Mature Erythrocyte Surface Antigen Protein Identified in the Serum of *Plasmodium falciparum*-Infected Patients

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Abstract. This study was performed to identify circulating *Plasmodium falciparum* proteins in patient serum, which may be useful as diagnostic markers. Depletion of highly abundant proteins from each pooled serum sample obtained from *P. falciparum*-infected patients and healthy individuals was performed using the Proteoseek Antibody-Based Albumin/IgG Removal Kit (Thermo Scientific, Rockford, IL). In analysis 1, the depleted serum was analyzed directly by NanoLC-MS/MS. In analysis 2, the depleted serum was separated by two-dimensional electrophoresis followed by western blot analysis. Subsequently, the selected band was analyzed by NanoLC-MS/MS. The result of analysis 1 revealed the presence of two mature erythrocyte surface antigen (MESA) proteins and chloroquine resistance transporter protein (PfCRT). In addition, analysis 2 revealed an antigenic 75-kDa band when the membrane was probed with purified IgG from the pooled serum obtained from *P. falciparum*-infected patients. MS/MS analysis of this protein band revealed fragments of *P. falciparum* MESA proteins. Thus, in this study, two different analyses revealed the presence of *Plasmodium* MESA protein in pooled serum from malaria patients; thus, this protein should be further investigated to determine its usefulness as a diagnostic marker.

INTRODUCTION

Malaria is an important global health problem. There were 627,000 deaths due to this disease in 2012, most of which occurred in the African continent.¹ There are five species of human *Plasmodium*, namely, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi*. *P. falciparum* is the most virulent, and this virulence is mainly due to its ability to grow in blood cells at all stages. Infection with *P. falciparum* can have severe consequences, such as poor pregnancy outcomes and cerebral malaria. The latter can cause obstruction of blood vessels in the brain due to adherence of the parasite to endothelial receptors and sequestration in deep vascular beds, which may lead to coma.

Despite the abundance of research on various aspects of malaria, investigations are still ongoing to discover markers to improve diagnosis, to further understand parasite virulence factors and to clarify the mechanism of malaria pathogenesis. In this regard, proteomic approaches have been actively used since the sequencing of the *P. falciparum* genome has been completed.²

Despite the challenge associated with the large dynamic range of proteins in serum samples, serum is still a rich source of potential biomarkers; thus, *Plasmodium* proteins in serum from malaria patients can be the basis for the development of serologic assays.³ The availability of gel- and liquid-based fractionation protocols, the large variety of immobilized pH gradient (IPG) strips and improvements in the solubilization of the vast majority of proteins in a given sample have led to improvements in two-dimensional electrophoresis (2-DE) of protein.⁴ In combination with mass spectrometry techniques, 2-DE is important in proteomics research for the discovery and identification of disease-associated markers.⁵

In this study, we investigated the presence of *P. falciparum* proteins in the serum of malaria patients with the aim of

identifying circulating proteins that may be useful as markers for diagnosis. Serum samples were depleted and analyzed using two approaches: direct mass spectrometry analysis and 2-DE followed by western blot and mass spectrometry.

MATERIALS AND METHODS

Serum samples. A total of 20 serum samples were selected from the archived serum samples. The Human Research Ethics Committee at Universiti Sains Malaysia permitted the use of these serum samples. Ten serum samples were obtained from Malaysian patients infected with *P. falciparum*, and 10 samples were obtained from archived healthy personnel at Institute for Research in Molecular Medicine, Universiti Sains Malaysia. The samples from malaria patients were from individuals confirmed to be positive for *P. falciparum* based on stained blood smears and real-time polymerase chain reaction, the latter used reported primers and probes.⁶ Table 1 shows the age of the patients and parasite count (parasites/uL blood) in each thick blood smear. The samples from malaria patients and healthy individuals were each pooled and depleted of albumin and IgG. Two analyses were performed. Analysis 1 involved direct analysis of the depleted sample using NanoLC-MS/MS (Waters, Milford, MA). Meanwhile, in analysis 2, the depleted pooled sera were separated using 2-DE followed by western blot analysis; subsequently, the selected band was analyzed using the same mass spectrometer method.

Depletion of albumin and IgG. Depletion was performed with the Proteoseek Antibody-Based Albumin/IgG Removal Kit (Thermo Scientific, Rockford, IL) according to the manufacturer's recommendations. Aliquots (6 μ L) of each of the pooled samples were placed in spin columns containing 605 μ L of gel slurry. Each column was capped, vortexed, and incubated for 30 minutes at room temperature on an orbital shaker. The column was then centrifuged at $1,000 \times g$ for 2 minutes, and the filtrate was retained.

Concentration of pooled serum from multiple depletions. To concentrate the depleted samples, 500 μ L of depleted serum was added to a spin column (Vivaspin 500, 3 kDa cutoff; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and

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TABLE 1
Age of patients and parasite count of thick blood smears

No.	Sample	Age	Parasite count (parasites/uL blood)		
			Asexual	Gametocytes	Total
1	L5	ND	172,800	1,680	174,480
2	L19	20	12,960	150	13,110
3	L31	53	11,360	0	11,360
4	L55	53	27,034	640	27,674
5	L58	38	115,555	800	116,355
6	L70	45	80,000	160	80,160
7	L71	14	9,680	0	9,680
8	S4	ND	22,880	0	22,880
9	S5	ND	12,480	320	12,800
10	S6	ND	33,116	80	33,196

ND = not described.

centrifuged at $15,000 \times g$ for 30 minutes at 4°C . Depleted serum was added continuously to the same spin column until all the depleted serum samples were pooled and concentrated to a final volume of 0.1–0.2 mL and the concentration reached ~ 1 mg.

Buffer exchange. Following the concentration of the samples, buffer exchange was performed to remove salt, which might interfere with the analysis, using an OFFGEL fractionator (Agilent Technologies, Santa Clara, CA). An equal volume of 10 mM Tris-HCl (pH 7.6) was added to the spin column containing the concentrated depleted serum. The sample was again centrifuged, and the process was repeated three times to remove $\sim 99\%$ of the initial salt content. The total protein content was determined using the RC DCTM assay kit (Bio-Rad, Hercules, CA).

First dimension separation. The first dimension separation involving isoelectric focusing (IE) was performed using an OFFGEL fractionator (Agilent Technologies). Ready-made IPG strips (GE Healthcare) that were 13 cm in length with a pH range of 4–7 were used. IPG strip rehydration solution (40 μL) was loaded into each of the 12 wells of the OFFGEL fractionator. Two wet electrode pads were placed on each protruding end of the IPG strip with no gap between the pad and the frame. The IPG gel was then allowed to swell for 15 minutes. After the rehydration step, a 150- μL sample containing ~ 1 mg of depleted serum protein was loaded into each well. Then, the cover seal was placed over the frame and pressed down gently on each well to ensure a proper fit. Subsequently, 10 μL of deionized distilled H_2O was pipetted onto the electrode pads at each end of the IPG gel. The tray was placed on the instrument platform, and 200 μL and 1 mL of cover fluid (mineral oil) was pipetted onto the anode end and the cathode end, respectively. After 1 minute, 200 μL of cover fluid was added to both ends of the IPG strip.

The fractionation was initiated with a maximum current of 50 μA and a maximum power of 200 mW. After 24 hours, the upper electrode pads were exchanged with fresh pads wetted in deionized water, and the cover fluid was refilled if necessary.

Second dimension separation. When the IE was completed, the liquid sample in each well was separately collected into labeled tubes. The second dimension separation was performed with a Mini-Protein 3 cell apparatus (Bio-Rad). A 16- μL aliquot of each fraction was mixed with 4 μL of $5\times$ sample buffer and directly loaded onto a 10% resolving gel.

Silver staining. Silver staining was performed according to a previously described protocol.⁴ At least 50 mL of each solution was prepared per gel. All steps were carried out at

room temperature with gentle shaking, and all solutions were prepared in double distilled water.

Purification of total IgG. A 600- μL aliquot of the pooled sera from *P. falciparum* patients was used for IgG purification using the Melon Gel IgG Spin Purification Kit (Thermo Scientific). The collected IgG was pooled and concentrated using a spin column (Vivaspin, 5 kDa cutoff) by centrifuging at $3,000 \times g$ for 60 minutes at 4°C until the total volume was reduced to ~ 500 μL .

Western blot. After sodium dodecyl sulfate polyacrylamide gel electrophoresis, the separated proteins were transferred onto a nitrocellulose membrane using a dry protein blotting apparatus (Bio-Rad, Hercules, CA) at 12 V for 30 minutes. After a blocking step, the membrane was incubated with the pooled *P. falciparum* serum at a dilution of 1:200 overnight at 4°C . The experiment was repeated using 0.25 $\mu\text{g}/\text{mL}$ IgG-purified *P. falciparum*-pooled serum as a primary antibody. Another set of western blotting experiments was performed using pooled healthy serum as a primary antibody. Immunodetection was performed after incubation for 1 hour with horseradish peroxidase-conjugated mouse anti-human IgG (Invitrogen, Waltham, MA at a 1:2,000 dilution. Substrate development was performed using enhanced chemiluminescence blotting reagent (Roche Diagnostics, Deutschland GMBH, Mannheim, Germany) and X-ray film (Kodak, Rochester, NY).

Colloidal blue stain. A Colloidal Blue staining kit (Thermo Scientific) was used to stain the gels for MS/MS analysis according to the manufacturer's instructions.

Protein digestion, mass spectrometry analyses and database search. Aliquots (200 μL ; 100 $\mu\text{g}/\mu\text{L}$ total protein) of the sample that had been depleted using the albumin/IgG removal kit (analysis 1) and the potential antigenic band (analysis 2) were analyzed using mass spectrometry at the Malaysia Genome Institute, Bangi, Selangor. Protein digestion was performed using standard protocols from Waters.⁷ For in-solution digestion (analysis 1), the depleted protein sample (100 μg) was diluted in 1.0 mL of 50 mM ammonium bicarbonate. Subsequently, it was concentrated to 100 μL using a spin column (MW cutoff of 3,000) and reduced with 5 μL of 100 mM 1,4-dithiothreitol (DTT). The sample was then heated for 30 minutes at 60°C , alkylated with 5 μL of 200 mM iodoacetamide and incubated at 25°C for 45 minutes in the dark. Proteolytic digestion was initiated with 2 μL of 1 $\mu\text{g}/\mu\text{L}$ trypsin (1:50, w/w) followed by incubation at 37°C overnight. Digestion was stopped by the addition of 2 μL of concentrated trifluoroacetic acid followed by incubation at 37°C for 20 minutes. The sample was then vortexed and centrifuged. The supernatant was collected and transferred to a clean microcentrifuge tube.

For protein digestion of the antigenic band (analysis 2), the gel was rinsed with distilled water, and the band of interest was excised and chopped into pieces (1×1 mm) using a clean scalpel. The gel pieces were rehydrated in 150 μL of 100 mM ammonium bicarbonate and heated at 37°C for 15 minutes to remove the stain. Then, 150 μL of acetonitrile was added, and the sample was vortexed and centrifuged. The supernatant was removed, and the gel was shrunk using a vacuum concentrator. This procedure was repeated several times until the stain was completely removed. The gel pieces were then soaked in 150 μL of water for 5 minutes, shrunk again in 450 μL of acetonitrile, and dried in a vacuum concentrator. Then, the

FIGURE 1. Comparison of depleted and nondepleted serum of pooled *Plasmodium falciparum* and pooled healthy serum samples. Lane M: unstained marker; lane 1: depleted healthy serum; lane 2: nondepleted healthy serum; lane 3: depleted *P. falciparum* serum; lane 4: nondepleted *P. falciparum* serum.

gel pieces were swelled by adding enough 1 mM DTT to completely cover the pieces. Subsequently, the sample was incubated at 56°C for 30 minutes. The gel pieces were then rehydrated, dried, and alkylated by adding enough 55 mM iodoacetamide to completely cover the pieces. Subsequently, the sample was incubated at 25°C for 45 minutes in the dark. The gel was rehydrated and dried, and 12.5 ng/μL (w/v) trypsin was added. The mixture was then incubated at 37°C overnight, and the digested peptides were recovered by adding 10 μL of 50% acetonitrile with 5% formic acid followed by sonication for 10 minutes. This process was repeated two to three times, and sample cleanup was performed using ZipTip™ (Millipore, Billerica, MS) immediately before mass spectrometry analysis. The peptides obtained from both types of digestion reactions were kept at -80°C until further analysis and for long-term storage.

The samples for analyses 1 and 2 were analyzed using NanoLC-MS/MS (Waters). The experiment was conducted using a 1.5-hour reverse-phase gradient at 300 nL/minutes (1–50% solvent B) on a nanoACQUITY UPLC® system (Waters). The aqueous mobile phase (solvent A) was water with 0.1% formic acid, and the organic mobile phase (solvent B) was acetonitrile with 0.1% formic acid. Peptide separation was performed with a nanoACQUITY 1.7 μm BEH130 C₁₈ NanoEase™ 75 μm × 200 mm column. Each sample was run in duplicate. Mass spectrometry analysis of the separated peptides was performed using a Q-TOF Synapt G2 HDMS (Waters). The mass spectrometer was programmed to step

TABLE 3

Western blot results for depleted and fractionated *Plasmodium falciparum* serum samples

Antigen	Primary antibody	Secondary antibody	Unique band
Depleted and fractionated <i>P. falciparum</i>	<i>P. falciparum</i> serum	IgG	–
Depleted and fractionated <i>P. falciparum</i>	Purified IgG from <i>P. falciparum</i>	IgG	~75 kDa
Depleted and fractionated <i>P. falciparum</i>	Healthy serum	IgG	–

between normal (6 eV) and elevated (15–40 eV) collision energies on the gas cell using a scan time of 1.0 seconds per function over *m/z* 50–1990. All experiments were performed in electrospray ionisation (ESI) positive mode.

The raw data were processed and searched using ProteinLynx Global Server (PLGS) version 2.4. Protein identifications were assigned by searching against the *Plasmodium* protein database available at UniProt. The mass error tolerance for the searched results was set automatically by the software; the precursor ion was set at 5 ppm, and mass error tolerance for the product ion was set at 20 ppm. Peptide identifications were restricted to tryptic peptides with no more than one missed cleavage. Cysteine carbamidomethylation was considered as a fixed peptide modification, whereas methionine oxidation, asparagine deamination and glutamine deamination were considered as variable peptide modifications. Peptides with PLGS scores greater than 200 and sequence coverage over 25% were considered as significant.

RESULTS

As depicted in Figure 1, most of the albumin and IgG were depleted from the pooled serum sample. The albumin and IgG appeared as very high-intensity bands in the nondepleted sample (lanes 2 and 4), whereas in the depleted sample, the intensities of these two proteins bands were greatly reduced (lanes 1 and 3).

As stated above, peptides with greater than 200 PLGS scores and more than 25% sequence coverage were considered significant. Thus in analysis 1, although a total of 112 proteins were detected; only three significant *Plasmodium* proteins were identified: mature parasite-infected erythrocyte surface antigen (MESA) or erythrocyte membrane protein 2 (PfEMP2), phosphoprotein 300 fragment of MESA protein, and chloroquine resistance transporter (PfCRT) fragment (Table 2). In analysis 2, three types of primary antibodies were used; the results of western blotting of the 12 OFFGEL fractions are summarized in Table 3. An antigenic protein

TABLE 2

Identification of *Plasmodium falciparum* proteins using mass-spectrometry and database search analyses (analysis 1)

No.	Accession number	Protein description	Protein score	Molecular mass	Matched peptide	Protein sequence coverage (%)
1	Q06166	Mature parasite-infected erythrocyte surface antigen MESA or PfEMP2 OS <i>P. falciparum</i>	558	195,479	39	28.78
2	Q86BT4	Phosphoprotein 300 Fragment OS <i>P. falciparum</i> GN MESA PE 4 SV 1	570	71,898	31	41.71
3	D5L5S1	PfCRT Fragment OS <i>P. falciparum</i>	414	48,592	5	69.32

MESA = mature erythrocyte surface antigen; PfCRT = chloroquine resistance transporter proteins; PfEMP2 = erythrocyte membrane protein 2.

FIGURE 2. Western blot analysis of OFFGEL fractionated depleted serum from *Plasmodium falciparum*-infected patients, incubated with different primary antibodies. (A) Western blot performed using IgG-purified *P. falciparum* pooled serum as the primary antibody; the results showed an antigenic protein band (~75 kDa) in lane 2 which is from OFFGEL well no. 5 (pH 5.25). (B) Western blot performed using pooled healthy serum as the primary antibody, the results showed absence of the ~75 kDa band in lane 2. Each lane represents fractions of different pH from the OFFGEL wells: Lane 1: well 4, pH 5; lane 2: well 5, pH 5.25; lane 3: well 6, pH 5.5; lane 4: well 7, pH 5.75; lane 5: well 8, pH 6; lane 6: well 9, pH 6.25; lane 7: well 10, pH 6.5; lane 8: well 11, pH 6.75; lane 9: well 12, pH 7.

with a molecular mass of ~75 kDa from fraction 5 (pH 5.25) was observed when the membrane was incubated with IgG purified from serum samples derived from *P. falciparum*-infected patients (Figure 2). The same result was obtained on two blots performed on different days. Analysis of the 75-kDa antigenic band by NanoLC-MS/MS revealed four fragments of the *P. falciparum* MESA protein (Table 4). Thus, the results of this study showed that MESA protein was observed in both analyses 1 and 2.

DISCUSSION

In a previous study on the *Plasmodium* proteome using 2-DE, 349 spots were detected, and 57 and 49 proteins were determined to be from the *Plasmodium* ring and trophozoite stages, respectively, by mass spectrometry analysis.⁸ A comparative proteomic study was also performed on *Plasmodium* at four different stages of the parasite life cycle (i.e., sporozoite, merozoite, trophozoite, and gametocyte stages) using multidimensional protein identification technology. The results of this study revealed over 2,400 *Plasmodium* proteins that were consistent with the physiology of each stage.⁹ Unlike most previous proteomic studies on *Plasmodium*, which used parasite proteins derived from cultures, this study used parasite proteins derived from the sera of malaria patients. The use of serum samples may enable the identification of effectors of parasite virulence that are present at the protein level,

which is relevant for the development of new detection tools and anti-malarials.¹⁰

Analysis 1, which involved direct NanoLC-MS/MS of whole depleted serum from *P. falciparum*-infected patients, showed that two of the three proteins identified were fragments of MESA protein identified as phosphoprotein 300 and PfEMP2. In analysis 2, the depleted proteins were assessed by 2-DE followed by western blot. A liquid-based IE apparatus was used instead of the traditional gel-based IE because it allowed for the loading of a large amount of protein (maximum of 5 mg), which increases the chances of isolating circulating *Plasmodium* proteins. Western blot analysis detected an antigenic protein band with an approximate molecular mass of 75 kDa that was not present in the blot probed with healthy serum. Mass spectrometry analysis of the gel band revealed four *Plasmodium* proteins, and all were fragments of MESA protein. Thus, in this study, both analyses 1 and 2 identified MESA protein in the pooled serum of malaria patients, which may indicate that MESA protein has potential diagnostic value.

MESA protein, also known as PfEMP2, is a protein with a molecular mass of 250–300 kDa. It may be implicated in malaria virulence due to its ability to modify the host cell, including altering the rigidity of the membrane.¹¹ MESA is exported from the parasite to the cytoplasmic face of the infected red cell and forms a strong noncovalent interaction with host red cell protein 4.1. Protein 4.1 is important in

TABLE 4
Identification of 75 kDa *Plasmodium falciparum* proteins using mass spectrometry and database search analyses (analysis 2)

Protein band	Accession number	Protein description	Protein score	Molecular mass	Matched peptide	Protein sequence coverage (%)
75kDa	Q25920	Mature parasite-infected erythrocyte surface antigen OS <i>P. falciparum</i> GN MESA PE 2 SV 1	557	177,185	38	35.76
	Q8I492	Mature parasite-infected erythrocyte surface antigen MESA or PfEMP2 OS <i>P. falciparum</i> isol	552	168,287	36	30.06
	Q06166	Mature parasite-infected erythrocyte surface antigen OS <i>P. falciparum</i> PE 4 SV 2	558	195,479	39	28.78
	Q86BT4	Phosphoprotein 300 Fragment OS <i>P. falciparum</i> GN mesa PE 4 SV 1	570	71,898	31	41.71

MESA = mature erythrocyte surface antigen; PfEMP2 = erythrocyte membrane protein 2.

regulating the mechanical properties of the red cell membrane, and the binding of MESA to this protein aids in the survival of the parasite. This binding is specific to a region that is involved in the formation of the ternary complex with p55 and glycophorin C.^{12–14} The specific function of MESA is unknown. However, the interruption of the MESA-4.1 protein-protein interaction causes accumulation of unbound MESA, which becomes toxic in the red blood cell cytoplasm and leads to parasite death. Thus, novel antimalarial therapies may take advantage of these phenomena.¹⁵

MESA protein is the same size as PfEMP1 and exhibits the same association with the host cell membrane. However, the topological arrangements of PfEMP1 and PfEMP2 in the membrane are different.¹² PfEMP2 is located beneath the erythrocyte membrane, while PfEMP1 is located on the surface of the infected cells. PfEMP2 localizes either in the erythrocyte cytoplasm or under the membrane knobs in association with electron-dense material.¹² The size of the MESA protein varies in different isolates, which might be due to an alteration in the number of repeats.¹³ The molecular mass of the MESA protein detected in this study was less than 200 kDa, which is different from its theoretical molecular mass of 250–300 kDa; this is likely due to partial proteolytic breakdown of the protein. Several previous studies have assessed this protein; some have examined the role of MESA/PfEMP2 in malarial infection of erythrocytes,¹⁶ while others have explored the structural and functional domains of MESA.¹³

It is interesting that only two proteins were identified in the pooled malaria serum sample. *P. falciparum* histidine-rich protein 2 (PfHRP2), a protein known to circulate in plasma of infected humans¹⁷ was not detected; neither were other nonstage-specific cell surface *P. falciparum* proteins. This may be due to the use of the albumin/IgG depletion column, whereby it may have led to one of the following consequences: 1) there was binding of those proteins to albumin, which were removed when the serum sample was depleted of albumin; 2) those proteins may be very immunogenic and circulate as immune complexes, thus were removed when the serum sample was depleted of IgG. With regard to the latter, the western blots in this study may be thus detecting antigens that were poorly immunogenic. Nevertheless in developing an antigen detection test, the selected antigen need not be very immunogenic to the host; it is more important that the monoclonal and/or polyclonal antibody raised against the antigen can be used in developing such a test.

CONCLUSION

In this study, seven *P. falciparum* proteins were identified in patient serum samples, including PfCRT and six fragments of MESA protein. MESA protein was identified using two types of analysis. Because of resource limitations, we could not perform the analyses on individual serum samples. In the future, targeted mass spectrometry may be performed to verify the presence of MESA protein in individual serum samples from *P. falciparum* patients. The samples should include patients with both high and low *P. falciparum* parasitemia. This would be important toward quantitative assessment of parasitemia in an endemic area where many infected people may be test-positive. It may also be valuable to investigate the presence of MESA protein in the serum of patients infected with other *Plasmodium* species. In conclusion, this study

provided evidence that circulating MESA protein is present in *P. falciparum* patients; thus, MESA may be potentially useful as a diagnostic marker.

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