Imperfect Duplicate Insertions Type of Mutations in Plasmepsin V Modulates Binding Properties of PEXEL Motifs of Export Proteins in Indian *Plasmodium vivax*

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Abstract

**Introduction:** Plasmepsin V (PM-V) have functionally conserved orthologues across the *Plasmodium* genus who’s binding and antigenic processing at the PEXEL motifs for export about 200–300 essential proteins is important for the virulence and viability of the causative *Plasmodium* species. This study was undertaken to determine *P. vivax* plasmepsin V Ind (PvPM-V-Ind) PEXEL motif export pathway for pathogenicity-related proteins/antigens export thereby altering *plasmodium* exportome during erythrocytic stages.

**Method:** We identify and characterize *Plasmodium vivax* plasmepsin-V-Ind (mutant) gene by cloning, sequence analysis, in *silico* bioinformatic protocols and structural modeling predictions based on docking studies on binding capacity with PEXEL motifs processing in terms of binding and accessibility of export proteins.

**Results:** Cloning and sequence analysis for genetic diversity demonstrates PvPM-V-Ind (mutant) gene is highly conserved among all isolates from different geographical regions of India. Imperfect duplicate insertion types of mutations (SVSE from 246–249 AA and SLSE from 266–269 AA) were identified among all Indian isolates in comparison to *P. vivax* Sal-1 (PvPM-V-Sal 1) isolate. In *silico* bioinformatics interaction studies of PEXEL peptide and active enzyme reveal that PvPM-V-Ind (mutant) is only active in endoplasmic reticulum lumen and membrane embedding is essential for activation of plasmepsin V. Structural modeling predictions based on docking studies with PEXEL motif show significant variation in substrate protein binding of these imperfect mutations with data mined PEXEL sequences. The predicted variation in the docking score and interacting amino acids of PvPM-V-Ind (mutant) proteins with PEXL and lopinavir suggests a modulation in the activity of PvPM-V in terms of binding and accessibility at these sites.

**Conclusion/Significance:** Our functional modeled validation of PvPM-V-Ind (mutant) imperfect duplicate insertions with data mined PEXEL sequences leading to altered binding and substrate accessibility of the enzyme makes it a plausible target to investigate export mechanisms for *in silico* virtual screening and novel pharmacophore designing.

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Introduction

Malaria, a global parasitic disease, caused by *Plasmodium* species, affects approximately 300 million people throughout the tropical and subtropical countries and causes considerable morbidity and mortality with estimated 800,000 deaths worldwide each year [1]. *Plasmodium falciparum* and *Plasmodium vivax* are considered as the two important human malaria parasites. *Plasmodium falciparum*, a virulent form of malaria, is responsible for 1 to 2 million deaths annually, mostly in children under the age of 5 years. *Plasmodium vivax* is responsible for 50–60% of all malaria cases in Western Pacific and South East Asian countries of which India is a major contributor to this burden [2,3]. Although, in comparison of *P. falciparum* the deaths due to *P. vivax* are rare, however socio-economic impact of *P. vivax* malaria is enormous [4] and several recent reports recognized *P. vivax* induced malaria as a severe and fatal malaria [5–9]. Furthermore, in light of the emergence of chloroquine and multidrug resistance in *P. vivax* malaria [5,10] and emergence of *P. vivax* strains with lower sensitivity to recent antimalarial therapy [11] there is an urgent need to develop a control strategy to identify new targets for human malaria parasite *Plasmodium vivax*.

The manifestation of malaria is heavily linked to the growth and development of the virulent form of *Plasmodium* inside the infected erythrocytes. In order to overcome the host responses, *Plasmodium* remodels red blood cell architecture and machinery, allowing the
export of hundreds of effector proteins beyond the parasitophorous vacuole membrane (PVM) [9–14]. Among the variety of Plasmodium effector enzymes, the family of aspartic proteases (plasmepsins) plays a key role in a wide variety of cellular processes including the export of plasmodium proteins which are essential for malaria parasite growth/survival and have been considered as promising targets for the development of novel chemotherapeutics [15–19].

The primary analysis of Plasmodium falciparum genome has led to the identification of at least 10 members of aspartic proteases (plasmepsins) family of proteins [18]. In contrast to P. falciparum, P. vivax genome sequence database analyses have shown that P. vivax has 7 orthologues of plasmepsins, PfPM-IV–PfPM-X [20–21]. Although similar to the P. falciparum, plasmepsins of P. vivax have also been considered as most promising anti-malarial drug targets, however because of the lack of in vitro culture system, the relative role of plasmepsins has not been yet fully examined in P. vivax. In this context, we have recently examined the structural properties and conservation of PfPM-IV in P. vivax from Indian isolates [22].

Unlike other plasmepsins, plasmepsin V, IX and X are not located in the food vacuole and plasmepsin-V is a unique and highly specialized aspartic protease with specific localization and function [14]. Fractional and solubilization experiments have demonstrated that plasmepsin-V is an integral membrane protein and it is distinct from those previously characterized plasmepsins. Plasmepsin-V is believed to be involved in the processing of the PEXEL motif (Plasmodium Export Element) and is essential for protein/antigen export [23]. PEXEL, a conserved and N-terminal amino acid motif, when cleaved and acetylated in the endoplasmic reticulum translocates proteins into the host cells [13,24, and 25]. Recent studies suggest that PfPM-V is a PEXEL protease, which could be a unique antimalarial drug target against P. falciparum infection. However, in case of P. vivax, such studies on the PfPM-V are limited and need attention to examine the genetic, structural and functional properties.

In this study, we examined genetic polymorphism, molecular nature and structural properties of P. vivax PfPM-V gene isolated from different geographical regions of India in order to determine if this export pathway are conserved in Plasmodium vivax. We performed an extensive in silico analysis to compare substrate binding with data mined PEXEL sequences from P. vivax exported proteins in order to develop an experimental system for studying functional modeled validation of these export processes to understand underlying effect of mutations on the activation of enzyme in ER without N-terminal processing as reported previously [14,26]. Our molecular and in silico studies add support for conservation of export pathway in P. vivax and predict a new putative plausible mechanism of immune evasion by P. vivax. Our results show that a variation in antigenic processing might be a key for emergence of more virulent type strains of P. vivax as differential antigen profile is known to be involved in immune evasion. PfPM-V based functional prediction data provides new insights into the design of new chemotherapeutic agents and diagnostic markers against malaria vivax infection.

Materials and Methods

Study Design

The study was carried out on P. vivax samples from different geographical regions of India to evaluate a plausible role of PfPM-V-Ind gene in genetic, structural and functional terms. This study was performed in three sequential steps namely molecular (genetic diversity and phylogenetic analysis), in silico structural analysis, PEXEL motif selection and docking studies with known inhibitors.

The study was conducted under the protocol reviewed and approved by the Institutional Scientific Advisory Committee (SAC) and Institutional Human Ethical Committee. Written informed consent was obtained from all the volunteers prior to the collection of P. vivax positive blood samples and human subject’s guidelines were followed. This manuscript is approved by Institutional publication committee having approval number 019/2012.

Study area and patient selection

The present study was conducted in seven different geographical regions of India having different topographical habitats viz. Bangalore, Chennai, Delhi, Goa, Nadiad, Rourkela and Sonapur as depicted in our earlier report [22]. The Centers selected for the study also had different international exposure i.e. Delhi, Chennai & Bangalore being urban commercial centers, Goa an international tourist destination and Nadiad, Rourkela & Sonapur suburban cities with low migratory population flux. P. vivax-infected patients, who were willing to participate and fitted the enrolment criteria [22] as per our earlier report, were included in this study as per WHO protocol [27].

P. vivax sample collection

P. vivax +ve blood samples were collected from patients (either sex) who were visiting NIMR Malaria Clinics in seven different geographical regions of India as described in our earlier report [22]. Briefly, the blood samples were screened microscopically (thick and thin smears) for the presence of P. vivax +ve malaria. 2–3 drops of finger prick blood from the patients having a minimum parasitaemia (0.05 to 0.5%) were collected on 3-mm filter paper (Whatman International Ltd., Maidstone, UK) as per our earlier report [22].

DNA amplification and PCR cloning

P. vivax genomic DNA was extracted from the blood samples collected on filter paper using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). For PCR amplification, a set of PfPM-V gene specific primers of PfPM-V (5′-ATGGTGGAG- GAGTTGGGGCCTGGGT-3′ and 5′-CTACG- CATCGCCGCGGGGCGCTGTCGCGGAGG-3′), were used [28] to amplify a complete gene sequence. Furthermore, another pair of specific primers targeting specific smaller segment of gene were also designed i.e. PfPM-V-5.2 (5′-GGGCGATTTGAGGAGTGCCTTCCT TTGCGACGACG-3′) and 5′-CGTTTGCTCATCTT- CAATCGCTTAT-3′. The PCR products were resolved on a 1% agarose gel.

Cloning of PfPM-V gene and sequencing

PfPM-V-Ind PCR amplified products were gel-purified by using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), ligated into the P derived cloning vector (Qiagen, Hilden, Germany) and transformed into competent E. coli DH5α cells as per the manufacturer’s recommended protocol. Positive recombinant clones were sequenced in both directions on an ABI 3730 Genetic Analyzer (PE Applied Biosystems). For sequence validation, two independent sequencing reactions of each clone were performed.

Sequence homology and Phylogenetic analysis

All nucleotide sequences of P. vivax PfPM-V-Ind isolates encoding each gene were submitted to NCBI GenBank (accession numbers GU569930 to GU569933) for public domain use. The verified sequences were translated to amino acid and aligned with PfPM-V of different regions of India to mark functional domain.
The translated protein sequences of \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\). \( P\)
PvPM-V-Ind is a highly conserved single copy aspartic protease gene having imperfect duplication insertions

In order to examine the molecular nature and genetic diversity of the PvPM-V, the PCR amplified products from different geographical regions of India were sequenced and analyzed through comparative bioinformatics analysis. This gene carries an open reading frame of 1635 bp, which is predicted to encode proteins of 544 amino acids, having a pro-domain region from 1–50 amino acids, a hing region in pro-domain from 45–48 AA, active side pocket from 77 to 88 and 318 to 329 AA with aspartic residues at 80 and 321 AA, aspartic protease signature at 226 to 239 and 438 to 453 AA, Tran-membrane domain from 497 to 529 AA and C-terminal tail from 529 to 544 amino acids (Fig. 1 and Fig. 2). We were unable to notice any polymorphism among any sequenced Indian isolate for the PvPM-V-Ind gene which showed a 100% amino acid sequence identity, indicating high degree of conservation among them. However, when we compared PvPM-V-Ind sequence with available P. vivax Sal-1 isolate which was used as control, interestingly we observed two unique mutations comprising insertions of three neutral and one acidic amino acids viz. SVSE from 246 to 249 positions and duplications of four amino acids i.e. SLSE from 266 to 269 positions, in all the Indian isolates tested (Fig. 1 and Fig. 2). The omnipresence of these mutations in PvPM-V-Ind gene in isolates from different geographical regions of India could imply that either Plasmodium vivax infections are all evolved from single ancestor isolated from rest of the world or Plasmodium vivax with

Figure 2. Clustal W multiple sequence alignment of plasmepsin-V orthologues within plasmodium genera. Plasmodium vivax PvPM-V-Ind is the mutant sequence from the Indian isolates. doi:10.1371/journal.pone.0060077.g002

Figure 3. Phylogenetic analysis (bootstrap) of plasmepsin -V using neighbor joining method. (A) Different orthologue sequences of plasmepsin-V from different pathogenic protozoan. Plasmodium vivax PvPM-V-Ind (PvPM-V-Ind) is the mutant sequence from the Indian isolates. (B) Cropped and zoomed in phylogenetic tree from Figure 3A showing Indian isolates to be a more evolved gene. doi:10.1371/journal.pone.0060077.g003
this polymorphism is more virulent and dominating emerging severe infections.

In order to measure the genetic relatedness and evolutionary events, we performed multiple sequence alignment analysis and compared *P*PM-V-Ind sequence with available plasmepsin-V sequences for other *Plasmodium* species in the database. *P*PM-V-Ind gene shows 98% homology with *P. vivax* Sal-1, 80% homology with *P. knowlesi*-strainH, 60% homology with *P. falciparum*, 58% homology with *P. yoelli*, [Fig. 2]. Plasmepsin-V multi sequence alignment analysis revealed that prodomain region of plasmepsin-V is most hyper variable among *Plasmodium* species 25–45 AA of *P*PM-V suggesting an absence of a conserved cleavage site while 45–65 amino acids were found to be highly conserved among all species of *Plasmodium*. Conserved and distinguished features of plasmepsin-V sequence have been summarized in the Figure 1.

Phylogenetic analysis suggested specialized role of this gene in *Plasmodium* genome as other apicomplexan like *Toxoplasma gondii* did not share much homology with *Plasmodium* plasmepsin-V. Though the orthologues had phylogenetic relationship similar to genomic phylogy but maximum similarity of plasmepsin-V to known orthologue was outside *Plasmodium* genera found less than 30% [Fig. 3A]. Other pathogenic protozoa did not show conservancy of plasmepsin-V orthologue in their respective genomes [Fig. 3A] however, this gene had distinct phylogeny in *Plasmodium* genera [Fig. 3B], not overlapping with other plasmepsins. Furthermore, the SPRING analysis showed that the polymorphism encountered in almost all Indian isolates is more evolved form of plasmepsin-V reported by previous workers. Sequence analysis suggests importance of this gene in *Plasmodium* genera as it is well conserved in the plasmodium genera but no conservancy with other closely related organisms.

**Figure 4. Structural cartoon representations of model of plasmepsin-V of *P. vivax*. (A) Complete model with C-terminal trans-membrane domain (purple), the n-terminal pro-domain (lime) inhibiting the complete active site cleft with aspartic acid residue (red) and side chains. (B) Displaying structural changes after the cleavage of c-terminal trans-membrane domain (purple). The N-terminal prodomain peptide (lime) frees complete active site cleft after folding at hinge and competitively interacts with same amino acid residue side chains of the model as that of C-terminal in the native structure. doi:10.1371/journal.pone.0060077.g004**

**Figure 5. Structural alignment of PEXEL domains.** Showing high structural consensus in the side chain topology in even dissimilar sequences. Brown PVX10368 (tsekdfsdkkeeyKFIEDsnfkyikelnwadc) PEXEL sequence and Purple PVX092305 (ksedlpskvdiklnKSLiDiIl-nynfnvndmgif) PEXEL sequence. doi:10.1371/journal.pone.0060077.g005
Out of these multiple events, trafficking across the PVM essentially requires additional sequence elements named *Plasmodium* export elements (PEXEL) [13]. Recently it has been shown that aspartyl protease plasmepsin-V activities are responsible for PEXEL processing in *P. falciparum* [23]. However, unlike *P. falciparum*, the functional studies on *P. vivax* plasmepsins have been very limited, primarily due to the lack in vitro culture. Therefore, to predict the possible functional properties and activation pathway of *P. vivax* plasmepsin-V, in the present study we took an opportunity of the available structural and functional database of *P. falciparum* plasmepsins and compared them with the predicted molecular model of *Pv* PM-V-Ind.

In order to examine the structural and functional relationship, we first compared the modeled structure of *Pv* PM-V-Ind isolate having C-score: −2.42, TM: 0.43±0.14, and RMSD: 13.4±4.1 Å with the *Pv* PM-V Sal-1 (i.e. wild) having C-score: −2.46, TM: 0.43±0.14, and RMSD: 13.5±4.1 Å. This analysis showed very compact structural similarities to other aspartic proteases and the N-terminal prodomain region of *Pv* PM-V seems to block active site, enabling higher substrate specificity of *Pv* PM-V. Therefore, in order to predict a model with more *in vivo* similarity where it could be embedded in the membrane; the transmembrane domain in C-terminal tail was cleaved off from the main model of *Pv* PM-V-Ind.

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Therefore, in order to predict a model with more *in vivo* similarity where it could be embedded in the membrane; the transmembrane domain in C-terminal tail was cleaved off from the main model in functional protein. The resulting models (*Pv* PM-V Sal-1 (i.e. wild type); C-score: −2.66, TM: 0.46±0.15, RMSD: 12.3±4.3 Å and *Pv* PM-V-Ind (i.e. mutant); C-score: −2.43, TM: 0.45±0.15, RMSD: 12.6±4.3 Å) showed an overall effect of transmembrane domain removal, enabling more stable than complete sequence models. In comparison to complete sequence model the most significant and consistent change observed in both tail deleted *Pv* PM-V Sal-1 (wild type) and tail deleted *Pv* PM-V-Ind (mutant) was folding of N-terminal pro-domain region at a hinge region (45–48 amino acids) and thus freeing active site pocket for substrate binding [Fig. 4A and 4B]. The N-terminal prodomain sequence after folding at hinge competitively interact with same amino acid residue side chains of the model as that of C-terminal in the native structure (Fig. 4b). The pocket formed by amino acids
cleavage once embedded in the ER membrane. In other reports, protein may becomes activated without self prodomain and somehow frees active site, indicating that prodomain could be involved in re-shuffling of prodomain to the tail deleted model.

Russo et.al [14] pulled down hsP70 of ER with recombinant hsp70 of ER with recombinant protein which could be involved in re-shuffling of prodomain to free active site in membrane embedded protein [14]. Earlier studies [26] in P. falciparum plasmepsin-V showed the presence of plasmepsin-V in ER as well as in cytosol, while the activity was shown specifically in the ER only [24]. Taken together all this information, it can be postulated that embedded C-terminal protein part in the membrane, brings about some structural changes mediated by ER chaperons rendering it active. Russo et al [14] has also showed the activity with uncleaved GFP tagged protein while in contrast Boddey et al [23] failed to show activity in native protein suggesting GFP tag interferes in pro-domain structure and somehow frees active site, indicating that prodomain may be involved to inhibits enzyme function and as per other studies, protein may becomes activated without self prodomain cleavage once embedded in the ER membrane.

**Table 1.** The predicted variations in the interacting amino acid residues side chains of P.vivax PM-V Sal-1 (wild) and P.vivax PM-V-Ind (mutant) with PEXEL and only known inhibitor Lopinavir.

| Legend | Interacting amino acid residues side chains for P.vivax PM-V Sal-1 (Wild)| Interacting amino acid residues side chains for P.vivax PM-V-Ind (Mutant) |
|--------|-------------------------------------------------|-------------------------------------------------|
| PEXEL  | KR: 78 ILE, 80* ASP, 139 TYR, 188 VAL, 315 GLY, 342 ASN, 374 SER, 376 ILE, 424 SER, 426 TRP, 427 CYS, 428 LYS, 439 ILE | KR: 78 ILE, 80* ASP, 139 TYR, 180 PHE, 188 VAL, 315 GLY, 342 SER, 344 ASN, 410 ARG, 474 ARG |
|        | GALVLMIFWPSTCYNQ: 83 SER, 286 TYR, 313* ASP. | GALVLMIFWPSTCYNQ: 83 SER, 294 TYR, 321* ASP, 442 VAL. |
|        | LI: 430 ILE, 434 VAL. | LI: 430 ILE, 434 VAL. |
|        | DEQ: 85 SER, 135 TYR, 137 GLN, 197 GLN. | DEQ: 85 SER, 135 TYR, 137 GLN, 167 ARG, 197 GLN. |
|        | Lopinavir 58GLU, 60ALA, 61TYR, 78ILE, 80*ASP, 139TYR, 140CYS, 141GLU, 176GLU, 178SER, 179LEU, 183GLN, 315GLY, 317THR, 318PHE, 320HIS, 341MET, 342ASN, 374SER, 376ILE, 424SER, 426TRP, 427CYS, 428LYS, 439ILE | Lopinavir 58GLU, 60ALA, 61TYR, 78ILE, 80*ASP, 139TYR, 140CYS, 141GLU, 176GLU, 178SER, 179LEU, 183GLN, 315GLY, 317THR, 326PHE, 328HIS, 349MET, 350ASN, 382SER, 384ILE, 430LYS, 432SER, 434TRP, 463LYS, 447ILE. |

Variations in the interacting amino acids speculate that a modulation in the activity of plasmepsin-V might have resulted from imperfect duplicate mutations.

Mutations in P.vivax PM-V-Ind shows significant effect on PEXEL motif substrate specificity and known inhibitor binding

Next, we attempted to predict the possible involvement of two unique insertions/mutations on the substrate binding activity of P.vivax PM-V-Ind. The plasmepsin-V structure shows that the active site is quite large and canal like for binding peptide substrates. Therefore, we predict that a small change in the sequence may affect active site architecture, thereby modifying binding and processivity of resulting enzyme. As PEXEL peptides are known substrates of plasmepsin-V therefore in silico molecular interaction studies of PEXEL peptide and active enzyme may reveal differences in the active site/substrate binding domains. Although, PEXEL peptides have low sequence similarity among themselves, however they always tends to form a right handed helix which could be structurally superimposed displaying high similarity [Fig. 5]. Modeled PEXEL peptides docked in central canal like active sites, the target site of cleavage i.e. between third and fourth AA of PEXEL motif clearly exposed to active aspartyl side chains [Fig. 6A and 6B]. Docking analysis showed unique changes/variation in the interacting amino acids (Table 1). Further, in order to compare the P.vivax PM-V-Ind (mutant) and P.vivax PM-V Sal-1 (wild) active sites, the comparative docking scores of all the five tight PEXEL [Table 2] and lose PEXEL were tabulated [Table 3]. The docking scores of

| Gene       | PEXEL sequences* | Position | Global energy (Wild)/(PvPM-V Sal-1) | Global energy (Mutant)/(PvPM-V-Ind) | ACE Native (PvPM-V Sal-1) | ACE Mutant (PvPM-V-Ind) |
|------------|------------------|----------|-------------------------------------|-------------------------------------|----------------------------|-------------------------|
| PVX_102130 | ssslcmsnislaekhKSLMQeckedlnlmpaltn | 171–175 | −56.26 | −65.32 | −17.64 | −4.72 |
| PVX_107755 | eyevklikaddddyKNICDiegscldntkdvai | 79–83 | −50.44 | −17.90 | −9.99 | −13–32 |
| PVX_108770 | eaddetuestosgqRIIFEhlpayfeqflikeda | 27–31 | −60.33 | −77.43 | −11.14 | −12.08 |
| PVX_09447 | vastetveikkkKyKIIQLhpdkshiseeek | 30–34 | −66.04 | −63.55 | −8.33 | −12.79 |
| PVX_115455 | sldadafqielpngRTLAEketskaqeqgffe | 85–89 | −65.64 | −36.21 | −12.14 | −7.90 |

*The five true PEXEL used for the docking studies displaying the gene number and position of PEXEL motif on the gene. The true PEXEL used in this study tightly follows the true PEXEL formula i.e. [KR][GAVLMIFWPSTCYNQ][LI][GAVLMIFWPSTCYNQ] [DEQ]--.

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**Table 2.** The comparative docking score of true PEXEL with P.vivax PM-V Sal-1 (wild) and P.vivax PM-V-Ind (mutant).
PvPM-V-Ind (mutant) and PvPM-V Sal-1 (wild type) clearly shows a lot of variation in Global energy as well as in ACE scores in case of both tight as well as loose PEXELs. In order to further analyze the effect of insertion/mutation on the active site domain of PvPM-V-Ind, only known inhibitor of plasmepsin-V i.e lopinavir [14] was molecularly docked with plasmepsin-V tail delete models [Fig. 7].

### Table 3. The comparative docking score of loose PEXEL with PvPM-V Sal-1 (wild) and PvPM-V-Ind (mutant).

| Gene          | PEXEL sequences*                                      | Position | Global energy | ACE Native | ACE Mutant |
|---------------|-------------------------------------------------------|----------|---------------|------------|------------|
| PVX_118695    | llkdkiqkkinkmIGIKQHglqmndlfngnldk                    | 113–117  | −63.24        | −6.97      | −4.88      |
| PVX_112630    | lyylhtihilddttlyKKLDEnvkdpsikietcsf                  | 37.41    | −69.85        | −12.11     | −15.21     |
| PVX_107750    | nmnknkknmnknknmNYIDDrgrvntefcmdn                    | 173–177  | −57.54        | −11.33     | −11.08     |
| PVX_104190    | skdkeiskdkiskKKLDINknezatyddikhr                   | 57–61    | −75.92        | −5.41      | −8.06      |
| PVX_103660    | pdkellinylqkelKDLFDffedyqmgkkkeian                  | 166–170  | −91.1         | −17.74     | −7.86      |

*The loose PEXEL used in this study follows the PEXEL formula i.e. [KR][LI][DEQ], where capital letters denote individual amino acids, multiple amino acids in brackets[XZ] represent ambiguity in pattern and full stop (.) means any amino acid.

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**Figure 7. Structural representations with Lopinavir interactions.** (A) Displaying active site of the PvPM-V Sal-1 (wild type) and its interaction with only known inhibitor lopinavir (red) (B) Displaying active site of the PvPM-V-Ind (mutant) and its interaction with known inhibitor lopinavir (red). The docked lopinavir (red) showing interaction with hydrophobic amino acid residue (white) of the active site, while hydrophilic residues: acidic (red), Basic (blue) and neutral (green) comprise of the docking site. There are clear overall changes in structure as well as in interacting amino acids with the ligand.

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Docking analysis again showed unique changes/variation in the interacting amino acids (Table 1) and docking scores for PmPV-Sal1 (wild type) and PmPV-Ind (mutant) structures (Fig. 7a & 7b). Therefore, the predicted variation in the docking score and interacting amino acids of PmPV-Sal1 (wild) and PmPV-Ind (mutant) proteins with both PEXEL and lopinavir suggests a modification in the activity of PmPV-Ind might have resulted from this mutation.

Various reports from India have shown lower sensitivity of rapid diagnostic tests (80–85%) [42] which are based on erythrocytic stage antigens. This could be a result of this type widespread plasmsin-V polymorphism or selection of this polymorphism by impairing diagnosis. Plasmsin-V may be a unique drug target, as it is conserved in all Plasmodium species and is also a single copy essential gene. The consensus architecture of PEXEL side chains can be used to design a novel inhibitor/pharmacophore specific to PmPV. Similar polymorphisms may be screened in Plasmodium falciparum/ cultivable parasites as it has not been done so far & could reveal mutualional impact of PmPV on antigenic profile of mutants.

Conclusions

Genetic polymorphism of the PmPV could be a novel tactic to change antigenic profile as plasmsin-V has been shown to be key enzyme for antigenic protein export. The omnipresence of this imperfect duplicate insertions type mutations in different geographical regions of India (PmPV-Ind) could imply that either Plasmodium vivax infections are all evolved from single ancestor isolated from rest of the world or Plasmodium vivax with this polymorphism is more virulent and dominating emerging infections. Sequence analysis suggests importance of this gene in Plasmodium genera as it is well conserved in the same but no conservancy with other closely related organisms. Overriding host cellular functions by exporting as many as 200–300 proteins are a characteristic feature of Plasmodium sp. [43,45] thus a conservancy and essentiality of this gene is due to this specialized function. Exported proteins, as identified by the PEXEL motif, play a major role in Plasmodium virulence and facilitate the parasite’s survival in the host cell. Polymorphism in PmPV-Ind gene could possibly have a wider impact by favoring or limiting export of certain PEXEL proteins thereby changing the antigenic profile. Comprehensive knowledge of their diversity and evolution will help to unravel the emergence of the high pathogenicity of P. vivax, and may allow the identification of novel targets for malaria therapy.

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Author Contributions

Interpretation of data: MR YG PKT AS. Provided facilities and scientific environment for experimental work: AS. Read and approved the final manuscript: MR SV YG PKT AS. Conceived and designed the experiments: MR AS. Performed the experiments: MR SV. Analyzed the data: MR YG SV. Wrote the paper: MR YG PKT AS.

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