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New molecular detection methods of malaria parasites with multiple genes from genomes

Himanshu Gupta[a], Shikha Srivastava[a], Sima Chaudhari[a], Thanvanthri G. Vasudevan[a], Manjunath H. Hande[b], Sydney C. D'souza[c], Shashikiran Umakanth[d], Kapaettu Satyamoorthy[a,*]

[a] Department of Biotechnology, School of Life Sciences, Manipal University, Manipal, Karnataka, India
[b] Department of Medicine, Kasturba Medical College, Manipal University, Manipal, Karnataka, India
[c] Department of Medicine, Kasturba Medical College, Manipal University, Mangalore, Karnataka, India
[d] Department of Medicine, Dr. TMA Pai Hospital, Udupi, Melaka Manipal Medical College, Manipal University, Manipal, India

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ABSTRACT

For the effective control of malaria, development of sensitive, accurate and rapid tool to diagnose and manage the disease is essential. In humans subjects, the severe form of malaria is caused by *Plasmodium falciparum* (*Pf*) and *Plasmodium vivax* (*Pv*) and there is need to identify these parasites in acute, chronic and latent (during and post-infection) stages of the disease. In this study, we report a species specific and sensitive diagnostic method for the detection of *Pf* and *Pv* in humans. First, we identified intra and intergenic multiloci short stretch of 152 (PfMLS152) and 110 (PvMLS110) nucleotides which is present up to 44 and 34 times in the genomes of *Pf* and *Pv* respectively. We developed the single-step amplification-based method using isolated DNA or from lysed red blood cells for the detection of the two malaria parasites. The limit of detection of real-time polymerase chain reaction based assays were 0.1copyof parasite/μl for PfMLS152 and PvMLS110 target sequences. Next, we have tested 250 clinically suspected cases of malaria to validate the method. Sensitivity and specificity for both targets were 100% compared to the quantitative buffy coat microscopy analysis and real-time PCR (*Pf*-chloroquine resistance transporter (PfCRT) and *Pv*-lactate dehydrogenase (PvLDH)) based assays. The sensitivity of microscopy and real-time PCR (PfCRT and PvLDH primers) assays were 80.63%; 95%CI 75.22%–85.31%; $p < 0.05$ and 97.61%; 95%CI 94.50%–99.21%; $p < 0.05$ in detecting malaria infection respectively when compared to PfMLS152 and PvMLS110 targets to identify malaria infection in patients. These improved assays may have potential applications in evaluating malaria in asymptomatic patients, treatment, blood donors and in vaccine studies.

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1. Introduction

Malaria remains a significant and constant cause of morbidity and mortality worldwide. Over 3.3 billion people are at risk of malaria infection, out of which 1.2 billion are at increased risk of malaria disease. In 2014, 97 countries and territories had ongoing malaria transmission (World Malaria Report, 2014). The total number of malaria cases reported from India were 1.07 million in the year 2014. The regions of India that are most affected by malaria include Orissa, Chhattisgarh, West Bengal, Jharkhand and Karnataka. In Karnataka, the Dakshina Kannada District contributes to maximum number of malarial cases and deaths. In 2014, 719 deaths were reported from Dakshina Kannada District, which is approximately 2 fold of number of deaths in the year 2013 (NVBDCP Data Available at http://nvbdcp.gov.in/Doc/Malaria-situation-upto2011(P).pdf). Early detection and accurate diagnosis of the infection are essential requirements in endemic regions. Five species within the genus *Plasmodium* known to be significant transmitters of malaria in humans are *Plasmodium fal-*

Abbrevations: Pf, *Plasmodium falciparum*; Pv, *Plasmodium vivax*; Pm, *Plasmodium malariae*; Po, *Plasmodium ovale*; Pk, *Plasmodium knowlesi*; PfMLS152, *Plasmodium falciparum* multiloci short stretch of 152; PvMLS110, *Plasmodium vivax* multiloci short stretch of 110; PfCRT, *Plasmodium falciparum* chloroquine resistance transporter; Pf18SrRNA, *Plasmodium vivax* 18S ribosomal RNA; Pf18SrRNA, *Plasmodium falciparum* 18S ribosomal RNA; PvLDH, *Plasmodium vivax* lactate dehydrogenase; RDT, rapid diagnostic test; QBC, quantitative buffy coat; PCR, polymerase chain reaction.

* Corresponding author at: School of Life Sciences, Planetarium Complex, Manipal University, Manipal 576104, Karnataka, India.
E-mail address: ksatyamoorthy@manipal.edu (K. Satyamoorthy).

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Table 1
Distribution of PfMLS152 and PvMLS110 repeats in *Plasmodium falciparum* and *Plasmodium vivax* genomes respectively (PlasmoDB 9.2).

PfMLS152 repeats in *Plasmodium falciparum* genome		PvMLS110 repeats in *Plasmodium vivax* genome	
Chromosome Number	Chromosome coordinates	Chromosome Number	Chromosome coordinates
Chr1	36956-37107	Chr3	660684-660755
Chr1	79721-79872	Chr4	391283-391354391283-391354
Chr1	601786-601928	Chr5	345805-345924
Chr1	607410-607560	Chr5	309638-309757
Chr2	30998-31149	Chr5	226205-226294
Chr2	916371-916519	Chr5	131302-131376
Chr3	44312-44463	Chr5	192702-192800
Chr3	1030842-1030992	Chr7	564983-565051
Chr4	37512-37658	Chr8	1466453-1466545
Chr4	39984-40135	Chr8	128253-128328
Chr4	561677-561831	Chr8	826984-827047
Chr4	969051-969201	Chr8	1330268-1330355
Chr4	1173073-1173220	Chr8	385242-385319
Chr5	28286-28437	Chr8	385268-385330
Chr6	12665-12816	Chr8	1365492-1365558
Chr6	29637-29788	Chr9	1790668-1790752
Chr6	1353965-1354113	Chr10	1300410-1300510
Chr6	1374816-1374967	Chr10	868266-868352
Chr7	27913-28064	Chr10	227478-227547
Chr7	53466-53616	Chr11	560199-560278
Chr7	527348-527498	Chr11	1392727-1392801
Chr7	542926-543079	Chr11	1015391-1015455
Chr7	552177-552328	Chr12	2416647-2416732
Chr7	590345-590496	Chr12	2723461-2723520
Chr7	1417607-1417758	Chr12	1094022-1094120
Chr8	28486-28633	Chr13	743658-743731
Chr8	29746-29887	Chr13	430863-430935
Chr8	50769-50919	Chr13	1230259-1230320
Chr8	438881-439031	Chr13	430684-430787
Chr8	1435813-1435967	Chr13	1067317-1067390
Chr9	27715-27866	Chr14	2766267-2766336
Chr9	1495598-1495749	Chr14	2358117-2358207
Chr10	35994-36145	Chr14	2182773-2182870
Chr10	1642420-1642571	Chr14	603708-603819
Chr11	31425-31579		
Chr11	64672-64823		
Chr12	24324-24477		
Chr12	41770-41921		
Chr12	1702920-1703068		
Chr12	1727283-1727436		
Chr12	2241290-2241447		
Chr13	28617-28768		
Chr13	2884805-2884953		
Chr14	5176-5324		

ciparum (*Pf*), *Plasmodium vivax* (*Pv*), *Plasmodium malariae* (*Pm*), *Plasmodium ovale* (*Po*), and *Plasmodium knowlesi* (*Pk*). These three species *Pm*, *Po* and *Pk* have less global distribution and lower levels of morbidity and mortality in comparison to *Pf* and *Pv* (Barcus et al., 2007; Genton et al., 2008; Kochar et al., 2009; Mueller et al., 2009; Price et al., 2009; Tjitra et al., 2008). The gold standard laboratory method for the diagnosis of malaria is light microscopy of giemsa-stained blood films, which is cost-effective and requires a trained personnel. However, often low parasitemia and mixed infection can lead to misdiagnosis (Amexo et al., 2004). Other qualitative method being implemented in the malaria detection and control programs is immunochromatography-based rapid diagnostic test (RDT). RDTs, with a sensitivity of 100 parasites/μl (Moody, 2002), identify the parasite antigens such as HRP2, pLDH and pAldolase, and can only indicate the presence or absence of *Plasmodium* species. However, low parasitemia or mixed infection may also lead to misdiagnosis. Low parasite counts after treatment can be one of the causes of relapse and certain degree of sensitivity and specificity of the diagnostic test is required for parasite detection in such cases and in individuals with asymptomatic infection. Therefore, there is a need to continuously improve the existing diagnostic methods for enhanced performance.

The molecular detection of malaria-transmitting parasites has the capability of being highly accurate and sensitive, and remains as the best methods for detecting multiple species and subclinical infections (Bronzan et al., 2008; Erdman and Kain, 2008). The sequenced genomes of *Pf* and *Pv* present a great opportunity for improving the existing molecular diagnostic tools by identifying appropriate targets for more sensitive and specific detection. Of the five human-infecting malaria parasites, complete genomic information is available for three species—*Pf*, *Pv* and *Pk*. Molecular methods developed to detect *Plasmodium* species include the loop-mediated DNA amplification method, real-time PCR and multiplex PCR (Han et al., 2007; Notomi et al., 2000; Padley et al., 2003; Rougemont et al., 2004; Rubio et al., 2002; Snounou et al., 1993a; Taylor et al., 2010). However, the majority of molecular diagnostic tools still rely on 18S rRNA sequence (Han et al., 2007; Rougemont et al., 2004; Li et al., 1995; Singh et al., 1999; Snounou et al., 1993b). In order to identify new target DNA sequences to improve existing molecular diagnostic methods, we searched for multicopy DNA sequence that may be present in the parasite genome repeatedly and here we report identification of such sequences in *Pf* and *Pv* and its validation by conventional and real-time PCR assays.

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