Molecular analysis of reticulocyte binding protein-2 gene in *Plasmodium vivax* isolates from India

Surendra K Prajapati*, Pragati Kumari and Om P Singh

**Abstract**

**Background:** *Plasmodium vivax* reticulocyte binding protein-2 (PvRBP-2) is a promising candidate for development of vaccine against parasite. DNA sequence polymorphism in *pvrbp-2* which may hamper the vaccine development program has been identified in laboratory strains. Therefore, unraveling genetic polymorphism in *pvrbp-2* from field isolates is a prerequisite for success in vaccine development. This study was designed with a primary aim to uncover genetic polymorphism in *pvrbp-2* among *P. vivax* field isolates.

**Results:** Using virtual restriction mapping of *pvrbp-2* sequences, two restriction enzymes (AluI and ApolI) were selected for the development of *pvrbp-2* as a PCR-RFLP marker. Restriction fragment length polymorphism (RFLP) analysis revealed a high degree of genetic polymorphism in the *pvrbp-2* gene among field isolates of *P. vivax*. ApolI-RFLP was found to be more efficient in identifying the extent of genetic polymorphism in *pvrbp-2* compared to AluI-RFLP. Combined genotyping/haplotyping of RFLP pattern revealed a total of 36 distinct RFLP patterns among 83 *P. vivax* isolates analyzed. DNA sequence analysis also supports high degree of genetic polymorphism among field isolates of *P. vivax*. *Pvrbp-2* PCR-RFLP method is able to distinguish multiple infection up to 16.86% and it revealed a low level of shared genetic pool between more than two populations.

**Conclusion:** The study suggests that *pvrbp-2* is highly polymorphic genetic marker which can be used for population genetic analyses. RFLP analysis suggests presence of nearly similar proportion of *Sal-1* and *Belem* alleles in Indian *P. vivax* populations. The larger extent of genetic polymorphism identified from limited samples advocates to screen genetic polymorphism in *pvrbp-2* from malaria endemic geographical regions and countries for designing *pvrbp-2* based anti-malarial control measures.

**Background**

*Plasmodium vivax* is the most widely distributed human malaria parasite outside sub Sahara regions of Africa. Although mild with its prolonged and recurrent infection resulting in huge morbidity, the species can also be severe and fatal [1-6]. Annual burden is estimated to be about 70–80 million cases globally [7], however in India, *P. vivax* is responsible for about one million malaria cases annually, contributing 50–55% of total malaria cases.

Using molecular techniques, genetic diversity studies of malaria parasites accelerated substantially and provided a landmark in understanding parasite genetic diversity, evolution of pathogenicity and drug resistance, and transmission success. Identifying highly polymorphic marker is essential for studying genetic diversity, population structure, multiplicity of infection, and relapse and recrudescence infection etc. Till date, two types of molecular markers are in frequent use to unraveled genetic diversity from field isolates of *P. vivax*, these are tandem repeats markers [8,9] and antigen encoding genes [10-12].

Invasion of erythrocytes by malaria parasite is a complex and multi-step process. Merozoites of *P. vivax* primarily invade the reticulocytes [13] whereas *P. falciparum* can invade both mature RBC as well as reticulocytes [14,15]. The specificity in binding with reticulocytes is mediated by a set of proteins which are encoded by a gene family called reticulocyte binding protein where members of this family are found in malaria parasites of human, simian and rodent [16-19]. The major function of reticulocyte binding protein is seen during the initial steps of erythrocyte selection and invasion [17]. Evidence suggests that the
PvRBPs form a complex at the apical pole of the merozoite and confer the reticulocyte-specificity of *P. vivax* blood-stage infections, suggesting the essential role of RBP-II in selection and identification of reticulocyte for invasion [17]. Two *pvrbp*-2 genes have been characterized from *P. vivax* and are shown to be a promising vaccine candidate [20]; however, up to 12 putative *pvrbp* genes have been identified in *P. vivax* genome so far [21].

*Pvrbp*-2 is a promising vaccine target for the development of effective anti-malarial control measures [20]. However, genetic polymorphism at *pvrbp*-2 may hamper the efficacy of vaccine [22]. Therefore, investigation of genetic polymorphism at *pvrbp*-2 from geographical field isolates is an essential step. This study was designed to investigate the genetic polymorphism in *pvrbp*-2 using PCR-RFLP method in *P. vivax* field isolates from Indian subcontinent.

**Methods**

**Ethics statement**

This study was approved by the Ethics Committee of the National Institute of Malaria Research and all blood spots were collected with written consent of the patients and/or their legal guardians.

**Parasite collection and DNA extraction**

Ninety *P. vivax* field isolates collected between 2003–2006 from six geographical regions of the Indian subcontinent were analyzed (Figure 1). Finger prick blood from the symptomatic patients in active case detection surveys as well as from patient attending the clinics, was spotted on autoclaved Whatman filter paper strips (Number 3). The six geographical regions are Delhi (N=13), Nadiad of Gujarat (N=26), Panna of Madhya Pradesh (N=18), Rourkela of Odisha (N=16), Chennai of Tamil Nadu (N=10), and Kamrup of Assam (N=7). Details of individual study sites such as location, parasite and vector species prevalence, and disease transmission pattern are reported elsewhere [23] as well as given in Additional file 1. Genomic DNA was isolated from microscopically diagnosed vivax-positive blood spotted on Whatman filter paper (3 mm) strips using QIAamp mini DNA kit (Qiagen, Germany). Three punches (5 mm diameter) of dried blood spots were used for DNA isolation, as per the manufacturer’s instructions. DNA was eluted in 120 μl triple distilled autoclaved water and stored at −20°C for future use.

**Primer designing and PCR amplification**

Nested PCR primers for *pvrbp*-2 gene were designed manually using *pvrbp*-2 sequence available in GenBank (AY501887). These primers are RBP2-F (5’-gatgatcatttt-tatgctgac-3’), RBP2-R (5’-cagaatcggcaataatag-3’), RBP2-NF (5’-ttcccgcacacacaaggtag-3’), RBP2-NR (5’-gcgtagtgttt-tagctgccac-3’), RBP2-IR1 (5’-ttggacgcctatgcgattc-3’), RBP2-IR2 (5’-tttgccgataagtac-3’). Internal primers used for sequencing this fragment are IR1 and IR2 and the schematic diagram of gene showing location of primers is given in Figure 2. Optimized PCR conditions for primary PCR for amplification of *pvrbp*-2 were: initial denaturation 95°C/5 minute, denaturation 95°C/30 S annealing 50°C/30 S and extension at 68°C/2 minute for 35 cycles, and a final extension of 68°C/5 minute. The cycling conditions of nested PCR were similar to primary PCR except annealing temperature, which was 55°C. All PCR amplifications were carried out in a 20 μl reactions volume (Qiagen’s Master Mix) with 10 pM of each primer and 1.2 μl (~ 3–5 ng) of genomic DNA in primary PCR and 0.5-1 μl of primary PCR product in nested PCR.

**Restriction Fragment Length Polymorphism (RFLP)**

To determine the level of *pvrbp*-2 polymorphism, RFLP analysis was carried out using two restriction enzymes *Apol* and *Alul* (NEB Inc, USA). These enzymes were selected on the basis of maximum probability of enzymes cutting sites in the polymorphic region of *pvrbp*-2 and the feasibility to resolve digested PCR fragment on agarose gel. Virtual restriction mapping of *pvrbp*-2 was done using SeqBuilder module of DNA Lasergene 7.1 software for identification of suitable restriction enzymes for RFLP study. Four microliters of PCR product was digested with individual restriction enzyme. *Alul* digestion was incubated at 37°C for 4 hours whereas *Apol* was incubated at 50°C for overnight. In both digestions, heat inactivation for enzymes was given at 80°C/20 minutes. The restriction products were visualized on a 2.5 % agarose gel containing ethidium bromide. A consistent current at 0.75 m for 2.5 hrs were used for all agarose gel electrophoresis experiments to achieve consistency in RFLP fragment sizes.

**RFLP Genotyping and multiple infection typing**

Digested DNA fragments were assessed using Genetool software and all fragments were considered for genotyping of RFLP data. In RFLP analysis, the restriction pattern of each enzyme was typed where each different/unique RFLP pattern was assigned 1…n as an allele. Finally, RFLP patterns of *Apol* and *Alul* from each sample were combined to make a “haplotype or genotype”. This “haplotyping/ genotyping” method provides a high-resolution power for differentiating parasites compared with RFLP pattern of individual enzyme.

Multiple infection could only be detected by RFLP analysis since all samples show only a single PCR fragment. A sample was considered as multi-clone infection if the sum of the digested fragments (either *Apol*
or Alu (or both) size is greater than the size of the PCR fragment.

**Cloning, DNA sequencing, and sequence analysis**

DNA sequencing of limited samples was done in order to validate RFLP pattern as well as to differentiate Sal-1 and Belem alleles of pvrbp-2. PCR products from 13 samples (Nadiad; 7, Delhi; 1, Kamrup; 2, and Panna; 3) were purified using gel extraction kit (MDI, India) and cloned in pTZ257R/T vector (Fermentas, USA). Six of 13 samples were single clone in nature on the basis of pvrbp-2 RFLP analysis. Plasmid was purified using plasmid extraction kits (MDI, India) and purified plasmids were sequenced commercially (Macrogen Inc, Seoul, Korea) [24]. For DNA sequencing, each plasmid was sequenced with forward, reverse and internal primers.

DNA Lasergene software 7.1 (DNA Star Inc., USA) was used for editing raw DNA sequences (EditSeq module), with SeqMan module used for contig formation and ClustalW module for sequences alignment. DNA sequences of pvrbp-2 obtained from field isolates of *P. vivax* were deposited in GenBank (JN872360-JN872372).

**Results**

**Identification of genetic polymorphism using PCR-RFLP method**

A total of 90 *P. vivax* samples were analyzed where in all samples gave single clear amplification of ~2.0 kb fragment size and none of the PCR fragments showed size variation (Figure 3a). Amplified PCR fragment covers both coding and non-coding regions. The coding regions are marked by a 449–503 bp and 705–1946 bp

![Figure 1 Map of India showing study sites. N indicates number of sample from individual geographical region.](image)

![Figure 2 Diagrammatic representation of primers location on pvrbp-2 gene. Gray and black boxes indicate intron and exon respectively, and arrows indicate location of primers. F and R: forward and reverse primers of primary PCR respectively, NF and NR: forward and reverse primers of nested PCR respectively. IR1 and IR2 are internal sequencing primers.](image)
in the amplified PCR product (Figure 2). Virtual restriction mapping of pvrbp-2 sequence suggests the use of Apol and Alul restriction enzymes for RFLP analysis. Initially, five samples were digested with the above two enzymes to make sure that these enzymes can identify genetic polymorphism from field isolates. Interestingly, genetic polymorphism in Alul and Apol digestion was observed in selected five samples. Further, PCR products obtained from 83 P. vivax isolates were digested with Alul and Apol enzymes separately. RFLP pattern of pvrbp-2 gene with Alul and Apol has been shown in figure 3b & c respectively.

A substantial number of RFLP pattern for both enzymes were observed with respect to the Sal-1 strain based pvrbp-2 gene sequence. In total, 13 distinct Alul and 30 distinct Apol RFLP patterns were observed among 83 samples. RFLP analysis revealed mainly two distinct digestion patterns in field isolates by both enzymes. This suggests that each enzyme has two major types of digestion pattern. RFLP pattern of six samples was confirmed by DNA sequencing. Among six samples, DNA sequences of five samples were in agreement with RFLP data, however in a single sample (Pv-7) RFLP pattern of only Apol enzyme was not matched. This may be due to the sequencing of only one clone from each cloning experiment. The numbers of RFLP pattern of individual enzymes from all samples are listed in Table 1. The frequencies of Alul and Apol genotypes varied in field isolates (Figure 4). Further, combination of Alul and Apol RFLP patterns revealed a total of 36 distinct haplotypes/genotypes suggesting a high degree of genetic diversity in pvrbp-2 sequences in the field isolates of P. vivax.

Multiple infection and population genetic structure
In brief, if a mono-infection sample (infection of single species) show a single genotype, it is denoted as single-clone infection, but if it shows more than one genotype, it is denoted as a multi-clone/multiple infection. RFLP analysis using Alul, showed seven isolates (8.43%) to have multi-clone infection. In contrast, Apol showed 13 isolates (15.66%) as multi-clone infection. In total, 14 isolates (16.86%) were observed to have multi-clone infection. Four multi-clone samples were having both Sal-1 and Belem alleles, however remaining ten multi-clone isolates were infected with different genotypes of either Sal-1 or Belem alleles.

Genetic polymorphism was observed among all geographical regions of the Indian subcontinent. The total number of genotypes observed in Delhi, Nadiad, Panna, Rourkela, Chennai and Kamrup were 11, 14, 12, 7, 7 and 4 respectively. In every geographical region, genotypes were observed to be unique (local polymorphism) and shared in varied proportion (Figure 5). Allelic analysis shows a limited fraction of genotypes were observed to have been shared within 2–3 populations. Only a single genotype (14) was observed in five geographical regions, however, none of the shared genotypes was observed between six geographical regions. This study suggests a diverse pool of pvrbp-2 repertoire in all geographical regions. This study also uncovered many unique pvrbp-2 genotypes to exist among geographical regions.

DNA sequence polymorphism
To understand and support the high degree of genetic diversity observed in PCR-RFLP analysis in pvrbp-2, 13 random samples (Nadiad; 7, Delhi; 1, Kamrup; 2, and Panna; 3) were sequenced, of which six isolates were RFLP analyzed. DNA sequence analysis also revealed a high degree of genetic polymorphism such as indels/tandem repeats and single nucleotide polymorphism (SNPs) among field isolates of P. vivax. Two indels were found which were restricted to non-coding region. The tandem repeat consisted of six amino acids (PA/TT/VQKK).
revealed as 0–3 repeats in field isolates. A total of 178 SNPs were found, out of which 32 were in non-coding region while the remaining were in coding region. The observed higher number of SNPs was mainly due to the dimorphism between \textit{Sal-1} and \textit{Bellem} type alleles. Number of non-synonymous substitutions in coding region was higher (n=106) as compared to synonymous substitutions (n=46), which indicates that \textit{pvrbp-2} is under positive selection pressure. None of the SNP (synonymous or non-synonymous) was associated with frame shift mutation. Comparison of \textit{pvrbp-2} sequences from Indian field isolates with \textit{pvrbp-2} reference sequence (\textit{Sal-1}: \textit{P. vivax} strain) suggests a higher degree of DNA sequence polymorphism.

| Sample | \textit{Alu} RFLP Pattern (bp) | \textit{Alu} Genotype | Combined Genotype/number of isolates | Apol Genotype | Apol RFLP Pattern (bp) |
|--------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Pv-2   | 1200 410 280 90 | 1               | 1/8             | 1               | 650 450 180 170 160 140 120 80 |
| Pv-7   | 1200 430 280 90 | 2               | 2/2             | 2               | 650 180 170 160 130 120 |
| Pv-14  | 1500 1200 410 380 280 | 3       | 3/1             | 3               | 800 650 450 410 250 180 120 |
| ND-52  | 1500 380 80     | 4               | 4/2             | 4               | 800 410 250 180 120 90 |
| ND-51  | 1200 430 280 90 | 2               | 5/5             | 5               | 650 480 180 170 160 140 130 120 |
| ND-54  | 1200 410 280 180 90 | 5       | 6/1             | 6               | 650 450 180 170 160 140 130 120 |
| M-53   | 1500 380       | 6               | 7/3             | 7               | 800 410 250 180 120 |
| M-54   | 1200 430 380 90 | 7               | 8/1             | 7               | 800 410 250 180 120 |
| M-55   | 1200 430 280 90 | 2               | 9/1             | 8               | 850 800 750 180 170 120 80 |
| ND-57  | 1000 430 280 180 90 | 8       | 10/2            | 9               | 800 650 180 170 120 |
| RR-1   | 1200 430 280 90 | 2               | 11/2            | 10              | 800 480 180 170 140 120 80 |
| RR-18  | 1200 430 380 80 | 7               | 12/1            | 11              | 650 450 250 180 160 120 90 |
| M-51   | 1200 430 280 90 | 2               | 13/1            | 12              | 650 180 170 160 130 120 80 |
| M-57   | 1200 410 280 90 | 1               | 14/2            | 13              | 800 480 180 170 140 120 |
| M-58   | 1500 380       | 6               | 15/10           | 14              | 800 410 250 180 120 80 |
| M-59   | 1200 410 280 90 | 1               | 16/4            | 15              | 650 450 180 170 160 140 120 |
| ND-1   | 1200 430 280 90 | 2               | 17/3            | 16              | 650 480 180 170 160 140 120 |
| ND-2   | 1200 430 280 90 | 2               | 18/1            | 13              | 800 480 180 170 140 120 |
| ND-8   | 1200 430 280 90 | 2               | 19/1            | 17              | 650 180 170 160 120 |
| ND-12  | 1200 430 280 90 | 2               | 20/1            | 18              | 800 650 180 170 130 120 |
| ND-14  | 1200 430 410 280 90 | 9       | 21/1            | 19              | 800 650 450 180 170 140 130 120 |
| ND-29  | 1500 380 80     | 4               | 22/10           | 14              | 800 410 250 180 120 80 |
| P-5    | 1500 380 80     | 4               | 23/1            | 21              | 650 410 250 180 160 120 80 |
| R-12   | 1200 410 280 90 | 1               | 24/2            | 22              | 650 600 180 170 160 120 |
| R-18   | 1200 430 280 90 | 2               | 25/2            | 23              | 800 480 180 170 140 130 120 |
| A-385  | 1500 1200 430 380 280 | 10     | 26/1            | 24              | 800 650 410 250 180 170 120 |
| R-37   | 1200 430 280 90 | 2               | 27/1            | 25              | 800 650 480 180 170 120 |
| D1     | 1200 410 280 90 | 1               | 28/2            | 6               | 650 450 180 170 160 140 130 120 |
| D5     | 1200 430 280 90 | 2               | 29/2            | 26              | 650 600 180 170 160 130 120 |
| S2     | 1500 380       | 6               | 30/2            | 27              | 800 650 250 180 120 |
| S4     | 1200 430 280 90 | 2               | 31/1            | 28              | 650 450 180 170 160 140 130 120 |
| C1     | 1200 410 280 90 | 1               | 32/1            | 29              | 600 180 170 160 130 120 |
| C2     | 1200 410 280 90 | 1               | 33/1            | 28              | 600 450 180 170 160 140 130 120 |
| C5     | 1200 430 380   | 11              | 34/1            | 30              | 800 450 250 180 120 80 |
| C6     | 1500 1200 430 410 280 | 12     | 35/2            | 3               | 800 650 450 410 250 180 120 80 |
| C8     | 1200 400 380   | 13              | 36/1            | 14              | 800 410 250 180 120 80 |
Distinguishing Belem and Sal-1 alleles with RFLP

The virtual restriction mapping of pvrbp-2 sequences AluI and Apol enzymes reveals a distinct RFLP pattern of Belem and Sal-1 alleles. Virtual restriction mapping of pvrbp-2 with AluI revealed a distinct 1500 bp and 380 bp fragments for Belem allele. Similarly, virtual restriction mapping with Apol showed a distinct 250 bp fragment for Belem allele. The results of virtual restriction mapping of Belem and Sal-1 pvrbp-2 sequences with AluI and Apol enzymes were confirmed with RFLP analysis of field isolates. On the basis of RFLP patterns, all samples were categorized according to the Sal-1 and Belem type. Of the 83 P. vivax isolates analyzed, 38.55% (32/83) were Belem type, 56.63% (47/83) were Sal-1 type, and 4.82% (4/83) were mixed of both alleles (Table 2). Furthermore, comparison of RFLP pattern showed Sal-1 alleles to be more polymorphic (24/36) than Belem allele (12/36) in the natural parasite populations. Thus, dimorphism observed in sequence analysis could also be identified by simple PCR-RFLP method.

Discussion

Malaria eradication program is facing remarkable challenges due to spread of drug resistance and the complex population genetic structure of human malaria parasites. Gaining an insight into the genetic population structure of the parasites would provide valuable information for designing an improved malaria control strategy. The present study investigates genetic polymorphism in pvrbp-2 among field isolates of P. vivax using simple PCR-RFLP.

This is the first population based study of pvrbp-2 gene which revealed a high degree of polymorphism in field isolates of P. vivax. The sequence polymorphism reported in pvrbp-2 from four strains of P. vivax including Sal-1 and Belem [22] is supporting the extent of genetic polymorphism observed in pvrbp-2 in Indian isolates. The sequences of pvrbp-2 have shown a distinct dimorphism between Sal-1 and Belem alleles [22]. The dimorphism between Sal-1 and Belem strains of P. vivax has been reported earlier on the basis of pvmsp-1 [25].
Conclusions
The study suggests that pvrbp-2 is highly polymorphic genetic marker which can be used for population genetic analyses. RFLP analysis suggests presence of nearly similar proportion of Sal-1 and Belem alleles in Indian P. vivax populations. The larger extent of genetic polymorphism identified from limited samples advocates to screen genetic polymorphism in pvrbp-2 from malaria endemic geographical regions and countries for designing pvrbp-2 based anti-malarial control measures.

Additional file

Additional file 1: Detail information about study sites.

Competing interests
Authors declare that they don’t have competing interests.

Author’s contribution
SKP: Conceptual designing, experimental design and work, data analysis and manuscript writing, PK: Experimental work and data compilation, OPS: Overall supervision and manuscript writing. All authors read and approved the final manuscript.

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Table 2 Distribution of Pvrbp-2 based Sal-1 and Belem alleles in field isolates

| Geographical regions | Sample size (N) | Sal-1 | Belem | Both |
|----------------------|----------------|-------|-------|------|
| Delhi                | 13             | 8     | 5     |
| Nadiad               | 21             | 17    | 4     |
| Panna                | 18             | 7     | 11    |
| Rourkela             | 16             | 10    | 4     | 2    |
| Chennai              | 10             | 3     | 5     | 2    |
| Kamrup               | 5              | 2     | 3     |
| Total (n)            | 83             | 47    | 32    | 4    |

and the distinction between Sal-1 and Belem strains is entirely based on geographical location and allelic variation. The RFLP analysis of the present study using AluI and Apol enzymes revealed a high degree of genetic polymorphism among field isolates which was further supported by pvrbp-2 nucleotide sequence polymorphism data. From RFLP analysis, it is clear that Apol is identifying larger extent of genetic polymorphism in field isolates compared to AluI. This suggests that under limited resources, Apol alone can be used to resolved larger extent of existing genetic variation in pvrbp-2 in the field isolates. The genetic polymorphism displayed by various antigen-encoding genes and biochemical marker in Indian field isolates of P. vivax [26–32] is also supported by the genetic polymorphism observed in pvrbp-2.

Plasmodium vivax isolates from Indian subcontinent represents diverse pool of genetic variants such as Belem and Chesson alleles in pgm-1 [23], Belem and Sal-1 alleles in pvmsp-1 [30], and VK210 and VK247 in pvcsps [30]. Though, pvrbp-2 based Sal-1 and Belem alleles have not been identified from natural parasite populations, however present study uncovered both alleles in Indian P. vivax populations. As like other above genetic markers, pvrbp-2 also harbors both Sal-1 and Belem alleles in Indian populations however, their proportion varied between geographical regions.

Pvrbp-2 is a promising vaccine target for the development of effective anti-malarial control measure [20]. Identifying allelic polymorphism in pvrbp-2 within and between populations would certainly improve and extend the existing knowledge for development of anti-malaria control measure. The significance of this prospective study would be to uncover maximum number of hidden polymorphism. Several studies in recent past have shown many polymorphic forms in local population [10,12,31,33]. This study revealed genetic polymorphism in P. vivax populations which have been rarely shared between more than two populations which suggests that in the natural population, pvrbp-2 is diverse and this calls for thorough care to be taken while designing any anti-malarial strategy targeting pvrbp-2.
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