An immunomics approach for the analysis of natural antibody responses to \textit{Plasmodium vivax} infection†

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High throughput immunomics is a powerful platform to discover potential targets of host immunity and develop diagnostic tests for infectious diseases. We screened the sera of \textit{Plasmodium vivax}-exposed individuals to profile the antibody response to blood-stage antigens of \textit{P. vivax} using a \textit{P. vivax} protein microarray. A total of 1936 genes encoding the \textit{P. vivax} proteins were expressed, printed and screened with sera from \textit{P. vivax}-exposed individuals and normal subjects. Total of 151 (7.8\% of the 1936 targets) highly immunoreactive antigens were identified, including five well-characterized antigens of \textit{P. vivax} (ETRAMP11.2, P$v$34, SUB1, RAP2 and MSP4). Among the highly immunoreactive antigens, 5 antigens were predicted as adhesins by MAAP, and 11 antigens were predicted as merozoite invasion-related proteins based on homology with \textit{P. falciparum} proteins. There are 40 proteins that have serodiagnostic potential for antibody surveillance. These novel \textit{Plasmodium} antigens identified provide the clues for understanding host immune response to \textit{P. vivax} infection and the development of antibody surveillance tools.

Introduction

Unlike \textit{Plasmodium falciparum}, \textit{P. vivax} receives little research attention and financing, which results in important knowledge gaps and limitations on effective control of vivax malaria.\textsuperscript{1} Malaria from \textit{P. vivax} causes significant morbidity in South Asia, Southeast Asia and Latin America, with approximately 132 to 391 million clinical infections each year.\textsuperscript{2} In Africa, strong evidence showing that \textit{P. vivax} is capable of causing blood-stage infection and disease in Duffy-negative individuals illustrate that under some conditions, \textit{P. vivax} exhibits capacity for infecting human erythrocytes without the Duffy antigen.\textsuperscript{3} These factors highlight the critical need for effective vaccines and surveillance tools for the elimination of vivax malaria.

The efficient continuous \textit{in vitro} blood-stage culture of \textit{P. falciparum} has promoted the understanding of the parasite, however, there is no available culture system for \textit{P. vivax}.\textsuperscript{4} Much effort has been concerned with the transcriptome and genome of \textit{P. vivax} parasite in recent years, and characterizing the stage-specific transcriptome of the intraerythrocytic developmental cycle (IDC) of \textit{P. vivax} provided broad insights into the biology and gene functionalities of this parasite.\textsuperscript{5,6} The genomes of \textit{P. vivax} reference strains (Salvador I, IQ07, North Korean, India VII, Mauritania I and Brazil I) have been sequenced, and the genetic diversity of \textit{P. vivax} has been analyzed.\textsuperscript{7–9} All of the data showed that the gene families associated with the merozoite invasion or immune response modulation (e.g., the \textit{msp3}, \textit{v}r\textit{r} and \textit{msp7} gene family) displayed the highest genetic diversity.\textsuperscript{8,9} Previous work using \textit{P. vivax} protein microarrays studied only hundreds of proteins in an effort to characterize the human immune response and identify interesting antigens.\textsuperscript{10,11} Understanding human immunity to malaria parasites is crucial for successful intervention. The naturally acquired antibodies against \textit{P. falciparum} antigens such as PMSP1–19, PMSP3, PfAMA1 and PFGLURP,\textsuperscript{12} as well as the antigen members of the PfEBAs and PfRBLs are associated with protection.\textsuperscript{13,14} As with falciparum malaria, individuals having chronic exposure to
*P. vivax* tend to develop some acquired immunity. In the previous reports, the IgG levels to the N terminus of PvMSP1, PvMSP3z, PvMSP9, PvAMA1 and rPV24 (PVX_002950) were negatively correlated with parasite levels, which collectively suggest that the antibodies against PvMSP1, PvMSP3z, PvMSP9, PvAMA1 and rPV24 are important and might be closely related to protection.\(^ {15-18} \) There are few studies showing clinical protection by IgG antibodies against *P. vivax* antigens because the knowledge of the complex life cycle of *P. vivax* is limited.\(^ {19} \) More investment and a greater effort toward the understanding of host immunity to *P. vivax* malaria are required.\(^ {20} \)

Serological parameters were shown in *P. falciparum* infections to offer an advantage for measuring the endemicity and malaria transmission dynamics because of overcoming sampling variations and the detection of persistent antibodies over months and years after infection.\(^ {21} \) Antibody detection might be useful in identifying established *P. vivax* infections, in which the blood-stage parasite density has fallen below the limits of light microscopy or antigen-detecting RDTs (rapid diagnostic tests), and they could be used to screen populations such as migrants or blood donors to identify asymptomatic individuals at risk of transmitting malaria.\(^ {22} \) There is an urgent need to accelerate the pace of discovery of specific immunogenic antigens of *P. vivax* using innovative screening approaches.

In this study, *in silico* data mining by comparative genomics combined with high-throughput profiling antibody using high density protein microarray screening was used to study responses against blood-stage *P. vivax* infection. A total of 151 highly immunoreactive antigens were identified, and there are 40 proteins that exhibit potential for antibody-surveillance applications.

### Materials and methods

#### Sample collection

The *P. vivax* malaria positive serum samples were collected from 15 patients (mean age, 32 year; range 18–62 year) in the Yunnan province, an area with low endemic malaria levels in the P. R. China. All the patients were experiencing fever (>37.5 °C) and were first-time reported, and the samples were microscopically positive for *P. vivax* (mean parasitemia, 0.078%; range 0.002–0.456%) and PCR confirmed for single *P. vivax* infection.\(^ {23} \) The serum samples from 10 unexposed individuals used as the negative controls in the study were collected in the Hangzhou, Zhejiang province, an area where malaria is not endemic. Thirty microliter of serum was stored using Whatman 903 cards for the microarray work.

#### Ethics statement

The study was approved by the Ethics Committee of the National Institute of Parasitic Diseases (NIPD), China CDC. The study protocol, potential risks and potential benefits were explained to the villagers. After an informed consent to participate in the study was given, field workers visiting the enrolled families provided detailed information to all the participants, and answered all questions from the participants. All the participants in a given household provided written informed consent.

### Enzyme-linked immunosorbert assay (ELISA)

To validate the immunoreactivity detected by the proteome microarrays, the serum samples from 15 cases of vivax malaria in the Yunnan province of the P. R. China and 10 serum samples from unexposed subjects were tested against a well-characterized *P. vivax* antigen, PvMSP1–42, by ELISA, as described previously.\(^ {24} \) The positive cut-off value was calculated as the mean optical density (OD) value of the normal controls plus 2 standard deviations (SD). The serum samples were screened by proteome microarrays as follows.

#### Serological profiling using protein microarrays

The *P. vivax* proteome microarrays were commercially prepared by Antigen Discovery, Inc. (Irvine, CA), and the preparation information was described in the ESI, † S1. A hole puncher was used to punch out a circle that is 1/4 in. (6 mm) in diameter (29.6 mm\(^ 2 \)) from the Whatman 903 Cards and one was placed into a 2 ml microcentrifuge tube, this equates to ~6.5 μl of serum. Prior to staining the *P. vivax* blood stage protein microarrays, the sera were eluted from filter paper using the following protocol. A tube with 10 mg lyophilized *E. coli* lysate was reconstituted by adding 1 ml 1× blocking buffer (Maine Manufacturing, Sanford ME USA) to make 1× BB/100% *E. coli* lysate (10 mg mL\(^ -1 \)). It was then diluted in 1× blocking buffer to make 1× blocking buffer/10% *E. coli* lysate (1 mg mL\(^ -1 \)) for the elution of all the samples. Subsequently, 1.3 mL of 1× BB/10% ECL was added to 1.7 mL microcentrifuge tube containing the punched out filter paper with serum, which resulted in the equivalent of a 1:200 dilution. Tubes were vortexed for 1 minute then incubated for 1 hour at room temperature with agitation. The diluted serum was incubated at room temperature for 30 minutes with constant mixing. The *P. vivax* blood stage protein microarrays were probed with the sera from the donors infected with *P. vivax* as well as sera from the healthy controls. The microarrays were rehydrated in 1× blocking buffer for 30 minutes and probed with the pretreated sera overnight at 4 °C with constant agitation. The slides were then washed 3 times with TTBS and incubated in biotin-labeled goat anti-human IgG Fc (Jackson Immuno Research Laboratories, West Grove PA USA) diluted from 1 to 1000 in 1× blocking buffer. After washing 3 times with TTBS, the antibodies were detected using Sensilight™ Streptavidin-P3 (Columbia Biosciences, Columbia NY USA). The slides were then washed 3 times with TTBS and 3 times with TBS followed by a final water wash. The slides were air dried after brief centrifugation and analyzed using a Perkin Elmer Scan Array Express HT microarray scanner (Perkin Elmer, Waltham MA USA). The intensities were quantified using Scan Array v4 software (Perkin Elmer, Waltham MA USA). All the signal intensities were corrected for the spot-specific background. Each chip contained negative control spots made with *E. coli* based Rapid Translation System 100 HY (RTS) without plasmid DNA; as well as positive controls spots such as anti-human IgG for the primary antibody, and human IgG for the
secondary antibody in serial dilutions. Antigens were considered “serodominant” if the mean intensity for the vivax patients was greater than the mean of the negative controls plus 3 SD of the mean of the negative controls.

Data analysis

The analysis was performed using the R statistical environment (http://www.r-project.org) and SAS (http://www.sas.com/) statistical software according to the recent report.25 The Benjamini–Hochberg method was used to correct the false discovery rate using the MULTTEST procedure in version 8.0 of SAS/STAT software.26 Statistical differences of p < 0.05 were considered significant. The heatmap of the antibody responses and the IDC transcription data were drawn using the TIGR multi-array experiment viewer (MeV) software.27 The bioinformatics data of the antibodies and the antibody responses and the software.26 Statistical differences of using the MULTTEST procedure in version 8.0 of SAS/STAT. The Hochberg method was used to correct the false discovery rate (Table S1 and S2, ESI†).

Results

The P. vivax blood stage protein expression

The expression of the P. vivax proteome was shown in ESI,† Fig. S1. A total of 89.5% (1733/1936) and 85.2% (1663/1936) of the P. vivax proteins tested positive for the anti-His antibody and anti-HA antibody, respectively. A total of 80.9% (1566/1936) of the P. vivax proteins tested positive for both anti-His/anti-HA antibodies, and 94.5% (1830/1936) tested positive for either of the antibodies.

Antibody profiling

The P. vivax blood stage protein microarrays were probed with the identical set of serum samples as those used in the PvMSP1–42 ELISA described above (ESI,† Fig. S2). Images created from the scans and colorized that display microarrays probed with serum from a vivax malaria patient and an unexposed subject are shown in Fig. 1A and B, respectively. The serum samples from the P. vivax-exposed individuals showed obvious reactivity against some of P. vivax proteins, whereas the serum samples from the unexposed subject showed low reactivity.

Immunomics profiles of the P. vivax blood stage protein microarrays

The profiles of the immunoreactivity against the 149 genes encoding the 151 ORFs (7.8% of the 1936 target proteins), representing the top-ranked immunogenic antigens, are shown in Fig. 2A. The signal intensities for the reactivity of each antigen by the individual serum samples are shown in a colorized matrix. Of the 151 high immunogenic P. vivax proteins, only 3 proteins (ETRAMP11.2, PV34 and SUB1) have been identified as immunogenic proteins in previous studies,10,11,32,33 and 2 proteins (RAP2 and MSP4) were considered as potential targets of host immunity to vivax malaria.34,35 Other proteins have not previously been described as immunologically reactive (Tables S1 and S2, ESI†). Forty of the 151 most immunoreactive proteins were considered as biomarkers for serodiagnosis, and 18 were proteins recognized by malaria serum samples with the area under the receiver operating characteristics (ROCs) curve (AUC) more than 0.95 (Table 1) (Fig. 2B).

Bioinformatics analysis of P. vivax immunoproteome

Of 149 genes coding 151 P. vivax immunogenic proteins, more than 50% have a transmembrane domain (57.7%, 86/149) and a signal peptide (63.8%, 95/149), which indicates that secreted and membrane proteins are involved in targeting the host immune response and that they play an important role in the erythrocyte stage, such as merozoite adhesion, parasite infected erythrocyte adhesion and pathogenesis (Fig. 3A and B). Approximately 53.0% of the gene coding P. vivax immunogenic proteins have the maximum gene expression pattern in the schizont
stage, and 55.0% of the *P. vivax* immunogenic proteins belong to the hypothetical proteins (Fig. 3C and D).

Among 149 genes coding 151 *P. vivax* immunogenic proteins, 98 have GO annotation, literature co-citation, or other annotated parasite-specific processes, e.g., there are 30 genes known to be involved in DNA replication (Fig. 4). There are 11 proteins involved in the merozoite invasion of red blood cells (RBC) and malaria pathogenesis (Table 2), respectively. Eight proteins involved in merozoite development and erythrocytic development are closely associated with the blood-stage of *P. vivax*. Four proteins (RAP2 and 3 hypothetical proteins) localized in the rhoptry, an important organelle during the invasion of RBC by merozoite, showed high immunogenicity.

Using MAAP software, 5 immunogenic *P. vivax* proteins were predicted to be adhesins, including MSP4, MSP7, a RAD protein and 2 conserved hypothetical proteins (Table 3). In comparison with the merozoite invasion-related proteins of *P. falciparum*, 11 immunogenic *P. vivax* proteins were merozoite invasion-related proteins, including RAP2, MSP4, MSP7, MTIP, SUB1, syntaxin and 5 conserved hypothetical proteins (Table 2). We analyzed the *P. vivax* microarray data through the IDC for the expression of the genes encoding the 11 merozoite invasion-related proteins. We found evidence for all of these proteins.

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**Table 1** List of the top immunogenic proteins of *Plasmodium vivax*

<table>
<thead>
<tr>
<th>Gene IDa</th>
<th>Product description</th>
<th>AUCb</th>
<th>No. of positive (%)</th>
<th>SPc</th>
<th>TMDd</th>
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<tr>
<td>PVX_090095</td>
<td>Hypothetical protein, conserved</td>
<td>1.00</td>
<td>15 (100.0)</td>
<td>N</td>
<td>0</td>
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<tr>
<td>PVX_003565</td>
<td>ETRAMP11.2</td>
<td>1.00</td>
<td>13 (86.7)</td>
<td>Y</td>
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<td>PVX_092885</td>
<td>Hypothetical protein, conserved</td>
<td>1.00</td>
<td>12 (80.0)</td>
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<td>4</td>
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<tr>
<td>PVX_087670</td>
<td>Hypothetical protein, conserved</td>
<td>1.00</td>
<td>10 (66.7)</td>
<td>N</td>
<td>1</td>
</tr>
<tr>
<td>PVX_089870</td>
<td>RAD protein (Pv-fam-e)</td>
<td>1.00</td>
<td>8 (53.3)</td>
<td>Y</td>
<td>0</td>
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<tr>
<td>PVX_096280</td>
<td>Hypothetical protein, conserved</td>
<td>1.00</td>
<td>4 (26.7)</td>
<td>Y</td>
<td>0</td>
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<tr>
<td>PVX_115430</td>
<td>Homolog to PfMAHRP1</td>
<td>0.99</td>
<td>12 (80.0)</td>
<td>N</td>
<td>1</td>
</tr>
<tr>
<td>PVX_090295</td>
<td>Hypothetical protein</td>
<td>0.98</td>
<td>12 (80.0)</td>
<td>N</td>
<td>1</td>
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<tr>
<td>PVX_116770</td>
<td>Nucleoside-diphosphatase mig-23, putative</td>
<td>0.98</td>
<td>11 (73.3)</td>
<td>Y</td>
<td>1</td>
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<tr>
<td>PVX_089785</td>
<td>RAD protein (Pv-fam-e)</td>
<td>0.98</td>
<td>8 (53.3)</td>
<td>Y</td>
<td>0</td>
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<tr>
<td>PVX_122915</td>
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<td>0.97</td>
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<tr>
<td>PVX_091815</td>
<td>Endoplasmic reticulum oxidoreductin, putative</td>
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<tr>
<td>PVX_095420</td>
<td>Inorganic pyrophosphatase, putative</td>
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<tr>
<td>PVX_089330</td>
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<td>7 (46.7)</td>
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<tr>
<td>PVX_090075</td>
<td>Pcr</td>
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<tr>
<td>PVX_121935</td>
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<td>PVX_101595</td>
<td>Hypothetical protein</td>
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<td>6 (40.0)</td>
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<td>2</td>
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<tr>
<td>PVX_118480</td>
<td>Delta-aminolevulinic acid dehydratase precursor, putative</td>
<td>0.95</td>
<td>5 (33.3)</td>
<td>Y</td>
<td>0</td>
</tr>
</tbody>
</table>

a Gene ID was obtained from PlasmoDB (http://www.plasmodb.org/plasmo/home.jsp). b AUC, the area under the receiver operating characteristics (ROCs) curve. c SP, signal peptide. d TMD, transmembrane domain.
and they show an expression pattern consistent with the involvement in the invasion or schizont stages of at least one isolate, peaking in the TP6 ~ TP9 post-invasion transcription (ESI,† Fig. S3).

Immunoproteome of *P. vivax* merozoite

Through antigen discovery by protein microarray, we can identify a set of immunogenic merozoite antigens of *P. vivax* from the current and previous studies.10,11,38 The merozoite surface proteins (MSPs) of *P. vivax* are the major family of immunogenic antigens, including the GPI-anchored MSPs (MSP1, MSP4, MSP8, and MSP10), the MSP3 family members, the MSP7 family members, and a 6-Cys s48/45 family member (Pv41), as well as their homolog proteins in the *P. falciparum* genome (Fig. 5).26,39,40 Both Pf12 and Pv12 are strongly recognized by immune sera from naturally infected patients and share similar localization in an apical organelle (rothry). The potential role of Pf12 and Pv12 is the involvement in host cell invasion and the establishment of infection.41,42 Duffy-binding protein (DBP) and apical merozoite antigen 1 (AMA1) are important vaccine candidates for blocking invasion, in addition to rhoptry-associated protein-2 (RAP2) and rhoptry protein (Pv34).43 The essential subtilisin-like serine protease SUB1 of *P. vivax* (PvSUB1), which plays a dual role in the egress from and invasion into host erythrocytes,34,43 was recognized by immune sera from naturally infected patients.

Discussion

Malaria caused by *P. falciparum* has a significant effect on human health and socioeconomic development in the developing countries. It has a high prevalence in Africa, whereas in Asia and the Americas, *P. vivax* malaria is more prevalent.5,46 Although non-falciparum parasites are often considered to cause only a mild disease, recent data show that *P. vivax* infections are associated with severe disease and mortality.47–49 In contrast to *P. falciparum*, for which the genomes of hundreds of isolates have now been sequenced or genotyped,50–52 only 6 *P. vivax* genomic reference strains (Salvador I, IQ07, North Korean, India VII, Mauritania I and Brazil I) have been completed.7–9 The current genome, transcriptome and proteome for *P. vivax* could be useful in the development of serodiagnostic and potential targets of host immunity in the future.5–7,16

Protein arrays were used to characterize the antibody reactivity profiles of *P. vivax* infection.10,38 Because of technological limitations,10,38 it is urgent to develop a proteome-wide microarray technology and discover the immunodominant proteins of *P. vivax*.73 Proteome-wide microarray technology has been well documented for characterizing the antibody reactivity profiles of *P. falciparum* infection in recent years.26,54–56 In this study, a blood stage proteome-wide microarray composing 1936 polypeptides of *P. vivax* was used to characterize the immunogenic profiles of *P. vivax* infection. Only a small amount of candidates overlap with previous immunogenic proteins from the *P. vivax*
blood-stage (e.g. AMA1, ETRAMP 11.2). Overall, 149 genes encoding 151 ORFs representing the top-ranked immunogenic antigens were identified. Unexpectedly, some GPI-anchored merozoite proteins and other merozoite proteins were shown to have low antigenicity, which may due to the low expression and low quality of these proteins by E. coli based cell-free expression system in comparison with the wheat germ based cell-free system.

In contrast to the other classes of blood-stage antigens, the GPI-anchored proteins appear to be essential for blood-stage parasite growth. With considerable data highlighting their potential as antibodies targets, our results place the 4 GPI-anchored merozoite
proteins among the most highly validated blood-stage vaccine targets. A GPI-anchored protein (Pv34) was among the key immunogenic proteins for P. vivax. The homolog protein in P. falciparum, Pf34, localized in the apical organelle of P. falciparum merozoites, shows a binding activity to erythrocytes and inhibits the invasion of RBCs by P. falciparum in vitro, which indicates that it is involved in the merozoite invasion of RBCs.

Parasite adhesins play important roles in the parasite invasion of the RBCs, sequestration or parasite–host interactions. In total, 137 adhesins were predicted in the P. vivax genome in contrast to 157 adhesins in the P. falciparum genome. Of which, 5 adhesins were identified as immunogenic proteins of P. vivax, including MSP4, a GPI-anchored epidermal growth factor (EGF)-like protein, and MSP7, a protein involved in the MSP1 associated complex on the P. falciparum merozoite surface. The MSP family is a group of merozoite surface proteins that are involved in the initial interaction between the merozoite and the host cell. Recently, the C-terminus of 3 MSP7 members has been reported as a conserved region, and could be an important target of host immunity to vivax malaria. Moreover, 11 members of the P. vivax MSP3 were expressed and characterized uniquely, and MSP3.7 was expressed exclusively at the apical end of the merozoites during late schizogony and in free merozoites, clearly differentiating this protein and its possible function from the other MSP3 family members.

Invasion of the host cell is an essential process for survival of the malaria parasite and is a key target for malaria intervention. A subnetwork of P. falciparum merozoite invasion-related proteins is obtained by a guilt-by-association prediction, which contains 418 proteins. We tried to identify the homolog genes with the P. falciparum merozoite invasion-related proteins and obtained 11 P. vivax merozoite invasion-related proteins. Of which, only one P. vivax protein (RAP2) of our immunogenic proteome was identified from the bioinformatics methodology.

In this study, we used a proteome microarray technology to screen the sera of P. vivax-exposed individuals. A total of 151 highly immunoreactive antigens were identified, including five well-characterized blood-stage antigens of P. vivax. Five antigens were predicted as adhesins of P. vivax by MAAP, and 11 antigens were predicted as merozoite invasion-related proteins of P. vivax in comparison with the functional genes of P. falciparum. These novel Plasmodium antigens identified provide the clues for understanding host immunity to P. vivax infection and the development of antibody surveillance tools.

### Table 3

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Product description</th>
<th>AUCb</th>
<th>No. of positive (%)</th>
<th>MAAP scorec</th>
<th>Max. exp. timing (h)</th>
<th>SPd</th>
<th>TMe</th>
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<tr>
<td>PVX_089765</td>
<td>RAD protein (Pv-fam-e)</td>
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<td>8 (53.3)</td>
<td>2.059</td>
<td>40</td>
<td>N</td>
<td>0</td>
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<td>PVX_003775</td>
<td>Merozoite surface protein 4 (MSP4), putative</td>
<td>0.83</td>
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<td>1.001</td>
<td>35</td>
<td>Y</td>
<td>0</td>
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<tr>
<td>PVX_084425</td>
<td>Hypothetical protein, conserved</td>
<td>0.94</td>
<td>13 (86.7)</td>
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<td>43</td>
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<td>0</td>
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<tr>
<td>PVX_082670</td>
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<td>0.76</td>
<td>8 (53.3)</td>
<td>0.734</td>
<td>43</td>
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<td>PVX_123455</td>
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<td>0.92</td>
<td>5 (33.3)</td>
<td>0.702</td>
<td>35</td>
<td>N</td>
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a Gene ID was obtained from PlasmoDB (http://www.plasmodb.org/plasmo/home.jsp). b AUC, the area under the receiver operating characteristics (ROCs) curve. c MAAP, malarial adhesins and adhesin-like proteins predictor. d SP, signal peptide. e TMD, transmembrane domain.
Authors’ contributions

Conceived and designed array: MG RW DM XL. Conceived and designed the experiments: JC DM WH. Performed the experiments: JC SC YW CJ TZ BX ME. Analyzed the data: JC HS DM. Contributed the reagents/materials/analysis tools: YW HS XM. Wrote the paper: JC DM WH.

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