Cloning, expression and characterization of lactate dehydrogenase from *Plasmodium vivax* and *Plasmodium knowlesi*, the human malaria parasites

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Submitted By

Vandana Singh

Under the Supervision of

Dr. (Mrs.) N. A. Kaushal  
(External Supervisor) 
Department of Parasitology 
CSIR-CDRI, Lucknow 
U.P., India

Prof. (Mrs.) Sushma Rathaur  
(Supervisor) 
Department of Biochemistry 
Banaras Hindu University 
Varanasi, U.P., India

DEPARTMENT OF BIOCHEMISTRY 
FACULTY OF SCIENCE 
BANARAS HINDU UNIVERSITY 
VARANASI-221005 
INDIA

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Abstract

Malaria is a parasitic disease which has severely affected the socio-economic conditions of the tropical and sub-tropical countries. Species-specific diagnosis of the parasite specific enzyme/antigen and subsequent cure of malaria is essential for control and effective management of the disease. *Plasmodium vivax*, earlier known for causing benign malaria is slowly transforming to the malignant form due to the increasing severity caused by it as well as the existence of dormant stage in *P. vivax*, having different drug. *Plasmodium knowlesi*, is the emerging human malaria parasite with their natural infection in humans, first reported in Malaysia. However, the *P. knowlesi* infections are limited only to some countries like China, Philippines, Thailand, Vietnam, Myanmar, Indonesia and Singapore. Thus, specific diagnostic tools are required to assess the public health importance of these malaria parasites in Southeast Asian countries. Most of the malaria diagnostic tests are either *P. falciparum* specific or pan-specific and some are *P. vivax* specific but with low sensitivity. Therefore, development of accurate, rapid and prompt diagnostic test is necessary for diagnosis of *P. vivax* and *P. knowlesi* infections. Three protein targets are widely being employed in the malaria rapid diagnostic tests (RDTs). These are *Plasmodium falciparum* histidine rich protein 2, Plasmodium lactate dehydrogenase and aldolase. Glycolytic enzyme, lactate dehydrogenase is one of the most important enzymes for malaria parasite for deriving their energy in the anaerobic environment of the host. Furthermore, Plasmodial LDH (pLDH) is biochemically, immunologically and structurally different from the mammalian and bacterial LDHs as demonstrated in many studies. Therefore, Plasmodial lactate dehydrogenase can be employed as the potential target for specific diagnosis and cure of malaria.

- In the present research work, lactate dehydrogenase of *Plasmodium knowlesi* and *Plasmodium vivax* (PkLDH and PvLDH) were selected in view of their defined role as chemotherapeutic and diagnostic target.
- Cloning and sub-cloning of lactate dehydrogenase genes from the genomic DNAs of *Plasmodium knowlesi* and *Plasmodium vivax* was done into suitable sequencing and expression systems. Homology search of PkLDH and PvLDH with LDHs of other Plasmodium species and human was done.
- Affinity purification of recombinant PkLDH and PvLDH was performed and determination of the kinetic parameters of recombinant PkLDH and PvLDH.
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- Determination of antigenicity of both the recombinant LDHs (rPkLDH and rPvLDH) bio-informatically and experimentally through immunological studies.
- Production of polyclonal antibodies against rPvLDH and rPkLDH and their use for immunochemical characterization of recombinant LDHs.
- Production of monoclonal antibodies against rPvLDH through hybridoma technology and characterization of monoclonals.
- Evaluation of polyclonals against rPkLDH/rPvLDH and monoclonals against rPvLDH in Sandwich ELISA for determining their diagnostic potentials.

1. Cloning and expression of *P. knowlesi* and *P. vivax* lactate dehydrogenase

The cloning and expression of LDH from two human malaria parasites, *P. knowlesi* (H strain) and *P. vivax* (Indian isolate) was performed to get the ample amount of recombinant proteins for their detailed studies. Initially, all the techniques were standardized with *P. knowlesi* LDH as it is closely related to its counterpart in *P. vivax* parasite and moreover it is the newly added species to the human malaria parasites group. Genomic DNAs of *P. knowlesi* H-strain and *P. vivax* Indian isolate were isolated from infected monkey and human blood respectively and PCR amplified as 0.9 Kb products using the gene specific primers at the optimized annealing temperature of 48°C. The amplified products were cloned at the EcoR1 site of pGEMT-Easy sequencing vector. Successful cloning of PkLDH and PvLDH genes in the pGEMT-Easy vector was confirmed by colony PCR of the positive white colonies as well as by EcoR1 restriction digestion of the purified recombinant plasmids. Sequence analyses of the cloned LDHs of *P. knowlesi* H-strain and *P. vivax* Indian isolate gave the open reading frames (ORFs) of 951 nucleotides encoding the deduced proteins of 316 amino acids. The nucleotide sequences of *P. knowlesi* H-strain and *P. vivax* Indian isolate LDHs were submitted to the GenBank (Accession numbers JF958130 and KF612322). The expression of the recombinant proteins was attained through cloning of the digested LDH genes in their respective expression vectors. The EcoRI and NotI digested PkLDH and BamHI and HindIII digested PvLDH genes were sub-cloned respectively in the pGEX-6-P1 and pET28a expression vectors and positive clones were confirmed by colony PCR and restriction digestion studies. The PkLDH was expressed as 60 kDa GST-tag protein and PvLDH as 34 kDa His-tag protein. The recombinant PkLDH and PvLDH were expressed in enzymatically active soluble form at optimum induction conditions of 0.5 mM IPTG at
25°C for 4 h and 0.1 mM IPTG at 30°C for 4 h respectively. The recombinant PkLDH was purified by affinity chromatography on GST-Trap Sepharose and Blue Sepharose columns while Ni-NTA Sepharose column was used to purify the recombinant PvLDH. The purified recombinant PkLDH and PvLDH were found to be immunogenic as evidenced by their immunoreactivity with polyclonal and monoclonal antibodies produced earlier against purified *P. knowlesi* LDH and *P. falciparum* LDH.

Therefore, in this chapter, we have cloned, over-expressed and purified the LDH from two human malaria parasites *P. knowlesi* (H strain) and *P. vivax* (Indian isolate).

2. **Computational and biochemical characterizations of plasmodial Lactate dehydrogenase**

Clustal W alignment of the amino acid sequence of the cloned PkLDH showed 96% homology with the *Plasmodium vivax* LDH, 90% with the LDH of *Plasmodium falciparum*, 89.9% with *P. malariae* LDH, 88.3% with *P. ovale* LDH and only 27% homology was observed with the human LDH-A. The cloned PvLDH revealed 96% homology with *P. knowlesi* LDH and 90% with *P. falciparum* LDH. Thus *P. vivax* LDH and *P. knowlesi* LDH shared maximum amino acid similarity. The alignment results suggested the application of LDH as the valid molecule for malaria specific diagnosis and chemotherapy. Furthermore, significant similarity of PvLDH was observed with the LDHs other *P. vivax* strains and isolates.

Phylogram of the LDHs of different *Plasmodium* species, Apicomplexans and human was constructed to study the evolutionary relationships among them. The *Plasmodium* and Apicomplexans LDHs showed divergence from the common ancestors and they are distantly related to the human LDH. Among, *Plasmodium* species, PkLDH and PvLDH showed much closeness to the *P. cynomolgi* LDH and among Apicomplexans, greater closeness was noticed with the *Babesia bovis* LDH.

The catalytic domain studies of the PkLDH and PvLDH using their amino acid sequences as the input showed the presence of the conserved regions. The key catalytic residues (R109, D168, R171, H195) and the characteristic five amino acid insert (DKEWN) (in the substrate specific loop) were present in the LDHs of *P. knowlesi* and *P. vivax* which are known for their presence in the LDHs of all *Plasmodium* species and thus proving them biochemically distinct from the human LDH. Furthermore, significant regions of
hydrophilicities and antigenicities were obtained in the PkLDH and PvLDH sequences in the antigenicity plots constructed using ChromasPro software. These regions were highly distinct from the antigenic regions in the human LDH. However no differences in the antigenic regions were observed for the PkLDH and PvLDH.

Optimum activity of recombinant pLDH was obtained at 37°C and pH 7.4 using 100 mM KCl as a catalyst metal ion. $K_m$ and $V_{max}$ values of the enzyme were calculated for NADH, APADH and pyruvate by using the Michaelis-Menten equation in GraphPad Prism 3.0 software. The $K_m$ values of rPkLDH and rPvLDH for pyruvate were 12.52 $\mu$M and 89.98 $\mu$M respectively. Lower $K_m$ values of both the pLDHs for pyruvate as a substrate as compared to the host LDH showed their higher affinity for pyruvate. Moreover, as expected recombinant pLDHs showed high affinity for the APADH as the substrate. Significant inhibition in the activity of recombinant Plasmodium LDH was recorded with the LDH inhibitor, gossypol.

Thus, from bioinformatics and kinetic studies suggested that structural and functional uniqueness of PkLDH and PvLDH could be taken as the rationale target for identifying new anti-malarial drug candidates as well as in designing the immunological assays for malaria diagnosis.

3. Immunochemical characterization of plasmodial lactate dehydrogenase using polyclonal and monoclonal antibodies

The purified recombinant PkLDH and PvLDH were found to be immunogenic as evidenced by the immunoreactivity with pan-specific anti-pLDH polyclonal and monoclonal antibodies produced previously against the purified P. knowlesi and P. falciparum LDHs. In the present study, the immunization of rabbits with rPkLDH and rPvLDH resulted in the production of hyperimmune polyclonal antibodies thereby suggesting the antigenic nature of both recombinant proteins (rPkLDH and rPvLDH). The anti-rPkLDH and anti-rPvLDH antibodies showed antibody titres of 1: 80,000 and 1: 64,000 respectively in ELISA against their respective recombinant parasite LDHs. The anti-rPkLDH polyclonal antibodies exhibited significantly high ELISA reactivities with native and recombinant LDHs and did not show any significant reactivity with the host LDH (hLDH). The immune rabbit serum bleed showing high antibody titres were pooled and used for the purification of IgGs for further studies. The strong recognition of 34 kDa band of rPkLDH and rPvLDH on immunoblotting by the respective antibodies also indicated the antigenicity of rPkLDH and
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rPvLDHs well as the quality of polyclonal antibodies produced in the present study against the recombinant antigens.

In view of antigenic differences of plasmodial LDH from the host LDH, as evidenced by immunochemical characterization of recombinant P. knowlesi and P. vivax LDHs, we have produced monoclonal antibodies against the recombinant P. vivax LDH. The efforts on production of monoclonal antibodies against recombinant P. vivax LDH were initiated by the immunization of BALB/c mice with rPvLDH. The mice showing high and consistent ELISA reactivity with rPvLDH were chosen for fusing with the myeloma partner. The 5 hybridoma clones producing monoclonal antibodies with high ELISA reactivities and recognizing 34 kDa band of rPvLDH in immunoblotting were characterized. The isotypic characterization of monoclonals revealed that all the five monoclonal antibodies (MoAb4, MoAb5, MoAb7, MoAb8 and MoAb9) are IgG1 isotype. All these 5 monoclonal antibodies exhibited high reactivities with rPvLDH but not with the analogous host enzyme suggesting their specificity to plasmodial LDH. Out of 5 monoclonal antibodies against rPvLDH, three monoclonals (MoAb4, MoAb5, MoAb9) showing high and consistent reactivity with rPvLDH were selected for further studies. On testing the culture supernatants of three monoclonals using the optimum antigen concentration of recombinant protein in ELISA, two monoclonals (MoAb4 and MoAb5) exhibited high ELISA reactivity as compared to the third monoclonal antibody (MoAb9). Almost similar results were obtained on testing the ascites fluids of three monoclonal antibodies in ELISA. On performing immunoblotting of recombinant P. vivax LDH employing these monoclonal antibodies, the MoAb4 and MoAb5 monoclonals strongly recognized the 34 kDa band of rPvLDH while less intense band was observed with MoAb9. These results were in agreement with ELISA reactivity of these monoclonals with rPvLDH. The three monoclonal antibodies (MoAb4, MoAb5 and MoAb9) showed high reactivity with the native and recombinant forms of LDHs from both P. vivax and P. knowlesi. However, no significant reactivities of these monoclonals were observed with the host LDH. These results suggest that the monoclonal antibodies produced against the recombinant P. vivax LDH, in the present study, are plasmodium pan-specific and directed towards the epitope(s) present in both P. vivax and P. knowlesi LDHs.

The diagnostic potential of monoclonal antibodies produced against recombinant P. vivax LDH was examined by Sandwich ELISA employing a combination of polyclonal antibody against rPvLDH/rPkLDH as capturing antibody and the three monoclonal antibodies
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against recombinant PvLDH for revealing the antigen. Two vivax LDH monoclonals (MoAb4 and MoAb5) were found better in revealing the antigen compared to MoAb9. The polyclonal-monoclonal based Sandwich ELISA was evaluated further for the detection of antigen using different dilutions of *P. knowlesi* infected monkey blood. The MoAb4 and MoAb5 monoclonal antibodies were more effective in revealing the parasite LDH in infected monkey blood as compared to MoAb9 monoclonal. Another combination of Sandwich ELISA involving the pan-specific monoclonal antibody against *P. falciparum* LDH (produced earlier in our lab) and anti-Pk/Pv LDH polyclonal was employed for the detection of parasite LDH in blood samples. The monoclonal-polyclonal based Sandwich ELISA could detect parasite LDH in malaria blood samples.

CONCLUSION

✓ Lactate dehydrogenase of *P. knowlesi* and *P. vivax* were successfully cloned, over-expressed and affinity purified.

✓ Amino acid sequence alignment studies of *P. knowlesi* and *P. vivax* lactate dehydrogenase with the other human malaria parasites and human LDHs showed their potential to be used as the chemotherapeutic and diagnostic targets for malaria.

✓ The recombinant PkLDH and PvLDH were found to be highly immunogenic as revealed by their high reactivities with anti-plasmodial LDH antibodies and the production of high titred polyclonal antibodies against recombinant PkLDH and PvLDH.

✓ The polyclonal antibodies produced against the recombinant PkLDH and PvLDH in combination with pan-specific monoclonal (anti-PfLDH) showed their diagnostic potential for recognition of parasite LDH in malaria blood samples.

✓ The monoclonal antibodies produced against rPvLDH were found to be Plasmodium pan-specific. However, differences in the amino acid sequences of *P. knowlesi* and *P. vivax* LDH’s, as observed in the present study, can be exploited in future for producing monoclonal antibodies specific to *P. knowlesi/P. vivax* LDH and subsequently use for specific diagnosis of *P. knowlesi/P. vivax* infection in humans.