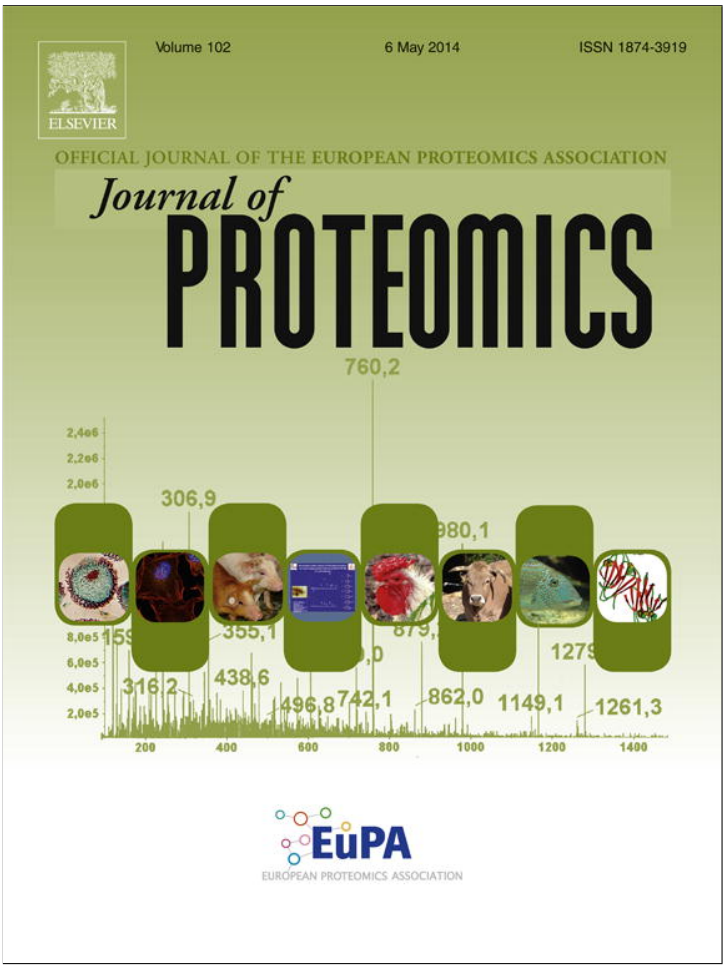

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Profiling the humoral immune responses to *Plasmodium vivax* infection and identification of candidate immunogenic rhoptry-associated membrane antigen (RAMA)

Feng Lu[a,b], *Jian Li*[a,b], *Bo Wang*[a], *Yang Cheng*[a], *Deok-Hoon Kong*[c], *Liwang Cui*[d], *Kwon-Soo Ha*[c], *Jetsumon Sattabongkot*[e], *Takafumi Tsuboi*[f,*], *Eun-Taek Han*[a,**]

[a]*Department of Medical Environmental Biology and Tropical Medicine, School of Medicine, Kangwon National University, Chunchon, Gangwon-do, Republic of Korea*
[b]*Jiangsu Institute of Parasitic Diseases, Key Laboratory on Technology for Parasitic Disease Prevention and Control, Ministry of Health, Wuxi, Jiangsu, People's Republic of China*
[c]*Department of Molecular and Cellular Biochemistry, School of Medicine, Kangwon National University, Chunchon, Gangwon-do, Republic of Korea*
[d]*Department of Entomology, The Pennsylvania State University, University Park, PA, USA*
[e]*Mahidol Vivax Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand*
[f]*Division of Malaria Research, Proteo-Science Center, Ehime University, Matsuyama, Ehime, Japan*

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ABSTRACT

Completion of sequencing of the *Plasmodium vivax* genome and transcriptome offers the chance to identify antigens among >5000 candidate proteins. To identify those *P. vivax* proteins that are immunogenic, a total of 152 candidate proteins (160 fragments) were expressed using a wheat germ cell-free system. The results of Western blot analysis showed that 92.5% (148/160) of the targets were expressed, and 96.6% (143/148) were in a soluble form with 67.7% of solubility rate. The proteins were screened by protein arrays with sera from 22 vivax malaria patients and 10 healthy individuals to confirm their immune profile, and 44 (27.5%, 44/160) highly reactive *P. vivax* antigens were identified. Overall, 5 candidates (rhoptry-associated membrane antigen [RAMA], Pv-fam-a and -b, EXP-1 and hypothetical protein PVX_084775) showed a positive reaction with >80% of patient sera, and 21 candidates with 50% to 80%. More than 23% of the highly immunoreactive proteins were hypothetical proteins, described for the first time in this study. One of the top immunogenic proteins, RAMA, was characterized and confirmed to be a serological marker of recent exposure to *P. vivax* infection. These novel immunoproteomes should greatly facilitate the identification of promising novel malaria antigens and may warrant further study.

Biological significance
The establishment of high-throughput cloning and expression systems has permitted the construction of protein arrays for proteome-wide study of *Plasmodium vivax*. In this study,

* *Correspondence to:* T. Tsuboi, Division of Malaria Research, Proteo-Science Center, Ehime University, 3 Bunkyo-cho, Matsuyama, Ehime 790-8577, Japan. Tel.: +81 89 927 8277; fax: +81 89 927 8528.
** *Correspondence to:* E.-T. Han, Department of Medical Environmental Biology and Tropical Medicine, School of Medicine, Kangwon National University, Hyoja2-dong, Chunchon, Gangwon-do 200-701, Republic of Korea. Tel.: +82 33 250 7941; fax: +82 33 255 8809.
E-mail addresses: tsuboi.takafumi.mb@ehime-u.ac.jp (T. Tsuboi), ethan@kangwon.ac.kr (E.-T. Han).

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high-throughput screening assays have been applied to investigate blood stage-specific immune proteomes from *P. vivax*. We identified 44 antigenic proteins from the 152 putative candidates, more than 23% of which were hypothetical proteins described for the first time in this study. In addition, PvRAMA was characterized further and confirmed to be a serological marker of exposure to infections. The expression of one-third of the selected antigenic genes were shifted between *P. vivax* and *Plasmodium falciparum*, suggesting that these genes may represent important factors associated with *P. vivax* selectivity for young erythrocytes and/or with immune evasion. These novel immune proteomes of the *P. vivax* blood stage provide a baseline for further prospective serological marker studies in malaria. These methods could be used to determine immunodominant candidate antigens from the *P. vivax* genome.

1. Introduction

Human malaria is caused by 5 species of the parasitic protozoan of the genus *Plasmodium*, the most common of which are *Plasmodium falciparum* and *Plasmodium vivax*. Although malaria caused by *P. falciparum* is a leading cause of mortality and morbidity globally, *P. vivax* is the most geographically widespread, causes significant morbidity and inflicts a huge socioeconomic burden in endemic countries [1,2]. Indeed, *P. vivax* chloroquine resistance has been demonstrated, especially in New Guinea [3], and a pattern of unusual clinical complications with fatality associated with *P. vivax* have been reported in East Asia and South America [4–6]. Severe vivax malaria is also found in areas of India and Brazil where *P. vivax* is still largely chloroquine sensitive [7]. Recently, *P. vivax* has come to the forefront of attention, with the malaria research agenda shifting from malaria control to elimination and eradication [8]. However, the serious gaps in basic information about this parasite may delay malaria elimination [1]. Furthermore, the unique aspects of the biology of *P. vivax* make it a great challenge [9,10], and comprehensive understanding of *P. vivax* parasite biology will be critical.

With a toolkit of stage-specific proteins encoded by about 5400 genes of this *Plasmodium* species, the parasites can survive and develop through multiple stages in erythrocytes [11]. The invasion process of merozoite parasites into erythrocytes requires several sequential steps and involves various surface and secretory organelles (micronemes, rhoptries and dense granules) and their associated proteins, which are mainly transcribed in late-stage parasites. Several proteins expressed during the erythrocytic stages, including merozoite surface protein 1 (MSP1), apical membrane antigen 1 (AMA1) and ring-infected erythrocyte surface antigen (RESA), have been confirmed as antigens and/or potential vaccine candidates [12–14]. Acquired immunity to symptomatic malaria in a human host is likely to involve responses against a multitude of antigens. Dissecting the relative contribution of each of these remains a great challenge.

To determine the targets for humoral immunity against malaria parasites, researchers are now focusing on functional genomic studies combined with the use of powerful relational databases and genome informatics, resulting in the identification of new antigens that trigger host immunity and new targets for drug and/or vaccine development. For *P. vivax*, the genome [15,16], the transcriptome [17,18], the proteome [19] and the development of advanced tools for functional genomics [20,21] provide clues to the putative immunogenicity of parasite proteins, which will not only suggest new ideas for parasite control but also allow the identification of new approaches. The ability to perform large-scale functional genomic studies depended on the invention of molecular tools such as high-throughput cloning and expression of target genes, tools that are now publicly available for post-genomic malaria research [16,22,23]. In our previous studies, In-Fusion cloning and the wheat-germ cell-free (WGCF) protein synthesis systems have demonstrated high efficiency for cloning and expression of *P. vivax* target genes, and protein arrays have been used to characterize the antibody reactivity profiles of *Plasmodium* infection by high-throughput screening [24–26]; together these enabled the genome-wide biochemical annotation of gene products. These results were shown to be a rapid way to scan comprehensively the humoral immunity of infected patients [27–29].

The human immune system reacts to malaria infection with an efficient production of antibodies to a large number of malarial antigens [30,31]. Some of these antibodies contribute to the acquisition of immunity to malaria, while others act primarily as markers of recent infection. Antibodies can, in principle, be measured using rapid diagnostic tests, so it is likely that a field-usable serological test for malaria could be developed. Serological measures of malaria exposure that have high-throughput and are relatively simple and can determine previous as well as concurrent infection could therefore be a very important new tool for monitoring and evaluation of national malaria programs [32,33]. While the intensive malaria control activities in endemic countries have resulted in a significant reduction in the burden of malaria infections and morbidity, new tools are required to refine this by discovering novel *Plasmodium* antigens that make suitable candidates for use as serological markers of recent exposure.

In this study, we have used in silico data mining by comparative genomics combined with In-Fusion cloning methods, the WGCF expression system and protein arrays for high-throughput profiling of humoral immune responses to *P. vivax* merozoite antigens. In addition to a set of orthologues of well-characterized *P. falciparum* blood-stage antigens, new hypothetical *P. vivax* proteins have been identified as novel serological markers of recent exposure to *P. vivax* infection.

2. Materials and methods

2.1. Plasma sample collection

Ninety-six plasma samples were collected in 2007–2010 from patients who were confirmed positive for *P. vivax* malaria via microscopy (mean number of parasites ± standard deviation, 5622 ± 6569 parasites/μl, range 10–33,500 parasites/μl) at local health centers and clinics in Gangwon and Gyeonggi Provinces within endemic areas of the Republic of Korea. Patients were aged from 20 to 55 years (mean age 28) and 90% of them were aged less than 30 years; 70% of the patients were male. Most of the patients were diagnosed 3–4 days after fever symptoms began and had had single malaria infection episodes during the previous 1 to 2 years. These vivax malaria patients were treated with a total of 1500 mg (base) chloroquine for 3 days, and then with 15 mg primaquine daily for 14 days under the standard treatment regimen. Ninety-six plasma samples taken from healthy personnel, who were confirmed negative for vivax malaria by microscopy and PCR methods, and had experienced no malaria episodes, were used as negative controls. This study was approved by the Institutional Review Board at Kangwon National University Hospital.

2.2. Selection of putative antigenic candidates

The malaria parasite genome information was derived from functional genomic databases PlasmoDB (http://plasmodb.org/plasmo) [34] and Research and Training in Tropical Diseases (TDR) targets (http://tdrtargets.org). The sources of antigenic candidates were: (1) well-known *P. vivax* proteins such as MSP, rhoptry protein, reticulocyte binding protein (RBP), and serine-repeat antigen (SERA); (2) orthologues of *P. falciparum* immunogenic proteins [35]; and (3) proteins with a signal peptide (SP) and/or 1–3 transmembrane domains (TM). Because of the limitations in identifying the open reading frame (ORF) of candidates, genes with multiple exons were BLAST-searched in the expressed sequence tag (EST) database to determine whether those targets were present in the cDNA library [36]. Those ORFs that were identified from the cDNA library (i.e., that included the start codon [methionine, M] of the ORF or for which a partial functional region existed in the ESTs database) were also selected.

2.3. In-Fusion cloning

Three types of starting materials were used as templates to generate clones as follows: (1) genomic DNA (gDNA); (2) double-stranded (ds) cDNA; and (3) cDNA library. Using the QIAamp DNA Blood Mini Kits (Qiagen, Hilden, Germany), gDNA was extracted from whole blood of vivax malaria patients that was collected from endemic areas of the Republic of Korea. *P. vivax* ds cDNA was prepared using the SMART cDNA synthesis kit (Clontech, Palo Alto, CA, USA), and the cDNA library was constructed from vivax malaria isolates obtained in Thailand as part of a previous study [36]. The ORFs with a single exon were amplified from gDNA; others were obtained from ds cDNA or the cDNA library. As the size of the PCR product significantly affects cloning efficiency, the lengths for insertion were limited to 3000 bp. Large target genes were divided into multiple overlapping sections with 45 nucleotide overlaps, and fragments were cloned from both terminal regions (N- or C-terminal) of the target sequence. The predicted signal peptide and partial GPI-anchor in the amino acid sequence were excluded from the expression construct [37,38]. In-Fusion PCR primers were designed with each gene-specific sense primer extending at the 5′-terminus, 5′-GGG CGG ATA TCT CGA G-3′, and antisense primer at the 3′-terminus, 5′-GCG GTA CCC GGG ATC C-3′. For PCR amplification of target ORFs, Platinum *Taq* DNA polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) was primarily used, followed by Phusion High-Fidelity DNA polymerase (Finnzymes, Espoo, Finland) for unamplified targets. The vector used for cloning was pEU-E01-His-TEV-MCS-N2 (pEU; CellFree Sciences, Matsuyama, Japan). In-Fusion cloning of PCR products was performed according to the manufacturer's instructions. The inserted nucleotide sequence was confirmed by sequence analysis (Genotech, Daejon, Korea). Highly purified plasmid DNA was prepared with the Midi Plus Ultrapure plasmid extraction system (Viogene, Taipei, Taiwan) according to the manufacturer's instructions.

2.4. Protein expression and Western blot

Highly purified plasmid DNA was used for in vitro transcription and subsequent translation by a 1 ml WGCF system using bilayer translation [23]. To assess the protein expression level and solubility, expressed histidine (His)-tagged proteins were detected by Western blotting probed with penta-His antibody (Qiagen) and secondary HRP-conjugated goat anti-mouse IgG (Thermo Fisher Scientific, Rockford, IL, USA) as described previously [24,26]. A purified His-tagged *P. vivax* circumsporozoite protein (PvCSP) chimera [39] was used to generate the standard curve. The ECL images were analyzed by the public domain *ImageJ* program (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/).

2.5. Preliminary screening

Eleven individual sera from vivax malaria patients were used for preliminary screening in well-type amine arrays. The procedure was performed as described in a previous report [26], and the fluorescence intensities of array spots were quantified by the fixed-circle method using ScanArray Express software (version 4.0; PerkinElmer, Boston, MA, USA) [24,26]. The positive cut-off value was calculated as the mean fluorescence intensity (MFI) value of reactivity of each patient's serum with 80% of the total proteins examined plus 3 SD, and the top-ranked proteins above the cut-off value of MFI for antibody reactivity were determined.

2.6. Comprehensive screening

The top-ranked recombinant proteins selected from the preliminary screening were coated onto a Ni^{2+}-chelated surface slide (Xenopore, Hawthorne, NJ, USA) and screened with sera from 22 vivax malaria patients and 10 healthy individuals. The procedure was as follows: 0.9 μl of each

crude *P. vivax* protein solution and positive control PvMSP1 (80 ng/μl) were spotted in duplicate wells of the arrays and incubated for 2 h at 37 °C, blocked with 5% BSA (Sigma-Aldrich, St. Louis, MO, USA) in PBS/Tween 20 (PBS-T) buffer for 1 h at 37 °C, loaded with sera from patients and healthy controls, and incubated for 1 h at 37 °C. Wells were then stained with Alexa Fluor 546 goat anti-human IgG (10 ng/μl; Invitrogen) in PBS-T buffer (1% BSA) for 1 h at 37 °C and scanned in a ScanArray GX laser confocal scanner (PerkinElmer). The positive cut-off value was calculated as the mean fluorescence intensity value of the negative controls plus 2 SD.

2.7. In silico expression level comparison of the antigenic candidate between P. vivax and P. falciparum

The gene transcription data from time points (TP) 1 to 9 of 3 vivax malaria isolates [17] were averaged and compared with the expression data at TP 9, 13, 17, 20, 24, 28, 35, 38 and 40 in the *P. falciparum* 3D7 or HB3 transcriptome (http://malaria.ucsf.edu/comparison/index.php). Pearson correlation coefficients (PCCs) were calculated and used for comparing the expression of the antigenic candidates of *P. vivax* and *P. falciparum*. The thresholds of PCCs for division of the genes based on their conservation of expression profiles between the *P. vivax* and *P. falciparum* intraerythrocytic developmental cycle (IDC) were defined in a previous study [18]. Briefly, high correlations (PCC 1 to 0.5) included genes with highly conserved IDC expression profiles, and low to no correlations (PCC 0.5 to −0.2) included genes with a partial shift. Negative correlations (PCC 0.2 to −1) included genes with a dramatic change in their IDC expression profiles between the two species.

2.8. Expression of recombinant proteins and production of polyclonal antibodies

One of the top-ranked immunogenic proteins, *P. vivax* rhoptry-associated membrane antigen (PvRAMA) (PlasmoDB ID: PVX_087885), identified from the high-throughput screening assays, was expressed on a large scale using the WGCF system. The 810 bp fragment (1384–2193 of the ORF), which was amplified with the gene-specific primers (5′-AAG GAG GCA GTG AAG AAG GG-3′ and 5′-TTA ATT GGT GAA ACA TAA CAA TCC G-3′), was inserted into pEU plasmid DNA. The highly purified plasmid was prepared using the Maxi Plus Ultrapure plasmid extraction system (Viogene). The recombinant protein was expressed in 12 wells of a 6-well plate scale WGCF system as described previously [23,40]. Recombinant PvRAMA proteins were purified using a Ni-nitrilotriacetic acid agarose column (Qiagen). Polyclonal anti-PvRAMA sera were produced from mice and rabbits immunized with the recombinant PvRAMA protein as reported previously [41]. The purified recombinant RAMA proteins and *P. vivax* parasite lysates were fractionated by SDS-PAGE. The human, mouse and rabbit sera used as primary antibodies, and the alkaline phosphatase-conjugated goat anti-human IgG (ICN-Cappel Inc., Aurora, OH, USA) and HRP-conjugated anti-mouse or anti-rabbit IgG antibodies (GE Healthcare) used as secondary antibodies, were used and developed as described in a previous report [41].

2.9. Indirect immunofluorescence assay and protein arrays

An indirect immunofluorescence assay (IFA) was performed to determine the subcellular localization of PvRAMA in *P. vivax* blood-stage parasites by confocal microscopy. The primary antibodies used were anti-PvRAMA serum at a 1:200 dilution with either anti-PvMSP1 serum (1:200 dilution, merozoite surface marker), anti-PvDBP serum (1:100 dilution, microneme marker), anti-PvRON2 serum (1:200 dilution, rhoptry neck marker) or PvRhopH2 serum (1:200 dilution, rhoptry body marker). Alexa 488- and Alexa 546-conjugated anti-mouse and anti-rabbit antibodies (1:500 dilution; Invitrogen) were used at 1:500 dilution as secondary antibodies, and 4′,6-diamidino-2-phenylindole (DAPI) (1 μg/ml, Invitrogen) was used for nuclear staining. Image analysis was performed using LSM 510 META software (Carl Zeiss AG, Jena, Germany).

Sera from the 96 vivax malaria patients and 96 healthy individuals mentioned above were tested against the recombinant PvRAMA protein using protein arrays. Recombinant PvRAMA protein (0.9 μl/spot, 20 ng/μl) was spotted to each well of a Ni^{2+}-chelated surface slide. The procedures were as described previously [40], and the positive cut-off value was calculated as the MFI value of the negative controls plus 2 SD.

2.10. Statistical analysis

Statistical analysis of significance of the data from the 2 groups was performed with 2-tailed unpaired *t*-tests using GraphPad Prism (version 5.0; GraphPad, San Diego, CA, USA), and <0.05 was considered significant. The prevalence of IgG antibodies reactive against the recombinant protein was also analyzed using GraphPad Prism. The calibration curve of protein expression, the correlation between the duplicate spots of protein arrays and antibody reactivity to different concentrations of *P. vivax* proteins were analyzed using Sigma Plot (version 10.0; Systat Software, San Jose, CA, USA). The hierarchical clustering of gene expression and antibody responses were calculated and drawn using Cluster and shown with TreeView (both http://rana.lbl.gov/EisenSoftware.htm). The Pearson correlation for the expression between the 2 species was analyzed with online software (http://pearsoncorrelation.com/) for PCC calculation.

3. Results

3.1. Selection of genes for putative antigenic proteins

In total, 187 genes (divided into 202 fragments) were selected, and their expression profiles in the IDC were analyzed from the reported transcriptome data [17] and clustered using a hierarchical algorithm (Supplementary Fig. 1). Most of the target genes shown in the heat map were highly expressed in the schizonts of blood-stage parasites. The putative *P. vivax* antigenic candidates selected based on the features of expression level and presence of predicted SP and TM domains are shown in a Venn diagram (Fig. 1). About 85% of the candidates were observed to show peak expression in the schizont stages. However, for 4 genes, which were classified as orthologues of *P. falciparum* genes or as *P. vivax* unique

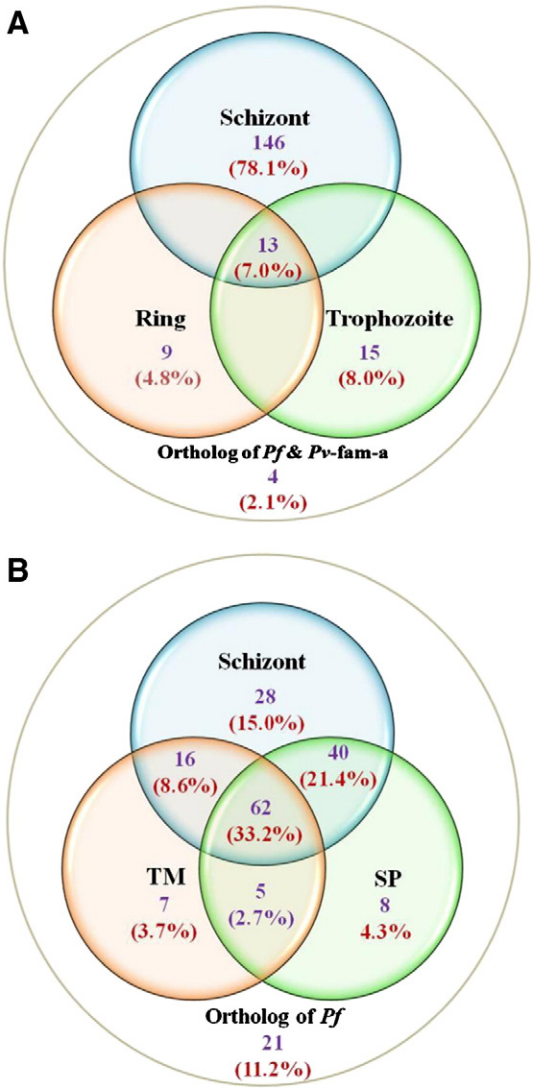

Fig. 1 – Venn diagram of the selected putative *P. vivax* antigenic candidates (total: 187). A) Transcription profiles of candidates selected in blood stage. B) Motif-specific profiles of candidates selected [17]. TM, transmembrane; SP, signal peptide.

genes (*Pv-fam-a*), no expression profile information was available in the PlasmoDB database. The majority (78%) of the antigenic candidates were highly expressed in schizonts and contained SP and/or TM. Based on their gene names or those of their homologues in other *Plasmodium* species (*P. falciparum* was preferred), the candidates were grouped into 12 categories (Supplementary Tables 1, 2). Hypothetical proteins were classified in the first category and consisted of 32.1% of the total candidates. The *Pv-fam-a* family included about 12% of candidates. The third group, including 23 (12%) of target genes, contained several types of enzymes including proteases, nucleases and kinases. Other well-known candidates included exported proteins, the 41K blood-stage antigen and the histidine-rich protein homolog KPRPC (HRKP). *Plasmodium* surface/membrane proteins constituted one of the categories for this study, so named because it included the target *P. vivax* genes or their *P. falciparum* homologues from PlasmoDB. *P. falciparum* orthologue proteins of *P. vivax* from the other groups, including GPI-anchored proteins, rhoptry proteins, *Plasmodium*-exported proteins, *P. vivax* RBP, erythrocyte membrane proteins, *Pv-fam-d* and SERA, were also selected and cloned.

3.2. In-Fusion cloning

The 202 fragments selected for PCR amplification ranged from 333 to 2947 bp in length (ORFs > 3000 bp were cloned as overlapping segments), with an average size of 1363 bp. Eighty-eight (44%) fragments were amplified from gDNA of Korean isolates, 2 (1%) from ds cDNA of a Korean isolate and 112 (55%) from the cDNA library from the Thai isolates. With the use of 2 types of polymerase enzymes, Platinum *Taq* and Phusion High-Fidelity DNA polymerases, most of the candidate ORFs (87%) were amplified. Target fragments of an average size of about 1300 bp were amplified successfully, but targets above 1766 bp were significantly more difficult to amplify ($p < 0.01$) (Fig. 2). One hundred sixty-nine (97%) PCR products were found to be ligated to the pEU-His vector (Fig. 2, Supplementary Table 2). The target gene sequences were analyzed and compared with that of the reference gene of *P. vivax* Sal-1 strain (PlasmoDB); 160 fragments were used for cell-free expression. Of the 160 fragments (152 genes) selected, gene sequences from 25 fragments (16%) were identical to those of Sal-1, and 54 (34%) were highly conserved (1–3 amino acid sequence variation). Conversely, 1 (1%, PVX_001015) had nonsynonymous mutations, and 7 (4%) had multiple mutations, mainly proteins belonging to the surface proteins group.

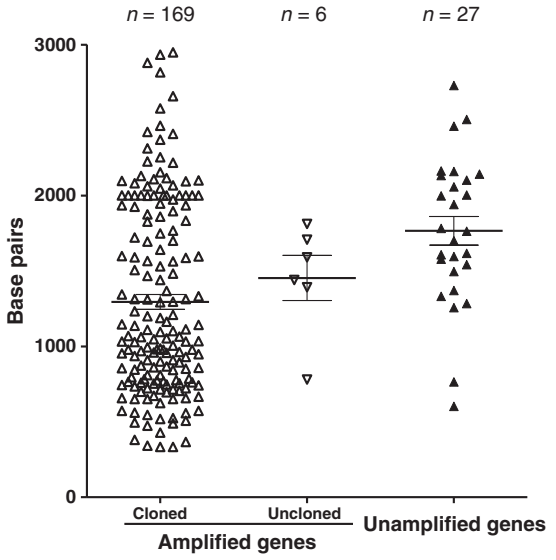

Fig. 2 – Size distribution of *P. vivax* genes selected for PCR amplification and In-Fusion cloning. Size distribution of amplified and unamplified ORFs was significantly different ($p < 0.01$). Size distribution of cloned and uncloned genes. There was no significant difference between these ($p = 0.54$). The horizontal lines show the mean and the upper and lower 95% confidence limits.

3.3. Recombinant protein expression

One hundred sixty fragments of selected *P. vivax* proteins were expressed using the high-throughput WGCF expression system. The expressed target proteins were evaluated in crude protein preparations by Western blot analysis (Fig. 3, Supplementary Table 2). The results indicated that 148 (93%) yielded proteins and 96.6% (143/148) were soluble, with an average concentration of 93.3 μg/ml (range 0.1–466.4 μg/ml). The expression levels of small-sized targets were significantly higher than those of larger-sized proteins ($p < 0.05$). Comparing the expressed group with both the lower-expressed and

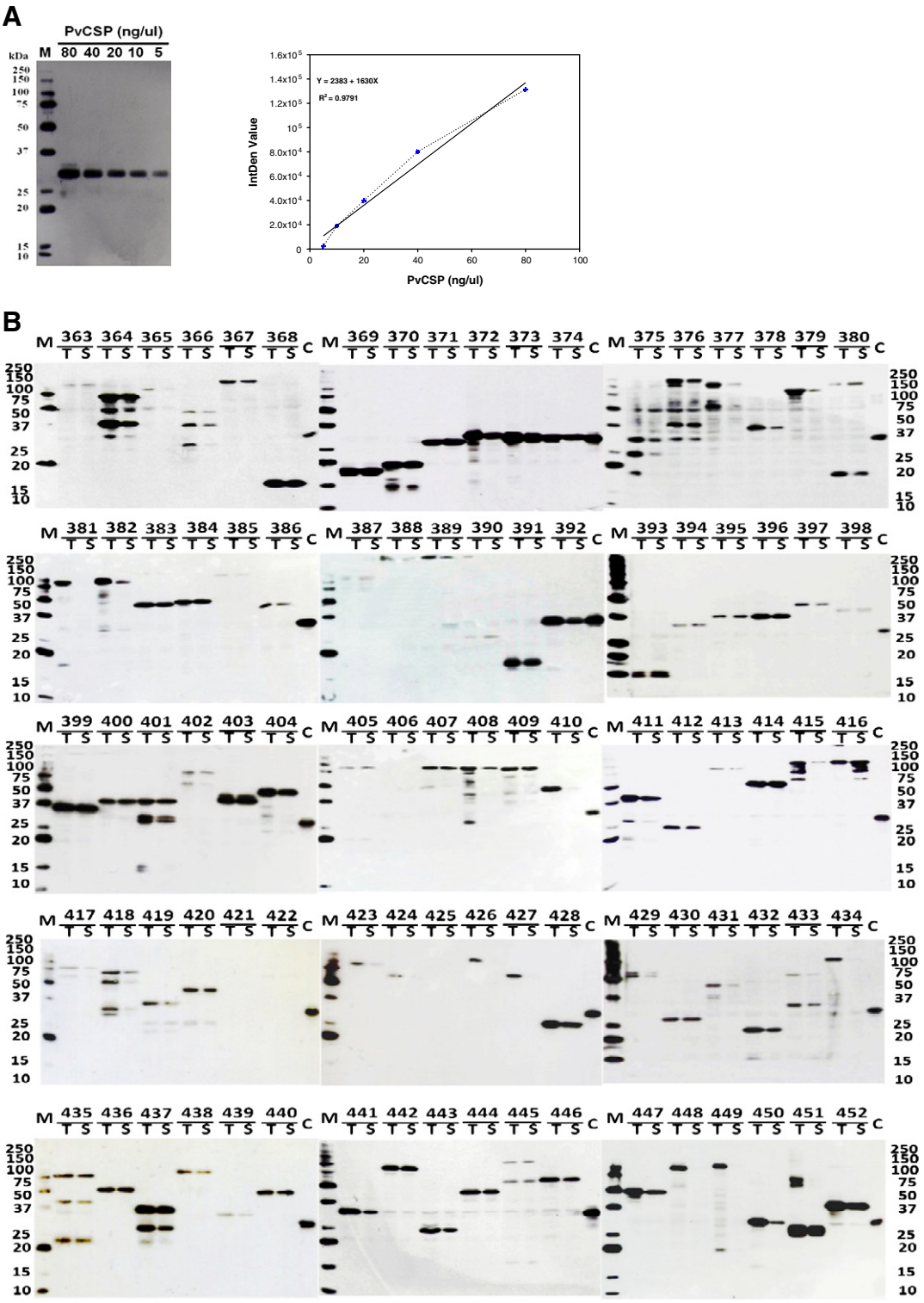

Fig. 3 – Analysis of the level of expressed *P. vivax* recombinant proteins by Western blot. A) Calibration curve of recombinant protein of PvCSP. B) Western blot analysis of the recombinant proteins. All proteins have two paired lanes, total fraction (T) and soluble fraction (S). M, protein marker; C, PvCSP recombinant proteins.

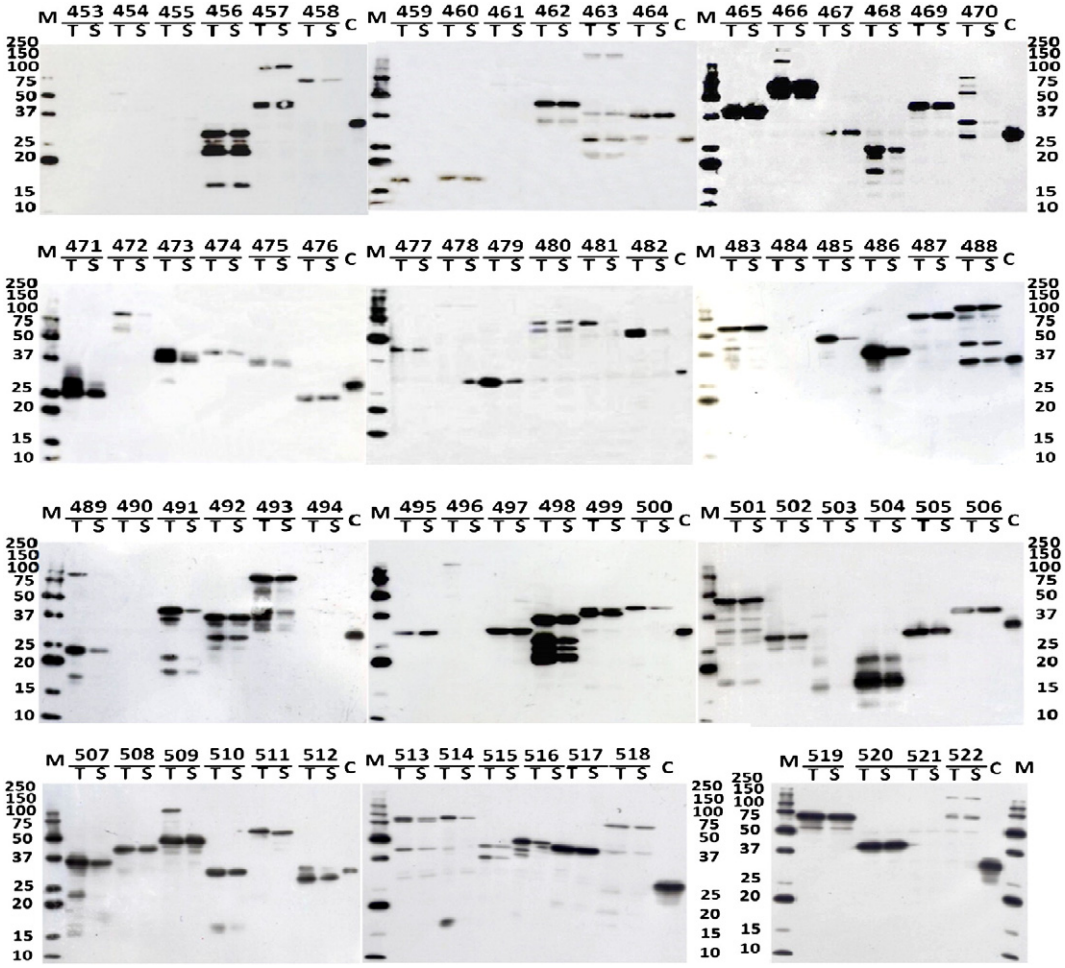

Fig. 3 (*continued*)

unexpressed groups, there was a significant difference between them in molecular weight (χ^2 test, $p < 0.05$) but no significant difference in pI ($p = 0.79$), A/T content, or the existence of SP and TM.

3.4. *Immunogenic profiles of patient sera against P. vivax proteins*

The efficiency of protein arrays for antibody profiling has been demonstrated in preliminary screening described in previous reports of our studies [24,26]. Eleven individual sera from vivax malaria patients were used for preliminary screening of the 160 recombinant proteins (Fig. 4A). The controls, including positive (PvMSP1-19), negative (mixed sera from negative samples) and blank (PBS only) controls and wheat germ extract (WGE), were used to analyze the reaction with pooled or individual sera. Of 160 recombinant protein antigens, 63 candidate proteins showed an MFI higher than the cut-off value in preliminary screening. Twenty-five proteins showed higher than cut-off MFI to at least 2 individual sera, and 38 were recognized by 1 individual serum only. Overall, 44 (27.5%, 44/160) of the top-ranked antigenic *P. vivax* proteins from the preliminary screening were identified and selected for comprehensive screening. Others, including 19 proteins with code numbers 371, 379, 381, 382, 383, 399, 401, 405, 414, 415, 424, 430, 453, 465, 468, 480 504, 510, and 517, were not included in further screening (Supplementary Table 2).

In the comprehensive screening, the 44 *P. vivax* proteins selected were screened with sera from 22 vivax malaria patients and 10 healthy individuals to confirm their antibody reactivity (Fig. 4B). Of the 44 (44 fragments) highly immunogenic *P. vivax* parasite proteins, 5 (RAMA [PVX_087885], HP [PVX_084775], Pv-fam-a [PVX_092995], EXP-1 [PVX_091700] and Pv-fam-b [PVX_093680]) reacted positively with >80% patient sera, 21 fragments (HP [PVX_087670], EXP-2 [PVX_116925], Pv-fam-a [PVX112670], Pv-fam-d [PVX_121910], SERA [PVX_003795], RBP-2 [PVX_090330], *Plasmodium* exported protein [PEXP, PVX_083560], PEXP [PVX_081845], Pv-fam-a [PVX_112675], MSP-5 [PVX_003770], aspartic protease PM5 [AP PM5, PVX_116695], serine esterase [SE, PVX_091436], HP [PVX_084815], putative erythrocyte membrane protein 3 [EMP3, PVX_092425], GPI-anchored micronemal antigen [GAMA, PVX_088910], glideosome-associated protein with multiple membrane spans 2 [GAPM2, PVX_090215], Pv-fam-a [PVX_090265], putative secreted ookinete protein 13 [PSOP13, PVX_080305], Pv-fam-a [PVX_112685], Pv52 [PVX_001015] and PvMSP3 [PVX_097715]) reacted positively with between 50% and 80% of sera, 12 fragments reacted positively with 30%–50% of sera, and 6 fragments reacted positively with <30% sera. More than 23% of the highly immunogenic proteins were

hypothetical proteins, which are described as immunologically reactive for the first time by this study, and these were ranked according to their positive reaction rate (Fig. 5, Table 1). A significant difference between the reactivity of proteins with positive sera and that with negative sera was found for 89% of the candidates ($p < 0.05$). The reliability of duplicate wells was examined and produced an $R^2 > 0.9$ for 40 (89%) proteins. Twelve proteins (PVX_097715, PVX_090330, PVX_089345, PVX_081845, PVX_001015, PVX_112680, PVX_092995, PVX_112670, PVX_112685, PVX_090265, PVX_112675 and PVX_121910) were expressed uniquely in *P. vivax* without a homologue in *P. falciparum* (Table 2). The others were clustered into 7 functionally related groups, including invasion, parasitophorous vacuole, Maurer's cleft proteins, and proteins of unknown function (Table 3). The transcriptional profiles were compared with those of the orthologues of *P. falciparum* by PCC analysis, except for the 5 candidates for which there were no paired transcriptional data (Table 3). The PCCs for others were calculated for gene pairs from 2 *Plasmodium* species. In summary, 11 genes showed high correlation (PCC 1 to 0.5), 6 genes showed low to no correlation (PCC 0.5 to −0.2), and 11 genes showed negative correlation (PCC −0.2 to −1). The results indicated that about half of the timing of the majority of biological functions in IDC is conserved between the 2 species. However, the transcriptional profiles for about half of the proteins, including most of the functionally related gene groups, were shifted between *P. falciparum* and *P. vivax*.

3.5. Molecular structure and recombinant protein of PvRAMA

The top-ranked and most widespread humoral responses (positive rate 90.9%) identified in this study were induced by PvRAMA and hypothetical protein PVX_084775. PvRAMA contains SP, TM and GPI motifs. Therefore, it is a more

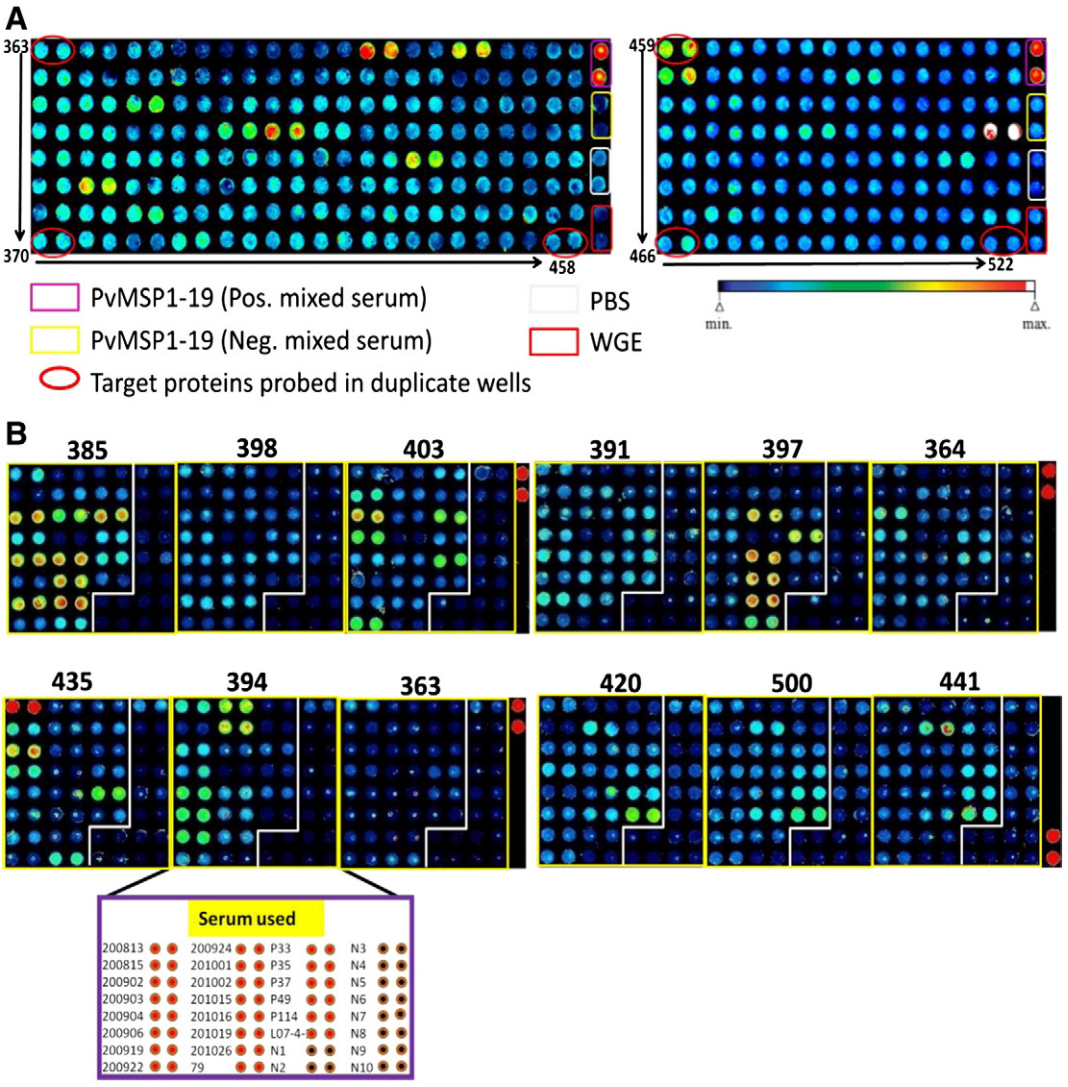

Fig. 4 – Immunoproteomics profiling of blood-stage *P. vivax* infection by high-throughput protein arrays. A) Preliminary screening to select antigenic *P. vivax* proteins. The protein arrays were probed with one *P. vivax* patient serum; a total of 11 proteins were studied. Wheat germ extract (WGE), PBS and PvMSP1-19 were used as controls. B) Comprehensive screening for the antibody profiling of *P. vivax* patient serum. Sera from 22 *P. vivax* patients and 10 healthy individuals were used. A total of 45 antigenic *P. vivax* proteins were studied.

attractive antigenic candidate than PVX_084775, which does not have these important structures. PvRAMA consists of an ORF of 2193 bp containing 6 exons, with the second and third exons comprising most of the coding sequence, 1155 and 765 bp, respectively (Fig. 6A). The predicted molecular weight of PvRAMA protein is 81.3 kDa, with 730 amino acids containing an N-terminal signal peptide (17 amino acids, positions 1–17) and a C-terminal GPI attachment site (22 amino acids, positions 708–730) as determined by the tool Big-PI (www.expasy.org) (Fig. 6B). Within the C-terminal part of PvRAMA, 3 short motifs, M1 (25 amino acids, amino acid positions 443–467), M2 (15 amino acids, amino acid positions 636–650) and M3 (24 amino acids, amino acid positions 677–700) are highly conserved in simian and rodent RAMA homologues, and these might represent functional motifs [42]. Mature *P. falciparum* RAMA (PfRAMA) protein has been confirmed to be a product of proteolytic cleavage of the full-length protein, and the protease responsible for this cleavage recognizes the sequence (D/E)SFL(Q/E) [43]. The closely related sequence (E/D/V/G)SFLE was also identified by amino acid sequence alignments and was repeated 8 times in the putative pre-mature form of PvRAMA (Fig. 6C).

A plasmid DNA clone encoding 269 amino acids in the C-terminal region of PvRAMA was constructed and used for recombinant protein expression by the WGCF system, and PvRAMA protein was purified by Ni-Sepharose column. SDS-PAGE analysis showed a target band at about 45 kDa (Fig. 7A), which is larger than the predicted size. This feature is similar to that of PfRAMA [44] and has been reported for a variety of proteins expressed by the WGCF system [45]. The recombinant PvRAMA was recognized by sera from *P. vivax* patients and not by sera from healthy controls (Fig. 7B). The immune mouse serum recognized the native PvRAMA of *P. vivax*, and reactivity was observed as 2 bands: a weaker band at 110 kDa and a predominant band at 40 kDa (Fig. 7C). Based on the analysis of homologues of RAMA in *P. falciparum* and *Plasmodium yoelii* [42,46], we propose that p110 is the primary translation product migrating at higher than its predicted size, whereas p40 is the mature form of PvRAMA.

3.6. Subcellular localization and antigenicity of PvRAMA

Double-labeling experiments were performed to confirm the subcellular localization of PvRAMA. As shown in Fig. 8, the

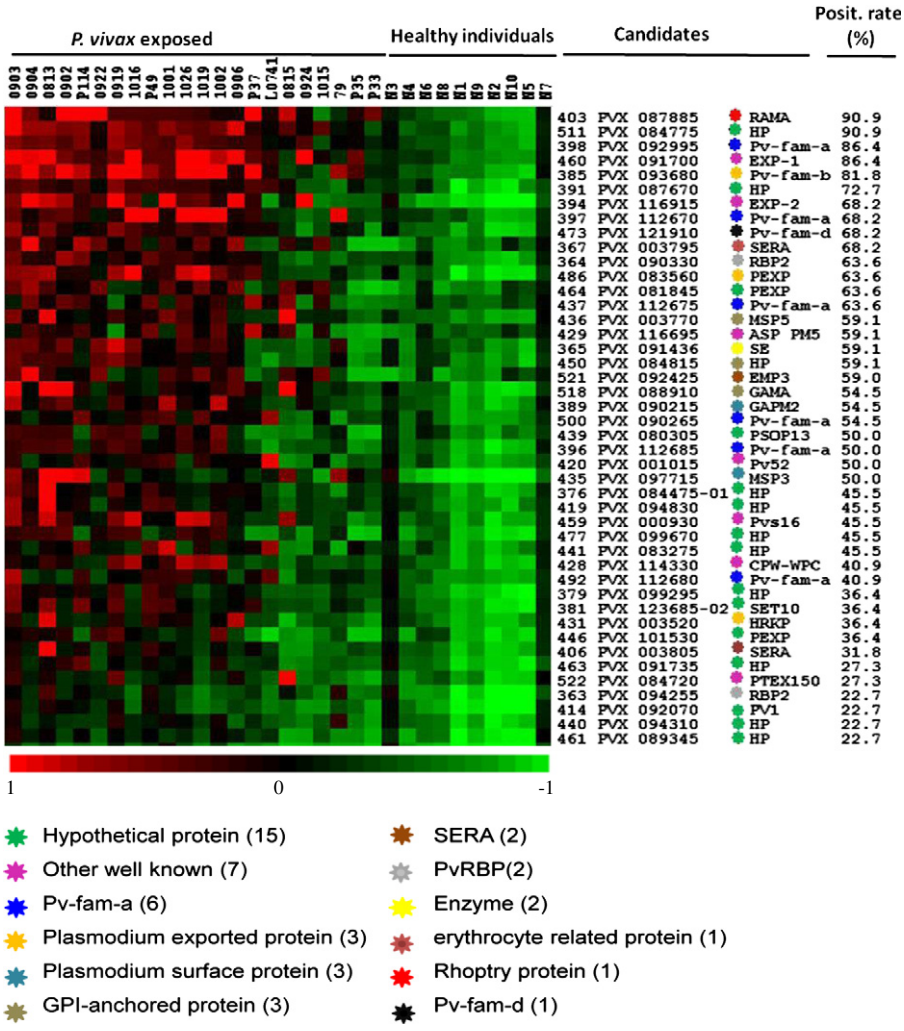

Fig. 5 – Immunoreactivity profiles of *P. vivax* antigenic proteins. High IgG antibody responses were detected in *P. vivax*-infected patient sera to a total of 41 proteins.

Table 1 – Features of *P. vivax* antigenic candidate proteins with high immunogenicity.

Pv gene ID	Annotation	MW (kDa)	SNP[a]	Solubility (%)	No. of positive (%)[b]	MFI	Features/structures
PVX_087885	RAMA	29.7	22	96.0	20 (90.9)	4302 ± 2801	Rhoptry protein, GPI-AP
PVX_084775	HP	43.1	1	71.6	20 (90.9)	3707 ± 1500	Unknown
PVX_092995	Pv-fam-a	39.7	32	83.4	19 (86.4)	3127 ± 797	Vaccine candidate
PVX_091700	EXP-1	12.5	2	74.9	19 (86.4)	5847 ± 4479	Vaccine candidate
PVX_093680	Pf-fam-b	80.6	12	44.8	18 (81.8)	6462 ± 4557	Exported protein
PVX_087670	HP	13.3	0	89.0	16 (72.7)	2680 ± 710	Unknown
PVX_116915	EXP-2	29.1	1	100	15 (68.2)	4077 ± 2774	Potential channel
PVX_112670	Pv-fam-a	35.3	2	50.6	15 (68.2)	4870 ± 4338	Vaccine candidate
PVX_121910	Pv-fam-d	31.0	4	36.3	15 (68.2)	2633 ± 851	Exported protein
PVX_003795	SERA	86.3	9	81.3	15 (68.2)	2932 ± 1652	Immune evasion
PVX_090330	RBP-2	65.1	127	68.6	14 (63.6)	2518 ± 866	RBP family
PVX_083560	PEXP	32.6	8	59.2	14 (63.6)	3303 ± 1725	Exported protein
PVX_081845	PEXP	36.9	3	100	14 (63.6)	2617 ± 982	Exported protein
PVX_112675	Pv-fam-a	31.0	18	81.1	14 (63.6)	2564 ± 975	Vaccine candidate
PVX_003770	MSP 5	36.5	70	92.6	13 (59.1)	3733 ± 5446	Vaccine candidate, GPI-AP
PVX_116695	ASP PM5	57.8	11	14.4	13 (59.1)	2484 ± 1031	PM5 family
PVX_091436	SE	75.3	19	13.6	13 (59.1)	2666 ± 1193	Unknown
PVX_084815	HP	26.4	11	24.5	13 (59.1)	2366 ± 859	GPI-AP
PVX_092425	EMP3	83.8	4	–	13 (59.1)	2681 ± 1277	Exported protein
PVX_088910	GAMA	81.0	18	56.6	12 (54.5)	5534 ± 9794	GPI-AP
PVX_090215	GAPM2	42.3	1	–	12 (54.5)	2642 ± 1165	IMC anchor
PVX_090265	Pv-fam-a	40.0	7	25.0	12 (54.5)	2391 ± 803	Vaccine candidate
PVX_080305	PSOP13	21.9	1	37.6	11 (50.0)	2233 ± 888	Unknown
PVX_112685	Pv-fam-a	32.4	5	91.2	11 (50.0)	2300 ± 781	Vaccine candidate
PVX_001015	Pv52	38.5	3	71.6	11 (50.0)	2385 ± 1141	Pfam domain
PVX_097715	MSP3	46.6	36	80.2	11 (50.0)	3768 ± 4698	Vaccine candidate
PVX_084475-01	HP	82.4	8	64.3	10 (45.5)	2531 ± 1416	Unknown
PVX_094830	HP	26.8	4	26.9	10 (45.5)	3683 ± 6639	Unknown
PVX_000930	Pvs16	11.8	0	1.5	10 (45.5)	3071 ± 1600	Potential drug target
PVX_099670	HP	40.1	13	54.5	10 (45.5)	2157 ± 824	Unknown
PVX_083275	HP	28.1	1	35.3	10 (45.5)	2407 ± 1113	Unknown
PVX_114330	CPW-WPC DCP	19.0	0	69.5	9 (40.9)	2608 ± 1274	Pfam domain
PVX_112680	Pv-fam-a	33.5	13	89.5	9 (40.9)	2332 ± 838	Vaccine candidate
PVX_099295	HP	74.5	2	10.1	8 (36.4)	2185 ± 678	Unknown
PVX_123685-02	SET10	78.6	8	1.4	8 (36.4)	2409 ± 829	Potential drug target
PVX_003520	HRKP	37.9	3	19.2	8 (36.4)	2283 ± 1242	Exported protein
PVX_101530	PEXP	34.1	25	63.6	8 (36.4)	1941 ± 805	Exported protein
PVX_003805	SERA	86.2	101	–	7 (31.8)	2547 ± 2764	Immune evasion
PVX_091735	HP	41.6	1	100	6 (27.3)	2003 ± 583	Unknown
PVX_084720	PTEX150	96.4	8	100	6 (27.3)	2494 ± 2632	Translocon component
PVX_094255	RBP-2	77.3	19	100	6 (22.7)	1870 ± 521	RBP family
PVX_092070	PV1	46.0	0	90.0	6 (22.7)	1759 ± 502	Potential drug target
PVX_094310	HP	43.5	11	100	6 (22.7)	1906 ± 486	Unknown
PVX_089345	HP	55.9	4	37.3	6 (22.7)	1799 ± 482	Unknown

MW, molecular weight; SP, signal peptide; TMD, transmembrane domain; HP, hypothetical protein; SET10, histone-lysine N-methyltransferase, H3 lysine-4 specific; PSOP13, secreted ookinete protein; CPW-WPC DCP, *P. falciparum* CPW-WPC domain contain protein; SERA, Serine-repeat antigen; GAMA, GPI-anchored micronemal antigen; HRKP, histidine-rich knob protein homolog KPRPC; KAHRP, knob associated histidine-rich protein; GAMP, glideosome associated protein with multiple membrane spans 2.

[a] The non-synonymous SNPs in 5 *P. vivax* genomic reference strains (Salvador I, North Korean, India VII, Mauritania I and Brazil I) were obtained from PlasmoDB (http://plasmodb.org/plasmo).

[b] Total number of positive antibody reaction with 22 patients serum samples.

PvRAMA fluorescence signal partially overlapped with that for the rhoptry marker PvRhopH2. In contrast, the PvRAMA fluorescence signal did not overlap with PvMSP1, the merozoite surface marker, PvDBP, the microneme marker or PvRON2, the rhoptry neck marker. These results suggest that PvRAMA can be considered to be a rhoptry body protein. Although immunoelectron microscopy study is indispensable for validation of the precise subcellular localization, these results are in agreement with those for PfRAMA, which have been confirmed by immunoelectron microscopy [46].

To evaluate the humoral immune response further, a protein array was used to screen for the presence of antibodies in human sera using purified recombinant PvRAMA protein. Antibody responses against PvRAMA were evaluated in serum samples from 96 patients infected with *P. vivax* and 96 healthy individuals. Sera of individuals infected with *P. vivax* showed a significantly higher total IgG MFI than did sera of malaria-naïve subjects (Fig. 9A, $p < 0.01$). The sera that were used for the comprehensive screening were also included, and the results of the two analyses were consistent. The

Table 2 – List of unique proteins expressed in *P. vivax* antigenic candidates.

Pv gene ID	Expression	Description	Putative function
PVX_097715[a]	LS	Merozoite surface protein 3 (MSP3)	Immune evasion/antigenicity
PVX_090330	LS	Reticulocyte binding protein 2 (RBP-2)	Reticulocyte binding
PVX_089345[a]	LS	Hypothetical protein (HP), conserved	Unknown
PVX_081845	ER/LS	Plasmodium exported protein (PEXP)	Unknown
PVX_001015[a]	LS	HP, conserved	Invasion
PVX_112680	LR	Tryptophan/threonine-rich antigen	Merozoite–erythrocyte interaction
PVX_092995	ER	Tryptophan-rich antigen (TRAg, Pv-fam-a)	Virulence/antigenicity
PVX_112670	ER/LS	TRAg (Pv-fam-a)	Virulence/antigenicity
PVX_112685	LS	TRAg (Pv-fam-a)	Virulence/antigenicity
PVX_090265	R	TRAg (Pv-fam-a)	Virulence/antigenicity
PVX_112675	ER/LS	TRAg (Pv-fam-a)	Virulence/antigenicity
PVX_121910	F	Pv-fam-d protein	Virulence/antigenicity

R, rings; ER, early rings; LR, late rings; LS, late schiznot; F, flat profile.
[a] Refers to proteins are present in any one species of *Plasmodium* except *P. falciparum*. Other proteins are only present in *P. vivax*.

prevalence of IgG antibodies against PvRAMA indicated a sensitivity of 63.5% (61/96; 95% confidence interval [CI] 53.6%–72.5%) and specificity of 95.8% (4/96; 95% CI 87.0%–97.1%). In addition, the AUC value for sera from *P. vivax* patients versus that from healthy individuals was 0.83 (Fig. 9A). Comparison of the prevalence of IgG against the positive control PvMSP1-19 in the same serum samples showed that detection of IgG antibodies against PvMSP1-19 had a sensitivity of 79.2% and a specificity of 94.8%, with an AUC of 0.89, a little higher IgG prevalence than that against PvRAMA (Fig. 9B).

4. Discussion

Malaria infection is associated with a strong host immune response, and antibodies against malarial antigens should be rapidly generated during the primary infection, efficiently boosted with submicroscopic parasite infection, and detectable for several months or years following parasite clearance. These circulating antibodies are thus indicative not only of concurrent but also of previous malaria parasite infections. For surveillance, specific antibodies against parasite antigens are very useful for detection of infection within the previous 12 months. However, appropriate antigens for vaccine research and development have been reported only recently [47]. Thus, there is an urgent need to develop novel serological tests as markers of exposure. In this study, we used a high-throughput cloning method, In-Fusion cloning, to prepare 160 constructs of *P. vivax* parasite proteins, of which 96% were successfully expressed using a WGCF-expression system. This large number of expressed proteins was then further screened with serum samples from *P. vivax*-exposed and -unexposed individuals by protein array. A total of 44 highly reactive *P. vivax* antigens were identified as serological markers of exposure to vivax parasite infection.

The candidates underwent a preliminary screening, and the top-ranked proteins were confirmed by secondary screening. The results of protein array-based screening were confirmed in a previous study to be highly correlated with those of conventional ELISA assays [26]; this study demonstrated that protein arrays are a useful tool for proteomic study. In this study, we screened the products of ~3% of the entire *P. vivax* genome (152 genes) for their capacity to be recognized by specific antibodies in *P. vivax* patient sera, to identify the immunoreactivity profiles of the putative blood-stage antigenic proteins. Within the top-ranked antigenic proteins identified by protein arrays, *P. vivax* blood-stage antigens, already well described as putative malaria vaccine candidates, were identified, including MSP5 (PVX_003770). The relatively conserved nature of the epidermal growth factor-like domain within these proteins suggests that immune responses targeting this region may induce cross-reactive antibodies [48]. One member of the *msp3* gene family, PVX_097715, has been shown to be immunogenic and partially to immunize nonhuman primates against blood-stage parasites. This family was significantly expanded in *P. vivax* compared with *P. falciparum*, perhaps as a means to enhancing immune evasion [17]. Two members of the *rbp2* gene family were demonstrated in this study to be immunogenic, and it is well known that the RBP family plays an important role in selective invasion of human reticulocytes by *P. vivax* parasites. These well-characterized antigens are verification that the screening assay method used here can effectively identify antigens.

The most attractive results in this study were the identification of 11 hypothetical proteins, which were confirmed to induce humoral immune responses against *P. vivax* infection but have never been described in *P. falciparum*. It is specifically worth mentioning that PVX_084775 was recognized with the highest positive rate (90.9%), similar to PvRAMA, by sera from *P. vivax*-infected patients. Based on the immunoreactivity data provided in this study, further characterization of these hypothetical proteins should be considered.

Another 6 candidates that were *P. falciparum* orthologues were described, but their functions were not well characterized. PfRAMA induced specific immune responses in individuals repetitively exposed to *P. falciparum* infection and may contain a protective epitope, which could be useful for inclusion in a multi-epitope vaccine against malaria [49]. PvRAMA also induced the highest humoral responses (positive rate 90.9%) of the candidates identified in this study. PvRAMA, one of the top antigenic proteins identified by the high-throughput screening assays, was characterized further. The predicted PvRAMA protein shared similar general structural features with its homologues in rodent, simian and other human malaria

Table 3 – Comparative analysis of *P. vivax* and *P. falciparum* IDC transcriptomes of the antigenic candidates.

Functional group	*P. vivax*	*P. falciparum*			PCCs[b]	Gene annotation
	Gene ID	Expression[a]	Gene ID	Expression[a]		
Unknown function	PVX_092425	S	PF11_0373	LS	0.872	HP; CPP
	PVX_084475	ES	PFL0445w	ES	0.705	HP; CPP
	PVX_094830	LS	PF10_0119	LS	0.787	HP; CPP
	PVX_003795	ES	PFB0330c	ES	0.721	SERA
	PVX_083560	R	PF13_0275	R	0.685	PEXP
	PVX_003805	ES	PFB0330c	ES	0.648	SERA
	PVX_091436	LS	PF11_0168a	S	0.635	HP; serine esterase
	PVX_088910	LS	PF08_0008	LS	0.591	HP; GAMA
	PVX_084775	LS	PF14_0333	LS	0.477	HP; CPP
	PVX_087670	ER	PF07_0007	R	0.391	HP; CPP
	PVX_099295	S	PFI0855w	ES	0.391	HP; CPP
	PVX_123685	ES	PFL1010c	S	0.155[c]	HP; SET10
	PVX_094310	LS	PF10_0027	F	−0.479[c]	HP; CPP
	PVX_003770	F	PFB0305c	LS	−0.475	Merozoite surface protein 5
	PVX_080305	LS	PFE0940c	F	−0.553	HP; PSOP13
	PVX_101530	ER/LS	PFE0040c	T	−0.560	PEXP; MESA
	PVX_091735	ES	PF11_0231	ER	−0.733	HP; CPP
	PVX_099670	ER/LS	PFI1230c	F	Absent	HP; CPP
	PVX_084815	S	PF14_0325	LS	Absent	HP; CPP
	PVX_114330	LS	PFF1170w	F	Absent	CPW-WPC DCP; CPP
	PVX_083275	LS	MAL13P1.237a	ND	Absent	HP; CPP
Cell invasion	PVX_094255	LS	PF13_0198	LS	0.576	Reticulocyte binding protein 2
	PVX_087885	ES	MAL7P1.208	S	Absent	Rhoptry-associated membrane antigen
Parasitophorous	PVX_116915	LS	PF14_0678	LR	−0.200	Exported protein 2
vacuole/Maurer's	PVX_084720	LS	PF14_0344	ER/LS	−0.253	HP; translocon component PTEX150
clefts proteins	PVX_092070	LS	PF11_0302	T	−0.397	HP; parasitophorous vacuolar protein 1
	PVX_000930	ES	PFD0310w	F	−0.493	Sexual stage antigen
	PVX_003520	ES	PFB0100c	R	−0.578	HRKP; KAHRP
	PVX_091700	LS	PF11_0224	ER	−0.806	Exported protein 1
Gliding motility	PVX_090215	S	PFD1110w	S	0.852	HP; GAPM2
Immune evasion	PVX_093680	R	PF08_0137	R	0.223	Phist protein (Pf-fam-b); PHISTc
Hemoglobin degradation	PVX_116695	ES	PF13_0133	F	−0.499	Aspartic protease PM5; plasmepsin V

PCCs, pearson correlation coefficients; HP, hypothetical protein; CPP, conserved *Plasmodium* protein; SET10, histone-lysine N-methyltransferase, H3 lysine-4 specific; PSOP13, secreted ookinete protein; CPW-WPC DCP, *P. falciparum* CPW-WPC domain contain protein; SERA, Serine-repeat antigen; GAMA, GPI-anchored micronemal antigen; MESA, mature parasite infected erythrocyte surface antigen; HRKP, histidine-rich knob protein homolog KPRPC; KAHRP, knob associated histidine-rich protein; GAMP, glideosome associated protein with multiple membrane spans 2.
R, rings; LS, ER, early rings; LR, late rings; S, schizont; ES, early scizont; LS, late schiznot; F, flat profile. ND, no data obtained.
[a] Corresponds to peak expression within each IDC transcriptome of *P. vivax*, *Pf 3D7* was selected for comparison.
[b] Absent, no detail expression data were found.
[c] *Pf HB3* was selected for comparison.

species. The C-terminal region of PvRAMA corresponds to the mature p60 form and p40 form of PfRAMA. Based on the results for PvRAMA identified in parasite extracts, we propose that PvRAMA is also subject to proteolytic processing that removes a large portion of the protein, and further study is required for experimental confirmation of the relationship between p40/PvRAMA and p110/PvRAMA. To determine whether PvRAMA is localized in the same cellular compartments as RAMA from other species, we performed double-staining IFA on thick blood films of Korean *P. vivax* isolates. Similar to PyRAMA [42], PvRAMA is co-localized with PvRhopH2 and is predicted also to be a rhoptry protein of *P. vivax* parasites. In addition, the fluorescence of PvRAMA does not overlap with that of a marker of rhoptry neck proteins (PvRON2). Thus, both PvRAMA and PvRhopH2 could be rhoptry body proteins, although this should be confirmed by immunoelectron microscopy study. The C-terminal region of PvRAMA that we expressed can be recognized specifically by sera from *P. vivax*-exposed patients. To confirm its antigenicity, we expanded the number of human sera tested and compared the results with those for PvMSP1-19. PvRAMA induced high antibody responses, although the reactivity was slightly weaker than that against PvMSP1-19. Antibodies towards this region are associated with a functional state of antimalarial immunity in humans, which makes PvRAMA a serological marker of exposure that should be further characterized.

Most of the gene products of *Plasmodium* are present in multiple developmental stages, including not only those gene products used for generic cellular processes but also factors mediating highly specialized *Plasmodium* functions [50]. This may indicate that the majority of the genome is utilized throughout the full lifecycle of the parasite and that only a small portion of the genome may actually be truly specific to a particular developmental stage. However, most of the transcriptome seen during the IDC correlates well with the function of the resultant proteins, and transcription of these genes is

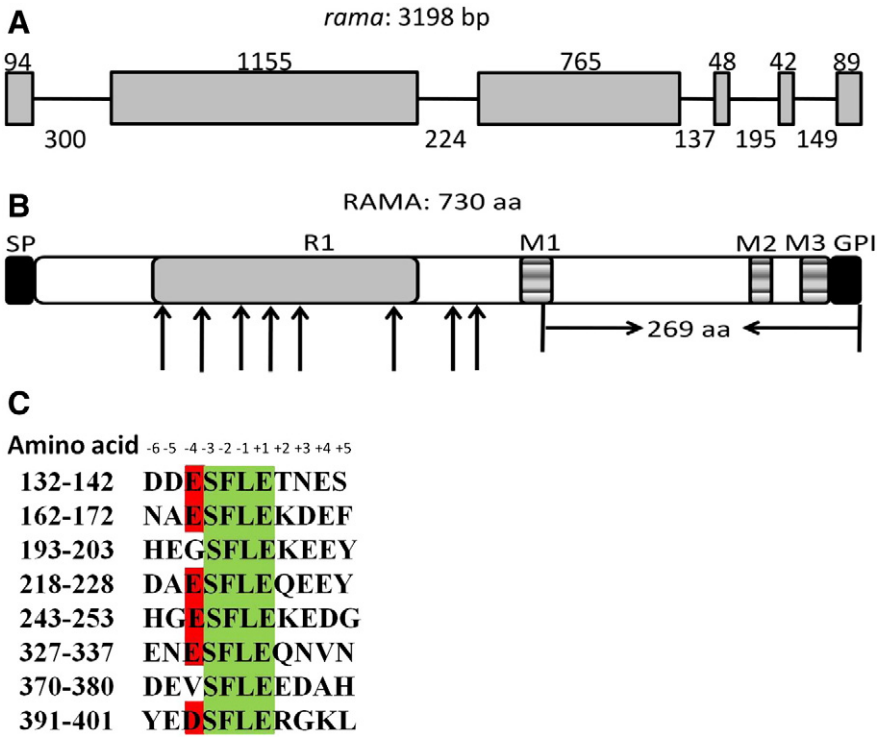

Fig. 6 – Primary structure of PvRAMA gene and protein. A) Gene structure of *rama*. Exon and intron sizes (in base pairs) are indicated above and below the diagram, respectively. B) Schematic of the predicted protein product, putative protease cleavage site and expressed fragment. Structural features are indicated as follows: the N-terminal signal peptide and the C-terminal GPI anchor attachment site are boxed in black, the conserved motifs (M1, M2 and M3) are boxed in gradient fill, putative cleavage sites are indicated with black arrows, and a 269 amino acid fragment expressed is also indicated. C) Amino acid alignment of putative protease cleavage sites within RAMA.

tightly controlled during IDC [51]. For example, genes with a profile of minimal transcription in the early blood stages and maximal transcription in the late stages are correlated with the apparent functions of these proteins during the invasion process or for trafficking proteins to infected erythrocyte surfaces [18,52,53].

More than one-third of the antigenic candidates selected in this study were found to have low to negative correlations between *P. vivax* and *P. falciparum*. The genes with shifted expression included PV and Maurer's cleft proteins and *Pvs16*. For example, in contrast to PfKAHRP (PF3D7_0202000), which is expressed during the ring stage, PvHRKP is expressed in the schizont stage. Comparison of expression revealed significant differences in the genes of the 2 species that are involved in crucial cellular functions that underpin the biological differences between them. PV-associated proteins that are shifted may be connected to the differences in rigidity and cytoadherence between *P. vivax* and *P.*

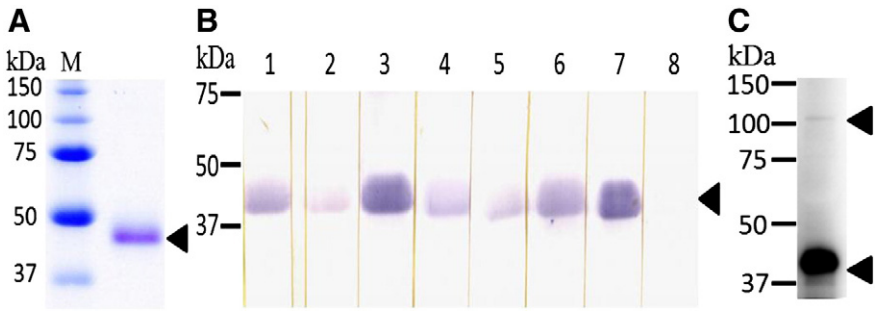

Fig. 7 – SDS-PAGE and Western blot analysis of the recombinant and native PvRAMA protein. A) SDS-PAGE analysis of purified recombinant PvRAMA protein. M, marker; Arrow indicates PvRAMA. B) Western blot analysis of recombinant PvRAMA protein with human sera. Lanes 1–7, reactivity of the recombinant PvRAMA protein with *P. vivax* patient sera; lane 8, nonrecognition of PvRAMA by serum from a healthy individual. C) Detection of the native PvRAMA protein in parasite lysate with immune mouse serum.

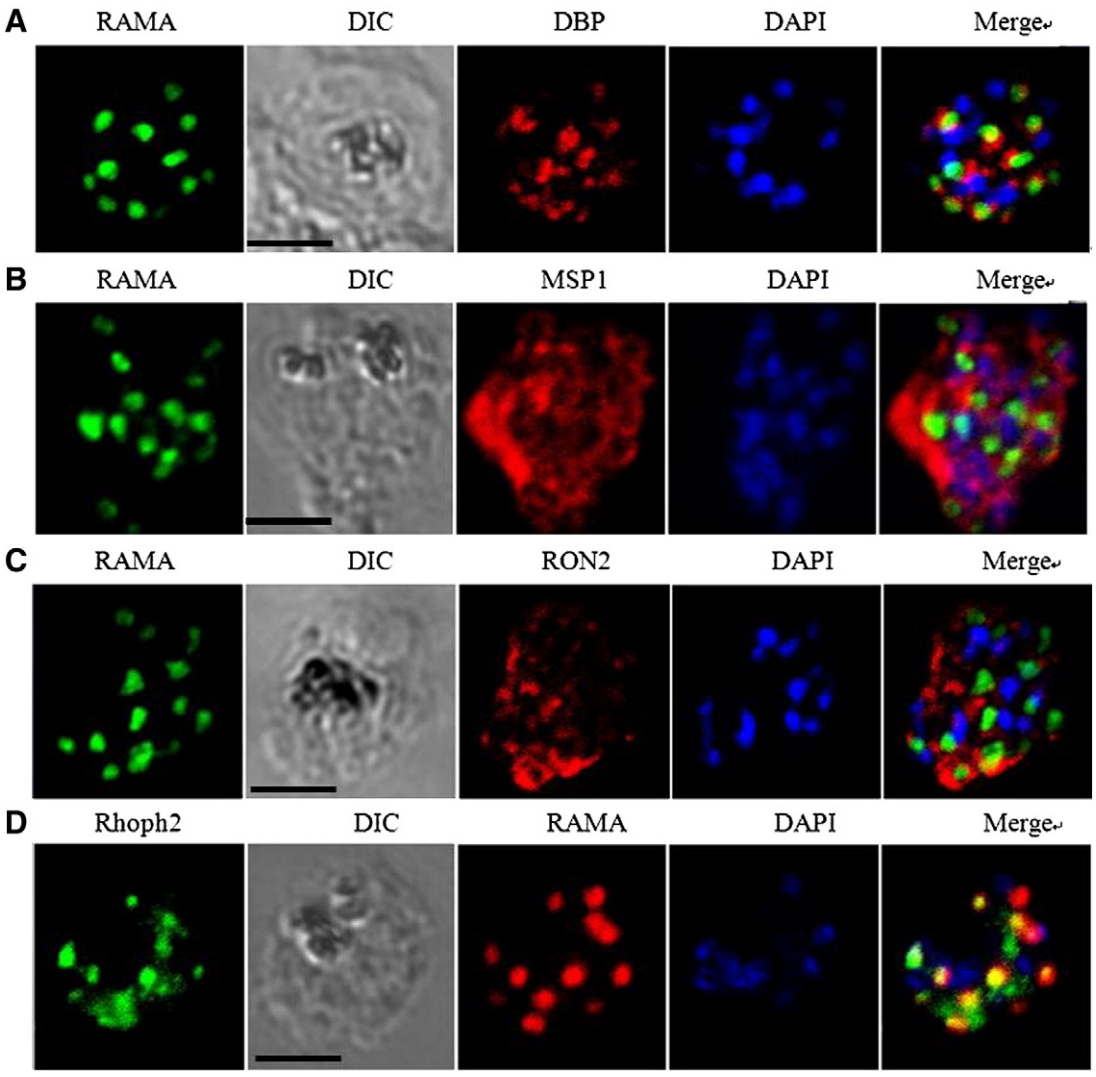

Fig. 8 – Subcellular localization of PvRAMA protein in the mature schizont stage of *P. vivax* parasites. Parasites were dual-labeled with antisera against PvRAMA and either A) PvDBP, B) PvMSP1, C) PvRON2 or D) PvRhoph2. Nuclei are visualized with DAPI in merged images. Bars represent 5 μm.

falciparum. The ring- and schizont-specific, non-syntenic and hypothetical genes expressed during the IDC of *P. vivax* may represent important factors associated with the *P. vivax* selectivity for young erythrocytes and/or immune evasion [17]. The results indicated that 12 antigenic candidates are unique genes of *P. vivax* and have no orthologues in *P. falciparum* (Table 2). This presentation of variant antigens is clearly different from that of *P. falciparum*, in which transcription of the majority of the antigenic gene families is silenced in the late stages [54–56]. The unique proteins in *P. vivax* could serve as potential candidates for developing *P. vivax*-specific tests.

Our results confirmed the advantages of this technique for producing high-quality eukaryotic proteins for structural and functional genomic studies [45,57]. This study also demonstrated that the protein array approach could effectively identify blood-stage antigen proteins of vivax parasites as serological markers of exposure to recent infection. Using these methods, many novel antigenic proteins were identified in this study, predominantly as markers of exposure. However, the study also provides basic information for further diagnosis, vaccine or drug development.

5. Conclusion

The establishment of high-throughput cloning and expression systems has permitted the construction of protein arrays for proteome-wide study of *P. vivax*. In this study, high-throughput screening assays have been applied to investigate blood-stage-specific immune proteomes from *P. vivax*. We identified 44 immunogenic proteins from the 152 putative candidates, more than 23% of which were hypothetical proteins described for the first time in this study. In addition, PvRAMA was characterized further and confirmed to be a serological marker for recent exposure to infection. The expression of one-third of the selected immunogenic proteins was shifted between *P. vivax* and *P. falciparum*, suggesting that these proteins may represent important factors associated with *P. vivax* selectivity for young erythrocytes and/or with

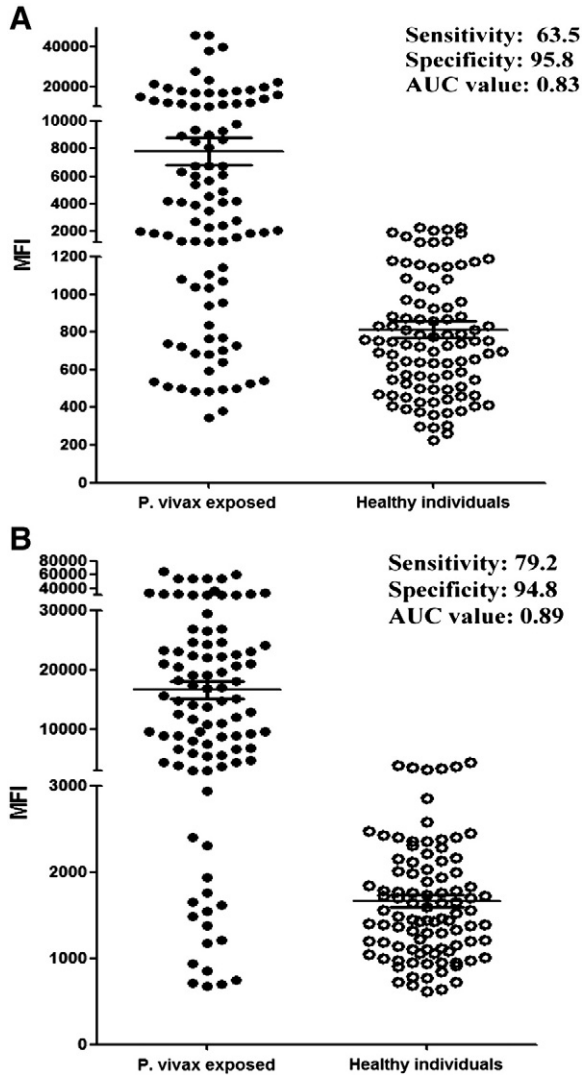

Fig. 9 – Comparison of IgG antibody responses to recombinant proteins PvRAMA and PvMSP1-19. A) PvRAMA. B) PvMSP1-19. Sera samples from *P. vivax*-infected patients (n = 96) and healthy individuals (n = 96) were used for measurement of antibody responses by protein array. The bar indicates mean value and 95% confidence interval. MFI, mean fluorescence intensity.

immune evasion. These novel immune proteomes of the *P. vivax* blood stage provide a baseline for further prospective serological marker studies in malaria. These methods could be used to determine immunodominant candidate antigens from the *P. vivax* genome.

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