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Determination of the *Plasmodium vivax* schizont stage proteome

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Abstract

With the genome of the malaria parasite *Plasmodium vivax* sequenced, it is important to determine the proteomes of the parasite in order to assist efforts in antigen and drug target discovery. Since a method for continuous culture of *P. vivax* parasite is not available, we tried to study the proteome of the erythrocytic stages using fresh parasite isolates from patients. In schizont-enriched samples, 316 proteins were confidently identified by tandem mass spectrometry. Almost 50% of the identified proteins were hypothetical, while other major categories include proteins with binding function, protein fate, protein synthesis, metabolism and cellular transport. To identify proteins that are recognized by host humoral immunity, parasite proteins were separated by two-dimensional gel electrophoresis and screened by Western blot using an immune serum from a *P. vivax* patient. Mass spectrometry analysis of protein spots recognized by the serum identified four potential antigens including PV24. The recombinant protein PV24 was recognized by antibodies from vivax malaria patients even during the convalescent period, indicating that PV24 could elicit long-lasting antibody responses in *P. vivax* patients.

Keywords

Antigen; Erythrocytic stage; Mass spectrometry; *Plasmodium vivax*; Proteome

1. Introduction

Plasmodium vivax is the most widespread human malaria parasite, which causes significant morbidity and socio-economic problems in endemic countries [1]. It has several unique characteristics that distinguish it from other human malaria parasite species. Most notably, *P. vivax* forms hypnozoites in hepatocytes, causing relapses of the disease [2]. *P. vivax* strains from the tropical and temperate zones can vary dramatically in terms of the pattern

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and frequency of the relapse. Moreover, *P. vivax* requires Duffy receptor on the red cell for invasion, and is thus absent in West Africa where Duffy negativity predominates [3]. It selectively invades reticulocytes [4], thus limiting parasitemias to low levels. Unlike *Plasmodium falciparum* infection that increases the rigidity of the host cell, *P. vivax* increases the size and deformability of infected red cells [5]. *P. vivax* also actively remodels the host cell, producing caveola-vesicle complexes along the plasmalemma in the infected erythrocyte cell, which are visible in Giemsa-stained smears as multiple red spots called “Schüffner's dots”. Apart from these characteristics, the ability of *P. vivax* to survive at much lower temperature has allowed this parasite to establish transmission foci in temperate zones. Despite that many of these unique features of *P. vivax* have been known for a long time, the underlying mechanisms remain poorly understood. Therefore, a better understanding of the fundamental biology of *P. vivax* is needed to effectively control and eventually eradicate this parasite.

The task to eliminate malaria globally requires integrated control measures, one of which is development of vaccines against malaria parasites. Several leading candidate vaccines from *P. falciparum* have been tested in clinical trials but do not offer protection against other *Plasmodium* species [6]. The deployment of such vaccines against *P. falciparum* may cause an unexpected outcome in malaria epidemiology in areas of *P. vivax* and *P. falciparum* coexistence, and therefore, multi-subunit and multi-species vaccines are needed in such endemic areas. Whereas the *P. falciparum* vaccine candidate repertoire is well characterized [7], few *P. vivax* antigens are well defined. Therefore, antigen discovery is a prerequisite for the development of vaccines against *P. vivax*.

Completion of the genome [8], transcriptome [9] and proteome [10,11] of *P. falciparum* has played a significant role in advancing research on this parasite. In comparison, research on *P. vivax* malaria has lagged much behind. One major reason is the unavailability of a continuous *in vitro* culture system for *P. vivax*, although recent work showed promises in this direction [12]. With the recent deciphering of the *P. vivax* genome [13] and transcriptome [14], the parasite proteome remains to be determined. The advance in highly sensitive mass spectrometry (MS) offers an extraordinary opportunity to determine the proteome of the *P. vivax* parasite in the absence of large amount of experimental materials from a continuous *in vitro* culture. In this study, we attempted to study the proteome of the erythrocytic stages of *P. vivax* field isolates by highly accurate tandem mass spectrometry (MS/MS). As a way of antigen discovery, we further tried to identify parasite antigens that are recognized by host humoral immunity using an immune serum from a *P. vivax* malaria patient.

2. Materials and Methods

2. 1. Sample collection

Fresh *P. vivax* isolates were collected from 10 symptomatic malaria patients attending a malaria clinic in Mae Sot district, Tak Province, Thailand. Twenty milliliters of *P. vivax*-infected blood were collected from each patient. White blood cells were removed by passing infected blood through a Plasmodipur® filter. Parasites were cultured with McCoy's 5A medium supplemented with 25% human AB serum at 37°C under 5% CO₂ until they reached the schizont stage. To reduce contamination of red blood cell proteins, schizont-infected red blood cells were purified on 60% Percoll® and parasites were released by 0.01% saponin treatment. Parasite pellet was washed with phosphate buffered saline (PBS) pH 7.4 until the supernatant was clear and stored at -80°C for proteome analysis.

A total of 118 and 33 plasma samples were collected in 2001, 2002, and 2007-2009 from *P. vivax* and *P. falciparum* patients, respectively, who were attending an outpatient malaria

clinic in Mae Sot, or the Hospital of Tropical Diseases, Bangkok, Thailand. Among these patients, follow-up was conducted in four *P. vivax* cases, from whom plasma samples were collected at the time of acute infection, and in three and six months after treatment. During the follow-up, the participants did not experience malaria infections. Control plasma samples from malaria naïve donors were collected from a non-endemic area in Thailand.

The study protocol was approved by The Pennsylvania State University Institutional Review Board and the Ethical Review Committee of Mahidol University. Informed consent or assent was obtained from volunteers before the blood samples were collected.

2.2. Preparation of protein extracts

For proteome analysis, $\sim 10^9$ *P. vivax* schizonts were resuspended in 100 μ l of 100 mM Tris-HCl (pH 7.2) and sonicated on ice for four 10-sec pulses. Proteins were dissolved in SDS-PAGE loading buffer and separated on a linear gradient (4-20%, 1.5 mm thick) Tris-glycine mini gel (Invitrogen, USA). The gel was stained by Colloidal blue (Invitrogen) and protein bands were horizontally sliced into 16 sections (Fig. 1.) Each gel section was cut into ~ 1 mm cubes, destained in 50% acetonitrile (ACN) containing 25 mM NH_4HCO_3 (pH 8.0) until gel pieces became transparent, dehydrated in 100% ACN, and dried completely in a SpeedVac. Afterward, the samples were reduced in 45 mM dithiothreitol (DTT) and 25 mM NH_4HCO_3 (pH 8.0) for 45 min at 55°C and then alkylated in 100 mM iodoacetamide, 25 mM NH_4HCO_3 (pH 8.0) for 45 min at room temperature in the dark. All samples were washed twice in 50% ACN, 25 mM NH_4HCO_3 (pH 8.0) and dried in a SpeedVac. For in-gel digestion, samples were digested overnight at 37°C with 0.01 μ g/ μ l MS grade trypsin (Promega, Madison, Wisconsin, USA) in 50% ACN, 25 mM NH_4HCO_3 (pH 8.0). The resultant peptides were extracted twice with 100 μ l of 5% trifluoroacetic acid (TFA) in 50% ACN for 15 min. Samples were dried down completely by SpeedVac, and then resuspended in 200 μ l of deionized water. This procedure was repeated twice, with a third drying down halted when the remaining volume was approximately 10 μ l. Then 1% TFA was added to make the final concentration of TFA at 0.1%. The peptide samples were cleaned with SCX ZipTips (Millipore, Billerica, Massachusetts, USA) according to the manufacturer's instructions. The eluant was dried completely in a SpeedVac and resuspended in 15 μ l of 2% ACN and 0.1% TFA. Separation of the peptides were achieved by reverse-phase nanoflow liquid chromatography (LC) using a 150 $\times$ 0.1 mm chromolith caprod column injector loop (Merk, USA) on a Tempo LC matrix assisted laser desorption/ionization (MALDI) spotting system (ABI-MDS/Sciex) and eluted with a gradient of 2% ACN/0.1% TFA and 98% ACN/0.1% TFA, respectively.

2.3. Two-dimensional gel electrophoresis (2-DE) and Western blot

To identify parasite antigens, parasite proteins were subjected to 2-DE, immunoblotting, and LC/MS/MS analysis. Briefly, parasite crude extract (40 μ g/gel) was solubilized in 2-D rehydration buffer (8 M urea, 0.5% CHAPS, 60 mM DTT), and 0.5% ampholyte (pH 3–10), thoroughly mixed and centrifuged at 15000 $\times$ g for 10 min. The resultant supernatant was subjected to 2-DE. Isoelectric focusing was performed with pre-cast 7 cm Immobiline® dry strips (pH 3–10) using the Ettan IPGphor 3 apparatus (Amersham Bioscience AB, Uppsala, Sweden). The running protocol was as follows: step 1, 300 v, 200 Vh; step 2, 1000 v, 300 Vh; step 3, 5000 v, 1,400 Vh; and step 4, 5000 v, 2000 Vh. The focused strips were equilibrated in 10 ml of equilibration solution (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% DTT) for 15 min, followed by incubation in 10 ml equilibration solution containing 2.5% iodoacetamide for another 15 min. The equilibrated strips were loaded on 12% SDS-PAGE gels for the second dimension separation. One gel was silver-stained using a MS compatible PlusOne Silver staining kit (Amersham Bioscience), whereas the other was used for immunoblotting.

For Western blot, proteins were transferred to a 0.45 µm nitrocellulose membrane (Pharmacia Biotech, California, USA) under cooling conditions and constant voltage (100 V) for 4 h. The membrane was blocked with 5% skimmed milk in Tris-buffered saline (TBS, pH 7.2) overnight at 4°C. After washing with TBS containing 0.05% Tween 20, the membrane was incubated with an immune serum from a *P. vivax* patient at 1:10 dilution for 2 h, and then with HRP-conjugated goat anti-human IgG antibody at 1:500 dilution for 1 h. The blot was visualized by incubating with the HRP substrate (4-chloro-1-naphthol, methanol and H₂O₂ in TBS). Positive spots were picked from the silver-stained gel. Each gel spot was destained in 1% H₂O₂, 25 mM NH₄HCO₃ (pH 8.0) prior to in-gel trypsin digestion as described above.

2.4. MS analysis and database search

For MS analysis, the final eluant of the peptides separated by reverse phase nanoflow LC was mixed with the MALDI matrix [7 mg/ml recrystallized α-cyanohydroxycinnamic acid (Sigma), 2 mg/ml ammonium phosphate, 0.1% TFA in 50% ACN], and automatically spotted onto a stainless steel MALDI target plate and analyzed on an ABI 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, USA). The MS spectra were acquired from each sample spot using 500 laser shots from 40 random per spot using Reflectron Positive Ion mode with laser setting of 3200. The highest top ten peaks of each observed m/z value of each MS spectrum (excluding trypsin auto-digestion peaks) were chosen for subsequent MS/MS analysis with collision-induced dissociation fragmentation. Up to 2500 laser shots at the laser power 4200 were accumulated for each MS/MS spectrum.

For identifying parasite antigens, peptides from the immune-positive spots of the 2-DE were analyzed by LC/MS/MS using a SYNAPTTM HDMS mass spectrometer (Waters, Manchester, UK). Nanoscale LC separation of tryptic peptides was performed with a NanoAcquity system (Waters) equipped with a 5 µm, 180 µm × 200 µm symmetry C18 trap column and a 1.7 µm, 75 µm × 200 µm BEH130 C18 analytical reversed phase column (Waters). The samples were initially transferred with an aqueous 0.1% formic acid solution to the trap column with a flow rate of 3 µl/min for 3 min. Mobile phase A was water with 0.1% formic acid, and mobile phase B was 0.1% formic acid in ACN. The peptides were separated with a gradient of 2–40% mobile phase B over 30 min at a flow rate of 350 nl/min followed by a 10 min rinse with 80% of mobile phase B. The lock mass was delivered from the auxiliary pump of the NanoAcquity pump with a constant flow rate of 200 nl/min to the reference sprayer of the NanoLockSpray source of the mass spectrometer. After nanoelectrospray ionization, accurate MS/MS data were acquired.

The MS and MS/MS data were searched against the annotated *P. vivax* protein database from PlasmoDB (<http://www.plasmodb.org>) and non-redundant human protein database from National Center for Biotechnology Information. The MS and MS/MS data from MALDI-TOF/TOF and LC/MS/MS were analyzed by Protein Pilot software version 2.01 (Applied Biosystems/MDS Sciex) and ProteinLynx Global Server 2.2.5 (Waters), respectively. Search criteria were trypsin-cleaved peptides; 200 parts per million mass error tolerance in MS mode; 0.4 Da mass error tolerance for MS/MS fragments; fixed modification of carbamidomethylation of cysteines, and allowed (variable) modification of oxidation of methionine. Protein identification acceptance was ProteinPilot Unused Score of >1.3 (>95% confidence interval) plus <5% false discovery rate.

The presence of a signal peptide (SP) and transmembrane domain (TM) in the protein sequence is an important indicator of secretory or cell surface protein. We used SignalP 3.0 and TMHMM 2.0 (<http://www.cbs.dtu.dk>) to determine the SP and TM in the proteins. To predict the antigenicity of the proteins, linear B-cell epitope and antigenicity were predicted

using the BepiPred 1.0 and Kolarskar & Tongaonkar Antigenicity programs (www.immunepitope.org).

2.5. Expression of a recombinant protein

To express a putative antigen, PV24 (PVX_002950), identified from the 2-DE/MS analysis, cDNA was synthesized from 1 µg of *P. vivax* total RNA using Superscript®III reverse transcriptase (Invitrogen) [15]. Primers (5' ATGCGGATCCTACAATGCAAGCGAAAGACAAAATGG3' and 5' AGTCGTCGACGAACAAGTAGCCATAATATTTGG 3', *Bam*HI and *Sal*I sites underlined) were designed to amplify a 540 bp fragment (79-618 bp of the open reading frame). The PCR product was cloned in the expression vector pET-28 (a+) (Novagen, Darmstadt, Germany) after digestion with *Bam*HI and *Sal*I. The recombinant PV24 protein (rPV24) was expressed in *Escherichia coli* BL21 after induction by 1 mM isopropyl β-D-1-thiogalactopyranoside for 2 h. The His-tagged recombinant protein was purified by a denaturing method using the Ni-NTA agarose resin (Qiagen, Hilden, Germany). Eluted protein fractions were analyzed by 12% SDS-PAGE and Western blot analysis with mouse anti-His antibody at 1:1000 dilution. Purified recombinant protein was extensively dialyzed against PBS (pH 7.4) at 4°C.

2.6. ELISA

Griener flat-bottomed, 96-well microtiter plates were coated with 50 µl per well of 1 µg/ml of purified rPV24 or crude *P. vivax* parasite protein (10 µg/ml) overnight at 4°C. The plates were blocked for 2 h with 100 µl of PBS with 0.05% Tween 20 (PBST) containing 0.5% bovine serum albumin. After washing twice with PBST, 50 µl of human plasma diluted to 1:50 with the blocking buffer were added and incubated for 2 h at room temperature. Plates were washed thrice with PBST and incubated with 50 µl of HRP-conjugated goat anti-human IgG antibody (Caltag, USA) at 1:2,000 for 1 h. Afterwards, the plates were washed thrice with PBST, and developed with 2, 2', azino-diethylbenzothiazolinesulfonic acid (Kirkepaard & Perry Laboratories, USA) for 30 min. The optical density (OD) was read at 405 nm on an ELISA plate reader. Each sample was tested in duplicate. Mean OD given by naïve control sera plus three standard deviations (STD) was used as the cutoff OD value.

2.7. Statistical analysis

Statistical analysis was performed using the SPSS program (Version 11.5, Chicago, USA). The level of anti-rPV24 antibodies among plasma samples from *P. vivax* and *P. falciparum* patients, and naïve control were evaluated by the analysis of variance (ANOVA). The correlation between anti-rPV24 antibody level and parasitemia was determined by Spearman's rank test. The results were considered significant at $P < 0.05$.

3. Results

3.1. The proteome of *P. vivax* schizonts

Due to the lack of a suitable continuous *in vitro* culture method for *P. vivax*, we used field isolates obtained from infected patients for proteomic analysis. Our preliminary studies using parasite samples immediately after saponin lysis suggested that host protein contamination was too overwhelming to obtain an adequate parasite protein coverage. In order to circumvent this problem, parasites were cultured until they reached the schizont stage and purified by Percoll. To determine the schizont stage proteome, protein extracts were obtained from ~10⁹ schizonts, separated by SDS-PAGE and subjected to MS analysis by MALDI-TOF/TOF. Search of the protein databases with the MS/MS spectra identified 316 *P. vivax* proteins with at least 95% confidence (supporting information 1). Almost half

of the identified proteins (47%) were hypothetical proteins, among which 29% contained a putative SP and 37% contained at least one TM. Further, 15% of these hypothetical proteins contained both a SP and a TM. This result suggested that the identified proteins were either secretory or/and membrane associated, which might play important roles in parasite-host interactions (supporting information 2).

Eleven percent (36 in 316 proteins) of the identified proteins was unique as defined by the absence of orthologs in other human malaria parasites, *P. falciparum*, *P. ovale* and *P. malariae* (supporting information 3). The majority of the unique proteins (18 in 36 proteins) were hypothetical. In addition, 16 of 36 proteins did not have orthologs in other human malaria parasites and *P. knowlesi*, which provide potential targets for *P. vivax*-specific diagnosis.

The identified proteins were classified into functional classes based on the Munich Information Centre for Protein Sequences (MIPS) catalogue [16] (supporting information 4). Proteins were plotted as a function of their broad functional classification; only one class was assigned per protein in order to avoid redundancy (Fig. 2). The predominant class contained hypothetical proteins (47%). Other important functional classes of proteins were proteins with binding function (10%), and involved in protein fate (5%), protein synthesis (4%), metabolism (4%) and cellular transport (4%). These protein classes were mainly housekeeping proteins.

Enzymes of major metabolic pathways of the parasite including glycolysis, nucleic acid metabolism, and hemoglobin digestion were identified (Table 1). During parasite development, it modifies or exports proteins into red cell membrane which causes dramatic changes in host cell membrane composition, structure and function. Different transportation mechanisms of parasite have been reported, and several proteins shown to play significant roles in the transport machinery including Rab GTPases (Rab2, Rab5c, Rab6 and Rab7) [17, 18], translocation protein complex (sec61, sec62 and SEC63), heat shock protein (Hsp70 and HSP101) and EXP2 [19-22] were identified in the schizont stage proteome. Several exported proteins potentially involved in immune evasion and host cell invasion including *vir* and erythrocyte membrane protein 3 were identified [23, 24]. The *P. vivax* tryptophan rich antigen (PvtrAg) or Pv-fam-a is one of the most abundant parasite protein families with 34 predicted members (www.plasmodb.org), among which 11 members were detected.

Since our parasite samples were enriched in schizonts, schizont-specific proteins were readily detected. These include proteins associated with merozoite surface and invasive organelles such as merozoite surface proteins (MSP1 [25], MSP5 [26], MSP7 [27], and MSP8 [28]), apical membrane antigen 1 (AMA1) [29], reticulocyte binding protein [30], rhoptry proteins (RAP1 [31], RAP 2 [32], RhopH2, EXP2, and CLAG), and actinomyosin motors (actin, myosin) [33-35]. The parasite samples also contained gametocytes and several sexual stage proteins were detected, including the *P. falciparum* orthologs of male fertility protein Pf47 (PVX_083240), Pfs16 (PVX_000930) and transmission-blocking vaccine candidate Pfs230 (PVX_000995) [36-38].

Parasite adhesins are of particular importance due to their roles in parasite invasion, sequestration or parasite-host interactions [24]. The *P. vivax* genome was predicted to encode 137 adhesins [39], of which 11 were detected in our schizont stage proteome and six were hypothetical proteins (Table 2). Some of the adhesion proteins such as MSP1 and AMA1 are known to involve in host cell invasion [40]. Glycosylphosphatidylinositol (GPI) anchor is the major carbohydrate modification of proteins mostly in schizont-stage parasites. Nine putative GPI-anchored proteins were detected in the schizont stage proteome (Table 3), which represent 10% of total GPI-anchored proteins predicted in the *P. vivax* genome. Four

of the nine proteins were known GPI-anchored proteins. Some of these GPI-anchored proteins were known to be involved in parasite-host interaction and invasion, and are promising vaccine candidates [41-43]. As a potential novel antigen, PVX_088910 encodes a hypothetical protein with maximum expression in late-stage parasites. Its *P. falciparum* ortholog (PF08_0008) is a GPI-anchored micronemal antigen shown to mediate binding to red blood cell surface, indicating involvement in the host cell invasion process.

3.2. Identification of novel *P. vivax* antigens

In order to identify parasite proteins that are recognized by host humoral immunity, 2-DE immunoblot of protein extract from schizont stage parasites was performed. Immune serum from an acute *P. vivax* infected patient showing a high antibody level to *P. vivax* lysate as determined by ELISA (data not shown) was used in Western blot analysis. Three immune-reactive protein spots were recognized by the antiserum. The positive protein spots were excised from the silver stained 2-DE gel for MS/MS analysis (Fig. 4). Four proteins were detected, corresponding to the protein products of PVX_002950, PVX_087070, PVX_095015 and PVX_092835 genes (Table 4). Antigenicity prediction suggested that all four proteins contained linear B-cell epitopes and had antigenicity score above the threshold. Of the four proteins, only PVX_002950 contained predicted SP and TM.

3.3. Expression and purification of rPV24

PVX_002950 is predicted to encode a 24 kDa hypothetical protein with 206 amino acids, referred to as PV24, which shared 41% similarities with the *P. falciparum* ortholog (PFB0515w). This gene encodes a putative dolichol-linked oligosaccharide biosynthesis enzyme. Microarray analysis indicates that it is expressed throughout the *P. vivax* erythrocytic cycle [14]. Since the predicted cleavage site of the SP is between amino acids 26 and 27, we decided to express the 180-residue recombinant protein without the first 26-residue SP sequence. The rPV24 expressed in *E. coli* had a molecular size of ~26 kDa on SDS-PAGE gel and reacted with antiserum against 6X His-tag, which was consistent with the predicted molecular size (26.4 kDa) of rPV24 (Fig. 4a and 4b). Moreover, rPV24 reacted with the immune serum previously used in the 2-DE immunoblot (Fig. 4c).

3.4. Antigenicity of rPV24

To determine the antigenicity of rPV24, ELISA was performed by testing rPV24 protein using plasma samples from *P. vivax* and *P. falciparum* patients, and malaria naïve donors. The reactivity of plasma from *P. vivax* patients was significantly higher than that of the naïve group ($P < 0.001$), and from that of plasma from *P. falciparum* patients ($P < 0.001$) (Fig. 5a). Compared with the naïve group, the reactivity to rPV24 of plasma from *P. falciparum* infected patients was not significantly different ($P > 0.05$). Plasma samples from *P. vivax* patients collected in different years (2001-2009) did not show significant differences in terms of reactivity with rPV24 ($P > 0.05$) (Fig. 5b). Ninety-nine percent of plasma samples from *P. vivax* patients showed reactivity to rPV24 above the cutoff value. Interestingly, the reactivity of plasma samples from all follow-up cases (3 and 6 months post treatment) was also higher than the cutoff level (Fig. 5c). In addition, there was a significant inverse correlation between the level of anti-rPV24 antibody and the *P. vivax* parasitemia (correlation coefficient = -0.372, $P = 0.039$). Altogether, these results indicated that the PV24 was strongly antigenic and elicited long-lasting immunity in *P. vivax* patients.

4. Discussion

With the completion of *P. vivax* genome sequencing and transcriptome projects, it is important to determine the *P. vivax* proteomes. Due to host protein contamination in other erythrocytic stages, we only attempted to purify schizont stage for proteomic analysis.

Consequently, our proteome was enriched in schizont stage proteins. In schizonts, 1,212 transcripts were detected (www.plasmodb.org), among which 316 proteins were detected in our proteomic analysis. Of those proteins, 65% overlapped with *P. falciparum* proteins [11]. Over half of the proteins identified were abundant proteins with putative functions in metabolism, protein synthesis, cellular transport and binding, etc. The hypothetical proteins accounting for almost 50% of this proteome could be a key to unravel the unique biology of *P. vivax*. In addition, numerous hypothetical proteins containing predicted SP (45) and/or TM (52) were discovered, some of which may represent potential antigens. Thirty six (11%) out of 316 proteins were unique in *P. vivax*, as defined by the absence of orthologs in other human malaria parasites. Given that most of the rapid diagnostic tests are based on the pan-*Plasmodium* antigens (e.g., lactate dehydrogenase and aldolase) [44], the unique proteins in *P. vivax* could serve as potential candidates for developing *P. vivax*-specific tests.

Transcriptome analysis of *P. vivax* revealed that members of *vir* and *pvtrag* gene families have two distinct phases of transcription, immediately after invasion and during schizogony [14]. Protein members of these gene families (10 *pvtrag* and 4 *vir*) detected in the schizont stage proteome appeared to be derived from the first wave of gene expression since all of them showed maximum transcription at the ring stage. The cognate proteins of 52 *vir* and 13 *pvtrag* genes with maximum transcription in schizont stage could not be detected in our schizont stage proteome possibly due to a delay in protein translation.

Antimalarial vaccines are an important component for the integrated approaches to malaria control during the malaria elimination phase [45]. In particular, malaria vaccine development efforts need to target other malaria parasite species, especially *P. vivax*. Currently, there are only two *P. vivax* vaccine candidates being tested in clinical trials, circumsporozoite protein and transmission-blocking vaccine candidate pvs25 [46]. Compared with *P. falciparum*, the number of vaccine candidates in *P. vivax* is very limited, and antigen discovery is essential for *P. vivax* vaccine development. In the schizont stage proteome, we have identified several potential membrane proteins that could serve as vaccine candidates, including those that are predicted adhesions and GPI-anchored proteins. These groups include antigenic proteins such as MSP1 and AMA1 that are already in the vaccine development pipeline. In addition, we used a MS-based method to identify *P. vivax* antigens, which proved to be a viable approach to *vivax* antigen discovery. In our schizont-stage proteome, four antigens were identified by a *P. vivax* patient serum. We have further characterized one of these proteins, PV24, and showed that *P. vivax* infection induced long-lasting reactivity with this protein, and antibody titers against this protein could still be detected during the convalescence stage six months after successful treatment of the primary infection. During malaria infection, T cells play a central role in the regulation of immune responses and the formation of immunologic memory which helps control and eliminate the infection [47]. For antigen discovery, we need to understand how the potential antigens elicit humoral and cellular immune responses during natural infection [48]. In this study, the persistence of anti-PV24 antibodies in *P. vivax* patients implies the involvement of T cells in the production of antibodies against this protein [49]. Our study has demonstrated PV24 as a usefulness marker for malaria epidemiology. Whether PV24 could induce protective immunity against malaria infection and serve as a vaccine candidate awaits further investigations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1. SDS-PAGE analysis of *P. vivax* lysate. Lysate from $\sim 10^9$ parasites were separated on 12% polyacrylamide gel and stained with colloidal blue. Molecular standards are shown in lane 1. Protein bands were horizontally sliced into 16 sections (lane 2, S1-S16) before subjected to in-gel trypsin digestion and MS analysis.

Fig 2.
Pie chart showing the distribution of functional classes of identified *P. vivax* proteins as defined by the MIPS catalogue.

Fig 3.
2-DE analysis of blood stage proteins of *P. vivax* (a) Silver stained gel, and (b) immunoblot with an immune serum of *P. vivax* patient. Circles indicate proteins of *P. vivax* reacted with the immune serum.

Fig 4.

Purification of rPV24 using Ni-NTA chromatography (a) SDS-PAGE analysis and (b) chemiluminescence analysis of immunoblot using anti-His antibody. Lane 1 = molecular weight standards, lane 2 = whole lysate, lane 3 = soluble fraction, lane 4 = flow-through fraction, lane 5 = last washing fraction, and lane 6 and 7 = eluted fractions (c) Immunoblot analysis of rPV24 with the immune serum from a *P. vivax* patient. Lane 1 = molecular weight standards, lane 2 = Ponceu S staining of rPV24 protein, and lane 3 = immunoblot of rPV24. Arrows indicate purified rPV24.

Fig 5.

Determination of antibody levels in plasma samples to rPV24 by ELISA. (a) Different groups of human plasmas; sera of *P. vivax* infected patients (PV), malaria naïve sera (N), and sera of *P. falciparum* infected patients (PF). (b) plasma from PV collected in different years compared with N. (c) Antibody levels during acute infection and convalescence period collected at 3 and 6 months after treatment (dash line indicates cut-off level at mean + 3 standard deviation).

Table 1The detected *P. vivax* proteins involved in parasite's metabolic pathways

Gene ID	Enzyme	EC no. ^a	Reaction catalyzed
<i>Glycolysis</i>			
PVX_095015	Enolase	4.2.1.11	Glycerate-2P ↔ Phosphoenol-pyruvate
PVX_114445	Pyruvate kinase	2.7.1.40	Phosphoenol-pyruvate + ADP ↔ Pyruvate + ATP
PVX_118495	Triosephosphate isomerase	5.3.1.1	Glyceraldehyde-3P ↔ Glycerone-P
PVX_118255	Fructose 1,6-bisphosphate aldolase	4.1.2.13	Aldolase ↔ Glyceraldehyde-3P
PVX_116630	Lactate dehydrogenase	1.1.1.27	Pyruvate + NADH+H ⁺ ↔ Lactate + NAD ⁺
<i>Nucleic acid biosynthesis</i>			
Purine salvage pathway			
PVX_111245	Adenosine deaminase	3.5.4.4	Adenosine + H ₂ O ↔ Inosine + NH ₃
PVX_092535	Adenylate and Guanylate cyclase catalytic domain containing protein	4.6.1.2	GTP ↔ 3',5'-cyclic GMP + PPi
PVX_094840	Hypoxanthine phosphoribosyltransferase	2.4.2.8	Hypoxanthine + PRPP ↔ IMP + PPi
Pyrimidine metabolism			
PVX_083135	Aspartate carbamoyltransferase	2.1.3.2	L-Aspartate + Carmoyl-P ↔ N-carbamoyl-L-Aspartate + Pi
<i>TCA cycle</i>			
PVX_084960	ATP-specific succinyl-CoA synthetase beta subunit	6.2.1.5	ATP + Succinate + CoA ↔ ADP + Orthophosphate + Succinyl-CoA
<i>Hemoglobin digestion</i>			
PVX_115000	Falcilysin	3.4.24.-	Acting on peptide bonds
PVX_097935	Subtilisin-like protease precursor	3.4.21.62	Hydrolyzes peptide amides
PVX_086040	Aspartic protease PM4	3.4.23.B14	Cleavage of hemoglobin
PVX_122425	M1-family aminopeptidase	3.4.11.2	Release of an N-terminal amino acid from a peptide

Plasmodium metabolic pathways can be found at <http://siteshujiacl/malaria>.^aEnzyme Commission (EC) numbers of *P. falciparum* orthologs.

Table 2

Plasmodium vivax adhesins predicted by MAAP (Malaria adhesins and adhesion like proteins predictor) [39].

Accession Number	Annotation	score ^a	SP	TM
PVX_097950	Hypothetical protein, conserved	0.742	0	0
PVX_096995	Tryptophan-rich antigen (Pv-fam-a)	0.740	0	1
PVX_084720	Hypothetical protein, conserved	0.935	1	0
PVX_092275	Apical merozoite antigen 1	1.001	0	1
PVX_099980	Major blood-stage surface antigen Pv200	1.001	1	1
PVX_096245	Hypothetical protein, conserved	1.008	0	0
PVX_117060	Hypothetical protein, conserved	1.027	0	0
PVX_083560	Hypothetical protein, conserved	1.242	0	0
PVX_112670	Tryptophan-rich antigen (Pv-fam-a)	1.615	1	0
PVX_096070	Early transcribed membrane protein (ETRAMP)	2.113	1	1
PVX_003555	Hypothetical protein, conserved	2.213	0	0

^a *P. vivax* proteins with Pmaap score of ≥ 0.7 were predicted as adhesins.

Table 3

GPI-anchored proteins identified in the schizont-stage proteome.

Accession number	Annotation
PVX_083135	Aspartate carbamoyltransferase, putative
PVX_088910	Hypothetical protein, conserved
PVX_097625	Merozoite surface protein 8, putative
PVX_099320	Acid phosphatase, putative
PVX_099980	Merozoite surface protein 1
PVX_100835	Hypothetical protein, conserved
PVX_110895	ADP/ATP transporter on adenylate translocase, putative
PVX_113775	Membrane protein pf12 precursor, putative
PVX_122545	COP-coated vesicle membrane protein p24 precursor, putative

Table 4

Plasmodium vivax proteins recognized by immune serum from *P. vivax* patient identified by LC/MS/MS

Gene ID	Description	Sequence	MW (kDa)	pI	SP	TM
PVX_002950	hypothetical protein	LIKDSNISFHFFYANNDP LSR	24.1	9.81	1	3
PVX_087070	hypothetical protein	APPTQGEMLLLLVR	110.1	6.53	0	No
PVX_095015	enolase	AAVPSGASTGIYEALRLR	48.8	9.70	0	No
PVX_092835	hypothetical protein	LGKSNKR, QKAKQVK, ENAERSK, AKISMFK, TNVKKNR, IFEREGK, HYKTNVK, DNKLGKSNK, TSENVNOSK, NILDEIAVK, GSTVNTYILK, QSKVSLKPIK, VKINLNNPVK, MHVFDLDDKAK, KNRFTIETR, KMHVFDLDDKAK	41.3	10.13	0	No