Comparative clinical transcriptome of *pir* genes in severe *Plasmodium vivax* malaria

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Abstract

Vir genes, a multigene family in *Plasmodium vivax* that are a part of a larger superfamily of genes called the *pir* (Plasmodium interspersed repeat) genes have been reported earlier to be potentially involved in cyto-adherence and evasion of splenic clearance. *Plasmodium vivax*, historically characterized as a "benign" malaria parasite, has been associated with clinical outcomes including hepatic dysfunction, renal failure, and cerebral malaria in India and several global regions. It constitutes an economic burden and presents a public health challenge alongside other Plasmodium species. Here, we present a part of global transcriptomic studies using custom designed microarrays, that compare the transcriptome of the parasite responsible for severe *Plasmodium vivax* manifestations, specifically hepatic dysfunction and cerebral malaria from India, with an emphasis on the *pir* genes, some of which are reported to play a role in cyto-adherence. 23 patients with *Plasmodium vivax* malaria (Uncomplicated=6, Hepatic dysfunction=12 and Cerebral malaria=5) were subjected to microarray hybridization and the data so obtained showed a wide range of *pir* subfamilies have been differentially expressed. Upregulation has been seen in 24 *pir* genes in cerebral malaria samples (n=5) and 28 genes in hepatic dysfunction samples (n=12) belonging to different subfamily in at least 50% of the patient samples. Out of the upregulated *pir* genes in cerebral malaria manifestation, members of *vir* subfamily E and *pvpir* H are maximum in number whereas in hepatic dysfunction manifestation, members of *vir* subfamily E and C comprise a significant proportion.

Keywords: *Plasmodium vivax*, *pir*, *vir*, *pvpir*, cerebral malaria, hepatic dysfunction, 15K microarray

1. Introduction

Malaria is a fatal disease known since ancient times to humanity (1). Five parasitic protozoa species from the genus Plasmodium: *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale,* and *Plasmodium knowlesi* causes this disease in humans (2). *P. vivax* is the primary cause of malaria outside of Africa and the second most common cause of malaria globally, after *P. falciparum* (3). An estimated 7 million persons are infected with *P. vivax* annually. In the past, *P. vivax* malaria was thought to be benign, especially in comparison to *P. falciparum* malaria. Over the past twenty years, there has been a significant increase in the reports of severe disease manifestations caused by *P. vivax* mono-infections (4–13). Severe disease manifestations by this parasite includes cerebral malaria (CM), hepatic dysfunction (HDYS), renal complication and others, as per the World Health Organization Criteria for severity (14).

In *P. vivax*, a multigene family known as the *vir* has been linked to the parasite's capacity for immune system escape and cytoadherence. These *vir* genes are a part of a larger superfamily of genes called the *pir* (Plasmodium interspersed repeat) genes (15). At first, 346 *vir* genes were found in the genome of *P. vivax*; these genes were primarily found in subtelomeric regions and were categorized into 12 subfamilies (16). Later, with the help of computational approaches this multigene family was redefined into *vir* genes; the newly defined *pvpir* genes (A, D and H subfamily); and the unclustered and unclassified genes (17). Over a 1000 *pir* genes have been found in *P. vivax* genomes thus far (18). Expression studies of these *pir* genes revealed that individual isolates in patients expressed a large number of *pir* genes from various subfamilies (17,19,20). This vast diversity of PIR antigens has been suggested as allowing the parasite to evade the host immune system (19) and some of these have been also hypothesized to help in aiding the parasite survival from splenic clearance (21). Studies on a few of the VIR proteins showed that they may have distinct subcellular locations and, thus, distinct roles. PVX_108770 of the *vir* subfamily C (22) and Pv11101_18 of the *vir* subfamily E (23) may be involved in mediating the parasite's cyto-adhesion phenotype.

Microarrays and RNA Sequencing are two examples of high throughput technologies that are frequently used for studying the genetic responses that underlie host-parasite interactions. Microarrays have been used in the past to study many biological processes in Plasmodium spp. (24,25) and have now become a universal tool for the study of the expression of thousands of genes (26,27). We have earlier, performed a series of custom designed 15K microarray

experimentations (28) for *P. vivax* field isolates causing severe or non-severe malaria. Intrigued by the enormous potential of *pir* genes as one of the key players in the pathogenesis of *P. vivax*, we have in the current study, analysed the expression patterns of *pir* genes for the first time in *P. vivax* isolates showing cerebral malaria (CM) and hepatic dysfunction (HDYS) manifestations.

2. Materials and methods

2.1 Patient details

We obtained blood samples from 23 patients with malaria caused by *P. vivax*. The categorization of severe malaria into cerebral malaria and hepatic dysfunction cases was according to WHO guidelines (WHO, 2015, 2010) (14). The patients were diagnosed as either complicated (PVC, n=17) or uncomplicated malaria (PVU, n=6). Under the complicated category (n=17), 12 were jaundice/hepatic dysfunction cases and 5 were cerebral malaria cases. Clinical profile of all the patients included in this study are presented in the Supplementary Table S1. All hepatic dysfunction cases had serum bilirubin >3 mg/dL and all cerebral malaria cases had Glasgow coma scale, ranging from 7 to 9, as per WHO criteria. All patients with severe malaria underwent appropriate blood tests to rule out viral hepatitis, dengue, typhoid, leptospirosis, infectious mononucleosis, and HIV. All the tests were negative, indicating that the reported symptoms were solely due to *P. vivax* infection.

2.2 Blood sample collection

Venous blood samples (~5 ml) were taken from *P. vivax* infected patients with their informed consent at S.P. Medical College, Bikaner, India, during the year 2007 to 2010. Microscopy and rapid diagnostic tests (RDTs) for malaria (OptiMal test, Diamed AG, Cressiersur Morat, Switzerland; Falcivax test, Zephyr Biomedical System, Goa, India) were used for a preliminary screening. Blood within 15 mins of collection was subjected to density gradient-based separation (Histopaque 1077, Sigma Aldrich, USA) to separate the peripheral blood mononuclear cells (PBMCs) from the infected and uninfected erythrocytes in accordance with the manufacturer's instructions. Both fractions were washed with phosphate buffered saline (PBS) twice and lysed with 4 volumes of Tri-reagent (Sigma Aldrich, USA) and kept at -80°C. Subsequently, samples were transported in cold chain to BITS Pilani and processed.

2.3 Total RNA preparation from patient's blood sample

Total RNA and DNA were extracted from complicated (n=17) and uncomplicated (n=6) malaria blood samples, following the manufacturer's protocol (Tri-Reagent, Sigma Aldrich, USA). The DNA was resuspended in 90 µl TE buffer and stored at -20°C. The RNA was resuspended in 90 µl of DEPC-treated deionized water and stored at -80°C. The quality of the RNA samples was assessed using RNA 6000 Nano Lab Chip on the 2100 Bioanalyzer (Agilent, Palo Alto, CA) and formaldehyde-based denaturing agarose gel electrophoresis. The quality and the quantity of the total RNA from samples were evaluated separately using NanoDrop® ND-1000 UV-Vis Spectrophotometer (Nanodrop technologies, Rockland, USA).

2.4 Diagnostic PCR to confirm Plasmodium vivax infection

The isolated DNA of all the 23 malaria-infected patients were assessed by 18S rRNA based multiplex PCR (29,30) to confirm *P. vivax* infection and rule out any chance of *P. falciparum* co-infection along with the results obtained from microscopic examination of PBF and RDTs. After the PCR reaction, the expected amplicon size for *P. vivax* would be ~500bp and if there were any *P. falciparum* co-infection, there would be an extra amplicon size of approximately 1400bp.

2.5 15K P. vivax 60mer microarray and experimentation

A total of 14342 validated probes representing 2160 and 4180 genes of *P. vivax* denoted in sense and antisense orientation was chosen and incorporated into the 15K microarray (28) from a previously custom-designed 244K microarray (31). 267 *pir* genes as per the classification of *Lopez et al.* (17) were represented in this microarray. The experimental procedures, normalization, and data processing have been described in detail by *Boopathi et al.* (28). Raw data from this microarray experimentation was submitted to GEO and is available under the series ID GSE301003 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE301003).

2.6 qPCR validation of microarray data

Quantitative real-time PCR was used to validate the microarray expression of a few genes (qPCR). Primer3 (32) was utilized to create the primers required for this purpose. The details of the primers are given in Table S2. DNA contamination was removed from total RNA, following the manufacturer's protocol using iScript™ gDNA Clear cDNA Synthesis Kit (BIO-RAD). The removal of DNA in RNA samples was verified by absence of DNA band in a 2% agarose gel after 40 cycles of PCR using seryl-tRNA synthetase primers. 500ng of total RNA

from uncomplicated (n=6; PVU-2, PVU-3, PVU-6, PVU-9, PVU-10 & PVU-16) and complicated (n=17; PVC-1, PVC-2, PVC-8, PVC-20, PVC-21, PVC-22, PVC-25, PVC-27, PVC-28, PVC-30, PVC-31, PVC-32, PVC-11, PVC-15, PVC-16, PVC-17 & PVC-18) samples was pooled at an equimolar concentration. Following the manufacturer's protocol, first strand cDNA synthesis was carried out using iScript™ gDNA Clear cDNA Synthesis Kit (BIO-RAD) in a total volume of 20µl. Quantitative real-time PCR was performed on a CFX Connect (BIO-RAD) real-time PCR cycler using iTaq Universal SYBR® Green Supermix (BIO-RAD). Seryl-tRNA synthetase gene was taken as an endogenous control. The PCR conditions of all the selected genes are given in Table S2. Log2 fold change expression was calculated to determine the status of the gene.

3. Results and Discussion

Plasmodium vivax, long described as a "benign" malaria parasite, has been linked to clinical consequences such as hepatic dysfunction, renal failure, and cerebral malaria in India and other regions of the world. To understand the severity of disease caused by *P. vivax*, the study of expression profile of *pir* genes is important as few of the *vir* genes have been reported earlier to be possibly involved in cytoadherence and evasion of splenic clearance. Here, in this study, with the help of a 15K custom designed microarray we analysed the difference between the expression pattern of *pir* superfamily members from patients with cerebral malaria (CM) versus hepatic dysfunction (HDYS) manifestation from Bikaner region of North-west India.

3.1 Analysis of the expression data of pir genes

In our analysis, we considered expression profile of *pir* genes in individual *P. vivax* severe malaria samples. There were 267 *pir* genes represented in the array. We applied a filtering criterion of transcript detection in at least 1 out of 11 samples in cerebral malaria samples and 1 out of 18 samples in hepatic dysfunction samples. A quantile normalization with baseline to median of control samples (*P. vivax* uncomplicated malaria samples; n=6) which resulted in differential expression profile of 260 *pir* genes in cerebral malaria samples and 259 *pir* genes in hepatic dysfunction cases (Supplementary sheet_2). The log2 fold change value of +1 was considered as upregulated and -1 was considered as downregulated. Based on the previous analysis by *Boopathi et al* (28) on other severe malaria samples, *pir* genes showing a 50% and above, upregulation and downregulation in patient samples have been considered in this study for both the manifestation.

On analysing upregulated *pir* genes, 24 genes in cerebral malaria samples (n=5) and 28 genes in hepatic dysfunction samples (n=12) belonging to different subfamily have been shown to be upregulated in at least 50% of the patient samples (Figure 1 and 2; Supplementary sheet_3). Under cerebral malaria manifestation members of *vir* subfamily B, C, E, and G; *pvpir* subfamily H and A as well as unclassified and unclustered genes have been upregulated in patients sample ranging from 60-80% (Figure 2; Supplementary sheet_3). Out of these 24 upregulated *pir* genes in cerebral malaria manifestation, members of *vir* subfamily E (33.33%) and *pvpir* H (25%) are maximum in number. Under hepatic dysfunction manifestation members of *vir* subfamily B, C, E, G, and I; *pvpir* subfamily H and A as well as unclustered genes have been upregulated in patients sample ranging from 50-91.7% (Figure 2; Supplementary sheet_3). Out of the 28 upregulated *pir* genes in hepatic dysfunction manifestation, members of *vir* subfamily E (28.57%) and C (21.42%) are maximum in number.

On analysing downregulated *pir* genes, 28 genes in cerebral malaria samples (n=5) and 34 genes in hepatic dysfunction samples (n=12) belonging to different subfamily have been shown to be downregulated in at least 50% of the patient samples (Supplementary sheet_3). Under cerebral malaria manifestation members of *vir* subfamily B, C, E, and G; *pvpir* subfamily H as well as unclassified and unclustered genes have been downregulated in patients sample ranging from 60-100% (Supplementary sheet_3). Out of these 28 downregulated *pir* genes in cerebral malaria manifestation, members of *vir* subfamily E (50%) and *pvpir* H (14.2%) are maximum in number. Under hepatic dysfunction manifestation members of *vir* subfamily C, E, G, and K; *pvpir* subfamily D and H as well as unclassified and unclustered *pir* genes have been downregulated in patients sample ranging from 50-91.7% (Supplementary sheet_3). Out of the 34 downregulated *pir* genes in hepatic dysfunction manifestation, members of *vir* subfamily E (64.7%) and C (8.8%) are maximum in number.

Further, microarray results of four *pir* genes were also confirmed by real-time qPCR experimentation shown in Figure 3. Real time qPCR experiment confirms upregulation of two genes (PVX_086865 in HDYS & PVX_097530 in CM) and downregulation of two gene (PVX_017645 in HDYS & PVX_104180 in CM) from array data (Supplementary sheet_3). Overall, real time qPCR analysis validated the expression pattern of selected genes as seen in the microarray data.

3.2 Comparison of the expressed pir genes between cerebral malaria and hepatic dysfunction cases

Out of the top 50% and above upregulated *pir* genes in severe malaria samples, 7 *pir* genes belonging to the *vir* subfamily C, G and E; *pvpir* subfamily H and unclustered are common among cerebral malaria and hepatic dysfunction cases (Figure 4a; Supplementary sheet_3). In cerebral malaria manifestation 17 *pir* genes belonging to the *vir* subfamily B, C, and E; *pvpir* subfamily A and H; unclustered and unclassified are unique (Figure 4a; Supplementary sheet_3). In hepatic dysfunction manifestation 21 *pir* genes belonging to the *vir* subfamily B, C, E, G, and I; *pvpir* subfamily A and H; unclustered are unique (Figure 4a; Supplementary sheet_3).

Out of the top 50% and above downregulated genes in severe malaria samples, 13 *pir* genes belonging to the *vir* subfamily C, E and G; *pvpir* subfamily H; unclustered and unclassified are common among cerebral malaria and hepatic dysfunction cases (Figure 4b; Supplementary sheet_3). In cerebral malaria manifestation 15 *pir* genes belonging to the *vir* subfamily B, C, E and G; *pvpir* subfamily H are unique (Figure 4b; Supplementary sheet_3). In hepatic dysfunction manifestation 21 *pir* genes belonging to the *vir* subfamily C, E, and K; *pvpir* subfamily D and H; unclustered are unique (Figure 4b, Supplementary sheet_3).

A very important finding from our analysis is that the specific genes belonging to individual families which, have been upregulated in over 50% of the sample studied are unique between the disease manifestations (Table 1).

Conclusion

In this study we have reported the differences in the transcriptomic profile of *pir* genes in *P. vivax* cerebral malaria and hepatic dysfunction cases. The upregulation of these *pir* genes might be involved in some crucial role in pathogenesis. One of the possible roles of *pir* genes is that it might show cytoadherence with host cell receptors as reported in *Bernabeau et al* (22) and *Schappo et al* (23). An interesting observation of this study is that the *vir* gene documented to have shown binding, PVX_108770, vir_C, in the reported study has shown 50% and above upregulation in the patient samples with hepatic dysfunction manifestation in our microarray data (Figure 2; Table 1). Different sub-cellular localisation of PIR proteins has been also shown suggesting the other possible functions of PIR proteins. Future studies focusing on interaction of the specific upregulated PIR proteins with host receptors could elucidate the potential

mechanisms of sequestration and cyto-adherence, which could explain the disease manifestation (Table 1).

Competing interests

The authors declare they have no conflicts of interest.

Author contributions

A.D., conceived the study, organized the material, was the principal investigator of a project from DBT and ICMR, India, and networked to facilitate the outcome. D.K.K. and S.K.K., performed the clinical investigations. A.D., P.A.B., S.S., and P.R., designed the experiments involved in this study. P.A.B., performed RNA isolation, quality check and microarray data analysis. S.S., and P.R., performed the real time qPCR experiment. S.S., and A.D., drafted the manuscript. P.A.B., and S.S., are equal contributors to this work. S.S., P.R., and S.T., designed the tables, graphs and figures for this paper. All authors have read and approved the manuscript.

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Supplementary data

Supplementary data_1

Supplementary sheet_1

Supplementary sheet_2

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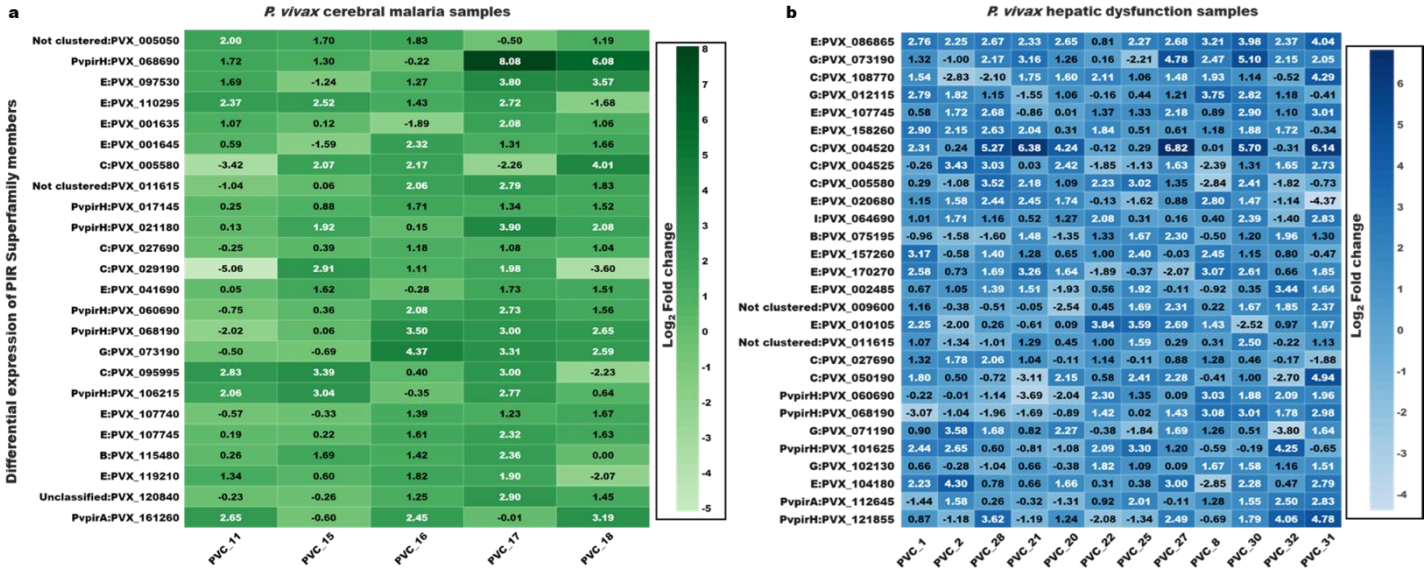

Fig 1. Heat Map expression profile of upregulated *pir* superfamily members in at least 50% and above *P. vivax* severe malaria samples (n=17) compared against uncomplicated malaria samples (n=6). (a) *P. vivax* cerebral malaria samples (n=5). (b) *P. vivax* hepatic dysfunction samples (n=12).

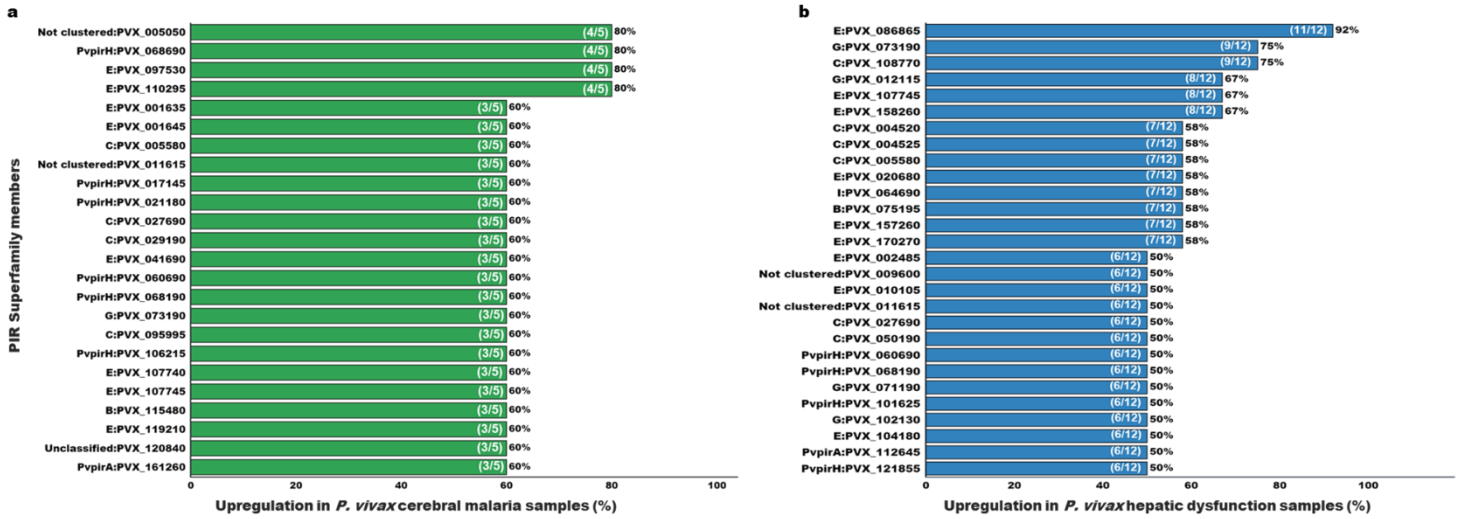

Fig 2. Upregulation of *pir* superfamily members in at least 50% and above *P. vivax* severe malaria samples (n=17) compared against uncomplicated malaria samples (n=6). (a) *P. vivax* cerebral malaria samples (n=5). (b) *P. vivax* hepatic dysfunction samples (n=12).

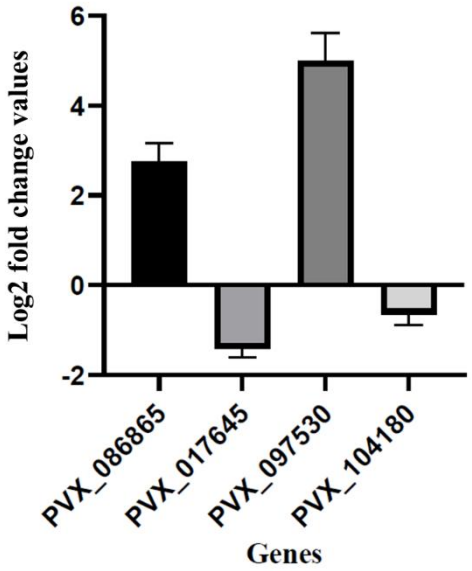

Fig 3. Validation of some *pir* genes by qPCR. Seryl tRNA synthetase was used as a reference gene. X-axis, represents genes selected for validation, Y-axis, represents Log2 fold change expression (mean ± corrected S.D.). PVX_086865, hypothetical protein (*vir* E); PVX_017645, variable surface protein Vir35 (*Pvpir* D); PVX_097530, variable surface protein Vir22/12-related (*vir* E); PVX_104180, variable surface protein Vir12 (*vir* E).

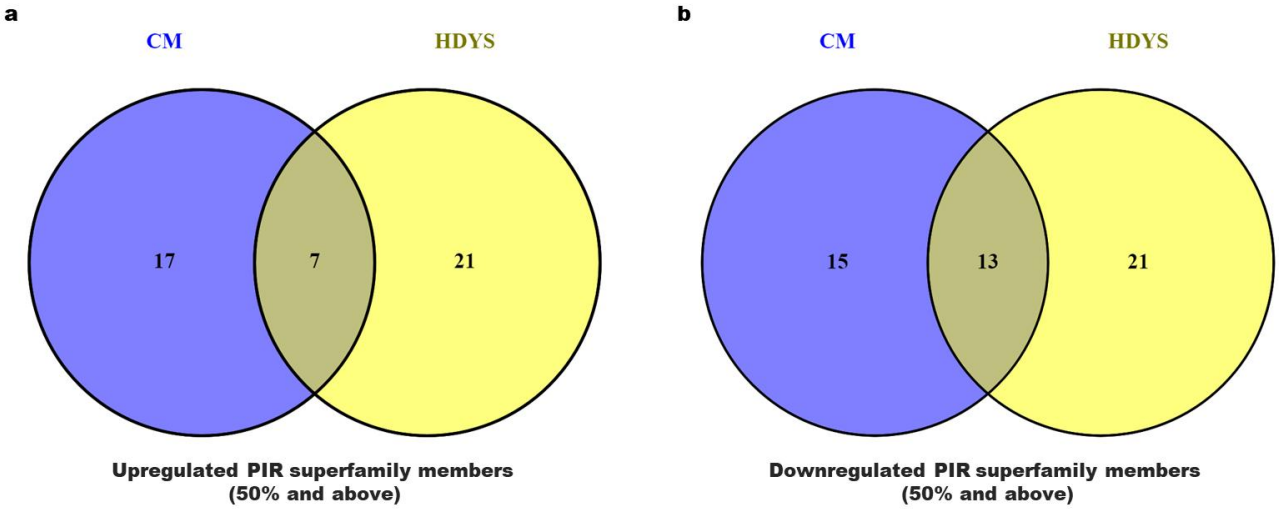

Fig 4. Distribution of *pir* superfamily members among cerebral malaria (n=5) and hepatic dysfunction (n=12) on the basis of 50% and above (a) upregulation and (b) downregulation.

		Upregulation in *P. vivax* isolates showing **Hepatic Dysfunction (n=12)** 12PVC vs 6PVU		Upregulation in *P. vivax* isolates showing **Cerebral Malaria (n=5)** 5PVC vs 6PVU	
Gene Symbol	PIR subfamily	No. of samples	%	No. of samples	%
PVX_086865	**E**	**11**	**92**	**2**	**40**
PVX_073190	G	9	75	3	60
PVX_108770	**C**	**9**	**75**	**1**	**20**
PVX_012115	**G**	**8**	**67**	**2**	**40**
PVX_107745	E	8	67	3	60
PVX_158260	**E**	**8**	**67**	**2**	**40**
PVX_004520	**C**	**7**	**58**	**2**	**40**
PVX_004525	**C**	**7**	**58**	**2**	**40**
PVX_005580	C	7	58	3	60
PVX_020680	**E**	**7**	**58**	**Nil**	**Nil**
PVX_064690	**I**	**7**	**58**	**2**	**40**
PVX_075195	**B**	**7**	**58**	**2**	**40**
PVX_157260	**E**	**7**	**58**	**2**	**40**
PVX_170270	**E**	**7**	**58**	**2**	**40**
PVX_002485	**E**	**6**	**50**	**Nil**	**Nil**
PVX_009600	**NC**	**6**	**50**	**1**	**20**
PVX_010105	**E**	**6**	**50**	**1**	**20**
PVX_011615	NC	6	50	3	60
PVX_027690	C	6	50	3	60
PVX_050190	**C**	**6**	**50**	**1**	**20**
PVX_071190	**G**	**6**	**50**	**1**	**20**

		Upregulation in *P. vivax* isolates showing **Hepatic Dysfunction (n=12)** 12PVC vs 6PVU		Upregulation in *P. vivax* isolates showing **Cerebral Malaria (n=5)** 5PVC vs 6PVU	
Gene Symbol	PIR subfamily	No. of samples	%	No. of samples	%
PVX_102130	**G**	**6**	**50**	**1**	**20**
PVX_104180	**E**	**6**	**50**	**Nil**	**Nil**
PVX_060690	PvpirH	6	50	3	60
PVX_068190	PvpirH	6	50	3	60
PVX_101625	**PvpirH**	**6**	**50**	**1**	**20**
PVX_112645	**PvpirA**	**6**	**50**	**Nil**	**Nil**
PVX_121855	**PvpirH**	**6**	**50**	**1**	**20**
PVX_005047	**NC**	**4**	**33**	**4**	**80**
PVX_068690	**PvpirH**	**4**	**33**	**4**	**80**
PVX_097530	**E**	**3**	**25**	**4**	**80**
PVX_110295	**E**	**3**	**25**	**4**	**80**
PVX_001635	**E**	**3**	**25**	**3**	**60**
PVX_001645	**E**	**4**	**33**	**3**	**60**
PVX_017145	**PvpirH**	**5**	**42**	**3**	**60**
PVX_021180	**PvpirH**	**2**	**17**	**3**	**60**
PVX_029190	**C**	**5**	**42**	**3**	**60**
PVX_041690	**E**	**4**	**33**	**3**	**60**
PVX_095995	**C**	**5**	**42**	**3**	**60**
PVX_106215	**PvpirH**	**4**	**33**	**3**	**60**
PVX_107740	**E**	**3**	**25**	**3**	**60**
PVX_115480	**B**	**1**	**8**	**3**	**60**
PVX_119210	**E**	**3**	**25**	**3**	**60**
PVX_161260	**PvpirA**	**4**	**33**	**3**	**60**

At least 50% upregulation in Hepatic Dysfunction and less than 50% upregulation in Cerebral malaria samples

At least 50% upregulation in Cerebral malaria and less than 50% upregulation in Hepatic Dysfunction samples

Table 1. Complication specific *pir* gene upregulation. This table shows the distinct genes upregulated in the analyzed severe malaria cases. The individual genes upregulated between CM and HDYS in 50 % or more of the samples analyzed are distinct in case of the individual severe disease manifestations.