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Plasmodium falciparum and *Plasmodium vivax* specific lactate dehydrogenase: Genetic polymorphism study from Indian isolates

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ABSTRACT

Control and eradication of malaria is hindered by the acquisition of drug resistance by *Plasmodium* species. This has necessitated a persistent search for novel drugs and more efficient targets. *Plasmodium* species specific lactate dehydrogenase is one of the potential therapeutic and diagnostic targets, because of its indispensable role in endoerythrocytic stage of the parasite. A target molecule that is highly conserved in the parasite population can be more effectively used in diagnostics and therapeutics, hence, in the present study polymorphism in PfLDH (*Plasmodium falciparum* specific LDH) and PvLDH (*Plasmodium vivax* specific LDH) genes was analyzed using PCR-single strand confirmation polymorphism (PCR-SSCP) and sequencing. Forty-six *P. falciparum* and thirty-five *P. vivax* samples were screened from different states of India. Our findings have revealed presence of a single PfLDH genotype and six PvLDH genotypes among the studied samples. Interestingly, along with synonymous substitutions, nonsynonymous substitutions were reported to be present for the first time in the PvLDH genotypes. Further, through amino acid sequence alignment and homology modeling studies we observed that the catalytic residues were conserved in all PvLDH genotypes and the nonsynonymous substitutions have not altered the enzyme structure significantly. Evolutionary genetics studies have confirmed that PfLDH and PvLDH loci are under strong purifying selection. Phylogenetic analysis of the pLDH gene sequences revealed that *P. falciparum* compared to *P. vivax*, has recent origin. The study therefore supports PfLDH and PvLDH as suitable therapeutic and diagnostic targets as well as phylogenetic markers to understand the genealogy of malaria species.

1. Introduction

Malaria is one of the foremost public health problems in India. Approximately two-thirds of all confirmed malaria cases in the Southeast Asia region were reported in India and country's 23% population resides in high malaria transmission areas (WHO, 2011). The challenge of malaria control and eradication is getting difficult with temporally increasing resistance to chloroquine and sulphadoxine–pyrimethamine since 1978 (Shah et al., 2011). In such scenario, inexpensive yet efficient disease management by proper diagnosis of true malaria cases is required to control the irrational use of antimalarial drugs (WHO, 2000).

The gold standard of malaria diagnosis, microscopic examination, requires instrumentation and expertise. These limitations of microscopic examination in field applications led the advent of immunochromatography-based malaria Rapid Diagnostic Tests (RDTs) with the potential advantages of simple, swift and precise diagnosis of malaria (Murray et al., 2003). The most common antigens targeted by these assays are: histidine-rich protein-2 (HRP-2), aldolase and *Plasmodium* specific lactate dehydrogenase (pLDH). pLDH is expressed at high levels in the blood-stage parasite and has significant structural differences, compared to human LDH (Brown et al., 2004). pLDH based RDT's have advantages over HRP-2, such as absence of the prozone effect (Gillet et al., 2009) and direct correlation between the level of parasitemia and titer of pLDH antigen in the patient's blood (Piper et al., 1999). Moreover, pLDH is vital for the survival of parasites in erythrocytic phase and plant extracts or synthetic compounds with pLDH inhibitory activity exhibit significant parasiticidal activity (Choi et al., 2007; Gomez et al., 1997; Keluskar and Ingle, 2012). Being substantially different from human ortholog, pLDH is considered as a good target for developing novel antimalarial drugs (Padmanaban et al., 2007).

Pharmacogenetic studies have provided strong evidence of linkage between variable drug response and polymorphism in the

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genes encoding drug targets (Johnson, 2003). Similar linkage was observed between the polymorphism in a diagnostic target and the sensitivity of a concern diagnostic kit. For example, genetic polymorphism in *Plasmodium falciparum* histidine-rich protein 2 was observed to influence the sensitivity of the RDT targeting it (Baker et al., 2005; Lee et al., 2006). Polymorphism studies of pLDH at both global (Talman et al., 2007) and regional level (Shin et al., 2013) have reported conserved as well as variable regions in pLDH genes and the extent of variation was also different in different *Plasmodium* species. India has high burden and unique distribution of *P. falciparum* and *Plasmodium vivax* malaria cases (Singh et al., 2009). Studying distribution of pLDH polymorphism in India, would provide a strong base to consider it as a good therapeutic and diagnostic target. Present study therefore aimed to analyze extant polymorphism in PfLDH (*P. falciparum* specific LDH) and PvLDH (*P. vivax* specific LDH) genes from different states of India. This was carried out by using PCR-Single Strand Conformation Polymorphism (PCR-SSCP) as a primary screening method, followed by sequence and model structure analysis of LDH from representative samples. Furthermore, the obtained pLDH sequences were used in the phylogenetic analysis of *Plasmodium* species.

2. Materials and methods

2.1. Samples

Blood from malaria patients was used as source of *P. falciparum* and *P. vivax* samples. Twenty *P. falciparum* and thirty-five *P. vivax* samples were collected from malaria endemic states of India (Odisha; Karnataka and Goa). These samples were collected from March to May and from September to November (peak transmission seasons for malaria infections in India) in 2007 and 2008. The necessary clearance for blood collection was obtained from the ethical committee of National Institute of Malaria Research (NIMR), New Delhi and informed consents were received from each patient. Blood samples collected were confirmed by microscopy for positivity of infection (Moody and Chiodini, 2000) and diagnosed for single infection of *P. falciparum* or *P. vivax* by PCR analysis (Gupta et al., 2010).

Additionally, a *P. falciparum* standard strain 3D7 and 25 *P. falciparum* isolates from different endemic and mesoendemic states of India (Odisha, Tamil Nadu, Mizoram, Meghalaya, Rajasthan, Chhattisgarh, Uttar Pradesh, Gujarat), maintained in the malaria parasite bank (NIMR, New Delhi), were also included. Thus, a total of 81 samples that include 45 *P. falciparum* samples, a *P. falciparum* standard strain and 35 *P. vivax* samples were used in the study (Fig. 1).

2.2. PCR-SSCP analysis

Genomic DNA was extracted from blood samples using QIAamp mini DNA extraction kit (Qiagen, Germany) according to manufacturer's instructions. Primers were designed using *P. falciparum* 3D7 *ldh* (GenBank ID: XM_001349953) and *P. vivax* Sal1 *ldh* (GenBank ID: XM_001615570.1) sequences as templates. DNA fragments to be used for SSCP analysis were amplified by a nested PCR approach wherein, initially 951 bp DNA fragments, coding for the entire PfLDH and PvLDH open reading frame (ORF), were amplified by a PfF and PfR primer pair and a PvF and PvR primer pair respectively (Table 1). These PCR products were then used as templates to amplify internal fragments in PfLDH (183 bp and 192 bp) and PvLDH (159 bp and 188 bp) genes using primers described in Table 1. The PCR mixture (50 μl) contained 50 pmol of each primer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, approximately 100 ng of genomic DNA and 2.5 U of *Pfu* polymerase with proofreading activity. PCR amplification was carried out by a following method: initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 30 s, respective annealing temperature for each primer pair (Table 1) for 30 s, extension at 72 °C for 75 s for amplification of complete ORFs and 30 s for amplification of internal fragments; followed by a final extension at 72 °C for 10 min. The purity and molecular weight of PCR products were determined by resolving on a native PAGE gel and silver staining. SSCP analysis was carried out as described by Sambrook and Russel (2001) with modifications. In brief, 2 μl of PCR products were denatured in 18 μl of gel loading dye (98% deionised formamide, 20 mM EDTA and 0.05% xylene cyanol) at 94 °C for 10 min and immediately plunged in ice. The samples were then loaded on 8% native PAGE gels of 0.75 mm thickness and 15 cm length. Single stranded DNA conformers (SSC) were resolved by electophoresis using DCode Universal Mutation Detection System (Bio-Rad Laboratories, USA) at constant 70 V, 25 °C for 12 h. Band pattern of SSC was visualized by silver staining of polyacrylamide gels. Relative electrophoretic mobility of each SSC was calculated by the AlphaEaseFC version 4.0 software using a 50 bp O'range ruler (Fermentas Inc.) as a reference. Samples were genotyped on the basis of similarity in the band pattern of the SSC. Abundance of each genotype among the samples was calculated as% occurrence.

2.3. Sequence and structure comparison

DNA fragments of 951 bp, coding for PfLDH and PvLDH ORFs, were cloned in *Escherichia coli* DH5α using a CloneJET PCR cloning Kit (MBI, Fermentas Inc., USA) and sequenced from both directions (SPA sequencing services, Merk Inc., Bengaluru, India) using pJET1.2 sequencing primers included in the cloning kit. Sequences were edited in a Chromas Pro Software version 1.49 beta (Technelysium Pty Ltd., Australia) to remove terminal vector sequences and submitted to the GenBank database (Accession numbers JN547218–JN547226).

Nucleotide and protein sequences of PfLDH and PvLDH genes, were aligned using a Clone Manager 7 (Scientific & Educational Software, USA) and compared with reported Pf LDH (XM_001349953) and PvLDH (XM_001615570.1) gene sequences. Protein structures for PvLDH genotypes with polymorphic amino acid sequence were predicted by homology modeling and their quality were estimated by QMEAN score (Benkert et al., 2011) in SWISS-MODEL workspace (Arnold et al., 2006). The PvLDH model structure of each genotype was compared to the crystal structure of PvLDH (PDB ID: 2A92) using Accelrys DS Visualizer (v2. 0.1.7347).

2.4. Phylogenetic analysis

The PfLDH and PvLDH sequences obtained in this study (Section 2.3) were used for the evolutionary and phylogenetic analysis of *Plasmodium* species. Following *Plasmodium* LDH sequences from Genbank and ENA were also included in the study: *P. falciparum* (XM_001349953, DQ198262, EU330208, DQ198261, DQ825436, M93720, EU589948 and EU589947), *P. vivax* (XM_001615570, GU078391, DQ060151, FJ527750, EU262983, EU589951, EU589952, EU589953, EU589954, EU589955, EU589956 and EU589957), *Plasmodium malariae* (ENA|AY486059), *Plasmodium ovale* (ENA|AAS77571), *Plasmodium reichenowi* (ENA|BAD73968), *Plasmodium knowlesi* (ENA|AEL88505), *Plasmodium berghei* (ENA|AAR99063), *Plasmodium chabaudi chabaudi* (ENA|CAH79796), *Plasmodium yoelii yoelii* (ENA|EAA15666), *Plasmodium* species from chimpanzee clade (ENA|ADN3213, ENA|ADN32195 and ENA|ADN32189) and *Plasmodium* species from gorilla clade (ENA|ADN32192, ENA|ADN32186 and ENA|ADN32204).

The number of intra and inter specific synonymous and non-synonymous sites in PfLDH and PvLDH loci were estimated using online tool for McDonald and Kreitman test (Egea et al., 2008).