP, Tatem AJ, Noor AM, Kabaria CW, Manh BH, Elyazar IR, Brooker S, Smith DL, Moyeed RA & Snow RW, A world malaria map: *Plasmodium falciparum* endemicity in 2007. *PLoS Med*. 6 (2009) 286.
- Imwong M, Suwannasin K, Kunasol C, Sutawong K, Mayxay M, Rekol H, Smithuis FM, Hlaing TM, Tun KM, van der Pluijm RW, Tripura R, Miotto O, Menard D, Dhorda M, Day NPJ, White NJ & Dondorp AM, The spread of artemisinin-resistant *Plasmodium falciparum* in the Greater Mekong subregion: a molecular epidemiology observational study. *Lancet Infect Dis*, 17 (2017) 491. doi: 10.1016/S1473-3099(17)30048-8.
- Millar SB & Cox-Singh J, Human infections with *Plasmodium knowlesi*--zoonotic malaria. *Clin Microbiol Infect*, 21 (2015) 640. doi: 10.1016/j.cmi.2015.03.017.
- Karl S, Laman M, Moore BR, Benjamin JM, Salib M, Lorry L, Maripal S, Siba P, Robinson LJ, Mueller I & Davis TM, Risk factors for *Plasmodium falciparum* and *Plasmodium vivax* gametocyte carriage in Papua New Guinean children with uncomplicated malaria. *Acta Trop*, 160 (2016) 1. doi: 10.1016/j.actatropica.2016.04.002.
- Cao P, Klonis N, Zaloumis S, Dogovski C, Xie SC, Saralamba S, White LJ, Fowkes FJI, Tilley L, Simpson JA & McCaw JM, A dynamic stress model explains the delayed drug effect in artemisinin treatment of *Plasmodium falciparum*. *Antimicrob Agents Chemother*, 61 (2017) e00618-17. doi: 10.1128/AAC.00618-17.
- Snounou G & Singh B, Nested PCR analysis of *Plasmodium* parasites. *Methods Mol Med*, 72 (2002) 189.
- Tham JM, Lee SH, Tan TM, Ting RC & Kara UA, Detection and Species Determination of Malaria Parasites by PCR:

- Comparison with Microscopy and with ParaSight-F and ICT Malaria Pf Tests in a Clinical Environment. *J Clin Microbiol*, 37 (1999) 1269.
- 14 Kamau E, Tolbert LS, Kortepeter L, Pratt M, Nyakoe N, Muringo L, Ogutu B, Waitumbi JN & Ockenhouse CF, Development of a Highly Sensitive Genus-Specific Quantitative Reverse Transcriptase Real-Time PCR Assay for Detection and Quantitation of *Plasmodium* by Amplifying RNA and DNA of the 18S rRNA Genes. *J Clin Microbiol*, 49 (2011) 2946.
- 15 Rajul Parikh, Annie Mathai, Shefali Parikh, G Chandra Sekhar & Ravi Thomas, Understanding and using sensitivity, specificity and predictive values. *Indian J Ophthalmol*, 56 (2008) 45.
- 16 Kersting S, Rausch V, Bier FF & von Nickisch-Rosenegk M, Rapid detection of *Plasmodium falciparum* with isothermal recombinase polymerase amplification and lateral flow analysis. *Malar J*, 13 (2014) 99. doi: 10.1186/1475-2875-13-99.
- 17 Zimmerman PA, Mehlotlotra RK, Kasehagen LJ & Kazura TW, why do we need to know more about mixed *Plasmodium* species infections in humans? *Trends Parasitol*, 20 (2004) 440.
- 18 Gupta B, Gupta P, Sharma A, Singh V, Dash AP & Das A, High proportion of mixed-species *Plasmodium* infections in India revealed by PCR diagnostic assay. *Trop Med Int Health*, 15 (2010) 819.
- 19 Krishna S, Bharti PK, Chandel HS, Ahmad A, Kumar R, Singh PP, Singh MP & Singh N, Detection of Mixed Infections with *Plasmodium* spp. by PCR, India, 2014. *Emerg Infect Dis*, 21 (2015) 1853.
- 20 Maude RJ, Nguon C, Ly P, Bunkea T, Ngor P, Canavati de la Torre SE, White NJ, Dondorp AM, Day NP, White LJ & Chuor CM, Spatial and temporal epidemiology of clinical malaria in Cambodia 2004–2013. *Malar J*, 13 (2014) 385.
- 21 Niang M, Thiam LG, Sane R, Diagne N, Talla C, Doucoure S, Faye J, Diop F, Badiane A, Diouf B, Camara D, Diene-Sarr F, Sokhna C, Richard V & Toure-Balde A, Substantial asymptomatic submicroscopic *Plasmodium* carriage during dry season in low transmission areas in Senegal: Implications for malaria control and elimination. *PLoS One*, 12 (2017) e0182189.
- 22 Schneider P, Bousema JT, Gouagna LC, Otieno S, van de Vegte-Bolmer M, Omar SA & Sauerwein RW, Submicroscopic *Plasmodium falciparum* gametocyte densities frequently result in mosquito infection. *Am J Trop Med Hyg*, 76 (2007) 470.
- 23 Jain A, Kaushik R & Kaushik RM, Malarial hepatopathy: Clinical profile and association with other malarial complications. *Acta Trop*, 159 (2016) 95. doi: 10.1016/j.actatropica.2016.03.031.
- 24 Lopes SC, Albrecht L, Carvalho BO, Siqueira AM, Thomson-Luque R, Nogueira PA, Fernandez-Becerra C, Del Portillo HA, Russell BM, Rénia L, Lacerda MV & Costa FT, Paucity of *Plasmodium vivax* mature schizonts in peripheral blood are associated with their increased cytoadhesive potential. *J Infect Dis*, 209 (2014) 1403. doi: 10.1093/infdis/jiu018.