Dynamics of Asymptomatic *Plasmodium vivax* Infections and Duffy Binding Protein Polymorphisms in Relation to Parasitemia Levels in Papua New Guinean Children

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Abstract. The interaction between *Plasmodium vivax* Duffy binding protein II (PvDBPII) and human erythrocyte Duffy antigen is necessary for blood stage infections. However, PvDBPII is highly polymorphic. We recently observed that certain recombinant DBPII variants bind better to erythrocytes in vitro. To examine the hypothesis that haplotypes with enhanced binding have increased parasitemia levels, we followed 206 Papua New Guinean children biweekly for six months with a total of 713 *P. vivax* samples genotyped. Twenty-seven PvDBPII haplotypes were identified, and 3 haplotypes accounted for 57% of the infections. The relative frequencies of dominant haplotypes remained stable throughout the study. There was no significant association with PvDBPII alleles or haplotypes with *P. vivax* parasitemia. The dominant haplotype (26% of samples), however, corresponded to a high-binding haplotype. Thus, common haplotypes are not likely to have arisen from increased fitness as measured by greater parasitemia levels. The restricted number of common haplotypes increases the feasibility of a PvDBPII-based vaccine.

INTRODUCTION

Although *Plasmodium vivax* infrequently kills, it can have major deleterious effects on personal well-being, growth, and development, especially for children. Many host factors such as age, immunity, and genetic background determine the severity of *P. vivax* infection and disease. Parasite factors such as efficiency of erythrocyte invasion, ability to avoid host immune responses, and anti-malarial drug resistance affect host risk. Blood stage parasitemia reflects the outcome of the interplay between host and parasite risk factors.

*Plasmodium vivax* Duffy binding protein II (PvDBPII) is an important protein in the *P. vivax* life cycle. The interaction between the PvDBPII and the human erythrocyte Duffy antigen receptor for chemokines (DARC) is required for blood stage *P. vivax* infections; alternative erythrocyte invasion pathways into human erythrocytes have not been identified. The PvDBPII is highly polymorphic within the region required for binding. We have observed differences in the binding of different recombinant PvDBPII variants to Duffy-positive erythrocytes as measured with a fluorescence-activated cell binding assay (Martin KK and others, unpublished data). This enhanced binding may increase parasite virulence by greater invasion into erythrocytes as reflected by increased parasitemia levels and/or greater frequency in the parasite population. In support of this hypothesis, we previously observed that Papua New Guinean children infected with *P. vivax* containing PvDBPII with a lysine at residue 447 were more likely to be blood smear positive and had higher parasitemia levels compared with children infected with *P. vivax* parasites with PvDBPII with a serine at residue 447.

The present study examines the hypothesis that certain PvDBPII alleles and/or haplotypes, particularly those with enhanced binding in vitro, are more common and/or are associated with higher parasitemia levels in children.

To examine whether PvDBPII alleles influence *P. vivax* parasitemia, we conducted an in-depth examination of *P. vivax* infections in a cohort of 206 Papua New Guinean children 5–14 years of age (mean = 9.4 years) who were followed every two weeks over a six-month study. We recently described the study design and epidemiology of malaria infection in the cohort in depth. At the beginning of the study, the children were treated to clear blood stage malaria infections. In this longitudinal cohort study, *P. vivax* parasitemia was assessed by several methods including blood smear, a semi-quantitative post–polymerase chain reaction (PCR) ligase assay, and a quantitative real-time PCR (QRT-PCR). *Plasmodium vivax* PCR-positive samples were then genotyped to determine the predominant PvDBPII allele.

MATERIALS AND METHODS

Study site. This study was conducted at the Mugil and Megiar elementary and primary schools situated on the north coast of Papua New Guinea, 50 km north of Madang directly across from Kar Kar Island as described in detail. The region is endemic for four species of human malaria: *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. The catchment area of all schools is serviced by a single health center at Mugil run by the Catholic Health Services.

Study design. In May 2004, 206 children 4–14 years of age (mean = 9.4 years, 95% confidence interval [CI] = 9.2–9.6 years) attending the Mugil/Megiar community schools were enrolled in the study after informed parental consent was obtained. A baseline peripheral 10 mL of venous blood was collected into EDTA-Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ). Blood smears from all children were obtained. A short physical examination was conducted. All children irrespective of blood smear positivity were then treated with a seven-day course of artesunate according to Papua New Guinea national treatment guidelines (i.e., 4 mg/kg on day 1, 2 mg/kg at days 2–7; 5 treatments were directly observed) for the eradication of all malaria blood stage infections. Artesunate does not clear liver stage *Plasmodium* infections. Two weeks after the start of treatment, all children were examined for treatment success with a finger prick of 250-μL blood collected into an EDTA-Microtainer® tube (Becton Dickinson) for blood smear and PCR analysis. Every two weeks thereafter for six months, enrolled children were
followed-up at the community schools or in their village (when possible) if they were not present in school that day. Blood smear readings and a 250-µL blood sample were obtained at each follow-up and a short physical examination was conducted. Samples were also collected through the community health center or at school when a child presented with symptoms of malaria illness. Only five clinical cases of infection with *P. vivax* were observed in the study on the basis of the following criteria: *P. vivax* positive with a parasite density greater than 1,000 parasites/µL and blood smear–negative for *P. falciparum, P. malariae*, and *P. ovale*. Further details of field procedures and levels of infection and disease with all four types of malarial infections have been described elsewhere.¹² Study protocols and consent forms were reviewed and approved by the Veteran’s Affairs Research Service and the Papua New Guinea Medical Research Advisory Committee institutional review boards.

**Diagnostic testing and quantification of Plasmodium infections.** *Plasmodium* infections were identified by two methods: blood smear and a post-PCR oligonucleotide ligation assay (OLA). For each of the study participants, a blood smear and a 250-µL blood sample were obtained at each follow-up. Thick and thin blood smears were stained with 4% Giemsa for 20 minutes and examined under microscopy at 100× magnification with an oil immersion lens. The number of parasites per 200 leukocytes was counted for each sample in two independent examinations by trained microscopists. All slides showing discrepant results for the two readings (different *Plasmodium* species or densities observed) were read a third time to verify the results. Parasitemia for each sample was determined from a geometric mean of two or more non-discrepant readings and converted to parasites per microliter of blood using a standard leukocyte count of 8,000/µL of whole blood.¹² Parasitemia levels were normalized by log transformation of n + 1. Thus, parasitemia used in this study is defined as the geometric mean of the parasites/µL + 1.

The post-PCR OLA was based on amplification of the small subunit ribosomal RNA gene as described and was used for *Plasmodium* species diagnosis.¹²–¹⁵ The infection intensity of *P. vivax* was quantified using an RT-PCR as described.¹⁶ Only *P. vivax*-positive samples identified by OLA were quantified using the real-time PCR. All other samples were assumed to have a *P. vivax* parasitemia of zero.

**Plasmodium vivax Duffy binding protein genotyping.** The polymorphic *PvdbpII* gene was used as a marker for *P. vivax* strains. *PvdbpII* was genotyped at 14 polymorphic residues using a *PvdbpII* post-PCR OLA as described.¹⁷ Haplotypes for single *P. vivax* infections were constructed on the basis of the genotyping results for the 14 single nucleotide polymorphisms (SNPs) at 13 amino acids; 8 of the 27 haplotypes were observed previously in the same population. On the basis of the observed haplotypes for single infections, haplotypes for mixed *P. vivax* infections (more than two *PvdbpII* variants were found) were inferred using the predominant allele at each SNP as described.¹⁷ Novel haplotypes not observed in single infections were listed as unclassified (5.3%). A subset of samples (n = 76) was further sequenced to verify genotyping results. Phylogenetic analysis was used to investigate the relationship between the observed *PvdbpII* genotypes. The phylogenetic tree was estimated using the neighbor-joining method and maximum parsimony as implemented in the MEGA 3.1 program.¹⁸,¹⁹ The reliability of the neighbor-joining tree was assessed by 1,000 bootstrap pseudoreplications.

**RESULTS**

**Accuracy of the *PvdbpII* OLA on the basis of sequencing.** To determine the accuracy of the *PvdbpII* OLA assay, 76 samples (representing more than 10% of the total samples analyzed) were sequenced from the same PCR product that was used to perform the OLA. Although not all sequences could be evaluated at residues 308 (one sample could not be evaluated) or 503 (8 samples could not be evaluated) because these residues were located near the ends of the PCR product, we were able to compare the sequencing results from the 76 samples at all other residues at the 14 SNPs. Thus, 1,055 residues (76 samples times 14 SNPs minus 9 SNPs that could not be evaluated) were used to determine the accuracy of the *PvdbpII* assay. A total of 17 (17 of 1,055 [1.6%]) OLA errors were found at individual SNPs in 14 of the 76 samples. These OLA errors occurred in only 5 of the 14 SNPs examined. Most errors occurred at SNP I503K (n = 8) and remaining errors occurred at SNPs L333R (n = 3), K371E (n = 2), N417K (n = 1), and W437R (n = 3). These errors resulted in assignment of the incorrect haplotype for 11 samples (14%).

Sequence analysis of these same 76 samples identified three additional non-synonymous SNPs in more than one sample that have not been previously identified: T404R (2 of 76 samples [2.6%]), K4111 (2 of 76 samples [2.6%]), and I430T (5 of 76 samples [6.6%]).¹¹,²¹–²³ We also observed another synonymous mutation at residue R378R (C to A transition at the third position of the codon); we have also observed a C to T transition in this same position in this population.¹¹ In addition, three other SNPs were observed with each SNP likely representing PCR errors.²⁴

**Diversity of *P. vivax* in the population on the basis of *PvdbpII.* To assess the diversity and relative frequency of *PvdbpII* variants in the population, 751 (95%) of the *P. vivax* PCR and/or blood smear–positive samples (n = 788) were amplified using the *PvdbpII*-specific OLA assay and genotyped. A total of 712 samples were assigned to 1 of at least 27 different *PvdbpII* haplotypes and only the dominant haplotype was assessed in mixed infections (Table 1). There were 39 (5.3%) samples for which we could not reliably ascribe a
haplotype. The PvDBPII haplotypes clustered into three main groups on the basis of phylogenetic analysis using both neighbor-joining and maximum parsimony methods that gave similar trees. Two haplotypes did not fit into the same families with both methods: PNG-26 and PNG-27. The condensed neighbor-joining tree is shown in Figure 1. At least eight of these haplotypes were present in this same population four years prior to this study (PNG-AH, PNG-C, PNG-U, PNG-P, PNG-E, PNG-17, PNG-11, and PNG-9), which indicated that polymorphisms at these residues are stable over time.\(^{11}\)

The predominant PvDBPII haplotypes during the study were PNG-AH (26%), PNG-P (10.4%), and PNG-O (20.4%) (Table 1). These same haplotypes were most prevalent in the children at each of the two-week follow-up time points with remarkably consistent frequency throughout the study (Figure 2). The mean number of PvDBPII haplotypes observed in each child over the course of the study was 2.6 (95% CI = 2.3–2.8, range = 0–7 genotypes).

**Higher parasitemias in multiple-strain *P. vivax* infections.** The PvDBPII-specific OLA method quantifies the amount of each allele present in a given sample and is able to detect alleles that are at least 10% of the *P. vivax* parasite population. We were able to identify multiple strain infections in many individuals.\(^{17}\) Using this method, we found that at least 63.0% (469 of 745) of the *P. vivax*-positive samples had more than one haplotype present and thus were multiple strain infections. Within the age group enrolled in the study (4–14 years), the number of multiple strain infections tended to decrease with age (Z = 1.3, P = 0.09, by Cochran-Armitage trend test). *Plasmodium vivax* infections with more than one detectable PvDBPII haplotype had 2.1–2.5-fold greater parasitemia levels as determined by blood smears (F = 19.01, P < 0.0001) and QRT-PCR (F = 64.23, P < 0.0001) (Figure 3).

**Lack of an association between PvDBPII haplotype and *P. vivax* parasitemia.** The mean parasitemia levels based on blood smears and QRT-PCR varied between the 27 haplotypes observed (Table 1). This mean, however, was not adjusted to account for differences in age, multiple strain *P. vivax* infections, differing number of follow-up observations for each child, or the increased *P. vivax* positivity observed in some of the children. Using a mixed linear model that adjusted for these confounding factors, we found no association between PvDBPII haplotype and *P. vivax* parasitemia as measured by blood smears (F = 1.28, P = 0.16) or QRT-PCR (F = 0.64, P = 0.92).

Because we did not observe a single PvDBPII haplotype that affected *P. vivax* parasitemia, we clustered the haplotypes into three groups as defined by the main branches of the neighbor-joining phylogenetic tree (Figure 1). As shown in Table 2, parasitemia in the Sal-I group tended to be higher than the other two groups but this difference did not reach statistical significance for parasitemia as determined by blood smears (F = 2.01, P = 0.1381) or for parasitemia determined by QRT-PCR (F = 0.54, P = 0.5835).

Modeling of the PvDBPII based on the crystal structure of the *P. knowlesi* Duffy binding like (PkDBL) domain suggests that subdomain 2 (amino acids 303–406) contains the binding region.\(^{23}\) Thus, we hypothesized that certain haplotypes with

### Table 1

| Haplotype name | Amino acid haplotype† | No. observed (%)‡ | No. BS positive (%)§ | Mean BS parasitemia (SD)$|$ | Mean QRT-PCR parasitemia (SD)$|$ |
|----------------|-----------------------|------------------|---------------------|-----------------------------|-----------------------------|
| PNG-AH         | SLENGERKIRSK          | 195 (26.0)       | 75 (38.5)           | 6.1 (10.9)                  | 4.7 (4.6)                   |
| PNG-O          | SLKNGEKHNIWKI         | 153 (20.4)       | 46 (30.1)           | 3.7 (8.4)                  | 3.6 (4.1)                   |
| PNG-P          | SFKDGGKNHIRSK         | 78 (10.4)        | 30 (38.5)           | 5.7 (8.8)                  | 3.8 (4.0)                   |
| PNG-25         | SLKNGEKHNIIRKK        | 47 (6.5)         | 29 (61.7)           | 20.8 (13.9)                | 7.4 (6.3)                   |
| PNG-20         | SLENGEKKRIRKK         | 27 (3.6)         | 8 (29.6)            | 3.8 (8.3)                  | 2.6 (3.0)                   |
| PNG-11         | SFKDGGKNHIRKK         | 25 (3.3)         | 15 (52.0)           | 13.1 (13.7)                | 5.2 (3.4)                   |
| PNG-T          | SLKNGEKHNIWKI         | 24 (3.2)         | 6 (25.0)            | 3.2 (8.5)                  | 4.4 (3.7)                   |
| PNG-17         | SLENGEKHIRKK          | 18 (2.4)         | 6 (33.3)            | 4.9 (9.1)                  | 4.0 (4.1)                   |
| PNG-9          | SFKDGGKHIRKK          | 14 (1.9)         | 6 (42.9)            | 7.9 (12.4)                 | 9.8 (3.5)                   |
| PNG-27         | SLKNGEKHNIWSK         | 12 (1.6)         | 5 (41.7)            | 9.1 (20.3)                 | 4.4 (4.1)                   |
| PNG-24         | SLKNGEKHIRKKI         | 11 (1.5)         | 3 (27.3)            | 3.2 (7.5)                  | 3.2 (2.8)                   |
| PNG-1          | RLFKNGEHIRKR           | 11 (1.5)         | 5 (45.5)            | 8.6 (12.2)                 | 10.6 (4.0)                  |
| PNG-E          | RLKNGEKHRNWSK         | 11 (1.5)         | 6 (54.6)            | 11.5 (10.7)                | 2.4 (2.4)                   |
| PNG-C          | SLKNGERKIRSK          | 10 (1.3)         | 4 (40.0)            | 7.4 (11.7)                 | 7.0 (3.3)                   |
| PNG-26         | SLKNGEKHNRWSK         | 10 (1.3)         | 5 (50.0)            | 15.7 (20.1)                | 4.7 (8.6)                   |
| PNG-2          | RLFKNGEKRIRKK         | 9 (1.2)          | 1 (11.1)            | 1.7 (4.6)                  | 1.8 (1.7)                   |
| PNG-12         | SFKNGEHIRKKI          | 9 (1.2)          | 3 (33.3)            | 3.1 (8.1)                  | 3.3 (1.9)                   |
| PNG-3          | RLKNGEKHIRKKI         | 9 (1.2)          | 5 (55.6)            | 22.2 (26.9)                | 12.4 (14.9)                 |
| PNG-10         | SFKNGEHIRKR           | 8 (1.1)          | 2 (25.0)            | 3.3 (9.1)                  | 5.6 (2.8)                   |
| PNG-U          | SLKNGEHIRKR           | 7 (0.9)          | 1 (14.3)            | 2.0 (5.2)                  | 3.0 (2.1)                   |
| PNG-4          | RLKNGEKHNRSWK          | 5 (0.7)          | 4 (80.0)            | 41.5 (8.8)                 | 2.4 (3.5)                   |
| PNG-28         | RLKNGEKHNRSWI          | 5 (0.7)          | 3 (60.0)            | 14.7 (11.8)                | 3.7 (4.6)                   |
| PNG-29         | RLKNGEKNNRSWK          | 5 (0.7)          | 2 (40.0)            | 6.2 (12.3)                 | 4.5 (5.7)                   |
| Sal-I          | RLKNGEKNRSWI          | 5 (0.7)          | 3 (60.0)            | 12.2 (10.1)                | 4.6 (2.0)                   |
| PNG-23         | SLKNGEKHRSWK          | 2 (0.3)          | 1 (50.0)            | 10.3 (27.2)                | 2.2 (1.1)                   |
| PNG-7          | SFKNGEKRIRSK          | 1 (0.1)          | 1 (100)             | 92.5                       | 13.5                       |
| PNG-19         | SLENGEKRIRSK          | 1 (0.1)          | 0 (0.0)             | 0.0                        | 0.0                        |
| Unclassified   | Not determined         | 39 (5.2)         | 15 (38.5)           | 7.2 (14.4)                 | 5.7 (7.5)                   |
| Total          |                       | 751 (100)        | 288 (38.4)          | 6.1 (10.8)                 | 4.4 (4.5)                   |

† PvDBPII = *Plasmodium vivax* Duffy binding protein II; BS = blood smear; QRT-PCR = quantitative real-time-polymerase chain reaction.
‡ Number of samples observed during the study with each haplotype and percent of total *vivax*-positive samples genotyped.
§ Number of BS-positive samples observed during the study for each haplotype and percent of samples BS positive for each haplotype.
$|$ Mean BS parasitemia or mean RTO-PCR parasitemia = geometric mean of parasites/μL + 1.
variants in subdomain 2 may be better able to bind to DARC leading to higher parasitemia levels. All haplotypes that contained the same subdomain 2 were grouped together for analysis to give a total of 15 groups. Subdomain 2 includes SNPs 308–390. Thus, haplotypes PNG-3, -4, and -28 were grouped together; PNG-10, -11, and -12 were grouped together; PNG-17 and -19 were combined; PNG-26 and -27 were grouped together; PNG-O, -U, -T, -24, and -25 were combined; and Sal-1, PNG-E, and -2 were grouped together for analysis; all other haplotypes contained a unique subdomain 2 and were not grouped with others. This reclassification of haplotypes also failed to show any association with \( P.\) vivax parasitemia using a mixed linear model as determined by blood smears (\( F = 0.92, P = 0.5386 \)) or as determined by real-time PCR (\( F = 0.97, P = 0.4832 \)).

We recently observed that a recombinant fragment including subdomain 3 of \( \text{PvDBPII} \) binds better to DARC-positive erythrocytes than the full \( \text{PvDBPII} \), which indicated that this portion of the molecule contains the minimum essential binding motif (Martin KK and others, unpublished data). In addition, we observed that recombinant proteins for the corresponding PNG-AH and PNG-C haplotypes bind more DARC-positive erythrocytes than other haplotypes (Martin KK and others, unpublished data). We examined whether haplotypes of subdomain 3 (amino acids 406–528) are associated with higher parasitemias. All haplotypes that contained the same subdomain 3 were grouped together for analysis (Table 3). We found a wide range of parasitemia levels associated with different subdomain 3 haplotypes based on blood smears (3.2–92.0 parasites/µL; \( F = 2.57, P = 0.0083 \)). There was no association with haplotypes and parasitemia levels, however, based on QRT-PCR (\( F = 0.90, P = 0.5250 \)) (Table 3).

It is possible that individual polymorphic SNPs may influence parasitemia by altering the structure of the binding region or be directly associated with binding that may not be reflected in haplotype analysis.\(^{26,27}\) Previously, we have also observed an association between lysine at residue 447 and higher parasitemia in children in this same community.\(^{11}\) Thus, we examined the geometric mean blood smear and QRT-PCR for \( P.\) vivax parasitemia for each allele at all 13 common polymorphic loci of \( \text{PvDBPII} \) and found no statistically significant differences using the mixed linear model at any of the 13 loci.

**DISCUSSION**

The binding motif of \( \text{PvDBP} \) is highly polymorphic. This may become an obstacle for development of \( \text{PvDBPII} \) as a vaccine candidate antigen for the prevention of \( P.\) vivax infection. Presumably, these polymorphisms arose as a conse-
sequence of host immune selection pressure, or enhanced binding facilitating erythrocyte invasion, or a balance between both. Approximately 24% of (171/712) nucleotide residues of the \textit{PvdbpII} PCR product examined in this study have been shown to be polymorphic with approximately 20% (142/712) encoding non-synonymous mutations. Most of these SNPs are rare, and 13 SNPs contribute to most of the observed variants.

We identified at least 27 different \textit{PvDBPII} haplotypes in this population and found that 3 haplotypes accounted for 57% of the haplotypes observed during the six-month study period. Previous \textit{Pv} genotyping studies conducted in Papua New Guinea have also found \textit{P. vivax} infections to be complex and dynamic in individuals. These studies, however, have not systematically examined the frequencies of haplotypes in a cohort of the population. By genotyping \textit{P. vivax} infections in 206 children every two weeks for six months, we have found that the relative frequency of the three most dominant haplotypes remained fairly stable over this time interval (Figure 2). These observations are consistent with previous studies using PCR-based oligonucleotide probes for allele determination and recent findings using a different high-throughput genotyping method for the \textit{P. falciparum} merozoite surface protein (MSP-1).

At least eight of the \textit{PvDBPII} haplotypes (PNG-AH, PNG-O, PNG-P, PNG-T, PNG-E, PNG-C, PNG-U, and Sal-1) were observed four years earlier in the same population. We also identified 19 additional haplotypes in samples with single \textit{P. vivax} infections; an additional 39 samples (5.2%) could not be inferred based on the \textit{PvDBPII}-specific OLA. These unclassified \textit{PvDBPII} haplotypes may represent newly emerging or rare alleles that are at a low frequency in the population. A few of these unclassified samples had more than one \textit{PvDBPII} haplotype with similar concentrations, which made their definitive haplotype assignment impossible.

This study represents the first large-scale genotyping of the \textit{PvDBPII} using the OLA. Although 98.4% of the 14 SNPs examined by sequencing matched the OLA results, haplotype

<table>
<thead>
<tr>
<th>Phylogenic group</th>
<th>Haplotypes included</th>
<th>Mean BS parasitemia (SD)</th>
<th>Mean QRT-PCR parasitemia (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>AH, C, 20, 17, 2, 23, U, 26, 27, 9, P</td>
<td>5.8 (10.3)</td>
<td>4.3 (4.3)</td>
</tr>
<tr>
<td>A</td>
<td>7, 10, 12, 11, 24, 25, T, 19, O</td>
<td>5.6 (10.6)</td>
<td>4.3 (4.3)</td>
</tr>
<tr>
<td>Sal I</td>
<td>1, 3, 4, 28, 29, E, Sal I</td>
<td>13.4 (12.0)</td>
<td>5.2 (12.0)</td>
</tr>
</tbody>
</table>

* Subdomain 3 includes single nucleotide polymorphisms between 417 & 503.
† Number of samples observed during the study with each haplotype as the predominant haplotype determined.
‡ Mean blood smear parasitemia = geometric mean of parasites/µL + 1.
§ Mean QRT-PCR parasitemia = geometric mean of parasites/µL + 1.
reconstruction proved challenging because we were examining 14 SNPs, many of the infections contained multiple \textit{P. vivax} strains, and the OLA reaction at residue 503 was problematic. The error rate we observed (0.14), however, is similar to other error rates for haplotype reconstruction.\textsuperscript{36} We may be able to improve our haplotype reconstruction of the most abundant clone in multiple strain \textit{P. vivax} infections by using an algorithm for reconstructing haplotypes such as PHASE.\textsuperscript{37}

The PvDBPII OLA reactions for the 14 SNPs were chosen because these SNPs were present in the population with a frequency greater than 5\% in 2000.\textsuperscript{11,17,24} Sequence analysis of 76 samples showed one other novel SNP at residue I430T that occurred at a frequency of 6.6\% that should be included for future genotyping studies.

There are several potential explanations that could account for the dominant PvDBPII haplotypes that were observed in this population. First, certain haplotypes may bind better to the DARC, which may enhance parasite invasion and thus fitness as reflected by higher peripheral blood parasitemias. We found that recombinant PvDBPII corresponding to PNG-AH, the most common haplotype, bound more erythrocytes than recombinant protein corresponding to other variants (Martin KK and others, unpublished data). However, recombinant protein corresponding to the PNG-O and PNG-P variants, the next most frequent variants in the population, did not bind better to erythrocytes compared with less common variants (PNG-T and Sal-I). The relationship, therefore, of \textit{in vitro} binding with haplotype frequency in the population is complex. Also, there was no detectable relationship between levels of parasitemia and PvDBPII haplotype, which rejected our initial hypothesis that certain haplotypes may invade more successfully than others. The measurement of parasitemia levels, however, is an imprecise measure of parasite fitness. Factors such as varying levels of protective antibodies could affect parasitemia levels in this population of children with significant protection against clinical disease; only five children had clearly confirmed clinical disease caused by \textit{P. vivax}.\textsuperscript{12} It is still possible that slight differences exist in parasite invasion efficiency that we were unable to detect. Ultimately, the isolation of parasites from different strains and evaluation of their ability to invade and replicate \textit{in vitro} might better substantiate this hypothesis. Such experiments are now feasible because of greater success with short-term culture of \textit{P. vivax}.\textsuperscript{38–40}

A second explanation to account for dominant haplotypes is that certain PvDBPII haplotypes may be less immunogenic, thereby escaping the host immune response, which results in a greater frequency in the population. Although less immunogenic PvDBPII haplotypes may not result in an increased frequency in those children with little immunity because they are more susceptible to all strains, a less immunogenic haplotype may be able to escape immune detection in those individuals with stronger immunity towards \textit{P. vivax}, which leads to higher levels of this haplotype in the human population. Overall, this seems less likely because plasma antibodies from these 206 children prior to treatment recognized recombinant PvDBPII corresponding to the dominant PNG-AH variant just as well and if not better than recombinant proteins corresponding to less abundant haplotypes, e.g., PNG-T, PNG-O, PNG-P, and Sal-I. To better understand the relationship of haplotypes with immunity, we need to examine their distribution in younger, less immune children relative to older children. We appreciate, however, that plasma recognizing recombinant proteins may not reflect the development of functional antibody responses \textit{in vivo}. Thus, we are currently investigating functional antibody assays. Additionally, if PvDBPII were under strong immune selection with escape mutants, we might expect to see a more dynamic change in PvDBPII haplotypes in parasite populations. Certain variants may be eliminated with immune pressure and others could emerge that may not have been previously recognized by the host. This has been observed with \textit{P. falciparum} erythrocyte membrane protein-1.\textsuperscript{41} Instead, we observed a fairly consistent proportion of the different variants over the period of observation. Six months, however, may be an insufficient period of time to observe such changes. Of note, more than one-third of the children lacked any detectable antibody response to any of PvDBPII variants tested, even though these children appeared to have developed a high degree of immunity to clinical \textit{P. vivax} malaria, as indicated by the almost complete absence of clinical \textit{P. vivax} disease.\textsuperscript{12} This suggests that the PvDBPII is not highly immunogenic and significant naturally acquired immunity conferred by antibodies to DBPII may develop in only some individuals.

Alternatively, the haplotype frequencies may simply be stochastic. Parasites with certain PvDBPII haplotypes may have been introduced into this population by chance and persist by relapsing and enhanced transmission with occasional escape mutants because of relatively weak immune selection pressure. This possibility would be supported by showing spatial heterogeneity of the PvDBPII variants in the study populations in Papua New Guinea and by measuring the relative abundance of these dominant PvDBPII haplotypes from archival samples from the same populations in previous years.

In the present study, we observed that children infected with two or more strains of \textit{P. vivax} had parasitemia levels at least two times higher than those children with only one detectable strain of \textit{P. vivax}. Previously, we have shown that the prevalence of \textit{P. vivax} infections, the intensity of infection (as measured by parasitemia), and the percent of infections with more than one strain of vivax by both PvDBPII and \textit{P. vivax} merozoite surface protein 3a (PvMSP3a) peaks in children 5–9 years of age in this community and begins to decrease in children more than 9 years of age.\textsuperscript{11} The same trends were observed in the current study. An explanation for this observation is that as children acquire immunity to \textit{P. vivax} malaria, they more readily clear blood stage infections and thus are less likely to acquire multiple parasite strains detectable at any particular time. Measuring PvDBPII haplotypes will underestimate the complexity of parasite strains in an individual. In a subset of children, 5–14 different \textit{P. vivax} microsatellite markers were measured, which showed a higher complexity of infection than we were able to detect with the PvDBPII-specific OLA genotyping (King CL, Karunaweera, unpublished data).\textsuperscript{42} PvMSP3a restriction fragment length polymorphism analysis performed on samples collected from this same population four years earlier also support the observation that the complexity of \textit{P. vivax} infections is higher than we are able to detect with the OLA genotyping.\textsuperscript{24}

In conclusion, we observed many PvDBPII haplotypes present in this population with three dominant haplotypes. It is unlikely that certain haplotypes increased efficiency of blood stage infection as measured by increased peripheral blood parasitemia levels. Studies that evaluate the association
of the different haplotypes with cellular and humoral immunity to PvDBPII and their spatial and temporal pattern of distribution in humans and mosquitoes will provide further insights to explain the distribution of PvDBPII variants in parasite populations. If certain haplotypes are predominant across different age groups and locations, this would simplify development of a PvDBPII subunit vaccine.

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