

Measurement of naturally acquired humoral immune responses against the C-terminal region of the *Plasmodium vivax* MSP1 protein using protein arrays

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Abstract Protein arrays are powerful tools for antibody profiling and vaccine development against infectious agents. In the previous report, we successfully applied an antibody-based protein array for immunoprofiling of *Plasmodium vivax* infection. Herein, we developed a Ni-NTA surface based protein array to detect immune responses against the recombinant C-terminal region (19 and 42 kDa) of the *P. vivax* merozoite surface protein 1 (PvMSP1-19 and -42) from sera of vivax malaria patients.

The PvMSP1-19 arrays detected *P. vivax* in 112 of 130 (86.2%; 95% CI, 83.2–89.2%) microscopically positive samples and 2 false positives were obtained among 100 sera samples from healthy subjects (2.0%; 95% CI, 0.6–3.4%). These results were in concordance with results of enzyme-linked immunosorbent assays (ELISA). Kappa values represented excellent agreement for the recombinant PvMSP1-19 protein against sera samples as measured by protein arrays and ELISA (Kappa=0.904, 95% CI:

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0.849–0.960). The PvMSP1-42 protein arrays detected antibody response in 100 of 130 microscopically positive samples (76.9%; 95% CI, 72.4–86.8%) and 8 false positives were obtained in 100 healthy subjects (8.0%; 95% CI, 2.7–13.3%). There is no significant difference between the fluorescent intensity of antibody response to PvMSP1-19 and PvMSP1-42 in the positive sera samples ($P > 0.05$). The novel protein array platform may be used for profiling naturally acquired humoral immune responses to *P. vivax* infection.

Introduction

Among the malaria parasites that infect humans, *Plasmodium vivax* threatens almost 40% of the world's population, resulting in 132–391 million clinical infections each year (Price et al. 2007). In South and Southeast Asia, where the majority of vivax malaria infections occur, *P. vivax* accounts for up to 50% of malaria cases, with prevalence rates between 1% and 6% of the population. The emergence of chloroquine-resistant *Plasmodium falciparum* has reduced the proportion of malaria cases due to *P. vivax*; nevertheless, the absolute numbers of *P. vivax* remain high (Price et al. 2007; Mueller et al. 2009).

During the erythrocytic stages of malaria infection, potential targets for an immune response are free merozoites or intraerythrocytic parasites, and it is assumed that humoral antibody responses play a major role in blood-stage immunity (Langhorne et al. 2008). The mechanisms by which antibodies are effective include blockage of red blood cell (RBC) invasion by merozoites, antibody-dependent cellular killing mediated by cytophilic antibodies, and binding of antibodies to parasite-induced molecules on the RBC surface, thus leading to greater clearance of infected RBCs (Richie and Saul 2002). Antibodies are typically measured using an enzyme-linked immunosorbent assay (ELISA), a technique that requires high amounts of coating-antigens and immune sera when large numbers of samples must be screened (Fernandez-Becerra et al. 2010). To overcome these limitations, protein arrays with high-throughput capacity to detect specific antibody responses with minimal amounts of immune sera have been developed (Parekh and Richie 2007). This methodology has already been shown to be useful in the serodiagnosis and vaccine development of infectious diseases (Mezzasoma et al. 2002; Davies et al. 2005; Zhu et al. 2006; Felgner et al. 2009).

P. vivax merozoite surface protein 1 (PvMSP1) is a glycosylphosphatidylinositol (GPI)-anchored membrane protein expressed abundantly on the merozoite surface. Several studies have demonstrated the high antigenicity of the C-terminal region (19 or 42 kDa) of PvMSP1

(PvMSP1-19 and -42) worldwide, including countries such as Brazil, India, Sri Lanka, and Turkey (Cunha et al. 2001; Sachdeva et al. 2004; Wickramarachchi et al. 2007; Zeyrek et al. 2008). Protective efficacy has been observed in preclinical vaccine trials using *P. falciparum* recombinant C-terminal regions of MSP1 (PfMSP1-19), which has stimulated the study of *P. vivax* recombinant MSP1-19 (Collins et al. 1999). Partial protection *P. vivax* C-terminal region was observed in the first trial, and effective protection was achieved in the second trial (Perera et al. 1998).

In the present study, recombinant PvMSP1-19 and -42 were expressed using a wheat germ cell-free expression (WGCE) system. This technology is known to be a suitable system for decoding malaria genes without any codon optimization into biologically active malaria proteins (Tsuboi et al. 2008; Chen et al. 2010a). PvMSP1-19 and -42 were used to profile naturally acquired humoral immune responses in human patients infected with *P. vivax* in the Republic of Korea, and the protein array technology was validated.

Materials and methods

Patient samples

A total of 130 blood samples were obtained from patients who were confirmed positive for vivax malaria via microscopy at Korea University Ansan Hospital and at local health centers and clinics in Gyeonggi and Gangwon Provinces within endemic areas of the Republic of Korea. A total of 100 blood samples were taken from healthy patients who were confirmed negative for vivax malaria by microscopy, and these were used as controls. This study was approved by the Institutional Review Board at Kangwon National University Hospital.

Cloning of the gene encoding PvMSP1-19 and -42

The gene fragment encoding PvMSP1-19 was amplified by PCR from genomic DNA of the *P. vivax* Sal I strain, and cloned into the pEU-E01-His-Tev-N2 vector (CellFree Sciences, Matsuyama, Japan) at the *Xho* I and *Not* I sites. The inserted nucleotide sequence was confirmed using the ABI PRISM 310 Genetic Analyzer and a BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The gene fragment encoding PvMSP1-42 was amplified by PCR from genomic DNA of the *P. vivax* isolate from Republic of Korea, and cloned into the pEU-E01-His-Tev-N2 vector by In-Fusion cloning method (Chen et al. 2010a). Highly purified plasmid DNA is required for in vitro transcription and subsequent translation. Plasmid DNA was then prepared using the Maxi Plus™ Ultrapure plasmid extraction system (Viogene,

Taipei, Taiwan) according to the manufacturer's instructions. Purified DNA was eluted in $0.1\times$ TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and used for recombinant protein expression by WGCE.

Production of recombinant PvMSP1-19 and -42 using a wheat germ cell-free expression system

P. vivax MSP1-19 was expressed by 12 wells in a 6-well plate scale of the WGCE system using previously described bilayer translation reaction methods (Tsuboi et al. 2008; Chen et al. 2010a). The recombinant PvMSP1-19 protein was purified using a Ni-nitrilotriacetic acid agarose column (Qiagen, Valencia, CA, USA). PvMSP1-42 was expressed by a 1 ml WGCE system using the bilayer translation reaction method (Chen et al. 2010a). A WEPRO®1240H kit (CellFree Sciences) was used to synthesize PvMSP1-19 and -42.

SDS-PAGE and Western blot analysis

The recombinant PvMSP1-19 and -42 proteins were separated by SDS-PAGE under reducing conditions. The separated proteins were transferred to $0.45\ \mu\text{m}$ PVDF membranes (Millipore, Billerica, MA, USA) in a semi-dry transfer buffer (50 mM Tris, 190 mM glycine, 3.5 mM SDS, 20% methanol) at a constant 400 mA for 40 min using a semi-dry blotting system (ATTO Corp., Tokyo, Japan). After blocking with 5% skim milk in TBS/T, penta-His antibody (Qiagen, Valencia, CA, USA) and secondary alkaline phosphatase-conjugated goat anti-mouse IgG (ICN-Cappel, Costa Mesa, CA, USA) were used to detect His-tagged recombinant proteins. The immunoblots were incubated with a BCIP/NBT color development solution.

ELISA

To validate the immunoreactivity detected by protein arrays, sera from 130 vivax malaria patients in endemic areas of the Republic of Korea and 100 healthy individuals were tested against recombinant PvMSP1-19 protein by ELISA, as described previously (Mehrizi et al. 2009). The positive cut-off value was calculated as the mean optical density (OD) of normal controls plus 2 standard deviations (SD). Sera were screened by protein arrays as described below.

Serum screening using protein arrays

Sera from 130 vivax malaria patients and 100 healthy individuals were tested against the recombinant PvMSP1-19 protein using protein arrays. One microliter of recombinant PvMSP1-19 protein (40 ng/ μl) or crude PvMSP1-42 protein was spotted to each well of a Ni^{2+} chelated (Xenopore Corp., Hawthorne, NJ, USA) surface slide and

incubated for 2 h at 37°C. The arrays were first blocked with 5% BSA in PBS-T for 1 h at 37°C. Then they were probed with human serum (1:200) that was first pre-absorbed against wheat germ lysate (1:100) to block anti-wheat germ antibodies. The arrays were incubated with serum in PBS-T for 1 h at 37°C and antibodies were visualized with 10 ng/ μl Alexa Fluor 546 goat anti-human IgG (Invitrogen, Carlsbad, CA, USA) in PBS-T and scanned in a fluorescence scanner (ScanArray Express, PerkinElmer, Boston, MA, USA) (Park et al. 2009). Fluorescence intensities of array spots were quantified by the fixed circle method using ScanArray Express software (version 4.0, PerkinElmer). The positive cut-off value was calculated as the mean fluorescence intensity (MFI) value of the negative controls plus 2 SD.

Statistical analyses

Ninety-five percent confidence intervals (95% CI) were calculated according to a previous report (Chen et al. 2010b). The correlation between duplicate spots of protein arrays and the antibody reactivity of different recombinant PvMSP1 protein concentrations were analyzed using the Origin program (version 6.1, OriginLab). OD and MFI values from different samples were plotted using GraphPad Software version 5.0 (GraphPad Software, San Diego, CA, USA). Concordance in seropositivity responses between the two methods was evaluated using Kappa statistics. According to a previous report, excellent agreement was defined as a Kappa value >0.9 (Fernandez-Becerra et al. 2010).

Results

SDS-PAGE analysis showed that the band of purified recombinant PvMSP1-19 protein appeared at about 14 kDa, which is similar to the predicted size of PvMSP1-19 plus the amino acids of the multiple cloning site (MCS) remaining on the plasmid vector (Fig. 1a). Western blot analysis showed one specific band of PvMSP1-19 at 14 kDa (Fig. 1b). These results indicate that the PvMSP1-19 was successfully expressed by WGCE and purified. The result of western blot analysis of PvMSP1-42 was described previously (Chen et al. 2010a).

The efficiency of protein arrays for antibody profiling was evaluated using purified PvMSP1-19 (Fig. 2a, right panel). A novel concentration-dependent analysis method showed a correlation coefficient of 0.98 between fluorescence intensities and protein concentrations (Fig. 2b). Because a high concentration of protein coating may lead to saturation of the antigen-antibody reaction, we determined the appropriate concentration (40 ng/ μl) of protein coating for Ni^{2+} -chelated slides. One protein array probed

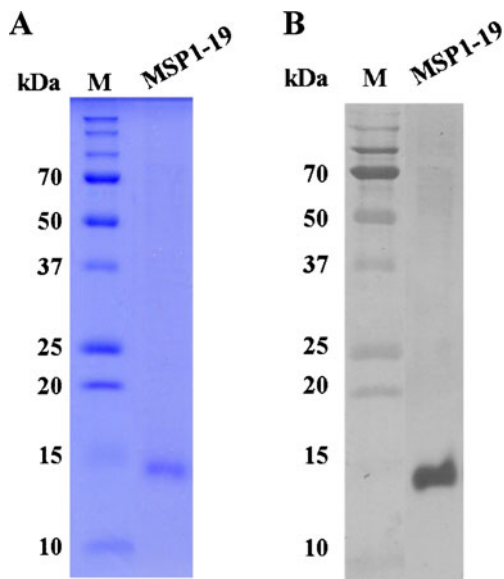
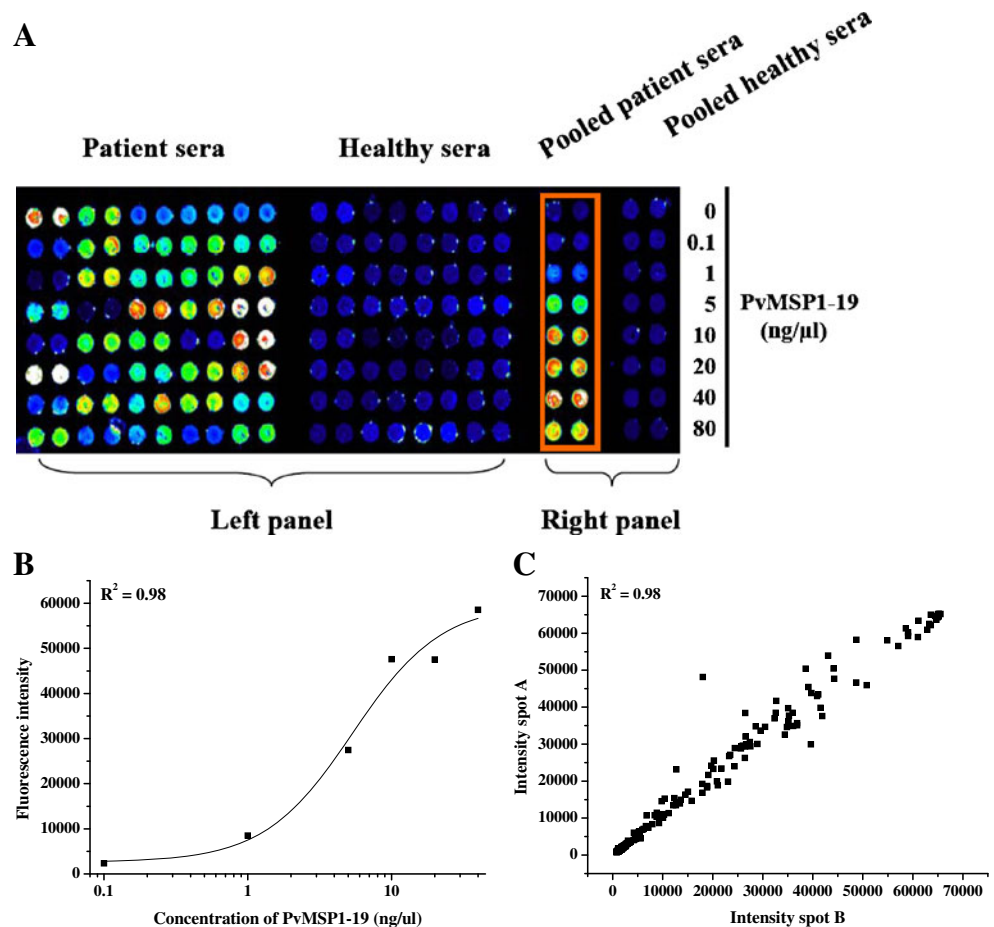


Fig. 1 SDS-PAGE and Western blot analysis of recombinant PvMSP1-19. **a** Recombinant PvMSP1-19 protein was stained with Coomassie brilliant blue. **b** Recombinant PvMSP1-19 protein was detected by anti-His antibody. M, protein marker

with serum from healthy individuals and vivax malaria patients is shown in the left panel of Fig. 2a. Sera from malaria-naïve subjects showed low reactivity, whereas that from *P. vivax*-exposed individuals showed obvious reactivity against PvMSP1-19 (Fig. 2a, left panel). When relative intensities of duplicate spots were plotted against each other, the resulting diagonal indicated a good reproducibility of spotting and detection of the immobilized proteins with a correlation coefficient of 0.98 (Fig. 2c).

Antibody responses against recombinant PvMSP1-19 in sera samples from 130 patients infected with *P. vivax* and 100 healthy individuals were detected using ELISA and protein arrays. Both methods showed apparent differences between the two groups of samples (Fig. 3a, b). The average OD value of samples from infected patients ($OD_{450\text{ nm}}=1.625$) was significantly higher than that of healthy individuals ($OD_{450\text{ nm}}=0.132$). In the same manner, the average fluorescent intensity value of sera samples from infected patients (fluorescent intensity=27,572 a.u.) was significantly higher than that of healthy individuals (fluorescent intensity=1,933 a.u.) (Table 1).

Fig. 2 Development of a protein array platform for profiling antibody responses to *P. vivax* infection. **a** Left panel Recombinant PvMSP1-19 (40 ng/μl) reacted with individual serum samples. Right panel Different concentrations of recombinant PvMSP1-19 protein were probed with pooled human serum (left duplicate, positive serum; right duplicate, negative serum). **b** Correlation between spot intensities and the concentration of recombinant PvMSP1-19. **c** Correlation between relative spot intensities of duplicates (*spot a* versus its duplicate *spot b*)



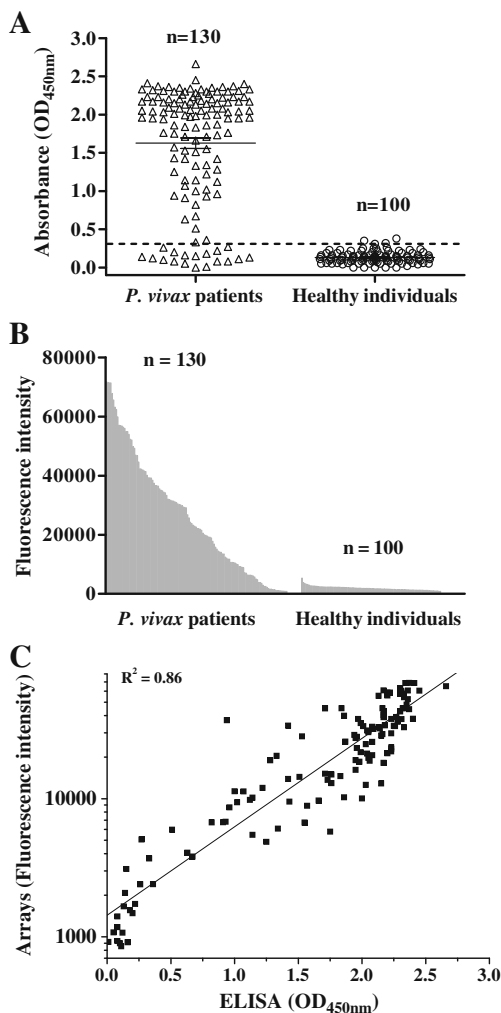


Fig. 3 IgG antibody responses to recombinant PvMSP1-19 protein using ELISA and protein arrays. Sera samples from *P. vivax*-infected patients ($n=130$) and healthy individuals ($n=100$) were measured by ELISA (a) and protein arrays (b). c Correlation between ELISA and protein arrays for antibody profiling

Both the ELISA and the protein arrays detected *P. vivax* in 112 of 130 of the microscopically confirmed positive samples (86.2%; 95% CI, 83.2–89.2%) (Table 2). Out of the 100 sera samples from healthy subjects, five false positives were obtained by ELISA (5.0%; 95% CI, 2.8–7.2%), whereas two false positives were obtained by

Table 1 Analysis of optical density (OD) values and mean fluorescence intensities (MFI) of IgG antibodies against PvMSP1-19 in sera samples using ELISA and protein arrays, respectively

Source of human serum samples	Total no. of samples	Screening methods for antibody profiling			
		ELISA (OD _{450 nm})		Protein arrays (MFI)	
		Mean	SD	Mean	SD
Vivax malaria patients	130	1.625	0.770	27,572	22,406
Healthy individuals	100	0.132	0.077	1,933	789

protein arrays (2.0%; 95% CI, 0.6–3.4%) (Table 2). The Kappa values represented excellent agreement for recombinant PvMSP1-19 proteins against sera samples measured by ELISA and protein arrays (Kappa=0.904, 95% CI: 0.849–0.960 and total agreement=95.2%).

The ELISA absorbance readings for antibody response against PvMSP1-19 in sera samples from *P. vivax*-infected patients were compared to the fluorescence measurements obtained by protein arrays. The ELISA and array data correlated well, with a correlation coefficient of 0.86 (Fig. 3c).

To test the efficiency of antibody response against crude *P. vivax* protein using Ni²⁺-chelated surface slides, crude His-tagged PvMSP1-42 was used for in situ binding and purification. One protein array probed with serum from healthy individuals and vivax malaria patients is shown in Fig. 4a. Sera from malaria-naïve subjects showed low reactivity, whereas those from *P. vivax*-exposed individuals showed obvious reactivity against PvMSP1-42 (Fig. 4a). When relative intensities of duplicate spots were plotted against each other, the resulting diagonal indicated a good reproducibility of spotting and detection of the immobilized proteins with a correlation coefficient of 0.98 (Fig. 4b). Protein arrays detected *P. vivax* in 100 of 130 sera samples from the microscopically positive samples (76.9%; 95% CI, 72.4–86.8%). From the 100 sera samples from healthy subjects, eight false positives were obtained by protein arrays (8.0%; 95% CI, 2.7–13.3%). There is no significant difference between the fluorescent intensity of antibody response to purified PvMSP1-19 and crude PvMSP1-42 in the positive sera samples ($P>0.05$).

Discussion

New methods for rapid gene cloning, and high-throughput cell-free protein expression are now publicly available in post-genomic malaria research (Gardner et al. 2002; Aguiar et al. 2004; Tsuboi et al. 2008). These methods promote the development of high-throughput assays for profiling immune responses against infectious diseases. Some work has focused on the development of subproteome or proteome arrays for smaller pathogens to identify immunodominant

Table 2 Frequency distribution of IgG antibodies against PvMSP1-19 in sera samples using ELISA and protein arrays, respectively

Source of human serum samples	Total no. of sample	Screening methods for antibody profiling			
		ELISA		Protein arrays	
		No. positive	Frequency (%) (95% CI)	No. positive	Frequency (%) (95% CI)
Malaria patients	130	112	86.2 (83.2–89.2)	112	86.2 (83.2–89.2)
Healthy individuals	100	5	5.0 (2.8–7.2)	2	2.0 (0.6–3.4)

Kappa values presented good agreement for the recombinant PvMSP1-19 protein against sera samples measured by ELISA and protein arrays (Kappa=0.904 and total agreement=95.2%)

antigens (Steller et al. 2005; Davies et al. 2007; Beare et al. 2008; Davies et al. 2008). The results verify this platform as a rapid way to comprehensively scan humoral immunity of vaccinated or infected humans and animals. Protein arrays have recently been used to characterize antibody reactivity profiles of *Plasmodium* infection (Sundaresh et al. 2006; Gray et al. 2007; Doolan et al. 2008; Chen et al. 2010a; Crompton et al. 2010).

P. vivax MSP1 is a polymorphic protein that is abundantly expressed on the merozoite surface. A processing mechanism may generate the C-terminal region of MSP1 (PvMSP1-19), which remains on the parasite surface where it appears to be essential for merozoite invasion (Cowman and Crabb 2006). PvMSP1-19 is normally purified from inclusion bodies using an *Escherichia coli* expression system (Rodrigues et al. 2005; Mehrizi et al. 2009). In the present study, the wheat germ cell-free-expressed PvMSP1-19 was purified from soluble fractions and showed positive reactivity with serum from humans naturally exposed to vivax malaria in the Republic of Korea. The antibody reactivities in this study were consistent with previously published results (Rodrigues et al. 2005; Mehrizi et al. 2009). Thus, these results indicate the proper immunogenicity of *P. vivax* proteins expressed using the wheat germ cell-free system.

We developed a protein array platform for immunoprofiling *P. vivax* infection. The concentration used for protein arrays (40 ng/spot) was five-fold lower than that for the ELISA (200 ng/well). This difference in concentrations is quite important to reduce the amount of coating antigen. Moreover, the amount of serum sample (1:200 dilution, 1 μ l) used for protein arrays was 200-fold lower than that used for ELISA (1:200 dilution, 200 μ l). This is a critical point for saving the amount of serum sample in the proteome-wide analysis of immune responses to infectious diseases (Felgner et al. 2009; Crompton et al. 2010).

We used protein arrays to characterize antibody reactivity profiles of *P. vivax* infection. From 230 sera samples of *P. vivax*-infected patients and healthy individuals confirmed by ELISA, protein arrays detected *P. vivax* in 112 of 130 (86.2%) microscopically confirmed positive samples. These results are consistent with the ELISA results, and the IgG frequency is consistent with previous reports in South Korea and Iran (Park et al. 2001; Mehrizi et al. 2009). Similar to the ELISA results, two false positives among 100 sera samples from healthy subjects were obtained using protein arrays. When we compared the results obtained by protein array and ELISA, the Kappa values represented excellent agreement for the recombinant PvMSP1-19 protein against sera samples

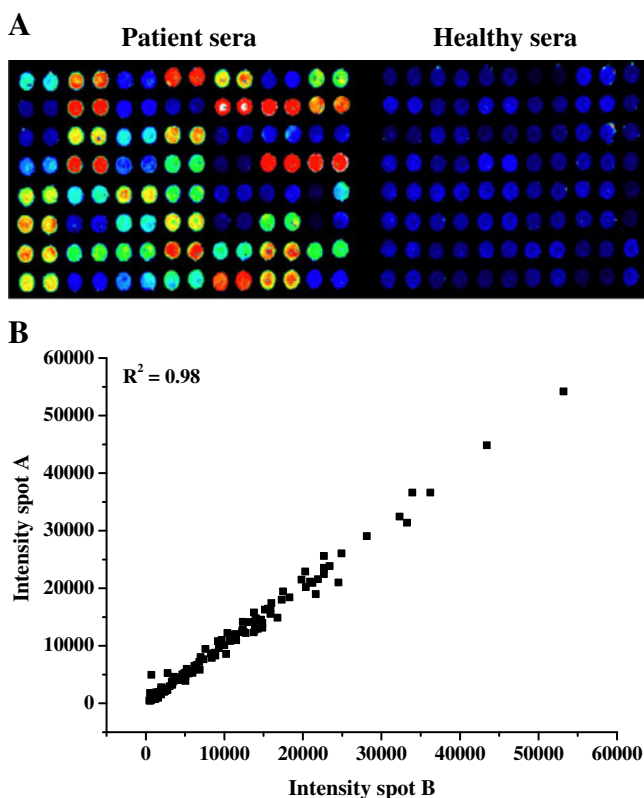


Fig. 4 Correlation between ELISA and protein arrays for antibody profiling. The ELISA absorbance readings for antibody responses against PvMSP1-19 in 130 sera samples from *P. vivax*-infected patients were compared to fluorescence measured by protein arrays. Protein array platform for profiling antibody responses to crude PvMSP1-42. **a** Crude recombinant PvMSP1-42 reacted with individual serum samples. **b** Correlation between relative spot intensities of duplicates (*spot a* versus its duplicate *spot b*)

(Kappa=0.904 and total agreement=95.2%). These results validate the protein arrays for profiling antibody responses to *P. vivax* infection.

In contrast with the use of nitrocellulose-coated glass slides in previous studies (Davies et al. 2005; Doolan et al. 2008; Crompton et al. 2010), we used Ni²⁺-chelated surface slides for in situ binding and purification of His-tagged recombinant proteins. When we analyzed the antibody response to crude PvMSP1-42 using Ni²⁺-chelated surface slides, it has the similar fluorescent intensity in comparison with purified PvMSP1-19 in the positive sera samples. These slides are especially powerful for coating with unpurified cell-free synthesized His-tagged proteins (data not shown). These slides have two major advantages for use with protein arrays. On one hand, it is possible to avoid the majority of printed protein (99%) derived from the lysate itself, which can compete with the expressed antigen for binding to the array surface. Higher background signals may be observed by cross-reacting serum antibodies to spotted lysate proteins on nitrocellulose-coated glass slides directly (Davies et al. 2005). On the other hand, tagged proteins are bound to the Ni²⁺-chelated surface in a uniform orientation and are not denatured. This means that they remain in the conformationally active form, thus providing optimal presentation to antibodies.

Analysis of serum reactivity profiles using protein arrays offers an opportunity to assess antibody responses against malarial antigens in a high-throughput manner. Furthermore, the novel protein array platform may be useful for profiling naturally acquired humoral immune responses to *P. vivax* infection.

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