

Efficacy of the Merozoite Surface Protein 1 of *Plasmodium Vivax* as an Antigen for ELISA to Diagnose Malaria

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Malaria is still a major health problem in Thailand and its incidence is currently rising in Korea. To identify a useful antigen for the diagnosis of malaria patients, a cDNA expression library from malaria parasites was constructed and screened out immunologically. One clone was selected in view of its predominant reactivity with the patient sera. The recombinant malaria parasite antigen (Pv30) with 27 kDa as a C-terminal His-tag fusion protein that was produced in *Escherichia coli* was identified through immunoblot analysis. The deduced amino acid sequence had the sequence homology with the merozoite surface protein 1 (MSP1) genes of *Plasmodium falciparum* and *P. yoelii*, each by 41% and 42%, respectively. Measurement of serum IgG and IgM antibody to Pv30 by enzyme-linked immunosorbent assay (ELISA) was evaluated as a serodiagnostic test for malaria patients in Thailand (endemic area) and Korea (recently reemerging area). The sensitivity of *P. vivax*, *P. falciparum*, and *P. malariae* was 96.3% (26/27), 90.6% (29/32), and 100% (6/6), respectively, and the specificity was 63.5% (40/63) in Thailand samples. The sensitivity of *P. vivax* was 98.8% (88/89), and the specificity was 96.6% (86/89) in Korean samples. Pv30 appears to be a good and reliable recombinant antigen for serodiagnosis of malaria in a nonendemic area.

Key Words: *Plasmodium vivax*, merozoite surface protein 1, ELISA, serodiagnostic test, sensitivity, specificity

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INTRODUCTION

Over 300 million people around the world, particularly in large populations residing in tropical regions, are at risk of having one or more episodes of malaria during their lifetime. Most of the infections are caused by *Plasmodium falciparum* or *P. vivax*, or sometimes by both plasmodial species.¹ Malaria is responsible for approximately 1.5 to 2.7 million deaths each year, which are predominantly in children living in Sub-Saharan Africa.²

The reemergence of *P. vivax* malaria has been reported in several countries, such as Peru, India, China, and Korea, and has become a serious public problem in these countries.³⁻⁵ Although Korea was declared to be free of malaria by the World Health Organization (WHO) in 1979,⁶ the reemergence of malaria has been apparent since 1993 among soldiers deployed near the De-Militarized Zone (DMZ) in the northern part of the country. Reemergence of a single case has resulted in an exponential effect in the northwestern part of the Korea, causing more than 1,700 cases in 1997 and an estimated 4,000 cases in 1998.^{7,8}

In Korea, one-third of all blood donors have traditionally been soldiers. However, since the *P. vivax* malaria epidemic began, the number of soldiers donating blood has sharply decreased, because more than half of all of the vivax malaria cases were occurring in soldiers.

Conventional microscopic examination of thin and thick blood films demonstrates the presence of the parasite. This method has been used to confirm the diagnosis of malaria, but it is a labor-intensive procedure and relies upon subjective interpretation. To overcome these limitations, fast and reliable methods for malaria diagnosis have been recently introduced. In this study, we examined the Pv30 antigen for the use in detecting malaria parasites in Korea, where reemergence has occurred, and in an endemic area in Thailand.

MATERIALS AND METHODS

Blood samples

Detection of the malaria parasites and characterization of the plasmodial species were performed by experienced microscopists who examined the Giemsa-stained, thick and thin blood films obtained from volunteers. Parasitemia in thick and thin blood films was estimated by counting the number of parasite-infected red blood cells in every 100 red blood cell. The number of asexual forms of malaria parasites corresponding to 500 red blood cells was counted on each thick blood film. Anti-Pv30 antibody levels were measured in sera of 89 individuals who had never been exposed to malaria parasites from Korea and in sera of 154 patients with microscopically confirmed malaria from Korea and Thailand. In order to study the sensitivity and specificity of the antibody test, 89 healthy individuals from nonendemic areas of Korea, 63 healthy individuals from endemic areas (Thailand samples) who were detected as negative for the malaria parasite by using the thin blood, and 154 malaria patients in Thailand and Korea were used. Sera from 37 individuals who had a recent illness of malaria from an endemic area in Thailand were collected one month after their recovery following treatment with chloroquine/primaquine. The convalescent malaria samples were negative for the malaria parasite on the blood smear.

Cloning of Pv30 expression vector

The construction of the pET21-Pv30 is shown

schematically in Fig. 1. A cDNA expression library of *P. vivax* was prepared from the blood of a patient with Korean *P. vivax* malaria. *P. vivax* was isolated from infected human blood by percoll gradient centrifugation.⁹ mRNA was isolated using the Oligotex^R mRNA Mini kit (Qiagen, Hilden, Germany). Double-stranded cDNA was synthesized from the mRNA with reverse transcriptase (cDNA synthesis kit; Stratagene, La Jolla, CA, USA), and a cDNA fragment of the MSP gene was amplified by PCR. The DNA sequence encoding the amino acids Tyr1522-Ser1729 was amplified by PCR.

To amplify the region from Tyr1522 to Ser1729, the PCR was performed using primers 1 (5'-GTG GAATTCGAGTACGAGTCC-3') and 3 (5'-TCTGT CGACAAGCTCCATGCA-3'). The underlined sequences in primers 1 and 3 are *EcoRI* and *Sall* restriction enzyme sites, respectively, which facilitate subsequent cloning. After initial denaturation (3 min at 94°C), 40 cycles of amplifications (94°C for 30 s, 58°C for 20 s, and 72°C for 1 min) were performed. The amplified DNA was ligated into an *EcoRI-Sall* digested pET21a vector (Fig. 1).

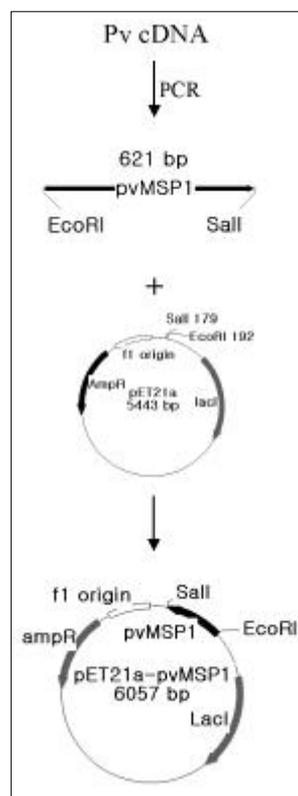


Fig. 1. Construction of pET21-Pv30. The PCR amplified DNA (PvMSP1) covering the region from Tyr1522 to Ser1729 of MSP1 was digested with *EcoRI* and *Sall* and ligated into pET21a.

Expression of Pv30

The recombinant protein encoding the region from Tyr1520 to Ser1729 was expressed. In order to overexpress recombinant Pv30, a single colony of transformed *E. coli* BL21(DE3) was inoculated into 5 ml of LB broth that contained ampicillin (100 µg/ml) and was grown at 37°C overnight. An overnight culture was diluted to a ratio of 1:50 using fresh medium and was grown at 37°C with vigorous shaking until the A600 reached approximately 0.8 before adding isopropyl-β-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM for the induction of protein. The cells were further grown at 37°C for 3 hrs and were harvested by centrifugation at 13,000 rpm for 5 min. The cell pellet was lysed and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Coomassie-stained SDS-PAGE showed that this recombinant protein had a molecular weight of 27 kDa. The Western blot analysis showed that the recombinant protein was

recognized by sera from individuals with patient malaria (Fig. 3).

The pET-Pv30, which contained a six-histidine residue at the C-terminal end, was purified from the culture supernatant by adsorption onto a nickel-nitrilotriacetic acid (Ni-NTA) column and was subsequently eluted with a phosphate buffer (pH 7.4). The eluted products were concentrated and used as an antigen in ELISA. The final products were confirmed by Western blotting using positive and negative human sera (Fig. 3).

Western blotting

The recombinant Pv30 protein was separated by 12% SDS-PAGE and was transferred to a nitrocellulose membrane. The membrane was blocked overnight with 5% non-fat milk in PBS at 4°C, was washed with PBS containing 0.05% Tween-20, was incubated with normal or malaria patient serum (1:100 dilution), and was washed and incubated with goat anti-human IgG and IgM that were

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PvMSP1: YESLVSKVNTYTDNLKQVINNCQLEKKEAEITVKKLQDYNKMDKLEFYKSEKKNNEVKS
PfMSP1: YNNLWNKIDDEYLINLAKINDCNVEKDEAHVKITKLSDLKAIIDDKIDLFRNHNDFEAIKK
PyMSP1: YETVIGQAEKSEBELQNRLLINYKNEKAEFEILTNNLEKYIQIDEKLDDEFVEHAENKHKHIA
PvMSP1: S-----GLLEKIMKSKLIKENESEKELLSQLLNVO-TQLLTMSSEHTCIDT-NVEDNAA
PfMSP1: LINDDTKKDMLGKLLSTGLV-QNFPNTIISKLIIEGK-FQDMLNISQHQCVKK-QCEPNSG
PyMSP1: S-----IALNNINKSGLVGESEKIKLAKMLNMDGMDLLGVDFKHVCVDTTRDIPKNAK
PvMSP1: CYRYLDGMEWRCLLTFKE-EGGKCVFPGSNVTCKDINNGGCAPBAECK----MTDS----N
PfMSP1: CFRHLDEREECKCLINYKQ-EGDKCVENPNPTCNEINNGCCDADAKC----TEEDSGSNGK
PyMSP1: CFRDDNGTEEWRCILGYKKGEGNTCVENNNPTCDINNGCCDPTASCONAESTENS----K

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Fig. 2. Deduced amino acid sequences of MSP1 from various malaria. PvMSP1; *P. vivax* (M60807), PfMSP1; *P. falciparum* (X03831), PyMSP1; *P. Yoelii* (J04668).

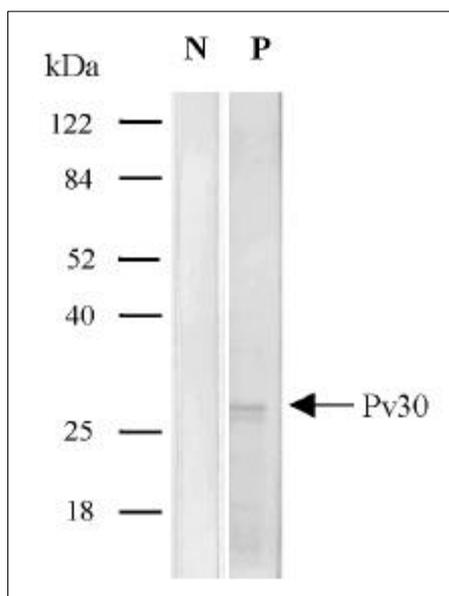


Fig. 3. Western blot analysis of recombinant Pv30. The recombinant Pv30 proteins were subjected to 12% SDS-PAGE and were transferred to a nitrocellulose membrane for Western blotting with a malaria patient serum (P) or a normal serum (N) described in MATERIALS AND METHODS. The arrow indicates the recombinant Pv30 reacting with a malaria patient serum.

conjugated with horseradish peroxidase (1:5,000 dilution). Color reactions were observed through the addition of diaminobenzidine (Sigma, St Louis, MO, USA).

ELISA

Purified Pv30 (0.1 μ g of antigen) (Humanbio Kit, Iksan, Korea) in phosphate buffered saline (PBS) was coated onto 96-well microtiter plates (Costar, Corning, NY) at 4°C overnight. The plates were washed with PBS containing 0.05% Tween20 (PBST), were blocked with 1% bovine serum albumin (Sigma) in PBST at 37°C for 1 hr, and then, were washed with PBST to remove unbound proteins. Test sera, which were diluted to a ratio of 1:20 using PBST, were added to the wells and were incubated at 37°C for 1 hr. Plates were then washed with PBST and incubated with horseradish peroxidase-conjugated anti-human IgG and IgM antibodies (Sigma) for 1 hr. Bound antibodies were detected following incubation with 100 μ l of the 3, 3', 5, 5'-tetramethylbenzidine peroxidase substrate (Kirkegaard & Perry Laboratories), which

was done using a microplate reader at a wavelength of 450 nm.

Results are shown as the mean \pm SD (standard deviations) from the triplicate experiments. Every anti-Pv30 ELISA experiment included at least three known negative samples and two positive patient samples.

RESULTS

Comparison of Pv30 with MSP-1 from several species of Plasmodia

The deduced amino acid sequence of Pv30 (GenBank Accession number: AF513000) was compared to the MSP1 of *P. falciparum*¹⁰ and *P. yoelii*^{11,12} (Fig. 2). The degree of sequence similarity of Pv30 was 42% and 41%, respectively.

Antibody levels of malaria patients

Western blotting of a malaria patient serum using recombinant Pv30 antigen revealed that a patient serum reacted with the recombinant Pv30 (Fig. 3). However, no reaction was shown in the normal serum. To assess the usefulness of the Pv30 antigen in the ELISA, a cut-off value of 0.09 for anti-Pv30 antibody levels was used in detecting malaria in Korea and Thailand. Because the secondary antibody recognized both IgG and IgM, the results are given as one value. As shown in Fig. 4A, when the mean + 2 SD of the antibody levels from 89 healthy Korean samples was regarded as the cut-off value (0.09) for positive reactions, the sensitivity of the test was 98.8% (88 of 89), and the specificity was 96.6% (86 of 89). The sensitivities of *P. vivax*, *P. falciparum*, and *P. malariae* were 96.3% (26/27), 90.6% (29/32), and 100% (6/6), respectively, and the specificity was 63.5% (40/63) in the Thailand samples (Fig. 4B).

Changes in antibody levels after treatment

Sera from patients from the endemic areas of Thailand included acute and convalescent malaria samples. The levels of antibody to Pv30 one month after malaria treatment were decreased in individuals who were infected with *P. vivax*

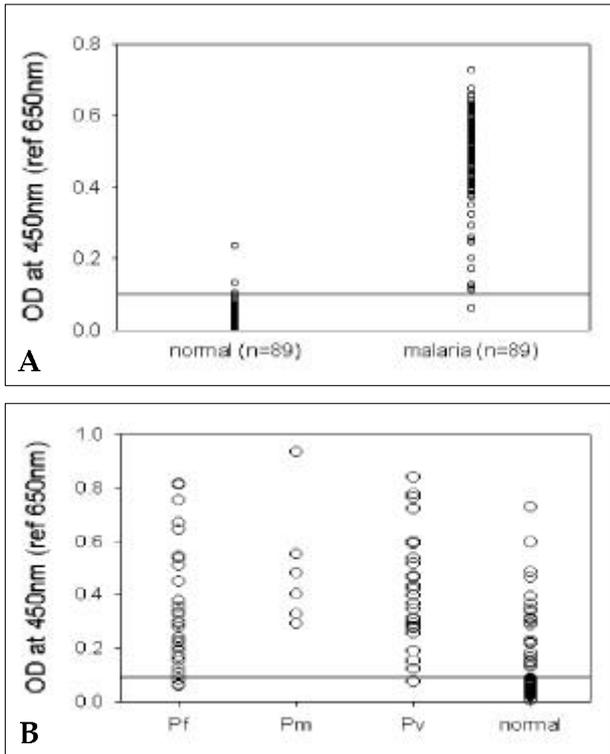


Fig. 4. Anti-Pv30 antibody levels of patients in each group. Results are shown for malaria patients and the healthy group in Korea (A) and Thailand (B, Pf; *P. falciparum*, Pm; *P. malariae*; Pv; *P. vivax*). The cut-off value for seropositivity was determined as the mean + 2 SD (0.09) of the antibody level from 89 healthy Korean samples.

(88.9%; 8/9), *P. falciparum* (76.9%; 20/26), and *P. malariae* (100%; 2/2), respectively (Fig. 5).

Relationship between number of malaria and anti-Pv30 antibody level

Results to determine whether there was a correlation between parasitemia and patient antibody levels to PV30 showed that there was no correlation (data not shown).

DISCUSSION

Clinical diagnosis of malaria infections requires microscopic observation of parasites on a Giemsa stained blood smear. Diagnosis of malaria by this method can be problematic since it requires up to 60 min of preparation time, is labor-intensive, and requires considerable expertise for its interpretation, particularly at low levels of parasitemia.

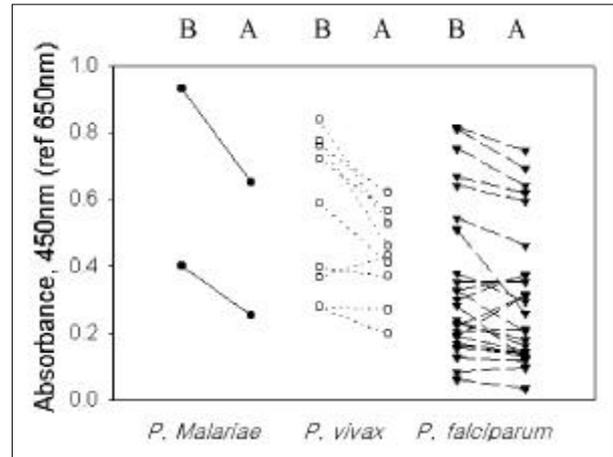


Fig. 5. Change in antibody levels (IgG and IgM) to Pv30 during infection or one month after treatment in samples of Thailand sera. B and A represent before and after treatment, respectively.

Antibody test kits of malaria have a diagnostic problem because it has a long persistent of antibody titer.^{13,14} Previous reports have implicated that the MSP1 of malaria is a good antigen for the diagnosis of malaria patients because it can elicit a significant protective immune response.¹⁵⁻¹⁷

In this study, we overexpressed a protein (Pv30) isolated from a *P. vivax* cDNA library and found that the purified recombinant protein was recognized by sera from individuals with patient malaria using the Western blot analysis. To assess the use of this protein for diagnostic analysis, which uses ELISA, we measured the levels of IgM and IgG antibodies from sera of 154 patients with malaria, 89 healthy individuals from areas of nonendemicity in Korea, and 63 healthy individuals from areas of endemicity in Thailand with recombinant Pv30. The ELISA results showed that the sensitivity and specificity of this test using Pv30 were 99% and 97%, respectively, for the sera samples from Korea. Although this assay was highly sensitive for other malaria serum samples from Thailand, the specificity was low, which suggests that the initial serum from a Korean patient that was used to isolate the Pv30 clone, may have recognized a clone that was particular to that strain of malaria. So, further screening of our library with sera from patients infected with other malaria species may give us additional clones that could be used to increase the speci-

ficity of the assay. In this study, the levels and frequency of antibodies against Pv30 antigen were similar in the malaria patients from the nonendemic areas of Korea to those from the endemic areas of Thailand.

To determine whether there was a significant decrease in antibody titers in patients treated for malaria, blood samples from treated patients were tested. We found that individuals with malaria infection before treatment had significantly higher antibody titers to the Pv30 compared to individuals treated one month after, suggesting that the antibody levels decreased rapidly after treatment. However, the antibody levels in almost all individuals after treatment were still positive to Pv30, which explains in part, the low specificity of healthy Thailand samples (Fig. 4B) and, consistency with previous reports.^{13,14} This also suggests that although Pv30 is useful for the diagnosis of malaria patients in a nonendemic area, it is of a lesser diagnostic value in an endemic area.

Comparison of the MSP1 amino acid sequences of *P. vivax*, *P. falciparum*, and *P. yoelii* revealed that this protein shows regions of strong similarity among all the species. Thus, it is not surprising that cross-reactivity with sera from patients infected with the different species of malaria was observed in the ELISA.

In conclusion, Pv30 is an adequate antigen for the serodiagnosis of malaria patients, since sufficient amounts of antibody are induced in almost all individuals with the malaria parasite infection at an early stage of symptom development.

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