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Amplification of LDH gene from Indian strains of *Plasmodium vivax*

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Abstract

Background & objectives: Plasmodium vivax is geographically widespread and responsible for > 50% of malaria cases in India. Increased drug resistance of the parasite highlights the immediate requirement of early and accurate diagnosis as well as new therapeutics. In view of this, the present study was undertaken to amplify *P. vivax* (Indian strains) lactate dehydrogenase gene (*Pv*LDH) which has been identified as a good target for antimalarials as well as diagnostics.

Methods: P. vivax infected clinical blood samples were collected from southern part of India and were tested with established diagnostic parameters (ICT, Giemsa staining). Total DNA was extracted from blood samples and subjected to PCR using two sets of primers, one for the amplification of full *Pv*LDH gene (951bp) and the other for a partial *Pv*LDH gene fragment (422bp), covering a variable antigenic region (140aa) as compared to other plasmodial species.

Results & conclusion: PCRs for both the full and partial gene targets were optimised and found to be consistent when tested on several *P. vivax* positive clinical samples. In addition, full gene PCR was found to specifically detect only *P. vivax* DNA and could be used as a specific molecular diagnostic tool. These amplified products can be cloned and expressed as a recombinant protein that might be useful for the development and screening of antimalarials as well as for diagnostic purposes.

Key words Amplification – antimalarial drugs – diagnosis – lactate dehydrogenase (LDH) – *Plasmodium vivax*

Introduction

Malaria, a disease caused by *Plasmodium* species, is one of the oldest and largest health challenges affecting 40% of the world's population[1]. It affects 300–500 million people and kills 1.5–2.7 million annually[2]. WHO forecasts 16% growth in malaria cases annually. These estimates rank malaria as one of the top three killers among infectious diseases[3]. The increasing incidences of malaria in tropical and subtropical countries reflect the development of drug resistant strains of *Plasmodium* and justify referring to malaria as a re-emerging disease[3–5]. Most of the molecular and biochemical studies have been done on *Plasmodium falciparum*, as it is the most deadly parasite among the four human *Plasmodia*. Even though *P. vivax* is responsible for tremendous morbidity due to the disease, little is known about its basic biological processes. *P. vivax* accounts for approximately 70–80 million cases annually[6]. In India >50% cases of malaria are due to *P. vivax*[7]. Although rarely fatal, it causes debilitating disease that severely affects the quality of life and economic productivity of its victims. *P. vivax* is of great importance as it is the most geographically widespread and is of common occurrence of all malarial *Plasmodia*. Early and accurate diagnosis and development of new antimalarials, whose targets differ from that of currently used drugs, remain the only available option in reducing morbidity and mortality due to malaria in tropical countries.

Plasmodium is homolactate fermenter and depends extensively upon anaerobic glucose metabolism for ATP production[8]. The parasite lactate dehydrogenase (pLDH), 316 amino acids tetrameric enzyme, is essential for energy production as it converts pyruvate to lactate while regenerating NAD^+ for continued use in glycolysis[9]. It has been previously reported that pLDH has notable structural and kinetic properties making it different from mammalian and bacterial LDH enzymes[10,11]. It has been identified both as potential antimalarial drug target and as an indicator of blood parasitaemia level in diagnosis[12,13].

In the present study, we report the amplification of complete (951bp) as well as partial (422bp) *P. vivax* LDH gene (*Pv*LDH).

Material & Methods

Collection of blood samples: P. vivax infected blood samples (2–3 ml) were collected in heparinised vials from southern part of India after testing with Now® ICT Kit (Binax Inc., USA). Thin and thick blood smears from each sample were prepared. The slides were stained with Giemsa stain and examined under light microscope to confirm *P. vivax* infection and to estimate parasitaemia level. The samples were stored at 4°C till further use. Clinical samples for the study were collected after obtaining prior permission from the authorities and informed consents from the patients.

Isolation of P. vivax genomic DNA: Vials containing blood samples were centrifuged at 1500 rpm for 10 min. Plasma and buffy coat of leucocytes were removed and RBCs (200 µl) were processed for the isolation of *Pv* genomic DNA by using a QIAamp® DNA Blood Mini Kit (Qiagen, Valencia, CA) as per manufacturer's instructions. For each DNA sample, absorbance at 260 nm was measured and DNA concentration calculated as A_{260} x 50 (µg/ml) x dilution factor. The integrity of DNA samples was monitored by agarose gel electrophoresis.

Amplification of PvLDH gene: Based on the partial LDH gene sequence of *P. vivax* strain Salvador I (GenBank accession no. AY486060), oligonucleotide primers NG3F-(5'TTGAACGTCTGCCCGAGA-GAT3') and NG4R (5'GCAACTGCCTCGTC-GAAC-TT3') were designed to amplify truncated region (422 bp/140aa) containing variable antigenic region as compared with *P. falciparum* (Fig. 1a). Antigenicity of *Pv*LDH was determined using software DNAstar Protean, WI (Fig. 1b). For the amplification of complete *Pv*LDH gene (951bp) specific primers NG1F (5'ATGACGCCGAAACCC-AAAAT3') and NG2R (5'TTAAATGAGCGCCTT-CATCC3') were constructed based on the sequence of *Pv*LDH Belem strain (GenBank accession no. DQ060151)[14].

All reactions were performed in 25 µl reaction volume using a thermal cycler (Applied Biosystems Ltd., USA). Reaction mixture used for the complete as well as partial gene amplification contained 1 x PCR buffer supplied with the enzyme (MBI Fermentas Inc., USA), 1.25 mM $MgCl_2$, 200 µM of dNTPs mix, 10 pmol of each primer, 50 ng of *Pv* genomic DNA and 1.25 units of *Taq* Polymerase. PCR programme was optimised to comprise an initial denaturation of 5 min at 94°C, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1.5 min, with a final extension step at 72°C for 10 min.

Prior to the amplification conditions described over here, reaction components and cycling conditions

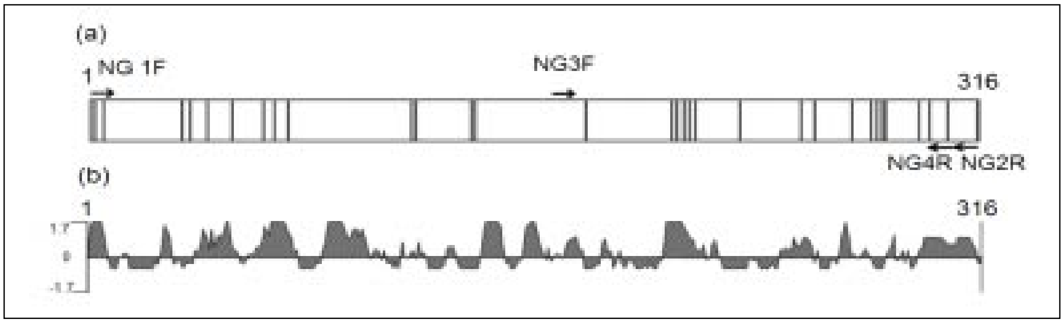

Fig. 1: (a) 316 amino acids long *Pv*LDH showing 31 variable residues (vertical lines) as compared to *Pf*LDH and the primers used; and (b) Antigenicity plot of corresponding regions of *Pv*LDH

were optimised to give reliable and reproducible results. Particularly $MgCl_2$ concentration (1.25–2.25 mM) and annealing temperature (50–60°C) gradients were used to determine the optimum conditions under which only the specific amplicon was produced. While optimisation, all the reaction components were kept constant except one variable at a time.

Analysis of PCR products: PCR amplified products were resolved on 1% agarose gel containing 0.5 mg/ml of ethidium bromide. The bands were visualised under UV light and documented using Alpha Innotech imaging system (San Leandro, CA).

Results

A total of 20 clinical samples were examined by microscopy of Giemsa stained blood smears. Of these 17 cases were having *P. vivax* infection. Three cases were positive for *P. falciparum*. Parasitaemia level was found to vary between 500 and 5000 parasites/µl. Genomic DNA extracted from different clinical samples was found to be intact by agarose gel electrophoresis (Fig. 2, few representatives shown). The yield of DNA obtained ranged from 1.5 to 3.0 µg. For PCR amplification, DNA isolated from an individual clinical sample was used as template. Fig. 1a shows the schematic diagram of 316aa long *Pv*LDH showing the primers used and variable residues (31aas, vertical lines) of *Pv*LDH as compared to *Pf*LDH. Multiple attempts to amplify the complete *Pv*LDH gene by primers based on the *Pf*LDH gene were unsuccessful. Full gene primers NG1F, NG2R designed based on the sequence of *Pv*LDH Belem strain, yielded the PCR product of expected size (951bp) and thus allowed the amplification of complete LDH gene (Fig. 3a). Primers NG3F–NG4R allowed the amplification of specific antigenic truncated region of *Pv*LDH giving the product of 422bp (Fig. 3b). The basic conditions for the amplification reaction were optimised for each primer set with regard to temperature of annealing and magnesium chloride concentration. Annealing temperature of 60°C gave best amplification for both sets of primers without any additional artefacts (Fig. 4a). The optimum concentration of $MgCl_2$ in the amplification of complete as well as partial LDH gene was found to be 1.25 mM (Fig. 4b). The optimised PCRs were tested on all *Pv*DNA taken for the study and found to be reproducible. The

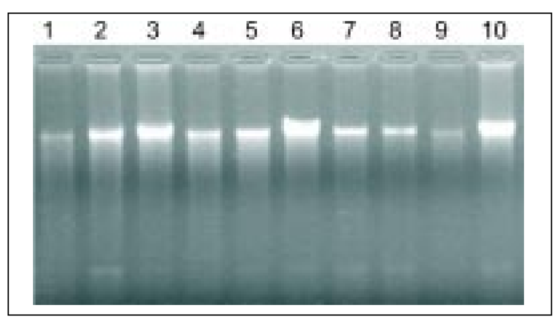

Fig. 2: Ethidium bromide stained gel of genomic DNA extracted from different clinical samples (Lane 1–10). 4 µl of genomic DNA was loaded to a 0.7% agarose gel

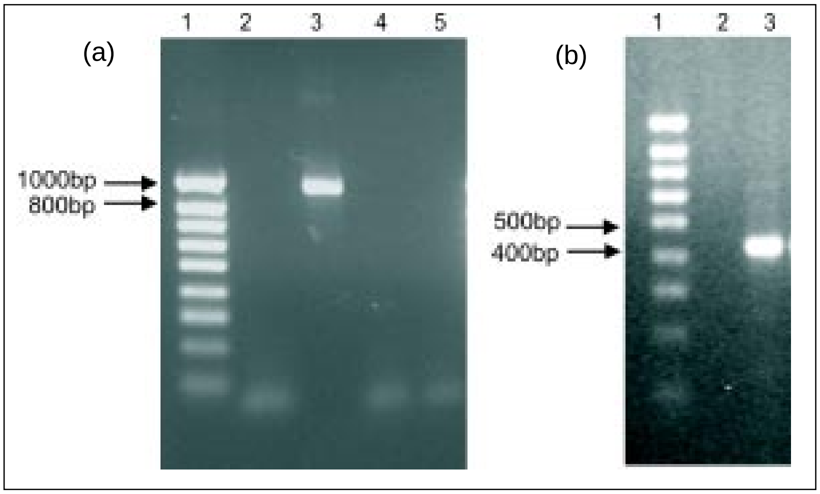

Fig. 3: (a) Amplified complete *Pv*LDH gene (951bp). Ladder (Lane 1), Blank (Lane 2) *P. vivax* (Lane 3), *P. falciparum* (Lane 4), Human DNA (Lane 5); and (b) Amplified partial *Pv*LDH gene (422bp). Ladder (Lane 1), Blank (Lane 2), *P. vivax* (Lane 3)

specificity of NG1F and NG2R primers was established by cross-checking on *Pf*DNA and human DNA. No amplification was obtained in any of these reactions.

Discussion

Parasitic enzymes, besides having a role in chemotherapy, have also been shown as a potential diagnostic antigens. *Pf*LDH has long been exploited for both purposes[15,16]. However, such studies have not been carried out with *Pv*LDH. In the present study, we report the amplification of complete *Pv*LDH gene from Indian strains of *P. vivax*. The amplified product has the potential to be expressed as recombinant protein that can be exploited for the screening as well as developing new antimalarials taking *Pv*LDH as a target. It is worth noting that NG1F, NG2R primers did not show any amplification with *P. falciparum* and human DNA.

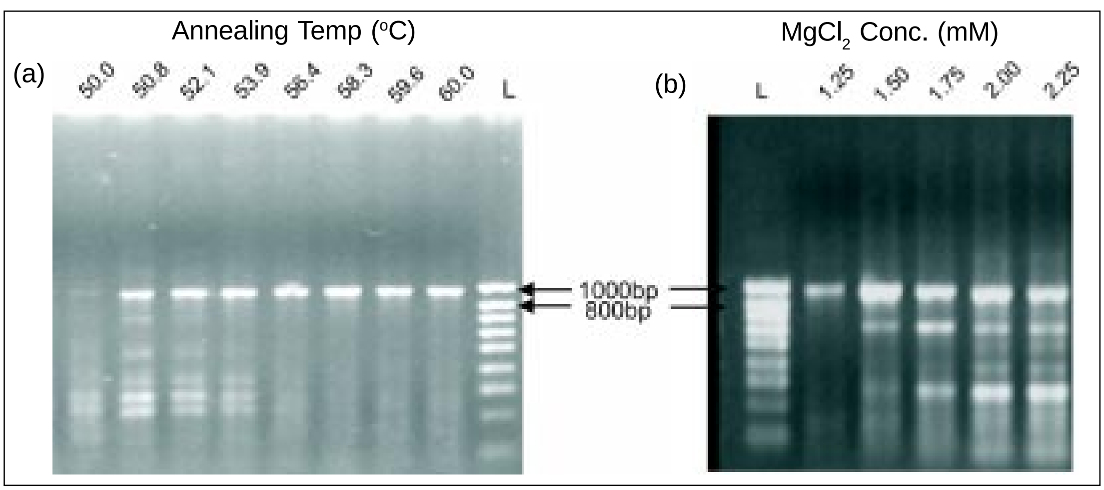

Fig. 4: Effect of primer/template annealing temperature (a) and $MgCl_2$ concentration (b) on amplification of complete *Pv*LDH gene (951bp)

A specific PCR product was only obtained when DNA from the corresponding species was present in the reaction. Thus, these primers were very specific to *Pv*LDH and did not cross react with *P. falciparum* or human DNA. Hence, the primers NG1F and NG2R could be used to specifically detect *P. vivax* by PCR. However, its diagnostic potential could only be ascertained if evaluated onto a large number of samples. As most of the rapid diagnostic systems available in the market can only differentiate between *falciparum* and non-*falciparum* malaria[17], a *P. vivax* specific detection system is essentially required. For this purpose, amplification of 422bp region of *Pv*LDH was attempted and successfully accomplished. This aspect could be further elaborated by cloning and expression of the amplified gene fragment and its utilisation as a potential antigenic recombinant protein for the detection of *Pv* infections.

In conclusion, we have reported here the amplification of *Pv*LDH gene from Indian isolates. Both standardised PCRs were found to be highly reproducible and gave the desired amplification with all *P. vivax* positive clinical samples screened.

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