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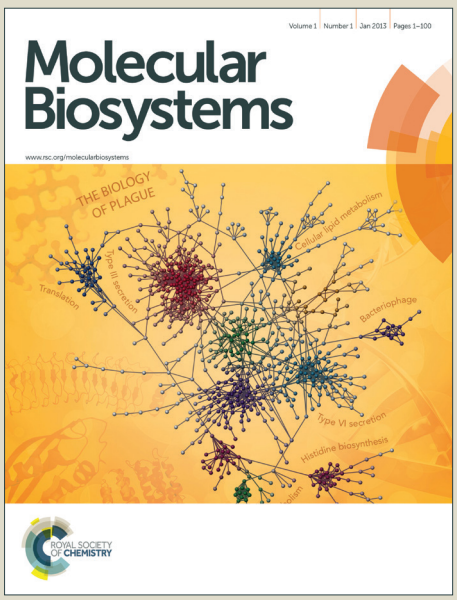

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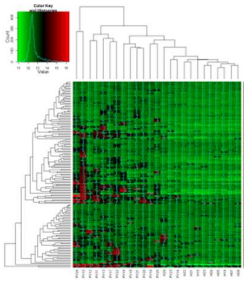

Plasmodium antigens identified by proteome microarrays provide the clues for understanding of host immune response to *Plasmodium vivax* infection.

An immunomics approach for the analysis of natural antibody responses to *Plasmodium vivax* infection

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Abstract

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 *P. vivax*-exposed individuals to profile the antibody response to blood-stage antigens of *Plasmodium vivax* using a partial proteome *P. vivax* protein microarray. A total of 1,936 genes encoding the *P. vivax* proteins were expressed, printed and screened with sera from *P. vivax*-exposed individuals and normal subjects. Total of 151 (7.8% of the 1,936 targets) highly immunoreactive antigens were identified, including five well-characterized antigens of *P. vivax* (ETRAMP11.2, Pv34, 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 to *P. falciparum* proteins. There are 40 proteins that have serodiagnostic potential for antibody surveillance. These novel *Plasmodium* antigens identified provide the clues for understanding of host immune response to *P. vivax* infection and development of antibody surveillance tools.

Keywords: *Plasmodium vivax*; antigen; antibody; immune response; immunomics

Introduction

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

The efficient continuous in vitro blood-stage culture of *P. falciparum* has promoted the understanding of the parasite, however, there is no available culture system for *P. vivax* [4]. Much effort has been concerned with the transcriptome and genome of *P. vivax* parasite in the recent years, and characterizing the stage-specific transcriptome of the intraerythrocytic developmental cycle (IDC) of *P. vivax* provided broad insights into the biology and gene functionalities of this parasite [5,6]. The *P. vivax* genomic reference strains (Salvador I, IQ07, North Korean, India VII, Mauritania I and Brazil I) have been sequenced, and the genetic diversity of *P. vivax* has been analyzed [7-9]. All of the data showed that the gene families associated with the merozoite invasion or immune response modulation (e.g., the *msp3*, *vir* and *msp7* gene family) displayed the highest genetic diversity [8,9]. Previous work using *P. vivax* protein microarrays looked at only hundreds of proteins in an effort to characterize the human immune

response and identify interesting antigens [10,11].

Understanding human immunity to malaria parasites is crucial for successful intervention. The naturally acquired antibodies to *P. falciparum* antigens such as PfMSP1-19, PfMSP3, PfAMA1 and PfGLURP [12], as well as the antigen members of the PfEBAs and PfRBLs are associated with protection [13,14]. As with falciparum malaria, individuals having chronic exposure to vivax malaria tend to develop some acquired immunity. In previous reports, the IgG levels to N terminus of PvMSP1, PvMSP3α, PvMSP9, PvAMA1 and rPV24 (PVX_002950) were negatively correlated with parasite levels, which collectively might suggest that the antibodies to PvMSP1, PvMSP3α, 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 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

Samples collection

The *P. vivax* malaria positive serum samples were collected from 15 patients (mean age, 32 yr; range 18-62 yr) in Yunnan province, an area with low endemic malaria levels in the P.R. China. All the patients were experiencing fever (> 37.5°C) and 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 Hangzhou, Zhejiang province, an area where malaria is not endemic. Thirty microliter of serum were 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 informed consent to participate in the study was given, field workers visiting the enrolled families provided detailed information to all the participants, and answered any questions from the participants. All the participants in a given household provided written informed consent.

Enzyme-linked immunosorbent assay (ELISA)

To validate the immunoreactivity detected by the proteome microarrays, the serum samples from 15 cases of vivax malaria in 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 deviation (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 Supplementary Text S1. A hole puncher was used to punch out a circle that is ¼ in. (6 mm) in diameter (29.6 mm^2) from the Whatman 903 Cards and placed 1 into a 2 ml microfuge tube, this equates to ~6.5 uls of serum. Prior to staining the *P. vivax* blood

stage protein microarrays, the sera were eluted from filter paper with the following protocol. Tube with 10 mg lyophilized *E. coli* lysate was reconstituted by adding 1mL 1X Blocking Buffer (Maine Manufacturing, Sanford ME USA) to make 1X BB/100% *E. coli* lysate (10 mg/mL). It was then diluted in 1X Blocking Buffer to make 1X Blocking Buffer/10% *E. coli* lysate (1 mg/mL) for the elution of all the samples. Subsequently, 1.3 mL of 1XBB/10% ECL was added to 1.7 mL microfuge 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 1X 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 in TTBS and incubated in biotin-labeled goat anti-human IgG Fcγ (Jackson Immuno Research Laboratories, West Grove PA USA) diluted 1 to 1,000 in 1X 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 in TTBS and 3 times in TBS followed by a final water wash. The slides were air dried after brief centrifugation and analyzed using a Perkin Elmer ScanArray Express HT microarray scanner (Perkin Elmer, Waltham MA USA). The intensities were quantified using ScanArray 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 control means 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 *P. vivax* genes/proteins were derived from the *Plasmodium* database (PlasmoDB, http://www.plasmodb.org/plasmo/home.jsp) [28]. The molecular function of the *P. vivax* immunogenic proteins was re-analyzed by gene ontology (GO) annotation [6]. MAAP was used to predict the adhesins of *P. vivax*, and the merozoite invasion-related proteins of *P. vivax* were predicted in comparison with the functional proteins of *P. falciparum* [29-31].

Results

The *P. vivax* blood stage protein expression

The expression of the *P. vivax* proteome was shown in supplementary Figure 1. A total of 89.5% (1,733/1,936) and 85.2% (1,663/1,936) of the *P. vivax* proteins tested positive for the anti-His antibody and anti-HA antibody, respectively. A total of 80.9% (1,566/1,936) of the *P. vivax* proteins tested positive for both anti-His/anti-HA antibodies, and 94.5% (1,830/1,936) 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 described above (Supplementary Figure 2). 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 Figures 1A&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 1,936 target proteins), representing the top-ranked immunogenic

antigens, are shown in Figure 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 (Table S1 and Table S2, Supporting Information). 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) (Figure 2B).

By searching the mass-spectra (MS) evidence from the peripheral blood of *P. vivax* infected patients and schizont proteome of *P. vivax*, 6 and 16 immunogenic *P. vivax* proteins, respectively, were shown with MS data [16,36]. A chromatin assembly factor 1 (PVX_081265), an early transcribed membrane protein (PVX_090230), a deoxyribose-phosphate aldolase (PVX_001945), a cell division cycle protein 48 homologue (PVX_114095), a heat shock protein (PVX_122065) and a conserved hypothetical protein (PVX_115450) were identified from peripheral blood of *P. vivax* infected patients [36]. A subtilisin-like protease precursor (SUB1, PVX_097935), rhoptry-associated protein 2 (RAP2, PVX_097590) and other 14 proteins were included in the schizont proteome dataset [16]. Especially, a homolog gene with a *P. falciparum* membrane associated histidine-rich protein (MAHRP1) PVX_115450, was

recognized by 12 malaria serum samples with ROCs of 0.99, which was identified from peripheral blood of a patient infected with *P. vivax* and the schizont proteome of *P. vivax* [37].

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 by the host immune response and that they play important role in the erythrocyte stage, such as merozoite adhesion, parasite infected erythrocyte adhesion and pathogenesis (Figure 3A&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 (Figure 3C&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 (Figure 4) [6]. 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) [30]. We analyzed the *P. vivax* microarray data through the IDC for the expression of the genes encoding the 11 merozoite invasion-related proteins [5]. We found evidence for all of these proteins, and they show an expression pattern consistent with involvement in the invasion or schizonts stages of at least one isolate, peaking in the TP6~TP9 post-invasion transcription (Supplementary Figure 3).

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* were 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 (Figure 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 (rhoptry). The potential role for Pf12 and Pv12 is 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 [44,45], was recognized by immune sera from naturally infected patients.

Discussion

Malaria caused by *P. falciparum* malaria 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 [2,46]. Although non-falciparum parasites are often considered to cause only 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 [10,39], it is urgent to develop a proteome-wide microarray technology and discover the immunodominant proteins

of *P. vivax* [53]. 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 1,936 polypeptides of *P. vivax* was used to characterize the immunomics 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) [10]. 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 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 Wheat germ based cell-free system [57].

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 [58]. 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* merozoites in vitro, which indicates that it is involved in the merozoite invasion of RBCs.

Parasite adhesins play important roles in parasite invasion of the RBCs, sequestration or parasite–host interactions [59,60]. In total, 137 adhesins were

predicted in the *P. vivax* genome in contrast to 157 adhesins in the *P. falciparum* genome [29]. 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 [61]. The MSP family is a group of merozoite surface proteins that are involved in the initial interaction between the merozoite and the host cell [30,58,62,63]. 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 [64].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 [65].

Invasion of the host cell is an essential process for survival of the malaria parasite and is a key target for malaria intervention [62]. A subnetwork of *P. falciparum* merozoite invasion-related proteins is obtained by a guilt-by-association prediction, which contains 418 proteins [30]. 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 [31]. The *P. vivax* merozoite invasion-related proteins are linked with the invasion-like apical organelle protein (RAP2), the GPI-anchored merozoite surface proteins (MSP4 and MSP7), the actin-myosin motor components (MTIP) and the merozoite egress and

328 invasion-related protease (SUB1).

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

337

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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.

Supporting Information

Text S1. Supplementary Materials and Methods.

Figure S1. Analysis of *P. vivax* proteins expression by proteome microarrays. (A) The *P. vivax* proteome microarrays probed with the anti-His_6-tag antibody. (B) The *P. vivax* proteome microarrays probed with the anti-HA antibody.

Figure S2. Analysis of antibody response to PvMSP1-42 by ELISA. The serum samples from 15 cases of vivax malaria in Yunnan province of the P.R. China and 10 serum samples from unexposed subjects were tested against PvMSP1-42 by ELISA.

Figure S3. Transcription pattern of 10 genes coded merozoite invasion-related proteins. The transcription data of the *P. vivax* genes were collected from the microarray results [5]. All of merozoite invasion-related proteins were shown an expression pattern consistent with involvement in the invasion or schizonts stages of

at least one isolate, peaking in the TP6~TP9 post-invasion transcription.

Table S1. List of 151 immunogenic proteins of *Plasmodium vivax*.

Table S2. AUC value and prevalence reactivities of 151 immunogenic proteins of *Plasmodium vivax*.

Table and figure legends

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

Table 2. Merozoite invasion-related proteins of *Plasmodium vivax* with immunogenicity.

Table 3. MAAP predicted adhesins of *Plasmodium vivax* with high immunogenicity.

Figure 1. Antibody profiling by proteome microarrays. (A) The *P. vivax* proteome microarrays reacted with serum from *P. vivax*-exposed individuals. (B) The *P. vivax* proteome microarrays reacted with serum from the unexposed subjects. Each chip included 16 subarrays (green box). Each subarray contained 8 negative control spots made with *E. coli* based Rapid Translation System 100 HY (RTS) without plasmid DNA (red boxes) and positive controls spots made with anti-human IgG for the primary antibody (yellow boxes).

Figure 2. Immunoreactivity profiles of immunogenic *P. vivax* proteins. A total of 151 antigens (149 genes) exhibited high IgG antibody responses to *P. vivax*-exposed individuals. (A) Immunoreactivity profiles of 151 immunogenic proteins with AUCs higher than 0.50. Each row shows the responses to a single *P. vivax* protein and each

column showing the responses of an individual (plasma sample) to each of these *P. vivax* proteins. (B) Forty of the 151 most immunoreactive proteins were considered as biomarkers for serodiagnostics with AUCs higher than 0.90.

Figure 3. Computational predictions for the *P. vivax* immunogenic proteins. (A) 57.7% of *P. vivax* immunogenic proteins have a transmembrane (TM) domain by TMHMM analysis. (B) 63.8% of *P. vivax* immunogenic proteins have signal peptide by SignalP analysis. (C) 53.0% of the gene coding *P. vivax* immunogenic proteins have the maximum gene expression pattern in the schizont stage. (D) 55.0% of the *P. vivax* immunogenic proteins belong to the hypothetical proteins.

Figure 4. GO annotation for the *P. vivax* immunogenic proteins. Among 149 genes coding 151 *P. vivax* immunogenic proteins, 98 have GO annotation, literature co-citation, or other annotated parasite-specific processes. There are 30 genes known to be involved in DNA replication and 11 genes involved in the merozoite invasion and pathogenesis, respectively. Eight proteins involved in merozoite development and erythrocytic development are closely associated with the blood-stage of *P. vivax*.

Figure 5. The immunoproteome of *P. vivax* merozoite identified by protein microarrays. The immunogenic proteins of the *P. vivax* merozoite with their respective location in the merozoite (the surface, rhoptries, micronemes and exonomes). The merozoite surface proteins of *P. vivax* were the major family of immunogenic antigens, which included 5 GPI-anchored MSPs and 9 peripheral surface proteins.

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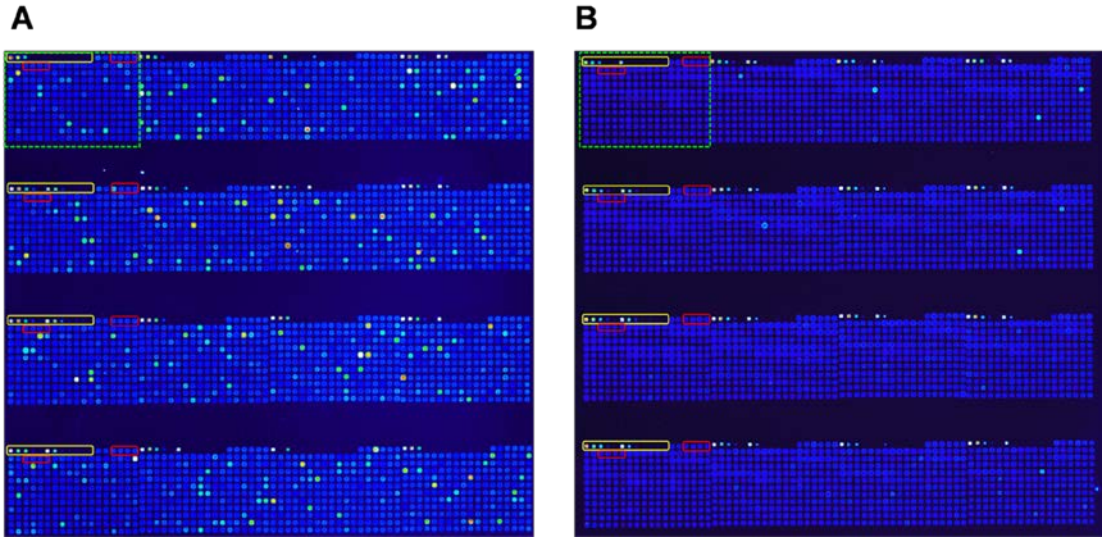
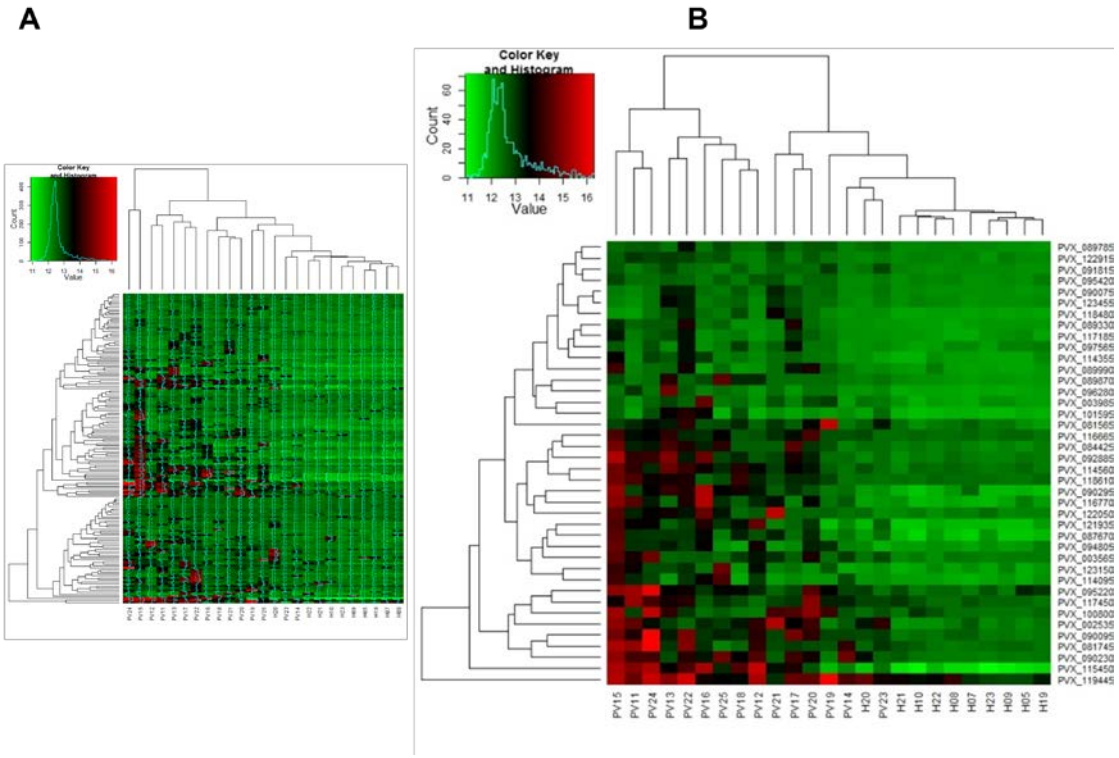


Figure 1



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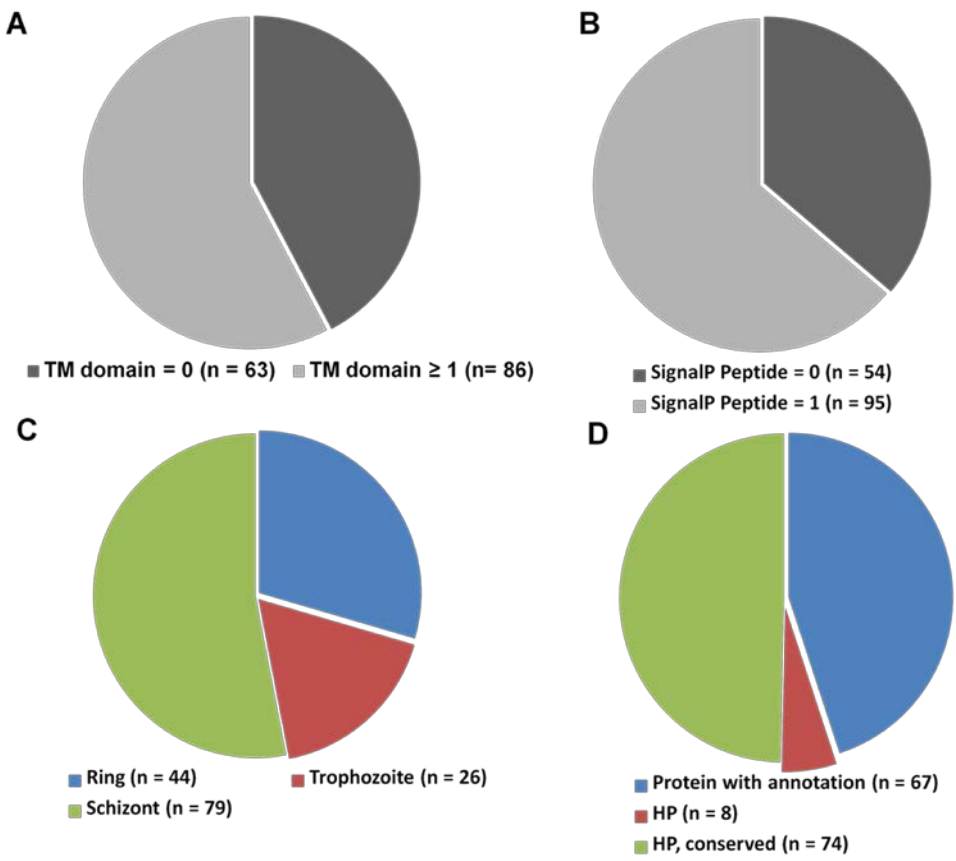
627 **Figure 2**



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629 **Figure 3**

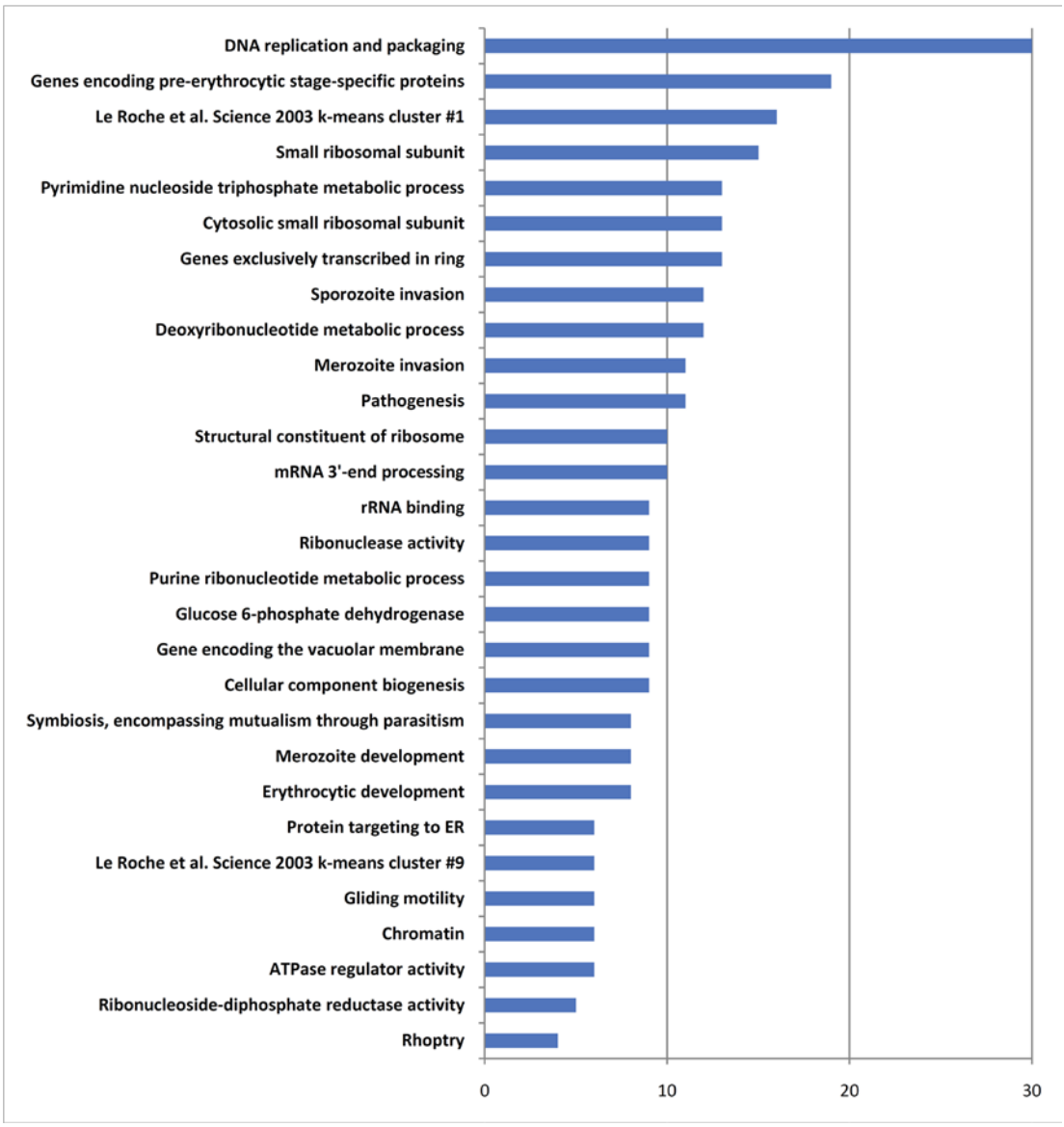
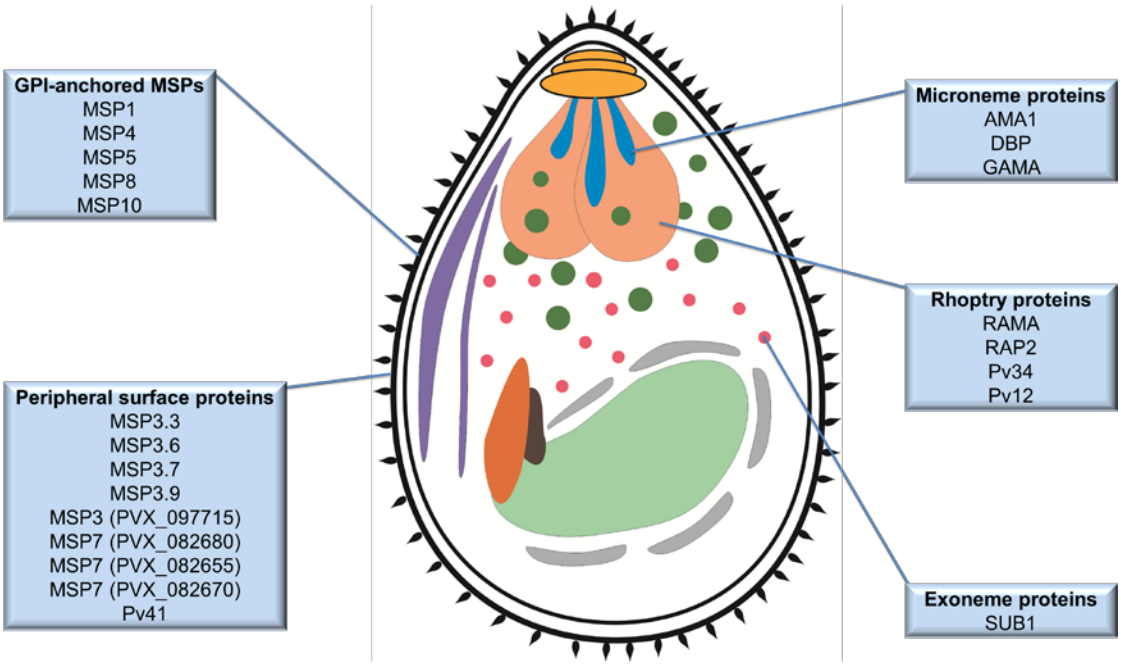


Figure 4



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633 **Figure 5**

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

Gene ID[a]	Product description	AUC[b]	No. of positive (%)	SP[c]	TMD[d]
PVX_090095	hypothetical protein, conserved	1.00	15 (100.0)	N	0
PVX_003565	ETRAMP11.2	1.00	13 (86.7)	Y	2
PVX_092885	hypothetical protein, conserved	1.00	12 (80.0)	N	4
PVX_087670	hypothetical protein, conserved	1.00	10 (66.7)	N	1
PVX_089870	RAD protein (Pv-fam-e)	1.00	8 (53.3)	Y	0
PVX_096280	hypothetical protein, conserved	1.00	4 (26.7)	Y	0
PVX_115450	homolog to PfMAHRP1	0.99	12 (80.0)	N	1
PVX_090295	hypothetical protein	0.98	12 (80.0)	N	1
PVX_116770	nucleoside-diphosphatase mig-23, putative	0.98	11 (73.3)	Y	1

PVX_089785	RAD protein (Pv-fam-e)	0.98	8 (53.3)	Y	0
PVX_122915	hypothetical protein, conserved	0.97	9 (60.0)	N	0
PVX_091815	endoplasmic reticulum oxidoreductin, putative	0.97	8 (53.3)	Y	1
PVX_095420	inorganic pyrophosphatase, putative	0.97	8 (53.3)	N	0
PVX_089330	hypothetical protein, conserved	0.97	7 (46.7)	Y	2
PVX_090075	Pv34	0.97	6 (40.0)	N	0
PVX_121935	hypothetical protein	0.96	12 (80.0)	Y	2
PVX_101595	hypothetical protein	0.95	6 (40.0)	N	2
PVX_118480	delta-aminolevulinic acid dehydratase precursor, putative	0.95	5 (33.3)	Y	0

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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] SP, signal peptide. [d] TMD, transmembrane domain.

Table 2. Merozoite invasion-related proteins of *Plasmodium vivax* with high immunogenicity

Gene ID[a]	Product description	AUC[b]	No. of positive (%)	*Pf* homolog	Max exp timing (hrs)	SP[c]	TM[d]
PVX_097590	rhoptry-associated protein 2 (RAP2)	0.86	10 (66.7)	PFE0075c	40	Y	0
PVX_088240	hypothetical protein, conserved	0.91	7 (46.7)	MAL8P1.135	43	Y	7
PVX_114355	hypothetical protein, conserved	0.93	6 (40.0)	PFF1210w	40	N	6
PVX_113355	hypothetical protein, conserved	0.95	8 (53.3)	PFF0185c	35	N	1
PVX_089695	hypothetical protein, conserved	0.70	7 (46.7)	PFD0715c	43	N	0
PVX_090075	Pv34	0.97	6 (40.0)	PFD0955w	40	N	0
PVX_003775	merozoite surface protein 4 (MSP4), putative	0.83	9 (60.0)	PFB0310c	35	Y	0
PVX_082670	merozoite surface protein 7 (MSP7), putative	0.76	8 (53.3)	PF13_0197	43	Y	0

PVX_101215	myosin A tail domain interacting protein MTIP, putative	0.96	15 (100.0)	PFL2225w	17	N	0
PVX_097935	subtilisin-like protease precursor (SUB1), putative	0.77	10 (66.7)	PFE0370c	40	Y	0
PVX_003985	syntaxin, putative	0.93	8 (53.3)	PFB0480w	43	N	1

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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] SP, signal peptide. [d] TMD, transmembrane domain.

Table 3. MAAP predicted adhesins of *Plasmodium vivax* with high immunogenicity

Gene ID[a]	Product description	AUC[b]	No. of positive (%)	MAAP score[c]	Max exp timing (hrs)	SP[d]	TM[e]
PVX_089765	RAD protein (Pv-fam-e)	0.93	8 (53.3)	2.059	40	N	0
PVX_003775	merozoite surface protein 4 (MSP4), putative	0.83	9 (60.0)	1.001	35	Y	0
PVX_084425	hypothetical protein, conserved	0.94	13 (86.7)	0.758	43	N	0
PVX_082670	merozoite surface protein 7 (MSP7), putative	0.76	8 (53.3)	0.734	43	Y	0
PVX_123455	hypothetical protein, conserved	0.92	5 (33.3)	0.702	35	N	2

691 [a] Gene ID was obtained from PlasmoDB (http://www.plasmodb.org/plasmo/home.jsp). [b] AUC, the area under the receiver operating

692 characteristics (ROCs) curve. [c] MAAP, malarial adhesins and adhesin-like proteins predictor. [d] SP, signal peptide. [e] TMD, transmembrane

693 domain.