Identification of a *vir*-orthologous immune evasion gene family from primate malaria parasites

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SUMMARY

The immune evasion gene family of malaria parasites encodes variant surface proteins that are expressed at the surface of infected erythrocytes and help the parasite in evading the host immune response by means of antigenic variation. The identification of *Plasmodium vivax vir* orthologous immune evasion gene family from primate malaria parasites would provide new insight into the evolution of virulence and pathogenesis. Three *vir* subfamilies viz. *vir-B, vir-D* and *vir-G* were successfully PCR amplified from primate malaria parasites, cloned and sequenced. DNA sequence analysis confirmed orthologues of *vir-D* subfamily in *Plasmodium cynomolgi, Plasmodium simium, Plasmodium simiovale* and *Plasmodium fieldi*. The identified *vir-D* orthologues are 1–9 distinct members of the immune evasion gene family which have 68–83% sequence identity with *vir-D* and 71·2–98·5% sequence identity within the members identified from primate malaria parasites. The absence of other *vir* subfamilies among primate malaria parasites reflects the limitations in the experimental approach. This study clearly identified the presence of *vir-D* like sequences in four species of *Plasmodium* infecting primates that would be useful in understanding the evolution of virulence in malaria parasites.

Key words: primate malaria parasites, immune evasion gene family, antigenic variation, virulence, pathogenesis.

INTRODUCTION

The rapid gene family expansion in phenotypically important genes indicates that adaptive natural selection favours accumulation of additional copies of genes. The genomes of the *Plasmodium* spp. present a model system to study lineage specific gene family evolution in response to host-pathogen interaction (*duffy binding like* and *reticulocyte binding protein* gene families), pathogenesis and virulence (*vir, cir, yir rifin/stevor, var* gene families), specific metabolism (*proteases, acyl carrier protein, kinases* etc.) and the pattern of evolutionary force that leads the evolution of specific biology (Carlton *et al*. 2008*a*). The genome sequences of seven *Plasmodium* spp. are available in the public domain (Carlton *et al*. 2002, 2008*a*,*b*; Gardner *et al*. 2002; Pain *et al*. 2008; Tachibana *et al*. 2012) and advances in bioinformatics tools accelerate studies on the evolution of gene families mediating specific malaria parasite phenotypes.

Comparative gene family sequence analysis would provide valuable information about the differential gene family expansion leading to specific biology of malaria parasites. This kind of comparative sequence analysis would also help in identifying unknown/novel members of a gene family from closely related *Plasmodium* spp. and to understand the pattern of gene family expansion in response to specific biology. In addition, how a gene family is evolving i.e. via gene gain or gene loss or both, can be understood by comparative gene sequence analysis.

At present, complete information about the framework of a *vir* orthologous gene family in primate malaria parasites other than *Plasmodium knowlesi*, is yet to be established. The majority of primate malaria parasites are closely related to *Plasmodium vivax* (Escalante and Ayala, 1994, 1995; Escalante *et al*. 1998) and a *vir* gene family orthologue among them is unknown. Therefore, identification of a *vir* orthologous immune evasion gene family from primate malaria parasites would provide valuable information to understand the evolution of virulence in malaria parasites. This study aimed to identify a *vir* orthologous immune evasion gene family from four primate malaria parasites (*Plasmodium cynomolgi, Plasmodium simium, Plasmodium fieldi* and *Plasmodium simiovale*).

MATERIALS AND METHODS

Parasite strains

Four primate malaria parasites viz. *P. cynomolgi, P. simium, P. simiovale* and *P. fieldi* were used for identification of a *vir*-orthologous immune evasion gene family. The genomic DNA of these parasites was obtained from Malaria Research and Reagent Reference Resource Center (MR4), Virginia, USA.

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Table 1. DNA sequencing of *Plasmodium vivax vir* gene family orthologues from primate malaria parasites

Primate malaria parasites	*P. vivax vir* gene subfamilies	Number of +ve clone sequenced	Number of unique sequences	Range of clones showing unique sequence	*Vir*-Orthologues
P. simium	*Vir-B*	ND	ND	ND	ND
	Vir-D	7	1	7	Yes
	Vir-G	ND	ND	ND	ND
P. cynomolgi	*Vir-B*	32	7	3–7	No
	Vir-D	32	9	2–5	Yes
	Vir-G	32	8	3–5	No
P. simiovale	*Vir-B*	32	7	3–6	No
	Vir-D	20	1	20	Yes
	Vir-G	32	10	2–5	No
P. fieldi	*Vir-B*	32	10	2–4	No
	Vir-D	20	1	20	Yes
	Vir-G	32	7	2–5	No

ND: Not done.

PCR amplification, purification and cloning

We employed a subfamily specific degenerate PCR strategy for amplification of *vir* gene family orthologues from primate malaria parasites. We have amplified eight subfamilies viz. *vir-A, B, C, D, E, G, K* and *L*; PCR primers and amplification protocols used were reported elsewhere (del Portillo *et al*. 2001; Fernandez-Becerra *et al*. 2005; Carlton *et al*. 2008*a*). The amplified PCR products were purified using a gel extraction kit (MDI, India) and cloned in TA-cloning vector (pTZ257R/T, Fermentas). Positive clones were confirmed by colony PCR method using M13 forward and reverse primers of plasmid. The degenerate primers amplified PCR product is likely to contain multiple sequences, therefore we have cloned the PCR product and sequenced up to 32 positive clones per sample.

Plasmid sequencing and sequence alignment

Plasmid was purified from overnight-grown positive clone bacteria, using a plasmid purification kit (MDI, India). Purified plasmids were sequenced with M13 forward and reverse primers. From a sequenced fragment, vector DNA sequences were trimmed from both 5′ and 3′ primes. Vector free DNA sequences were edited and aligned using DNASTAR Lasergene software version 7.0.0. Each unique sequence was submitted to GenBank.

Sequence identity and phylogenetic analysis

Percentage identity of identified DNA sequences with *vir-D* subfamily and within primate malaria parasites was done using DNASTAR Lasergene software. DNA sequence of each clone was BLAST at NCBI and Plasmodium genome database (PlasmoDB: www.plasmodb.org). DNA sequences showing identity either with *Plasmodium vivax vir* gene family or with *P. knowlesi kir/sicavar* gene family were included for further analysis and those sequences showing identity other than *Plasmodium* spp. were discarded. A Maximum Likelihood (ML) phylogenetic tree was constructed using newly identified DNA sequences along with *vir* subfamilies' specific reference sequences. MEGA software version 5.0 (Kumar *et al*. 2008) was used to construct an ML phylogenetic tree. To understand the topology of the ML phylogenetic tree, 1000 bootstrapping was done.

RESULTS

Degenerate PCR amplification, cloning and sequencing of immune evasion genes

Three subfamilies *vir-B, D* and *G* were successfully PCR amplified from *P. cynomolgi, P. simiovale* and *P. fieldi*. From *P. simiumo* only *vir-D* primer shows amplification. *Vir-D* amplified PCR product shows a single and expected PCR fragment of 550 bp whereas *vir-B* and *vir-G* primers show 800 and 300 bp amplicons respectively along with a few non-specific amplicons. The expected size of PCR product was gel purified, cloned and sequenced. Details of the number of positive clones sequenced are given in Table 1.

Identification of immune evasion gene family

Vir-B, vir-D and *vir-G* primers each generated a unique sequence that was represented by an average of three to ten clones (Table 1). These unique sequences were BLAST at NCBI and PlasmoDB to make sure of their identity with malaria parasites. BLAST search result shows that orthologues of *vir-D* subfamily were present in four primate malaria

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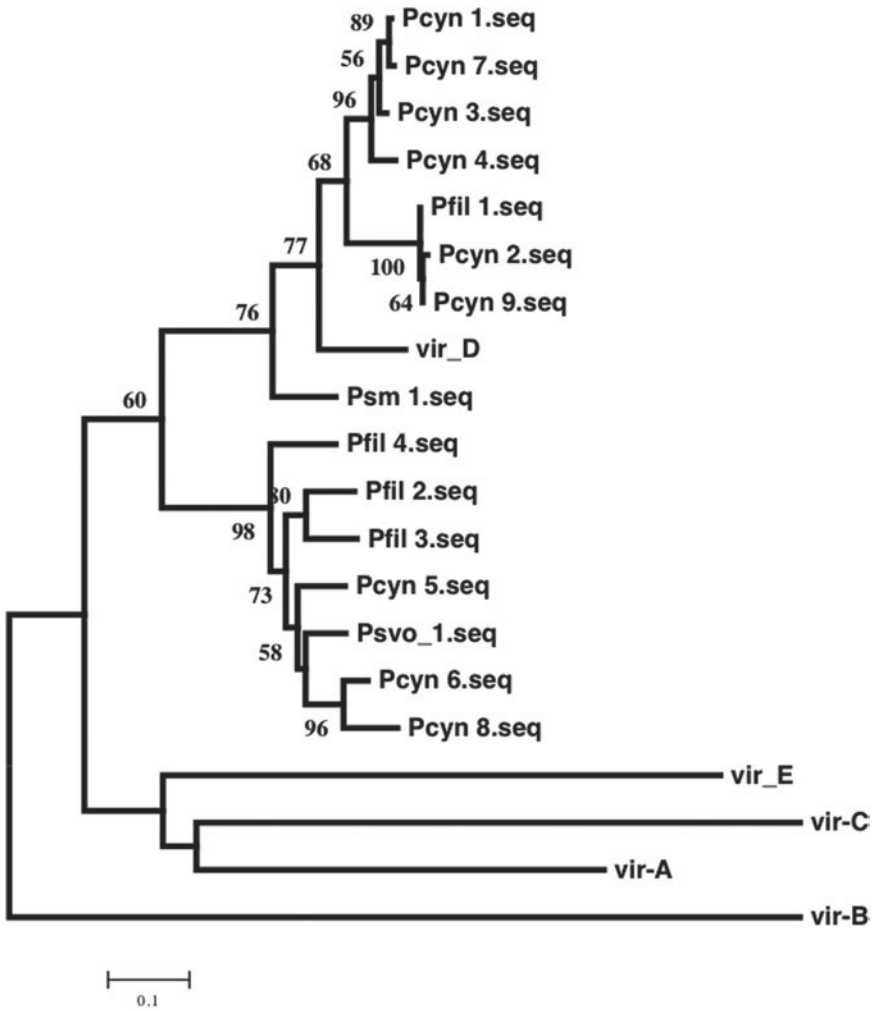

Fig. 1. Phylogenetic tree showing *vir-D* primer generated DNA sequences from primate malaria parasites are clustered with *vir-D* subfamily reference sequence. Pcyn: *Plasmodium cynomolgi*, Pfil: *P. fieldi*, Psm: *P. simium*, Psvo: *P. simiovale* and vir (A-E): *Plasmodium vivax vir* subfamilies reference sequences. Bootstrapping values are shown in percentage.

parasites however the number of unique sequences varied within species (Table 1). A maximum of nine members of a *vir-D* orthologous immune evasion gene family were identified from *P. cynomolgi*, four from *P. fieldi* and one each from *P. simium* and *P. simiovale*. *Vir-B* sequences identified from *P. cynomolgi, P. simiovale* and *P. fieldi* show no homology with *vir* gene family in BLAST search, rather they show homology with other genes in *P. vivax* and *P. knowlesi* genomes such as *lipoate synthase, lipoyl synthase* and hypothetical protein-encoding genes. This suggests that *vir-B* primer generated sequences from primate malaria parasites do not belong to an immune evasion gene family. Therefore, *vir-B* primer generated sequences were excluded from further analysis. None of the *vir-G* primer generated sequences were matched with malaria parasites, rather they show homology with host DNA that suggests contamination or non-specific amplification from host DNA and thus were excluded from the study. We have designated *vir* orthologous immune evasion gene family *as fir* for *P. fieldi, sir* for *P. simium* and *siir* for *P. simiovale*. The immune evasion gene family of *P. cynomolgi* has already been designated recently as *cyir* (Tachibana *et al*. 2012).

DNA sequence identity of immune evasion gene family

BLAST search confirmed *vir* orthologous sequences from primate malaria parasites were further analysed to confirm their identity with *vir-D* subfamily. We did an ML phylogenetic tree construction along with the reference sequences of *vir* subfamilies (*vir-A, B, C, D* and *E*). Interestingly, all sequences obtained from *vir-D* primer were clustered with *vir-D* reference sequence (Fig. 1). Since these sequences are orthologous to *vir* gene family, which is confirmed by BLAST search, therefore in the ML phylogenetic tree a non-*vir* gene sequence was not used as an out-group. ML phylogeny suggests that the obtained sequences are member of an immune evasion gene family of primate malaria parasites. The genetic identity of these new sequences with *vir* gene family of *P. vivax* and between primate malaria parasites is given in Fig. S1. The percentage

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DNA sequence identity analysis revealed 68–83% identity of primate malaria parasites with *P. vivax vir-D* sub-family. The percentage DNA sequence identity within members of *cyir* and *fir* was 71·2–98·5% and 70·9–71·9% respectively (Fig. S1).

DISCUSSION

In this study, we identified orthologues of the *P. vivax vir* gene family from four primate malaria parasites using a degenerate primer amplification, cloning and DNA sequencing approach. This study shows the presence of a *vir* orthologous immune evasion gene family in primate malaria parasites and suggests that a *vir-D* subfamily specific degenerate primer binding site is relatively more conserved among the parasites of the primate malaria clade and may be employed in other primate malaria parasites for the identification of *vir* orthologous immune evasion gene family.

The identification of *vir* gene family orthologues from primate malaria parasites was based on the close relationship between *P. vivax* and parasites of the primate malaria clade (Escalante and Ayala, 1994, 1995; Escalante *et al*. 1998). We assumed a higher degree of sequence homology of *vir* genes between these species and therefore a degenerate PCR amplification approach was employed. This approach has several limitations. Firstly, *vir* family members are hypervariable in nature (del Portillo *et al*. 2001; Merino *et al*. 2006). This may lead to non-specific amplification or no amplification in some cases. The failure of PCR amplification for *vir-A, vir-C* and *vir-E* subfamilies and non-specific amplification of *vir-B* and *vir-G* could be a possible reason. Secondly, use of *vir* subfamilies specific degenerate primers provide only a partial fragment of immune evasion genes (del Portillo *et al*. 2001). Thirdly, the degenerate PCR amplification approach may not be able to pinpoint the exact number of members in an immune evasion gene family from closely related species. Therefore, absence of other *vir* subfamilies members from four primate malaria parasites in the present study reflects experimental limitations and not necessarily the actual absence of these genes.

Antigenic variation in *Plasmodium* spp. is conferred by a larger repertoire of variant surface proteins which is encoded by an immune evasion gene family (Ferreira *et al*. 2004). The genome sequences of primate malaria clade parasites such as *P. vivax* (Carlton *et al*. 2008*a*), *P. knowlesi* (Pain *et al*. 2008) and *P. cynomolgi* (Tachibana *et al*. 2012) show a larger number of members in the immune evasion gene family that may have evolved to shape the specific pathogenesis. Primate malaria parasites are close relatives of one of the most prevalent human malaria parasites, *P. vivax*, therefore identification of *vir* orthologous genes from these parasites would provide an important insight into the evolution of virulence among *Plasmodium* spp. by comparative gene sequence analysis. Although this study has identified a limited number of immune evasion genes from four primate malaria parasites in comparison to the number of members that have been identified in *P. vivax* ($n = \sim 400$), *P. knowlesi* ($n = \sim 300$) and *P. cynomolgi* ($n = \sim 400$), their identification presents a clear picture of relatively higher conservation of *vir-D* subfamily specific degenerate primer binding sites in primate malaria parasites.

This study clearly identified the presence of *vir-D* like sequences in four species of *Plasmodium* infecting primates and suggests that a *vir-D* subfamily specific primer may be employed for the identification of *vir*-like genes from other primate malaria parasites. The absence of other *vir* orthologous members from primate malaria parasites reflects experimental limitation and not necessarily the actual absence of the genes. Further investigation leading to identification of complete members of an immune evasion gene family would be highly helpful in developing deep understanding of how virulence is being evolved among *Plasmodium* spp. and would be helpful for drug targeting.

CONFLICTS OF INTEREST

The authors declare that they do not have any conflicts of interest.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/S003118201300214X by article doi number.

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