Molecular Microbiology (2005) **58**(3), 648–658

doi:10.1111/j.1365-2958.2005.04850.x
First published online 22 September 2005

Variant proteins of *Plasmodium vivax* are not clonally expressed in natural infections

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Summary

***Plasmodium vivax* is the most widely distributed human malaria parasite and responsible for 70–80 million clinical cases each year and a large socio-economical burden. The sequence of a chromosome end from *P. vivax* revealed the existence of a multigene superfamily, termed *vir* (*P. vivax* variant antigens), that can be subdivided into different subfamilies based on sequence similarity analysis and which represents close to 10–20% of the coding sequences of the parasite. Here we show that there is a vast repertoire of *vir* genes abundantly expressed in isolates obtained from human patients, that different *vir* gene subfamilies are transcribed in mature asexual blood stages by individual parasites, that VIR proteins are not clonally expressed and that there is no significant difference in the recognition of VIR-tags by immune sera of first-infected patients compared with sera of multiple-infected patients. These data provide to our knowledge the first comprehensive study of *vir* genes and their encoding variant proteins in natural infections and thus constitute a baseline for future studies of this multigene superfamily. Moreover, whereas our data are consistent with a major role of *vir* genes in natural infections, they are inconsistent with a predominant role in the strict sense of antigenic variation.**

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Introduction

Plasmodium vivax is the most widely distributed human malaria parasite and responsible for 70–80 million clinical cases each year and large socio-economical burdens for countries such as Brazil where it is the most prevalent species (Mendis *et al*., 2001). Unfortunately, as a result of the fact that vivax malaria is not life-threatening, the impossibility of maintaining this parasite in continuous *in vitro* culture, the low parasitaemias associated with natural human infections and the difficulties in adapting wild isolates into monkeys, research on *P. vivax* remains largely neglected. Furthermore, because of the strict species-specificity of the naturally acquired antimalarial protective immune responses, it is likely that a vaccine against *P. falciparum* will not be active against *P. vivax*. Moreover, unlike *P. falciparum*, *P. vivax* invades, predominantly, if not exclusively, reticulocytes and it is generally accepted that it does not sequester in the deep capillaries of inner organs having an obligate passage of infected reticulocytes through the spleen. Together, these data call for a comprehensive research effort to study *P. vivax*, *per se*.

Malaria parasites establish chronic infections that persist for long periods of time, despite the concurrent presence of a strong immune response directed against parasite-encoded variant antigens exposed on the surface of the host erythrocyte [reviewed in the study by Kyes *et al*. (2001)]. To identify gene(s) encoding variant antigens in *P. vivax*, we constructed a representative genomic library of *P. vivax* in yeast artificial chromosomes (YAC) and screened it for the presence of telomeric YACs (Camargo *et al*., 1997). Telomeric YAC clones were investigated because, in *P. falciparum*, subtelomeric domains contain clusters of multigene families, such as *var*, *rif* and *stevor*, which are implicated in antigenic variation and cytoadherence (Gardner *et al*., 2002). Significantly, sequencing of a 155 771 bp telomeric YAC from this library revealed the existence of a multigene superfamily putatively involved in immune evasion and which repre-

sents close to 10–20% of coding sequences in the haploid genome of the parasite (del Portillo *et al*., 2001). Recently, homologous subtelomeric multigene families have also been described in the rodent malarias of *P. berghei*, *P. yoelii* and *P. chabaudi* (Janssen *et al*., 2002), and a new variant gene superfamilly (*Plasmodium* interspersed repeats, *pir*) together with *rif/stevor* in *P. falciparum*, *kir* in *P. knowlesi*, and the *cir/bir/yir* family in three rodent malarias, proposed (Janssen *et al*., 2004). An understanding at the molecular level of the function of malarial subtelomeric multigene families will undoubtedly contribute to unveil virulence in *Plasmodium* and to accelerate alternative control strategies against human malaria.

To date, there are no studies reported of the *vir* multigene superfamily of *P. vivax* in natural infections. The present study was designed to determine the genomic and expressed repertoire of *vir* genes among parasites obtained from human patients, to determine the pattern of expression of *vir* genes and VIR proteins by individual parasites and to characterize the naturally acquired humoral immune responses against different VIR proteins in first- and multiple-infected patients. These results are discussed with regard to the putative role of VIR proteins in the strict sense of antigenic variation, namely, the process by which parasitic protozoa built-in a programme that switches variant proteins from mutigene families against which our immune system produces variant-specific antibodies.

Results

Genomic and expressed vir *gene repertoires in natural parasite populations*

As *P. vivax* cannot be continuously cultured *in vitro*, we decided to examine both the genomic and expressed *vir* gene repertoires in natural infections using parasites obtained directly from patients. Parasite genomic DNA and total RNA were extracted from peripheral blood of three *P. vivax* patients from the Brazilian Amazon State of Rondônia. Nucleic acids were used as templates in polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) reactions using degenerate yet *vir*-specific oligonucleotides representing subfamilies termed A–E. These subfamilies had been predicted *in silico* by similarity analysis of *vir* gene sequences from a chromosome end of *P. vivax* (del Portillo *et al*., 2001). Amplified fragments were cloned into bacterial vectors and 5–10 different clones from each amplification were fully sequenced generating 252 *vir* clones representing ~152 kb of *vir* sequences and corresponding to 63 clones of subfamily A, 40 clones of subfamily B, 51 clones of subfamily C, 45 clones of subfamily D and 53 clones of subfamily E (Table S1, supplementary data). After similarity analysis, identical sequences from each patient were removed generating 146 unique sequences (GenBank Accession Numbers AY608742–AY608887). Dendrogram analyses from these unique sequences confirmed the existence of different subfamilies in natural parasite populations and revealed that the extent of allele polymorphism as well as the size and domain architecture of each subfamily varied among them (Figs 1 and S1, supplementary data). Thus, subfamilies A (90–124 aa), B (193–197 aa) and C (185–202 aa) were highly polymorphic with variant and conserved amino acids scattered throughout the entire domain. In contrast, subfamily D (173–178) was highly conserved with large blocks of complete amino acid conservation. Of interest, polymorphism in this subfamily was largely restricted to a single block of approximately 15 residues towards the C-terminus. Subfamily E contains the largest domain (246–271) and could be divided into a large conserved N-terminus block and a polymorphic C-terminus block. Multiple sequence alignments of these subfamilies revealed that there was not any one particular residue or sequence conserved among all of them. In contrast, within each subfamily, there were conserved residues in all alleles sequenced (Fig. S1, supplementary data).

We next analysed the repertoire of expressed *vir* genes from parasites obtained from these same patients (AY608815–AY608887). As expected, sequence analysis readily identified all subfamilies and revealed that independently of the *vir* subfamily or patient from which parasites were obtained, the expressed *vir* sequences had 10% or no redundancy at all (Table S1, supplementary data). Together, these data demonstrate that *vir* genes are structured into subfamilies displaying different sizes, extents of allele polymorphisms and domain structures. Most relevant, they also demonstrate that *P. vivax* parasites from different patients actively transcribe large and mostly non-overlapping *vir* gene repertoires in natural infections.

Expression of vir *genes by individual parasites*

Having established that *P. vivax* actively transcribes *vir* genes, we next determined the pattern of *vir* gene expression by individual parasites using single-cell RT-PCR. This technique has elegantly been used to solve the pattern of gene expression of multigene families in malaria (Chen *et al*., 1998; Preiser *et al*., 1999; Kaviratne *et al*., 2002). To this end, 20 ml of peripheral blood were withdrawn from three infected patients with high parasitaemias of 1% and containing mostly schizonts. Blood was then passed sequentially through either plasmodipur filters or cellulose CF11 colums to remove human leucocytes and through 57% nycodenz gradients to concentrate infected reticulocytes with mature stages (Camargo *et al*., 1997). Giemsa-

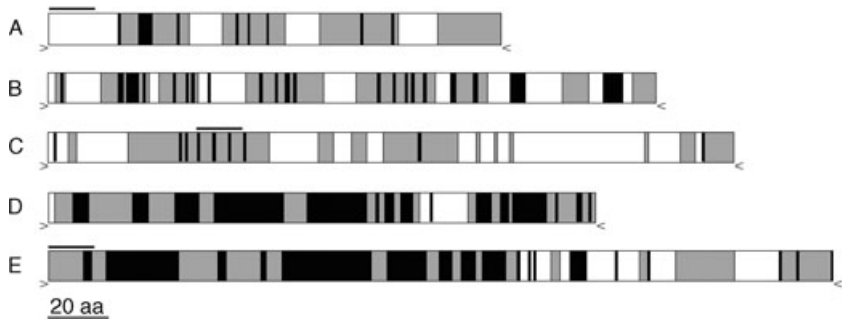

Fig. 1. *vir* genes are structured into subfamilies differing in their extent of allele polymorphisms and domain structures. Schematic diagram of domains structures of subfamilies A–E (del Portillo *et al*., 2001). Black boxes represent conserved blocks with 80–100% homology. Grey boxes represent semi-conserved blocks with 60–80% homology. White boxes represent polymorphic blocks with less than 60% homology. Bars above subfamilies A, C and E showed the relative position of the peptides used to obtain polyclonal antibodies. Arrows indicate the relative position of forward (>) and reverse (<) primers used in PCR and RT-PCR amplifications. Scale at the bottom of the figure refers to amino acids.

staining confirmed that concentrated infected reticulocytes in this gradient carried exclusively mature stages and that infected reticulocytes with ring stages in the pellet of the gradients contained individual parasites (data not shown). Next, individual infected reticulocytes were collected using a micromanipulator and single-cell RT-PCR analysis carried as described (Chen *et al*., 1998). As shown in Fig. 2, mature stages of *P. vivax* derived from individual parasites transcribe *vir* genes pertaining to more than one subfamily. For instance, individual parasites from the three patients amplified *vir* genes from subfamilies A and D (lane 5 patient 1, lane 4 patient 2, lane 6 patient 3), whereas individual infected reticulocytes from lanes 1 and 4 from patient 1 amplified genes from subfamilies A and C. Attempts to amplify more than two subfamilies from an individual parasite failed most likely because of the scarcity of material. Of notice, amplification of *vir* genes with these oligonucleotides from these subfamilies always amplify fragments of approximately the same sizes, including the doublet seen in subfamily C of patient 1, in spite of their sequences being different (not shown). This result however, was expected as fragments of similar sizes were amplified from genomic DNA (Fig. 2). Regardless, these data demonstrate that *vir* genes repre-

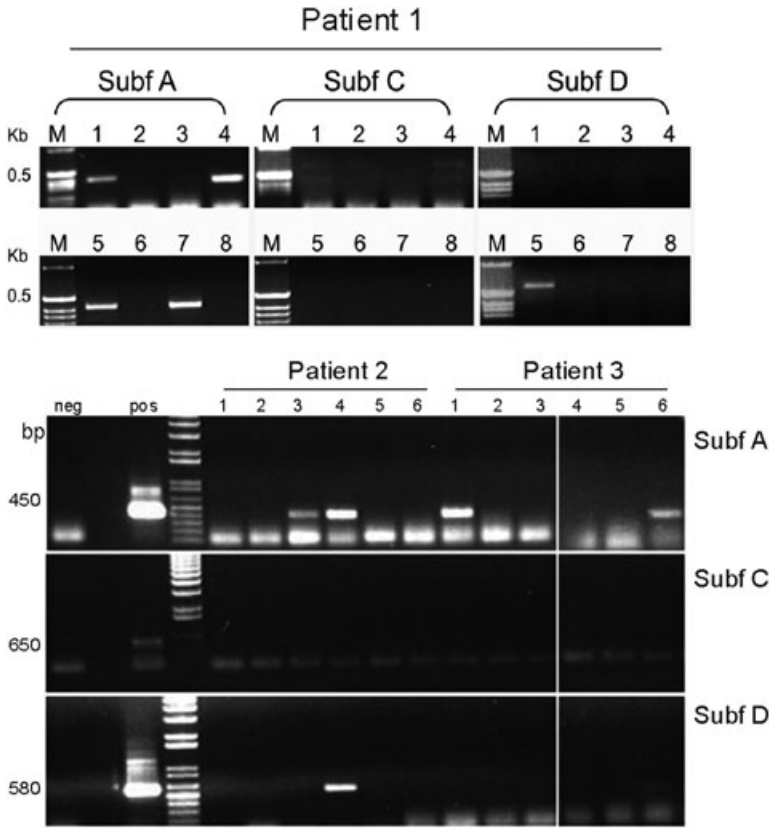

Fig. 2. Reverse transcription polymerase chain reaction analysis of single micromanipulated trophozoite-infected *P. vivax* reticulocytes. PCR fragments from three individual patients (patient 1–3) and individual trophozoites (patient 1, lanes 1–8 upper panel; patient 2, lanes 1–6, patient 3, lanes 1–6 lower panel) were resolved on 1.5% agarose gels stained with ethidium bromide. Primers for subfamilies A, C and D (del Portillo *et al*., 2001) were used in RT-PCR reactions as pair-combinations; all attempts to amplify simultaneously the three subfamilies failed. Molecular weight markers (M) in base pairs (bp) are shown to the left. Representative negative and positive controls are shown to the left on the lower panel.

senting at least two different subfamilies are transcribed concomitantly during mature stages by individual parasites.

Expression of VIR proteins in individually infected reticulocytes

The data gathered above suggested that VIR proteins were not clonally expressed. Lack of a continuous *in vitro* culture for *P. vivax* however, precluded the possibilities of using techniques such as radiolabelling and immunoprecipitation to determine whether VIR proteins are clonally expressed. In an attempt to circumvent these limitations, synthetic peptides representing *in silico* predicted conserved epitopes from subfamilies A, C and E were used to immunize rabbits, mice, rats and guinea pigs to produce polyclonal monospecific antibodies to use in laser confocal immunofluorescence. A similar approach had been used with a peptide from subfamily D to demonstrate that VIR proteins were located at the surface of infected reticulocytes (del Portillo *et al*., 2001). Enzyme-linked immunosorbant assay (ELISA) and Western blot assays were performed to unequivocally demonstrate the specificity of these new antibodies and validate their use in laser confocal microscopy. Firstly, we initially used the peptides representing each subfamily as coating antigens on ELISAs and showed that, as expected, antipeptide antibodies were specific against the peptide from which they had been produced (Fig. S2, supplementary data). Secondly, we showed that sera from infected patients reacted against these peptides demonstrating that they represent immunogenic epitopes in natural infections (Fig. S3, supplementary data). Thirdly, we purified by electroelution four different GST-VIR tags from subfamily A, five different GST-VIR tags from subfamily C, three different GST-VIR tags from subfamily D and six different GST-VIR tags from subfamily E from different patients and used them in Western blot analysis using the sera from the different animals immunized with peptides A, C and E. The results showed that anti-VIRA antibodies cross-react with a truncated GST-VIRD tag and anti-VIRC antibodies cross-react against GST-VIR tags from subfamily D indicating that combinations of peptide antibodies anti-VIRA/C and anti-VIRC/E antibodies could be used in laser confocal microscopy (Fig. 3A). Yet, these results were obtained with pools rather than individual tags and there was a weak reaction against the VIRA-GST tag by the anti-A antibody. Thus, we performed a new Western blot analysis of five individual VIRC tags and five VIRE tags and further demonstrated that anti-VIRC and anti-VIRE antibodies are highly cross-reactive within subfamilies but not across subfamilies (Fig. 3B). Although these results strongly suggested that these reagents were subfamily-specific, ultimate validation of their specificity had to be demonstrated with parasite extracts. To this end, *P. vivax* parasites were obtained directly from two human patients and protein extracts used in new Western blot analysis. As shown in Fig. 3C, anti-VIRC and anti-VIRE antibodies reacted differently and specifically against these extracts. Indeed, anti-VIRE antibodies reacted against major and single band of approximately 50 kDa whereas anti-VIRC antibodies reacted against several bands varying in size from 20 to 55 kDa. As expected, anti-PvMSP1 antibodies used as a positive control recognized MSP1 processed

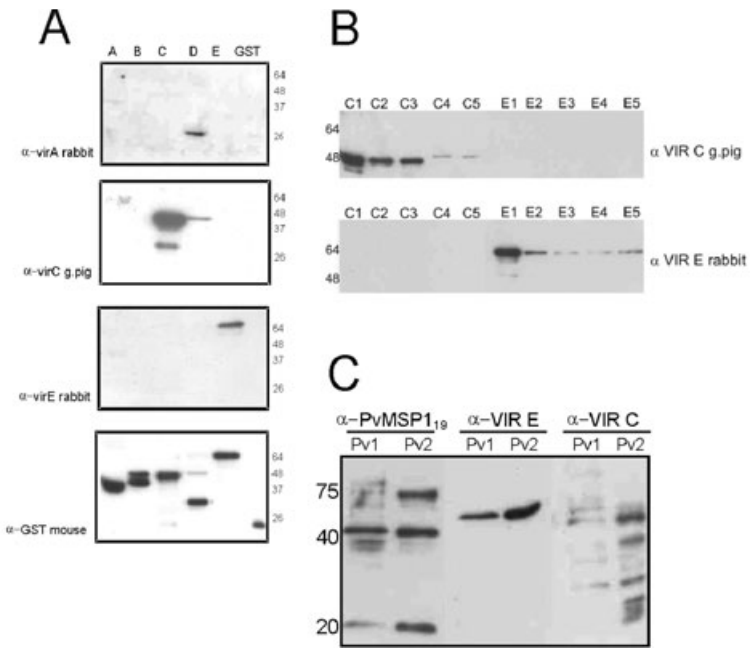

Fig. 3. Western blot analysis of the specificity of anti-peptide VIRA, VIRC and VIRE antibodies.
A. Four different GST-VIR tags from subfamily A, five different GST-VIR tags from subfamily C, three different GST-VIR tags from subfamily D and six different GST-VIR tags from subfamily E from different patients were purified by electroelution (see Fig. 5), tags from each subfamily were pooled and used in Western blot analysis using the anti-VIR A, C and E antibodies. GST alone was used as a negative control and anti-GST antibodies were used as positive control of loaded GST-tags.
B. Western blot analysis of individual VIRC (C1–C5) and VIRE (E1–E5) tags reacted against guinea pig anti-VIRC and rabbit ant-VIRE peptide antibodies.
C. Western blot analysis of *P. vivax* parasites extracts obtained from two different patients (Pv1 and Pv2) and reacted against rabbit anti-PvMSP1$_{19}$ antibodies, and anti-VIRC and anti-VIRE antibodies.
Molecular weight markers in kilo-Daltons are shown to the right of A and to the left of B and C. Assays were done as described in the study by Levitus *et al.* (1994).

polypeptides (del Portillo *et al*., 1988). All together, these results unequivocally demonstrate that anti-VIRC and anti-VIRE antibodies are VIR subfamily-specific, that within each subfamily they are cross-reactive and that they recognize specifically VIRC and VIRE proteins from parasite extracts of *P. vivax*. As such, they can be used in laser confocal micrsocopy to determine whether expression of VIR proteins is clonal in individual infected reticulocytes.

Figure 4 shows a representative result of such experiments using anti-VIRC and E antibodies. For these experiments, samples were washed in RPMI containing 10% albumax and fixed in methanol prior to transportation to São Paulo (non-endemic region). Logistic problems thus precluded the possibility of using live parasites. In spite of this technical limitation that was also recently reported in *P. falciparum* (Winter *et al*., 2005), the results showed that individual parasites do not express clonally VIRC and VIRE proteins. Similar results were obtained with anti-VIRA and anti-VIRC antibodies (Fig. S4, supplementary data). All controls using preimmune sera displayed much lower levels of fluorescence intensities under identical image capture conditions further validating the specificity of these results. Unexpectedly, when counting the infected reticulocytes that reacted against these antipeptide antibodies, we found that more than 95% gave positive fluorescence (Fig. S4, supplementary data). These results showed that the peptides used in the production of these polyclonal antibodies represent conserved subfamily-specific epitopes capable of eliciting cross-reacting antibodies and that unlike PfEMP1 proteins, VIR proteins are not clonally expressed by individually infected reticulocytes.

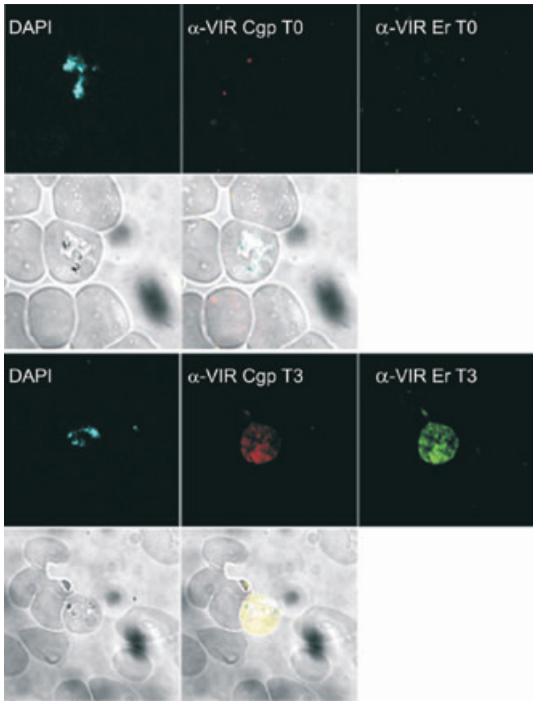

Fig. 4. Confocal laser scanning immunofluorescence of an individually infected *P. vivax* reticulocyte. Infected *P. vivax* reticulocytes were double-labelled with antisera to subfamily C (detected using a rhodamine-conjugated secondary antibody, KPL) and antiserum to subfamily E (detected using a fluoresceine-conjugated secondary antibody, KPL). Upper panel from left to right: DAPI staining, guinea pig preimmune serum (T0), rabbit preimmune serum (T0), phase contrast, overlay preimmune sera. Lower panel from left to right: DAPI staining, guinea pig anti-VIR C serum (third boost, T3), rabbit anti-VIR E serum (third boost, T3), phase contrast, overlay immune sera. Images were obtained from the same slide using the exact same image-capturing conditions.

Naturally acquired humoral immune responses against VIR proteins

Variant surface antigens of *P. falciparum* play a major role in antigenic variation and in the slow development of acquired immunity by the repeated exposure to different isolates (Miller *et al*., 1994; Kyes *et al*., 2001). As a first approximation to investigate this fundamental aspect in the biology of *P. vivax*, we studied the naturally acquired IgG responses of *P. vivax* patients during their acute attack against different VIR proteins. To this end, we generated a total of 22 GST-VIR tags comprising most of the variant region encoded in exon II from subfamilies A to E and after purifying, they were used in Western blot analysis and reacted with acute-phase sera from first-infected ($n = 6$) and multiple-infected ($n = 26$) *P. vivax* patients from Rondônia. Previous malaria attacks in multiple-infected patients varied from two to 10. Results demonstrated that 23 out of 32 patients (~72%) reacted against one or more VIR-tags (Figs 5 and S5). Of notice, none of the sera from seven normal volunteers from this same endemic region tested in parallel reacted against any VIR-tag, nor to GST alone (not shown). Interestingly, proteins from subfamily A were always and significantly more frequently recognized than all other subfamilies in pair-wise subfamily comparisons (A versus B $P = 0.00003$; A versus C $P = 0.00092$; A versus D $P = 0.00369$; A versus E $P = 0.00014$). Unexpectedly, there was no significant difference in the recognition of VIR-tags by immune sera of first-infected patients compared with sera of multiple-infected patients ($P = 0.82$). Furthermore, immune sera from three patients who were first-infected reacted against different VIR-tags from different subfamilies. These results thus confirm and extend the original observations that VIR proteins are immunogenic in natural infections (del Portillo *et al*., 2001).

Discussion

Here we describe studies demonstrating that there is a large repertoire of *vir* genes abundantly expressed in iso-

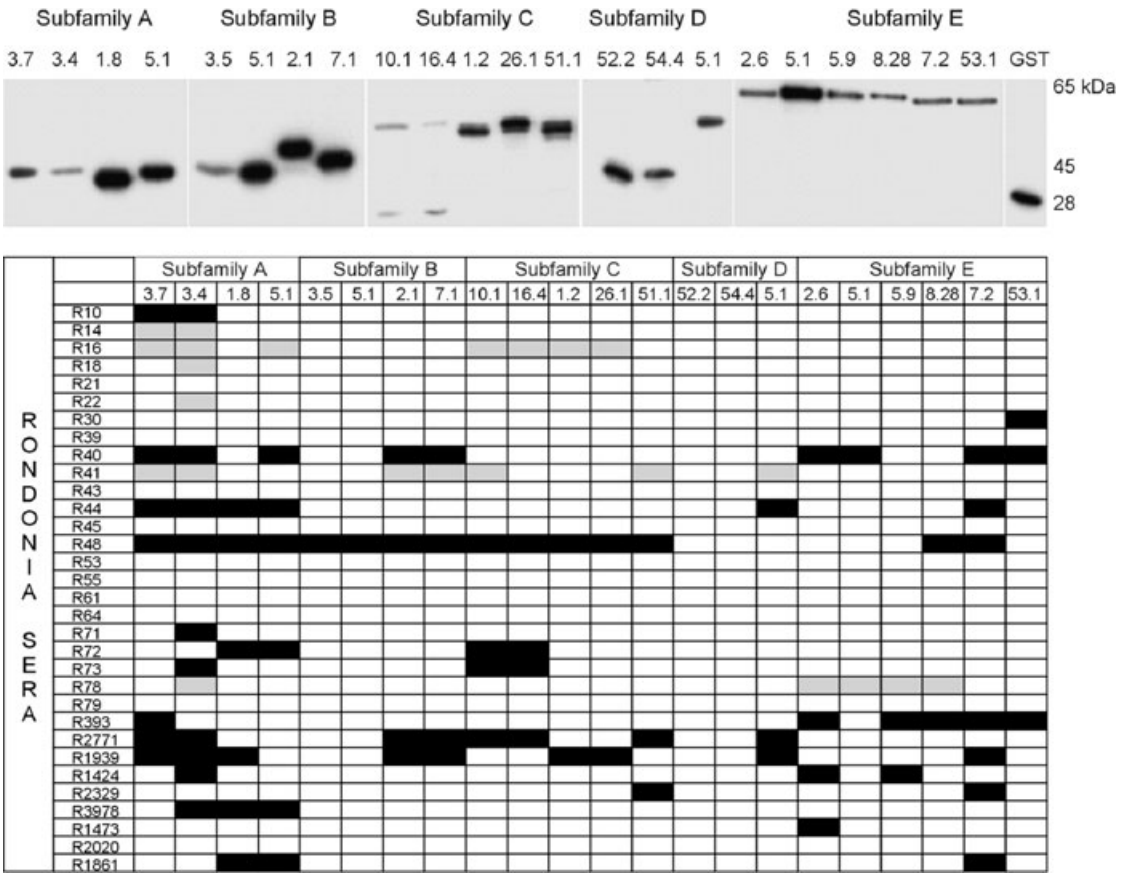

Fig. 5. Naturally acquired IgG responses of human patients against VIR proteins. Upper panel: Western blot analysis of 22 purified GST-VIR tags representing different subfamilies (A–E) using a monoclonal antibody to GST. Lower panel: schematic diagram of positive reactivity of immune sera from first-infected (grey boxes) and multiple-infected (black boxes) patients from Rondônia against these different GST-VIR tags. Molecular weights in kilo-Daltons (kDa) are shown to the right.

lates obtained from human patients. *vir* genes pertaining to different subfamilies were transcribed in mature asexual blood stages by individual parasites and VIR proteins were not clonally expressed. Furthermore, there was no significant difference in the recognition of VIR-tags by immune sera from first-infected patients compared with immune sera from multiple-infected patients. These data provide to our knowledge the first comprehensive study of *vir* genes in natural infections and thus constitute a baseline for future studies of this multigene superfamily. Moreover, the large expression, immunogenicity and variant nature of VIR proteins is consistent with a major role in natural infections. Yet, the data appear inconsistent with a predominant role in the strict sense of antigenic variation, namely, the process by which parasitic protozoa built-in a programme that switches variant proteins from mutigene families against which our immune system produces variant-specific antibodies. Further studies will be necessary to determine whether the function of *vir* genes is related to the unique aspects of vivax malaria with regard to the invasion of host reticulocytes and the lack of apparent sequestration, which imposes an obligate passage through the spleen.

The availability of the complete and assembled genome sequences of *P. falciparum* allowed the design of primers to amplify and sequenced variant genes from different isolates (Kyes *et al*., 1997; Taylor *et al*., 2000; Abdel-Latif *et al*., 2002). Using this approach, the genetic variation of *P. falciparum var* genes in natural infections from different endemic regions revealed a large global diversity with mostly non-overlapping genomic repertoires whose sequences cannot predict geographical origin (Ward *et al*., 1999; Kirchgatter *et al*., 2000; Fowler *et al*., 2002). Our studies of *vir* genes in natural infections were limited to the highly variant portion of exon II as this gene segment codes for the putative extracellular variant domain. Moreover, only the predicted subfamilies A–E representing 13 of the 32 *vir* genes sequence from a chromosome end were analysed as we were unable to design oligonucleotides capable of amplifying the remaining genes and there are no other assembled chromosome ends from *P. vivax*. Furthermore, the fact that there were identical

gDNA as well as cDNA sequences within each patient, clearly indicate that the primers used in this study are biased. In spite of these limitations, this study represents the first sequence analysis of *vir* genes from malaria in parasite populations obtained from human patients. More relevant, our results clearly demonstrate that, similar to *var* genes, there are large genomic repertoires of *vir* genes with limited overlap thus emphasizing the importance of *vir* gene diversity in natural infections. Complex non-coding tandem repeats at chromosome ends of *P. falciparum* have been proposed to promote homologous and ectopic recombinations favouring the generation of diversity of subtelomeric multigene families (Freitas-Junior *et al*., 2000). *P. vivax* seems to lack large arrays of tandem repeats at the chromosome ends but clearly has evolved alternative mechanisms to facilitate the generation of divergent repertoires of *vir* genes. Unfortunately, lack of a continuous *in vitro* culture for *P. vivax* still precludes functional analysis of the genome of this human malaria parasite.

Studies on the pattern of gene expression of multigene families in malaria using single-cell RT-PCR as the gold standard technique have described different patterns of gene expression for different multigene families (Chen *et al*., 1998; Preiser *et al*., 1999; Kaviratne *et al*., 2002). Thus, there is relaxed transcription of *var* genes during the ring stages and as the parasite matures there seems to be allelic exclusion of all but one of these transcripts that ultimately is translated and exported to the surface of infected red blood cells (IRBC) (Chen *et al*., 1998). In contrast, in spite of being localized on the same genome region and presumably the same chromatin context, peak transcription of a subset of different *stevor* genes coincides with the mid-trophozoite stages (Kaviratne *et al*., 2002). Moreover, although *rif* gene transcription has not been determined in individual parasites, Northern blot analysis revealed that it occurs for a short period of time in the transition between rings and pigmented trophozoites (Kyes *et al*., 1999). Remarkably, another distinct pattern of gene expression has been determined throughout the asexual blood stages of the multigene family p235 of *P. yoelii* (Preiser *et al*., 1999). Thus, individually infected red blood cells lack detectable transcription of p235 genes during the ring stages whereas transcription of different transcripts closely correlates with the number of nuclei present in individual parasites as they mature. Therefore, unique timings and patterns of transcription of these different malarial multigene families have now been established.

We used single-cell RT-PCR technology to determine the pattern of *vir* gene expression during late trophozoites/schizogony using infected reticulocytes obtained directly from three different human patients. Moreover, we used pair-combinations of degenerate yet subfamily-specific oligonucleotides (see *Experimental procedures*) to determine if more than one subfamily was concomintantly expressed. Of notice, these oligonucleotides amplify gene segments coding for the variant exon II of the different subfamilies and thus we have not addressed whether these genes are correctly spliced. Regardless, our results demonstrated that individual *P. vivax* mature trophozoite/schizonts concomitantly transcribes *vir* genes from at least two different subfamilies firmly establishing that expression of *vir* genes is not clonal during these blood stages. To the best of our knowledge, this is the first report of single-cell RT-PCR analysis of a subtelomeric multigene family using parasites obtained directly from human patients reinforcing the value of these data. Moreover, it opens the possibility of understanding control of gene expression of human subtelomeric multigene families in natural infections from individual parasites, which are still under immune pressure.

Laser confocal microscopy had previously demonstrated the localization of VIR proteins on the surface of infected reticulocytes (del Portillo *et al*., 2001), and the subcellular localization of STEVOR proteins, Sec31p proteins and SURFIN proteins (Akinola Adisa *et al*., 2001; Kaviratne *et al*., 2002; Winter *et al*., 2005). Using this same technology and different combinations of peptide antibodies against different VIR subfamilies, we were able to demonstrate that VIR proteins are expressed non-clonally by individually infected reticulocytes obtained from different patients. Of notice, the subcellular localization of these subfamilies was not unequivocally demonstrated. Thus, unlike the rim fluorescence detected at the surface of infected reticulocyte using anti-VIRD peptide antibodies (del Portillo *et al*., 2001), a more scattered pattern of fluorescence was detected with anti-VIRA-C-E peptide antibodies. Interestingly, a new subtelomeric *P. falciparum* multigene family termed SURFIN has been recently described and laser confocal microscopy demonstrated that members of this subfamily have different subcellular localizations (Winter *et al*., 2005). In addition, genomic and expressed *vir* sequences of parasites from different patients established varying extents of allele polymorphism among different subfamilies, which in turn have different predicted structures. It is thus tempted to speculate that VIR subfamilies have different subcellular localizations and current efforts are being done to address this possibility.

Of importance, in counting the number of individual cells reacting with antipeptide antibodies against subfamilies C and E, we found that more than 95% of infected cells showed fluorescence. Western blot analysis using anti-VIRE and anti-VIRC antibodies demonstrated that there is a large extent of cross-reactivity among subfamilies (Fig. 3B). Moreover, we analysed the sequences of these subfamilies generated in a larger study of parasites obtained from 17 other different patients (E.F. Merino and

H.A. del Portillo, unpublished). Remarkably, these particular peptide sequences from these two subfamilies were highly conserved in all the sequences analysed and which included 162 sequences from subfamily C, and 101 sequences from subfamily E. These data clearly explain the fact that most infected cells react against the different antipeptide antibodies and open rational avenues for the development of subunit VIR-based vaccines. Most important, these data demonstrate that conserved immunogenic VIR sequences capable of eliciting cross-reactive subfamily-specific antibodies are expressed in natural infections.

Acquired immunity in *falciparum* malaria is associated with exposure to different variant surface antigens, mostly PfEMP1 (Kyes *et al*., 2001). To date, no studies on acquired immunity against variant genes of *P. vivax* are available. We thus analysed the IgG responses of first-infected and multiple-infected *P. vivax* patients from the Brazilian Amazon State of Rondônia during their acute attacks against different VIR-tags. Malaria in this region is hypoendemic affecting mainly the adult male immigrant populations presenting infections that are invariably symptomatic or riverine populations of native Amazonians where asymptomatic infections are readily detected (Camargo *et al*., 1994; Alves *et al*., 2002). In the present study, immune sera pertained to immigrant adult populations with symptomatic infections. Moreover, although the number of genetic markers used in genetic population studies in Rondônia is very limited, different reports indicate that infections are mostly clonal (Hoffman *et al*., 2003). In spite of these epidemiological characteristics, immune sera from *P. vivax* first-infected patients reacted against different VIR-tags. As these results were generated using denatured GST-VIR tags in Western blot analysis, it was of importance to validate those using soluble GST-VIR tags in ELISAs. Indeed, using seven soluble GST-VIR tags representing the different subfamilies, ELISA and immune sera from 191 patients, one hundred of whom were first-infected patients, we have observed the same results (unpublished). The large extent of reactivity against VIR proteins in first-infected patients can be explained by the elicitation of cross-reacting antibodies to widely conserved VIR epitopes such as those described here for subfamilies C and E. Alternatively, mechanisms such as those recently proposed for PfEMP1 in which multiple infections with different variants are able to elicit cross-reacting antibodies (Gratepanche *et al*., 2003; Recker *et al*., 2004), can also be considered but this remains to be determined. Regardless, our data are inconsistent with the idea of long-term acquisition of immunity in malaria through the development of variant-specific antibodies against VIR proteins.

What is the function of VIR proteins? Perhaps the answer to this fundamental question resides in the unique biology of *P. vivax*, which predominantly, if not exclusively, invades reticulocytes (Kitchen, 1938; Galinski and Barnwel, 1995) and it is generally accepted that it does not cytoadhere in the deep capillaries of inner organs. In contrast to this idea, our present working hypothesis is that there is cytoadherence in *P. vivax*; yet, it is restricted to the barrier cells of the spleen where the main, if not unique function of VIR proteins, is spleen macrophage-clearance escape (del Portillo *et al*., 2004). While this proposition remains speculative, we hope that these data will further stimulate research on the complex interactions of *P. vivax*-infected reticulocytes and the spleen, which are central to a better understanding of how *P. vivax*, a non-lethal malaria, escapes the hosts' immune response and establishes chronic infections.

Experimental procedures

Study areas

Manaus and Porto Velho are the capital cities of the States of Amazonas and Rondônia where the 'Fundação de Medicina Tropical' (FMT/AM) and the 'Centro de Pesquisa em Medicina Tropical' (CEPEM), respectively, are located and where malaria patients participated in this study presented themselves for diagnosis and treatment.

Serum samples

A total of 32 serum samples pertaining to *P. vivax* patients from Rondônia and collected during their acute attack were used in this study. All patients, five females and 27 males, were volunteers ranging from 10 to 66 years old. Six patients were first-infected whereas the other reported 2–10 previous *P. vivax* attacks. Individual clinical and epidemiological information on these patients can be found in supplementary data (Table S2). In addition, the sera from seven healthy volunteers from Rondônia were used as controls in this study.

Parasite isolates

Plasmodium vivax isolates used to determine *vir* gene repertoires were obtained from three individual adults (code numbers MalDB80, MalDB81 and MalDB100) presenting themselves to CEPEM. Upon informed consent from these patients, 20 ml of peripheral blood were withdrawn from each patient and host leucocytes removed using Plasmodipur filters (EURO-DIAGNOSTICA, Arnhem, the Netherlands). Infected erythrocytes containing trophozoite and schizont stages were concentrated by centrifugation on a 57% Nycodenz gradient and parasite pellets stored frozen.

Plasmodium vivax isolates used in Western blot analysis were obtained from two individual adults presenting themselves to FMT/AM. Upon informed consent from these patients, 10 ml of peripheral blood were withdrawn from each patient and host leucocytes removed using cellulose CF11 columns. Infected erythrocytes containing trophozoite and schizont stages were concentrated by centrifugation on a

57% Percoll gradient and parasite pellets resuspended into 50 μl of cracking buffer. These studies received ethical clearance from the local and University of São Paulo's executive committees and all patients consented to donate their blood.

DNA/RNA extractions

Parasite genomic DNA was extracted following standard methodologies and stored at 4°C. Total parasite RNA was extracted using the TRIZOL reagent (Invitrogen) following the manufacturer's instructions, pellets resuspended in 20 μl of 100% formamide and stored at –70°C.

cDNA synthesis

As needed, 1 μg RNA aliquots were treated sequentially, in the same tube, with 1 U, 0.5 U and 0.5 U of DNase (Invitrogen) in final volumes of 10 μl, 15 μl and 20 μl, respectively, for 15 min each at room temperature. Reactions were stopped by adding EDTA to a final concentration of 2.5 mM and incubation at 65°C for 10 min. To guarantee the complete digestion of genomic DNA, 1 ml aliquots of DNase treated samples were amplified by PCR using primers of the different *vir* subfamilies (del Portillo *et al*., 2001). Only samples that did not amplify any products, and so were free of contaminating genomic *P. vivax* DNA, were used to prepare cDNA. cDNA was prepared using the GeneAmp RNA PCR Kit (Applied Biosystems) and random hexanucleotide primers according to the manufacturer's instructions. Single-stranded cDNAs were used as templates for new PCR rounds as described below.

Amplification, cloning and sequencing

Different *vir* subfamilies (A–E) were amplified from genomic DNA or cDNA by PCR using degenerate primers. Primers for subfamilies A, B and C have been previously described (del Portillo *et al*., 2001); subfamilies D and E were amplified using the following primers: forward, *virD* 5′-CG(W)TTATT(R)(K)CAAA(R)(M)(M)TGA(W)(Y)TG-3′, reverse, *virD* 5′-AATATATTCTTTTCTATTCATTTT-3′; forward, *virE* 5′-AA(Y)CAAGAA(W)TTTAT(S)AACTTTGT-3′, reverse, *virE* 5′-TACC(Y)TATATA(W)CGTTATTAGAGG-3′. We used annealing temperatures 5°C below the calculated Tm value of the primer pairs. PCR or RT-PCR fragments were resolved on 1% agarose gels, fragments excised, purified and cloned into pGEM-T easy (Promega) or TOPO TA Cloning (Invitrogen) vectors. Samples were sequenced using ABI PRISM BigDye terminator cycle sequencing kit version 2.0 (Applied Biosystems) and resolved and analysed in an ABI3700 96-capillary DNA sequencer (Applied Biosystems).

Computer analysis

Sequences were translated using the EditSeq Programme (DNAstar package; http://www.dnastar.com/) and submitted to BLASTP (Altschul *et al*., 1990). Alignments were done using CLUSTALX (Higgins *et al*., 1996) and manually edited with GeneDoc (http://www.psc.edu/biomed/genedoc/).

Micromanipulation and RT-PCR

Infected *P. vivax* blood was processed as described above and individual infected reticulocytes with mature forms from one patient (Pv1; Fig. 2) were collected by micromanipulation using 5 μm (HUMAGEN) micropipettes and an inverted light microscope (Nikon, model Eclipse TE 300) connected to a micromanipulator system NT-88 (Nikon/Narishige). RT-PCR was performed as described by Chen *et al*. (1998), with some modifications. Briefly, single infected cells were transferred to tubes containing 15 μl of RT mix (1× GeneAmp PCR buffer II, 1 mM dNTPs and 5 mM $MgCl_2$), frozen on dry ice and stored at –70°C. As needed, samples were heat-lysed, treated with 10 U of DNase (Stratagene) for 30 min at 37°C, and the RT reactions performed in a final volume of 20 μl. Four-microlitre aliquots were taken for PCR experiments using specific primers to amplify different *vir* subfamilies from the same tube. Fifty PCR cycles were performed, each consisting of denaturation for 30 s at 94°C, annealing for 90 s at 55°C (subfamily A)/50°C (subfamily C)/45°C (subfamily E), and elongation for 60 s at 72°C. For each experiment, a blank control and a control without reverse transcriptase were included to rule out the possibility of contamination and amplification attributed to the presence of genomic DNA.

To further exclude the possibility that these results were attributed to contaminant genomic DNA, individual infected reticulocytes with mature forms from two other patients were collected by micromanipulation using 5 μm (HUMAGEN) micropipettes, an inverted microscope Zeiss (Germany) model Axiovert 200, with DIC optics, and a micromanipulador Hydraulic, Narishige International, model MHW-3 (Tokyo, Japan). Equipments are mounted on a vibration isolation table from TMC (Peabody, MA, USA). Moreover, after releasing individual parasites by heat-lysis at 94°C for 3 min, 5 μl aliquots were left aside and used as negative RT-controls from each individual infected reticulocyte. Remaining 15 μl aliquots were treated with DNase as *vir* subfamilies amplified as above.

Laser confocal microscopy

Confocal microscopy was performed using a laser scanning confocal microscope (LSM 510 Zeiss). Smears were obtained as follows. After removal of 5 ml of peripheral blood from infected *P. vivax* patients, samples were centrifuged at 1200 r.p.m. per 3 min and the plasma and upper layers containing mostly leucocytes removed. One hundred-microlitre aliquots from pellets were then washed four times by centrifugation as above in RPMI containing 10% albumax. Smears were made on glass slides, air-dried, fixed in methanol at –20°C for 2 min, hot-dried immediately and stored individually wrapped at –70°C. Smears were probed with polyclonal monospecific antibodies raised against rabbits, guinea pigs, rats and mice immunized with peptides from different subfamilies: A (KKKKRKKRNYYDYGWC), C (QKDWRERKALY DYC) and E (RKFARNLKNISTILNDC). Peptides and antibodies were commercially produced (NeoSystem, FR). As controls, smears were probed with preimmune antisera. For dual labelled samples, the images were collected simultaneously using appropriate antibodies conjugated with fluorescein (KPL) or rhodamine (KPL). Images were visualized

using the software LSM Image Browser. Images sizes were 512 × 512 pixels. The area occupied by the parasite was defined by examination of transmission images.

GST-Vir tags, Western blot and ELISAs

Twenty-two different GST-fusion proteins expressing exon II and representing the different VIR subfamilies from three different patients (MalDB80 Accession Numbers AY608819, AY608843, AY608848, AY608849, AY608877, AY608879; MalDB81 Accession Numbers AY608826, AY608828, AY608844, AY608775, AY608875, AY608880, AY608810 and MalDB100 Accession Numbers AY608835, AY608846, AY608847, AY608860, AY608861, AY608799, AY608800, AY608885, AY608882) were cloned into pGEX-3X or pGEX4T-1 (Amersham Biosciences), expressed in *Escherichia coli* (BL21, Stratagene) after induction with 1 mM IPTG analysed by Western blot analysis under reducing conditions as previously described (Levitus *et al*., 1994). All GST fusion proteins remained as inclusion bodies and were purified by electroelution (Bio-Rad) prior to use in Western blot analysis.

Statistics

Statistical analysis was done using chi-squared Pearson's statistics and binomial exact test in the McNemar procedure (Kempthorne and Folks, 1971).

Acknowledgements

We are particularly grateful to Professor Fidel Zavala for critical reading of the manuscript. To Professor Maria das Graças Alecrim and Professor Luis Hildebrando Pereira da Silva for facilities at FMT/AM and CEPEM respectively. To Dr Monica Manso (FMT/AM) and Dr Juan Miguel Villalobos (CEPEM) and to Ms Yonne Chehuan Melo (FMT/AM) and Ms Joana d'Arc Neves (CEPEM) for immune sera and *P. vivax* samples. To Drs Karin Kirchgatter and Silvia di Santi (SUCEN) for *P. vivax* samples in São Paulo. To Dr Arthur Gruber (FMVZ–USP) and Dr Marta M. Teixeira (ICB–USP) for sequence facilities. To Professor Celia Garcia (IB–USP) and Dr Glaucia M. Machado Santelli (ICB–USP) for confocal facilities and to Mr Roberto C. Modia Junior and Mr Marcos M. Gazarini for helping in image acquisition. To Dr Rick Fairhurst (NIH) for technical advice in IFA and to Dr Edson Borges Junior for allowing the use of the micromanipulator facilities. The rabbit anti-PvMSP1 antibodies were obtained from the Malaria Research and Reference Reagent Resource Center (MR4). O.P. was a Diplomand from the laboratory of Professor Michael Lanzer. T.R.O. is a graduate student from FAPESP (No. 02/00039-0). I.S. receives support from FAPESP (01/08498-0). C.F.-B. receives support from CAPES (AUX-PRODOC-711/2002). The laboratory of H.A.P. is supported by FAPESP (01/09401-0) and CNPq (302572/2002-3).

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Multiple sequence alignment of the different VIR protein subfamilies.

Fig. S2. ELISAs of rabbit immune sera raised against peptides representing subfamilies A, C and E.

Fig. S3. ELISAs of human immune sera against peptides A, C and E.

Fig. S4. Confocal laser scanning immunofluorescence of individually infected *P. vivax* reticulocytes.

Fig. S5. Western blot analysis of the reactivity of human immune sera against GST-VIR tags from subfamilies A, C and E.

Table S1. Identification, redundancy and accession numbers of genomic and cDNA clones from the three patients (MalDB80, 81 and 100) from which the genomic and expressed *vir* gene repertoires were generated.

Table S2. Clinical and epidemiological data of patients who participated in this study.