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Genome-Scale Protein Microarray Comparison of Human Antibody Responses in *Plasmodium vivax* Relapse and Reinfection

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Abstract. Large scale antibody responses in *Plasmodium vivax* malaria remains unexplored in the endemic setting. Protein microarray analysis of asexual-stage *P. vivax* was used to identify antigens recognized in sera from residents of hypoendemic Peruvian Amazon. Over 24 months, of 106 participants, 91 had two symptomatic *P. vivax* malaria episodes, 11 had three episodes, 3 had four episodes, and 1 had five episodes. *Plasmodium vivax* relapse was distinguished from reinfection by a merozoite surface protein-3 α restriction fragment length polymorphism polymerase chain reaction (MSP3 α PCR-RFLP) assay. Notably, *P. vivax* reinfection subjects did not have higher reactivity to the entire set of recognized *P. vivax* blood-stage antigens than relapse subjects, regardless of the number of malaria episodes. The most highly recognized *P. vivax* proteins were MSP 4, 7, 8, and 10 (PVX_003775, PVX_082650, PVX_097625, and PVX_114145); sexual-stage antigen s16 (PVX_000930); early transcribed membrane protein (PVX_090230); tryptophan-rich antigen (Pv-fam-a) (PVX_092995); apical merozoite antigen 1 (PVX_092275); and proteins of unknown function (PVX_081830, PVX_117680, PVX_118705, PVX_121935, PVX_097730, PVX_110935, PVX_115450, and PVX_082475). Genes encoding reactive proteins exhibited a significant enrichment of non-synonymous nucleotide variation, an observation suggesting immune selection. These data identify candidates for seroepidemiological tools to support malaria elimination efforts in *P. vivax*-endemic regions.

INTRODUCTION

Plasmodium vivax malaria affects human populations in Asia, Latin America, and parts of Africa.^{1,2} *Plasmodium vivax* malaria is conventionally considered to be less of a public health concern than the more lethal malaria parasite *P. falciparum*, which dominates malaria-related mortality predominantly in sub-Saharan Africa.³ The biology of *P. vivax* fundamental differs from *P. falciparum* particularly with regard to how this species maintains itself in human populations, particularly the ability to relapse from the dormant liver forms, the hypnozoite, and its wider tolerance of temperature conditions enabling sporogonic development.³ For these reasons, and because the parasite cannot be propagated in vitro, studies of this form of malaria are neglected. In an era when malaria eradication has been put on the global health agenda,⁴ an exigent need has emerged to understand detailed aspects of *P. vivax*, including parasite antigen-specific human immune responses and to develop rapid tools to assess transmission on a population level.

Current estimates of the global prevalence of *P. vivax* infection are based on identifying potential regions of transmission using a geo-referenced parasite-prevalence rate combined with biological masking to indicate malaria-compatible transmission zones.⁵ Quantifying the global burden of *P. vivax* malaria is complicated by the biology of the parasite: *P. vivax* relapse from dormant hypnozoite liver stage over time and space—often asymptotically—which allows for human migration to

disperse infection within an endemic region or reintroduce the parasite into areas where malaria may have been eliminated. The potential range of *P. vivax* transmission is vast, the biology of the parasite complicates incidence and prevalence estimates, and the public health impact of *P. vivax* remains. Regional elimination and global eradication require accurate and population-deployable tools to estimate parasite prevalence and malarial disease incidence.^{1,2,4,6}

Because determining the presence of malaria parasitemia or exposure is time intensive, often insensitive, and expensive, interest has grown in using serological tools to monitor infection status and transmission dynamics—so-called seroepidemiology. This approach has been particularly useful for malaria^{7,8} and additionally for vector-borne diseases such as lymphatic filariasis in which mass drug administration campaigns have been carried out.^{9–14} Antigens for antibody detection have included lysates of in vitro grown *P. falciparum* schizonts^{8,15–20} and recombinant proteins based on vaccine candidates (circumsporozoite protein, merozoite surface protein 1 [MSP1], and apical membrane antigen-1 [AMA-1]) have been commonly used. By analogy, for seroepidemiological studies of *P. vivax*, recombinant PvMSP1 and PvAMA-1 have been used to determine seroprevalence of antibodies to *P. vivax* in populations of Vanuatu, Solomon Islands.⁷ More recently, a proof-of-principle array study using 152 predicted asexual-stage proteins was reported in which sera from Korean *P. vivax* malaria patients were analyzed for anti-*P. vivax* antibodies using a wheat germ expression system.²¹ Recent work from Papua New Guinea—where all four major *Plasmodium* spp. circulate at intense level—has led to the development of focused protein microarrays²² composed of both *P. falciparum* and *P. vivax* recombinant proteins. Because *P. vivax* samples from humans have low parasitemia, and only in limited quantities from non-human primates, seroepidemiology studies using

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P. vivax asexual-stage parasite lysates has only infrequently been done, hence recombinant proteins are essential for studying exposure to *P. vivax* in endemic populations.

To understand the *P. vivax* antigens predominantly recognized by the antibody response of naturally infected humans in a low-transmission region, we analyzed the serological reactivity of patients with *P. vivax* malaria using custom-made protein microarrays composed of asexual blood-stage antigens predicted by transcriptional profiling^{23,24} and heterologously produced by a prokaryotic cell-free expression system.^{25–27} With the validation of molecular tools to distinguish *P. vivax* relapses and reinfections that are common in our Amazonian village study population,^{28,29} we compared the level of IgG responses between consecutive episodes of symptomatic *P. vivax* malaria, in particular, to determine whether naturally acquired antibody responses were boosted by subsequent infection. More generally, we intended to systematically and comprehensively identify *P. vivax* antigens of potential use for seroepidemiological studies and possibly for identifying vaccine candidates.

MATERIALS AND METHODS

Ethics statement. This study was approved by the following institutional review boards: Ethical Committee of Universidad Peruana Cayetano Heredia, Lima, Peru; Ethical Committee of Asociación Benéfica PRISMA, Lima, Peru; the Directorate of Health of Loreto-Peru; the Humans Research Protection Program of the University of California at San Diego, La Jolla, CA; and the Institutional Review Board of the Johns Hopkins Bloomberg School of Public Health, Baltimore, MD. All participants provided written informed consent and were coded through all study participation. Specimens were also coded before protein microarray analysis.

Study sites. The field activities of this study were carried out from May 2005 to July 2008 in northeastern Peru, in villages near to the city of Iquitos in the province of Maynas, the capital of the Amazon Department of Loreto (map in Ref. 28). Considering the region's geographical isolation from the rest of Peru, health services within the surrounding areas of Iquitos are relatively good and accessible. Three health posts provide medical services to the study villages described as follows. Santo Tomas health post, located 16 km by road from Iquitos and surrounded by the Nanay River, is a referral health center for three villages: La Unión, 12 de Diciembre, and Santo Tomás, collectively 2,650 inhabitants. San Jose de Lupuna health post, located 10 km from Iquitos is accessible only through the Nanay River and is the referral center for four villages: San Pedro, Santa Rita, Fray Martín, and San Jose de Lupuna, with collectively 1,250 inhabitants. Padrecocha health post, located 6 km from Iquitos is accessible only through the Nanay River and is a referral health post for three villages: San Andrés, Nueva Vida, and Padrecocha, with collectively 1,800 inhabitants.

Blood sampling. Blood samples were collected from patients diagnosed by conventional light microscopy on site at Peruvian Ministry of Health establishments according to national norms. Patients identified as infected with only *P. vivax* (microscopy only) were invited to participate in the study. At each enrollment site, venous blood was collected in EDTA Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) and taken to the field project laboratory where samples were aliquoted and frozen at

–20°C. Samples were shipped on dry ice to Universidad Peruana Cayetano Heredia in Lima for molecular diagnostic analysis.

Molecular confirmation of *P. vivax* infection. DNA was extracted from 200 µL of thawed, anticoagulated whole blood using the Qiagen Blood Kit (Qiagen, Valencia, CA). The diagnosis of *P. vivax* infections was confirmed in all patients using a genus and species-specific nested polymerase chain reaction (PCR) assay based on an 18S ribosomal RNA gene fragment specific for the genus *Plasmodium*, as previously reported.^{28,30} A 1,200-bp (base pair) fragment allowed for *Plasmodium* genus-level identification; identification of *P. vivax* and *P. falciparum* species was done using a 120-bp fragment specific for *P. vivax* and a 205-bp fragment specific for *P. falciparum*; samples containing *P. falciparum* or mixed infections were not considered for further analysis, *P. malariae* or *P. ovale* were not found.

Molecular genotyping. Genotyping of *P. vivax* isolates was performed by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the *P. vivax* MSP3α (*PvMSP3α*) gene as previously described.³¹

Case definition and data analysis. Relapse was defined as identical *PvMSP3α* PCR-RFLP patterns when primary and subsequent infection parasites were compared. Reinfection was defined as different genotypes present in primary versus subsequent infection parasites by *PvMSP3α* PCR-RFLP patterns.^{28,29,32,33}

Protein microarray analysis using a *P. vivax* asexual blood-stage proteome array. A proteome array was designed containing 2,233 *P. vivax* recombinant proteins, representing 1,936 predicted *P. vivax* asexual blood-stage proteins and comprised as recently described.²² Proteins predicted to be secreted or present on the parasite surface, on the basis of the presence of a secretory signal peptide or transmembrane domains, were annotated as such in PlasmoDB^{34,35} and preferentially included. Additional selection for expression of *P. vivax* genes included expression microarray evidence for blood-stage expression.^{23,24} Putative cytoplasmic proteins, lacking both signal peptides and transmembrane domains, were also selected (isoelectric point [pI] < 7.6 for *P. vivax* genes). *Plasmodium vivax* *vir* genes were excluded because of their variability. Both single and multi-exon genes were selected. Exons of multi-exon genes were cloned and expressed separately. Furthermore, large genes or exons were further divided into overlapping segments to limit amplicon length between 300 and 3,000 nucleotides. Amplicons were labeled with the exon number and the total number of exons, such as “1o2” for exon 1 of a 2-exon gene. Genes that were further divided into segments were labeled s1, s2, and so forth.

The array was fabricated as previously described.³⁶ In brief, target sequences were amplified genes from *P. vivax* genomic DNA (Sal I strain [MRA-552, MR4]) and cloned into the PXT7 plasmid with a T7 transcription terminator and encoded 5' polyhistidine (HIS) and 3' hemagglutinin (HA) epitopes. Recombinant proteins were expressed by an *Escherichia coli* cell-free in vitro transcription and translation system (Rapid Translation System 100 High Yield [RTS 100 HY] kits from 5 PRIME, Gaithersburg, MD) according to the manufacturer's instructions. Proteome arrays were printed as previously described, with each recombinant protein spotted once in each array.³⁶ Each array contained 256 negative control spots made with an in vitro transcription–translation master mix without plasmid DNA. Once printed, recombinant protein expression

TABLE 1
Characteristics of participants and recurrent episodes (relapses plus reinfections)

	Number	Mean age (years) [min-max]	Gender (%)	Median age (years)	Mean time to relapse/reinfection (months) [(min-max)]	Mean lifetime malaria episodes
Participants	106	27 [3-83]	Female (48)	24	8.5 [1-24.2]	4.8
Recurrences	126				8.5 [1-24.2]	
Relapses	48	26 [3-83]	Female (38)	18	6 [1.2-20.4]	4.3
Reinfections	78	28 [5-76]	Female (49)	25	10 [1-24.2]	5.2

max = maximum; min = minimum.

was verified using anti-polyhistidine (clone His-1; Sigma-Aldrich, St. Louis, MO) and anti-hemagglutinin (clone 3F10; Roche, Indianapolis, IN) monoclonal antibodies, as previously described.³⁶ All signal intensities were corrected for spot-specific background. A recombinant protein was deemed to be present on the slide if its spot's mean fluorescence intensity was higher than the mean of the "no DNA" control spots plus two standard deviations.

Overall, 1,470 different *P. vivax* recombinant proteins (66%) were expressed with both His and HA tags, confirming that the majority of recombinant proteins were fully expressed. These represented 1,328 *P. vivax*-predicted asexual blood-stage proteins. Furthermore 1,949 *P. vivax* recombinant proteins (87%) were expressed with at least one tag suggesting that these recombinant proteins were at least partially expressed.²²

Microarray data analysis. A reactive protein was defined as a protein that had a signal intensity values > 2-fold the mean of the "no DNA" intensity values for the same sample (i.e., column on the matrix) in at least 10% of the samples in the *Pv*-infected group, *Pv* reinfected group or the *Pv* relapse group. The background-corrected signal intensity values for reactive antigens were log 2 transformed and normalized by subtracting the median of the "no DNA" spots in the log space. *t* tests were used to identify differentially reactive antigens. The Benjamini-Hochberg (BH) method was used for multiple test correction, which estimates the False Discovery Rate (FDR) and provide corrected *P* values. Statistical analysis and figure generation was performed in the R environment (www.r-project.org). Paired Student's *t* test was used to compare differences in total antibody responses between relapsed and reinfected subjects.

Analysis of genome-wide *P. vivax* single nucleotide polymorphisms. The genes encoding were compared with the four globally represented and annotated *P. vivax* genomes (parasites from Brazil, India, Mauritania, and North Korea), available at:

https://www.broadinstitute.org/annotation/genome/plasmodium_vivax/GenomeDescriptions.html (accessed May 5, 2015).

RESULTS

Number of *P. vivax* malaria episodes. In the study period, 106 participants were enrolled who had 1-5 episodes of symptomatic light microscope- and PCR-confirmed *P. vivax* malaria (Table 1, Figure 1). The median age was 24 years, main age was 27 years, and 48% of the subjects were women. Occupation and demographics of the study participants were typical of the region (Table 2).

All infected participants were given standard antimalarial treatment following guidelines from the Peruvian Ministry of Health (chloroquine for 3 days (10 mg/kg on days 1 and 2 and 5 mg/kg on day 3), plus primaquine (0.5 mg/kg/day for 7 days)). Of the 106 subjects, 91 had two episodes, 11 had three episodes, 3 had four episodes, and 1 had five episodes. Using PvMSP3 α genotyping, of the recurrent infection episodes, 48 had identical PvMSP3 α PCR-RFLP patterns (hereafter referred to as "genotype") compared with a previous infection leading to classification as relapse; 78 had different genotypes and were classified as reinfection. This classification is limited by the possibility of a subject harboring more than one *P. vivax* clone and hence overestimates the rate of reinfection compared with relapse. Subjects with *P. vivax* malaria tended to report more lifetime episodes of malaria than the episodes observed during the study period (Table 1). The average number of self-described malaria lifetime episodes for all participants was 4.8; for relapsing subjects, 4.3; and for reinfection subjects, 5.2.

Descriptive analysis of serological reactivity to *P. vivax* antigens using a protein microarray. Endemic controls were not used for this determination because they have substantial reactivity against *P. vivax* antigens and therefore are not an

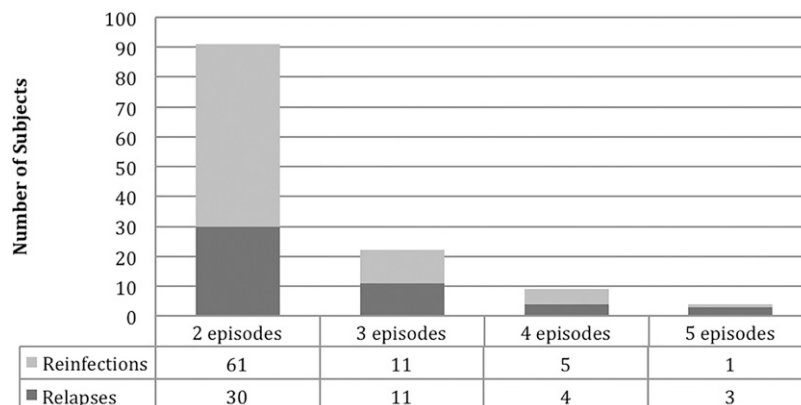


FIGURE 1. Number of *Plasmodium vivax* relapses and reinfections in patient population.

TABLE 2

Occupation/demographic characteristics of *Plasmodium vivax* malaria subjects

Occupation	Relapses (%)	Reinfections (%)	Total (%)
Student	23 (48)	25 (32)	48 (38)
Agriculture	7 (15)	12 (15)	19 (15)
Homemaker	6 (13)	20 (26)	26 (21)
Crafting	0 (0)	2 (3)	2 (2)
Transportation	1 (2)	3 (4)	4 (3)
Commerce	1 (2)	1 (1)	2 (2)
Construction	0 (0)	8 (10)	8 (6)
Unemployed	4 (8)	4 (5)	8 (6)
Fisherman	4 (8)	2 (3)	6 (5)
Logging	0 (0)	1 (1)	1 (1)
< 5 years old	2 (4)	0 (0)	2 (2)
Total	48 (100)	78 (100)	126 (100)

appropriate control for the discovery of markers of exposure as shown in the heat map of the reactive antigens (Figure 2). Therefore, differentially reactive antigens in the text indicates results in relation to the negative control group with no history of malaria ($N = 18$, U.S. residents with no history of malaria). Of the 2,233 *P. vivax* protein spots (derived from 1,470 genes) on the protein microarray (see Supplemental Table 1 for complete list of arrayed recombinant proteins), 304 proteins spots were reactive when comparing *P. vivax*-infected subjects to a negative control group after BH P value correction (Figure 2).

The most highly recognized differentially reactive *P. vivax* proteins were MSP 4, 7, 8, and 10 (PVX_003775, PVX_082650, PVX_097625, and PVX_114145); sexual-stage antigen s16 (PVX_000930); early transcribed membrane protein (ETRAPM) (PVX_090230); tryptophan-rich antigen (Pv-fam-a) (PVX_092995); AMA 1 (PVX_092275); and proteins of unknown function (PVX_081830, PVX_117680, PVX_118705, PVX_121935, PVX_097730, PVX_110935, PVX_115450, and PVX_082475). The top 10 differentially reactive proteins and gene for all *P. vivax*-infected subjects are listed in Table 3. The complete list of differentially reactive proteins is presented in Supplemental Table 2.

Reactive *P. vivax* antigens were not found more commonly in reinfection than in relapse and reinfection, and relapse had similar cumulative IgG signals. *Plasmodium vivax* parasitemia may reflect one of several clinical states, relapse, which may be symptomatic or, most often in the Peruvian Amazon region, asymptomatic new infection,³⁷ or a continuing previously un- or inadequately treated blood-stage infection. Subjects studied here represent either relapse or reinfection because blood-stage *P. vivax* is not known to be resistant to chloroquine in the region and all patients were treated with directly observed therapy (DOT) with standard therapy (chloroquine and primaquine).

The antigens recognized by *P. vivax*-infected subjects were not more likely to have higher cumulative signal for subjects that were reinfected than for subjects undergoing a relapse (Figure 3). In addition, reinfected subjects did not recognize more of the antigens (285) than did the subjects that relapsed (219). In this study, a new *P. vivax* infection was only determined for symptomatic subjects.

Operationally, subjects who develop a recurrent infection within 28 days after prior episode were considered as treatment failure or recrudescence and could potentially have been due to tolerance or resistance to treatment. In this study, no

subject developed a recurrent episode within 28 days after prior treatment. One subject developed a recurrent episode after 29 days but it had a different *PvMSP3 α* pattern, hence was considered a reinfection for this analysis.

Paired analysis reveals that relapse was not associated with a greater increase in reactivity than reinfection. Although looking at the infected, reinfected, and relapse groups allowed us to find markers of exposure, there were multiple confounding factors that complicated comparison of the reinfected and the relapse groups. One confounder is precise knowledge of previous to *P. vivax* infections before episode 1 in this study, which would be useful in understanding changes in antibody profile after reinfection versus relapse. The current dataset had 113 subjects for whom samples for episodes one and two were available, of which 73 were defined as reinfection and 40 as relapse. Paired analysis looking at the changes elicited by the second episode for relapse subjects did not show increase after episode 2 compared with reinfection subjects; this latter group appeared to have just as many recognized antigens decreasing at episode 2 as increasing (Figure 4). Comparing the deltas (Second Episode–First Episode for each subject) observed in the two subject groups, the deltas for the reactive antigens in the relapse group were not greater in scope and magnitude than the deltas in the relapse group (Figure 4).

Enrichment of genetic diversity of genes encoding differentially reactive antigens. The non-synonymous genetic diversity (π) of genes encoding the set of recognized *P. vivax* antigens was compared with coding sequences found in the *P. vivax* genome as a whole. This analysis, using globally representative *P. vivax* strains from Brazil, India, Mauritania, and North Korea, only included annotated genes; *vir* gene family members, hypothetical genes, were not included. The set of genes encoding the recognized *P. vivax* antigens as a whole (combining proteins differentially recognized by both relapse and reinfection patients) had a significantly higher level of single nucleotide polymorphism (SNP) diversity than the rest of the coding genes, as assessed by the Mann–Whitney test ($W = 249,177$, $P = 0.0007472$). Genes encoding the set of recognized proteins were significantly more likely to have 1 or more non-synonymous SNPs ($W = 249,791$, $P = 0.0006419$). In contrast, the level of synonymous SNPs in genes encoding recognized proteins was not significantly different ($W = 291,635$, $P = 0.3948$). This analysis suggests that positive immune selection for amino acid polymorphism in these *P. vivax* antigens has taken place.

DISCUSSION

This study is the first large-scale analysis in which immune responses to relapsed versus reinfection of *P. vivax* infection in a hypoendemic human population were compared. The first major finding was that there was no observable difference in seroreactivity between relapsed versus reinfection *P. vivax* infections. The second major finding is that we identified a large set of serodiagnostic antigens that have potential utility for seroepidemiological studies of transmission in *P. vivax*-endemic areas. Third, genome-scale analysis of the diversity of the serodiagnostic antigens recognized by *P. vivax*-infected subjects suggests that immune selection has led to antigenic changes in the parasite. These results collectively demonstrate that most of the antigens that could lead

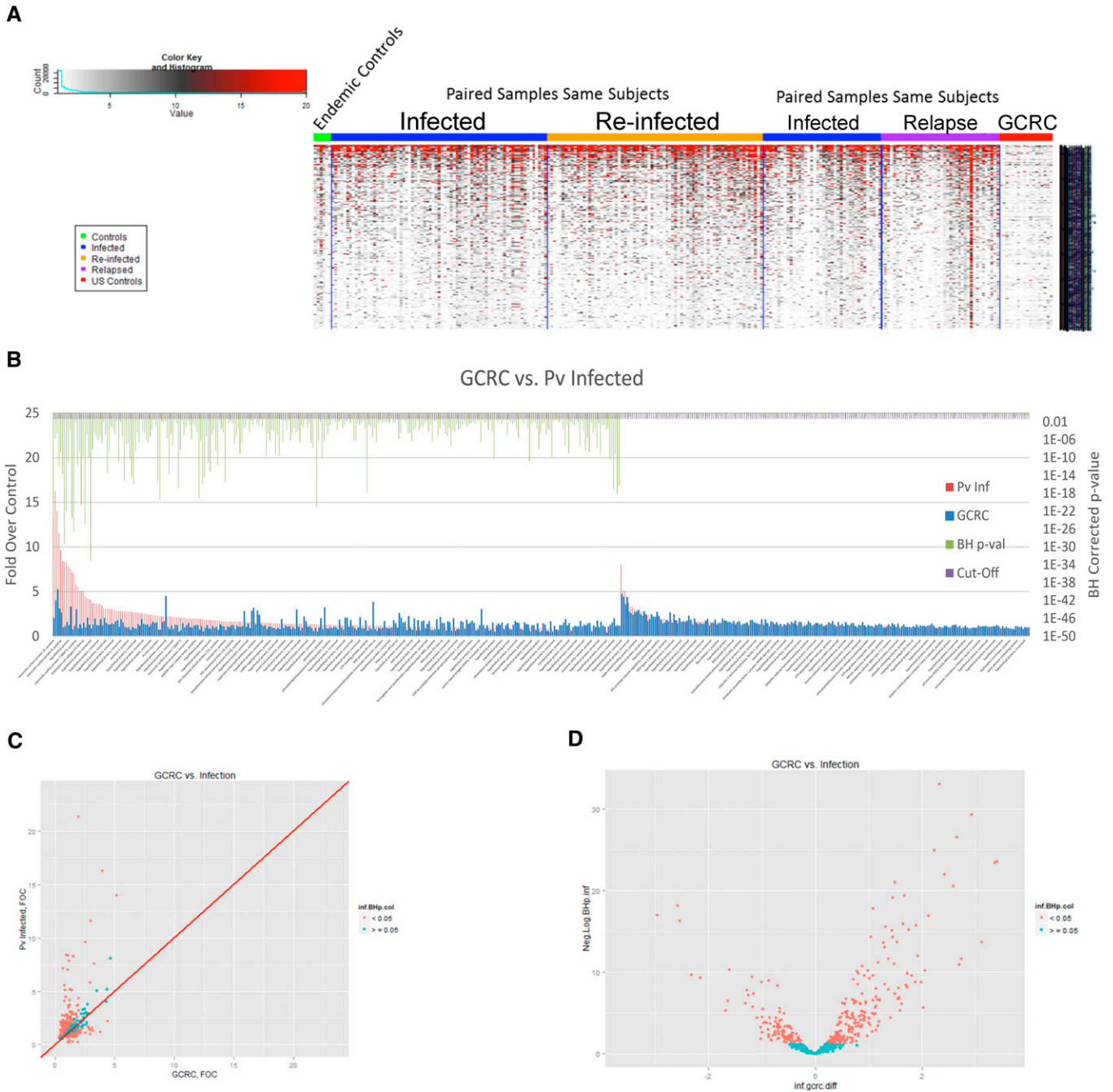


FIGURE 2. Protein microarray analysis comparing serological responses of *Plasmodium vivax* relapse vs. reinfection. **(A)** Key to heat map, indicating color associated with signal intensity (left). Displayed at the left of the heat map are results of uninfected controls from the Iquitos region (“endemic controls”); the middle two panels of the heat map show results of paired, sequential samples from *P. vivax* infection of same subjects comparing total Infected (this category does not distinguish relapse from re-infection) vs. Re-infected; the right two panels of the heat map show results of paired, sequential samples from *P. vivax* infected-subjects that compare total Infected vs. Relapse (based on shared molecular genotyping—see Methods). **(B)** Protein microarray analysis of sera from first observed episode of *Plasmodium vivax* malaria. Recognized proteins are indicated with a green bars above them on the left side of the figure, where the *P* value is less than 0.05 (corrected for multiple measures by the Benjamini Hochberg test (BH) *P* value). All *P. vivax*-infected (relapse plus reinfection) are indicated by (Pv Inf) and negative controls are non-malaria infected individuals from the USA controls (GRC, General Clinical Research Center). The full list of recognized proteins is in Supplementary Table 1. **(C)** Scatter plots with the GRC mean fold over control (FOC) values on the x-axis, the mean *P. vivax* Infected FOC values on the y-axis and the identity line in red are graphed (Pv antigens with BH *P* values < 0.05 are colored in salmon and the others in cyan). **(D)** Volcano plots with the log₂ (fold difference) on the x-axis and the -log₁₀(BH *P* value) on the y-axis (*P. vivax* antigens with BH *P* values < 0.05 are colored in salmon and the others in cyan) to illustrate the difference in antibody profiles between the groups.

TABLE 3
Top 10 reactive proteins recognized in *Plasmodium vivax* malaria patients

Protein	Exon	No. of exons	No. of tm domains	SignalP positive	Molecular weight (Da)	Isoelectric point	Selection criteria	Description
PVX_114145	1	1	0	Yes	52,341	4.79	SE_secreted	MSP10, putative
PVX_081830	2	2	0	No	56,607	4.64	ME_cytoplasmic	Hypothetical protein
PVX_117680	1	2	0	No	128,624	5.47	ME_cytoplasmic	Hypothetical protein, conserved
PVX_000930	1	2	2	Yes	15,068	10.46	ME_secreted	Sexual stage antigen s16, putative
PVX_097625	1	1	1	Yes	54,742	4.92	SE_secreted	MSP8, putative
PVX_118705	1	1	1	No	50,808	10.51	SE_secreted	Hypothetical protein, conserved
PVX_121935	2	2	2	Yes	27,735	8.85	ME_secreted	Hypothetical protein
PVX_097730	1	1	0	Yes	46,332	3.25	SE_secreted	Hypothetical protein
PVX_110935	1	1	0	Yes	42,780	3.7	SE_secreted	Hypothetical protein
PVX_082650	1	1	0	Yes	47,963	5.2	SE_secreted	MSP7 (MSP7), putative

MSP = merozoite surface protein; tm = transmembrane.

to important antibody responses are not being investigated; and immune responses differ between relapse and reinfection, which has important implications for understanding *P. vivax* epidemiology, transmission, and vaccine development.

Infections by malaria parasites are known to lead to broadly reactive anti-*Plasmodium* antibody responses.³⁸ How to analyze large-scale antibody responses in key areas of malaria research such as delineating target antigens of protective immunity or how to identify new proteins for seroepidemiological analyses of malaria transmission in endemic regions are important applications of current systems immunology methodologies.²⁷ In this study, sera from symptomatically

relapsed or reinfected *P. vivax*-infected subjects in the low-transmission region of the Peruvian Amazon were used to probe genome-scale *P. vivax* protein microarrays.

That the cumulative total antibody response was not greater in reinfection compared with relapsing infection has implications for understanding immunological mechanisms and consequences of relapsing *P. vivax* malaria. Previous studies have suggested that asymptomatic relapse is common in *P. vivax* malaria,³⁷ despite the more widely recognized phenomenon of relapse leading to symptomatic infection and the seeking of medical care. The present investigation provides evidence that reinfection resulting in clinical

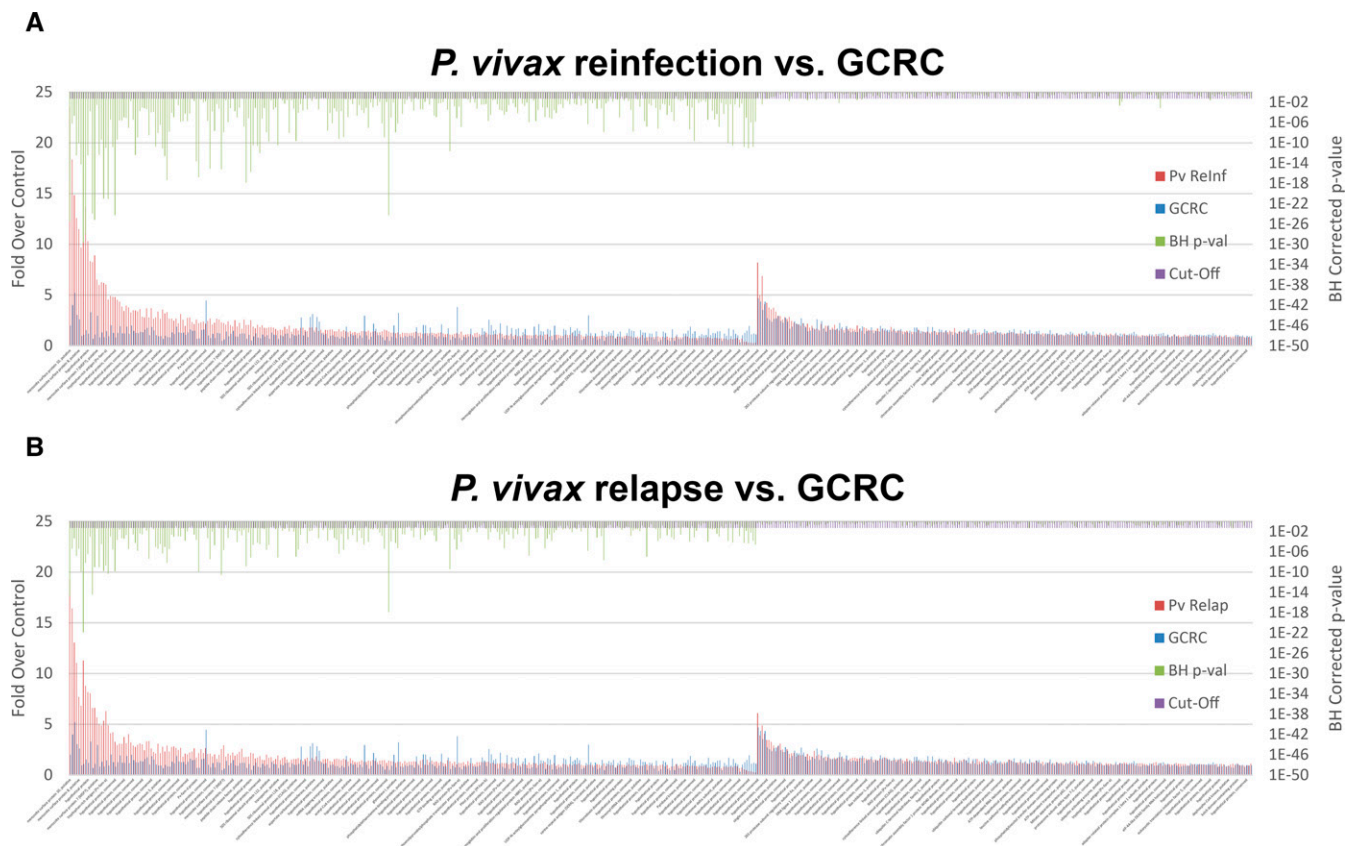


FIGURE 3. Protein microarray analysis comparing serological responses of subjects with *Plasmodium vivax* (*Pv*) reinfection to relapse (**A** and **B**, respectively). The number of reactive proteins and signal intensity of these proteins (top 10 proteins shown in Table 3) is not significantly different (paired Student's *t* test) in the reinfection group compared with the relapse group (complete list in Supplemental Table 2). For clarity, proteins that are not recognized are not shown. (**C**) The sum of intensities (*y* axis) for each sample (*x* axis), the median of these sums and a Volcano plot (**D**) demonstrates antibody titers and number of anti-*Pv* antigen antibodies.

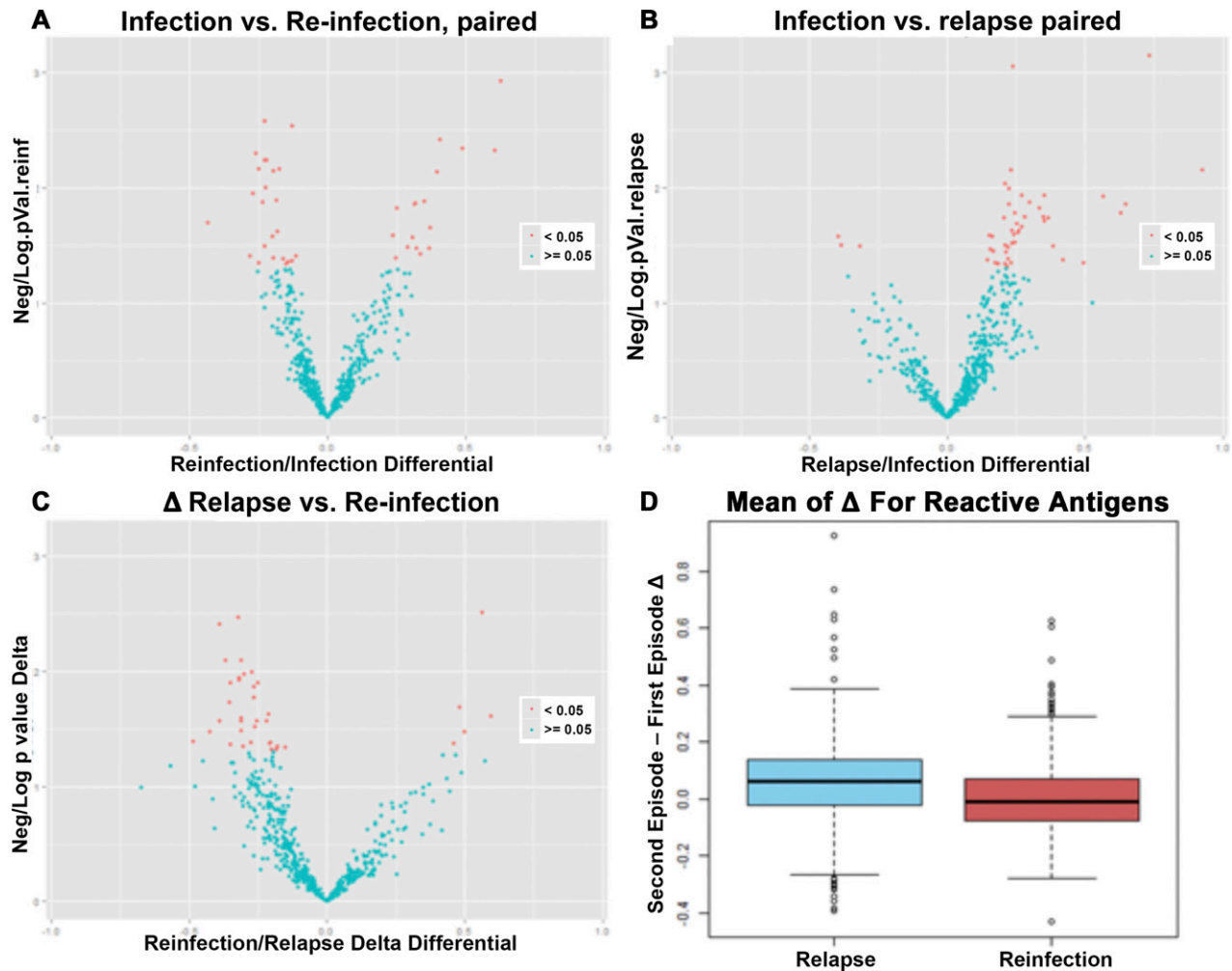


FIGURE 4. Paired protein microarray analysis comparing changes (indicated as delta) in serological response between episodes 1 and 2 of subjects with *Plasmodium vivax* (*Pv*) relapse or reinfection, as defined by MSP3 α PCR-RFLP. Volcano plots comparing episodes 1 and 2 for reinfection and relapse (**A** and **B**, respectively) show log₂ of the fold change on the x axis and the -log₁₀ (*P* value) on the y axis. (**C**) Comparison of reinfection to relapse using a volcano plot comparing the deltas for each subject and each antigen with the log₂ of the fold difference on the x axis and the -log₁₀ (*P* value) on the y axis. A boxplot of the mean of the deltas is shown. A boxplot of the mean of the deltas for the relapse group on the left and the reinfection group on the right. (**D**) illustrates the median and distribution of the mean of the deltas for the relapse group on the left and the reinfection group on the right.

symptoms is not associated with an enhanced immune memory-driven antibody response than relapsing symptomatic infection. This observation might be explained in several ways, but raises the possibility that any partially effective immune response to a previous *P. vivax* strain might have been maximized in terms of driving elevations of antibody response. Alternatively, it is also possible that lower parasitemia in relapse compared with reinfection might be associated with a lower antibody response, but the present data are not consistent with this possibility because the parasitemias were only semiquantitatively assessed (1–4 + system) in this study.

An important limitation of this study is the accurate classification of *P. vivax* relapse versus reinfection in patients at continued risk for new infections, an issue addressed in other reports when *P. vivax*-infected subjects leave a malaria-endemic region and experience what can definitively be classified as relapse in a malaria non-endemic place.^{39,40} Our classification of relapse is limited by the possibility that subjects may harbor more than one *P. vivax* clone. Hence our data conser-

vatively underestimate the rate of relapse, and for this reason the lack of difference in antibody responses between relapse and reinfection on a systems level should remain valid. However, there is still no reliable method to differentiate *P. vivax* relapse from reinfection in the endemic setting, an important area for future research.

This protein microarray analysis provides immediate tools to apply to seroepidemiology of malaria with regard to surveillance, control, and elimination. Future work should use these tools to establish the population decay kinetics of antibody responses to different specific antigens, thereby allowing estimates of timing of previous infection. Comparison of *E. coli* to eukaryotic recombinant protein expression systems may be important to improve the performance of such serological assays based on the protein microarray results. Such studies will be important because previous seroepidemiological studies have relied on blood-stage vaccine candidate antigens selected for high titer, immunodominant and sustained antibody responses rather than with regard to seroreactivity in human populations that would diminish over time. The use of

parasite lysates for enzyme-linked immunosorbent assay or immunofluorescent microscopy is not applicable to such studies of decay kinetics because of their heterogeneity and low sensitivity/specificity for inferring previous time of infection. The ideal antigen(s) for determining recent versus remote malaria transmission from mosquitos to residents of endemic areas would have the properties of being associated with rapid versus sustained decay kinetics.

The *P. vivax* antigen yielding the most consistently high-intensity reactivity on the protein array was PvMSP10; other top-ranked antigens included MSP7, MSP8 and several hypothetical proteins, and a putatively sexual-stage antigen s16. Antibodies to PvMSP10 have previously been readily detected in humans with *P. vivax* malaria^{41,42} using *E. coli*-expressed⁴¹ and wheat germ in vitro expression system-expressed protein.⁴² Studies with *P. falciparum* MSP10 demonstrated a lack of cross-reactivity with other MSPs (PfMSP-1, -4, -5 and -8), consistent with the 95% specificity for *P. vivax* infection found for antibodies detected against wheat germ-expressed PvMSP10.⁴² No experimental evidence has emerged to date regarding the viability of any *Plasmodium* spp. MSP10 as a candidate for an asexual blood-stage-based vaccine.

This large-scale analysis of antibody responses to *P. vivax* infection has identified a large set of serodiagnostic antigens with potential utility for seroepidemiological studies of transmission in endemic areas. This work sets the stage for testing these antigens in well-characterized human populations in diverse malaria-endemic settings around the world and for planning new approaches to malaria vaccine development aimed at the neglected parasite, *P. vivax*.

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Conflict of interest: The authors have read the journal's policy and declare the following potential conflicts: Philip Felgner has an equity interest in Antigen Discovery, Inc., which is developing products related to the research described in this article. In addition, this author serves on the advisory board of ADI and receives compensation for these services. The terms of this arrangement have been reviewed and approved by the University of California in accordance with its conflict of interest policies. Douglas Molina is an employee of Antigen Discovery, Inc. No other author declares a conflict of interest. This does not alter our adherence to ASTM/AJTMH journal policies on sharing data and materials.

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