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DNA Sequencing and Expression of the Circumsporozoite Protein of *Plasmodium vivax* Korean Isolate in *Escherichia coli*

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To obtain the recombinant circumsporozoite (CS) protein for the diagnosis of patients and seroepidemiology of *Plasmodium vivax* malaria which have been prevalent in northern part of Kyonggido, the CS protein gene was amplified by the polymerase chain reaction (PCR) from genomic DNA of the Korean vivax malaria patient. The gene consists of 1,123 nucleotides except signal peptide sequences and had an uninterrupted reading frame encoding a protein of 374 amino acids with a central region of 20 tandem repeats of the nonapeptide. The CS protein gene was expressed in *Escherichia coli* and purified, the molecular weight of recombinant CS protein was about 44 kDa (monomer) under denaturing purification and about 65 kDa (dimer) under native purification by SDS-PAGE. The purified recombinant CS protein which has antigenicity to malaria patients in Western blot analysis and Enzyme-linked immunosorbent assay, reacted only with the serum of *P. vivax* (PV210) infected malaria patients with no cross reaction to the *P. falciparum* malaria patient. The recombinant CS protein purified in this study will serve as a useful antigen to support the diagnosis of malaria patients and seroepidemiology.

Key words: *Plasmodium vivax*, DNA sequencing, circumsporozoite protein, PV210, expression, purification

Among many tropical diseases, malaria is prevalent worldwide and remains a major health problem. According to the World Health Organization (WHO) (1994), one third of the world population live in malaria endemic regions. Vivax malaria has been prevalent throughout the Republic of Korea (ROK) for many years. However, as a result of a national malaria eradication program with help from the WHO, the incidence of vivax malaria rapidly decreased (22, 23). Vivax malaria was believed to be eradicated in the ROK since the late 1970s, although two sporadic cases were detected in the 1980s (26).

In 1993, a vivax malaria case was detected among ROK army soldiers serving in the northern Kyonggido province (7). Subsequently, Cho *et al.* (1994) reported two civilian malaria patients infected with vivax malaria. Thereafter, a total of 2,198 patients were detected from 1994 to 1997 near the demilitarized zone (DMZ) centering around Paju-shi, Yonchon-gun, Chorwon-gun, Kimpo-shi, Kangwha-gun, Koyang-shi, and Tongduchon-shi (18).

It is expected that the outbreak of malaria in South Korea will continue in the future. Therefore, a method of diagnosis which can analyze many malaria samples at once is urgently needed. Microscopic examination, DNA hybridization (3, 14, 20), and Polymerase Chain Reaction (PCR)(4, 5, 15, 17, 27, 28) for parasite detection in man and enzyme-linked immunosorbent assay (ELISA)(31, 32) and indirect immunoflourescent antibody test (IFAT)(6, 9, 10, 11, 30) have been used in epidemic areas as serological tests. In Korea, the methods described above, except ELISA, have been established. ELISA has the advantage of being able to test many samples at once and this data can be compared with those of other laboratories. ELISA is a useful epidemiological tool to assess and monitor malaria endemicity, the level of malaria transmission, and the impact of malaria control measures (13). This study was conducted in order to obtain antigens for use in ELISA. First, we determined the expression of the CS protein, which is the major surface protein of the malaria sporozoite. The central one-third of the molecule consists of a repetitive amino acid sequence which contains the protein's immunodominant B cell

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epitopes (2, 21, 33) and T-cell epitopes that reside in the non-repeating portion of the protein (12).

Materials and Methods

Origin of *Plasmodium vivax* parasite

Microscopically confined *P. vivax*-infected blood was collected directly from a 16 year old individual living in Yonchon-gun, Kyonggi-do in 1996.

Bacterial strains, plasmids and media

Plasmid construction for the CS protein gene cloning was done in *E. coli* strain, INVαF', F' *end*Al *rec*A1 *hsd*R17(r_k^-, m_k^-) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 ø80*lac*ZΔM15Δ(*lac*ZYA*arg*F) U169λ$^-$ (Invitrogen Co.) and pCR^{TM}2.1 vector (Invitrogen Co.) was used for cloning the PCR product of the CS protein gene. Protein expression was done in *E. coli* strain M15, Nal^s Str^s Rif^s Lac$^-$ Ara$^-$ Gal$^-$ Mtl$^-$ F$^-$ RecA$^+$ Uvr$^+$ Lon$^+$ (Qiagen Co.) and pQE30 expression vector (Qiagen Co.) was used for expression of the CS protein. All *E. coli* strains were routinely grown in Luria-Bertani broth (LB broth: 1% tryptone, 0.5% yeast extract, 0.5% sodium chloride). Ampicillin (100 μg/ml) and/or kanamycin (25 μg/ml) were added to the LB media when necessary.

Primers

For sequencing and PCR of the CS protein gene, three sets of forward and reverse primers were synthesized according to Mann *et al.* (1994). The first set of primers were 41- and 42-mers: 5' tgtaaaacgacggccagtTCCCCACGCACTGCGGGCACAAT 3' (F1) and 5' caggaaacagctatgaccTTAATATGCACCGTGGAGGACGCC 3' (R1) and include M13 forward and reverse primer sequences at their 5' ends respectively. The second set of primers were nested to the first set. These also contained the M13 sequences at their 5' ends: 5' tgtaaaacgacggccagtAAAAAGGATGGAAAGAAAG 3' (37-mers, F2) and 5' caggaaacagctatgaccGACTTTTCATTTGGGGCA 3' (36-mers, R2). The third set of primers were 29- and 30-mers: 5' ggatccTCCCCACGCACTGCGGGCACAAT 3' (Fex1) and 5' ttcgaaTTAATATGCACCGTGGAGGACGCC 3' (Rex1), including *Bam*HI and *Hin*dIII restriction enzyme sites in their 5' ends.

Manipulation of plasmid DNA

Plasmid DNA was prepared from an overnight culture of *E. coli* by a Qiagen plasmid purification kit (Qiagen, Co.). Various restriction endonucleases and T4 DNA ligase were purchased from Promega Co. and TaKaRa Co. Enzyme reactions were performed according to the supplier's instructions. Agarose gel electrophoresis of DNA was performed using the procedure described by Sambrook *et al.* (25).

Preparation of CS protein gene

To prepare the CS protein gene, *P. vivax* genomic DNA was extracted from the whole blood of a malaria patient using QIAamp Blood Kit (Qiagen Co.). PCRs were performed with an AccuPower^{TM} PCR Premix (Bioneer Co.), 50 ng of purified genomic DNA, 40 pmoles of F1 (or Fex1) and R1 (or Rex1) primers each, and total volume adjusted to 20 μl with distilled water. The cycling conditions were as follows: denaturation at 94°C for 5 min, 35 cycles of 1 min at 94°C, 1 min at 49°C, 2 min at 72°C and finally incubation at 72°C for 5 min. For the second amplification, a sample of 2-5 μl of the first PCR products were transferred to a new AccuPower^{TM} PCR Premix tube containing F2 and R2 primers. The PCR products were confirmed under a UV transilluminator and purified with a Gene Clean Up Kit (Kisan Co.). The purified PCR products were ligated with a pCR2.1^{TM} cloning vector (Invitrogen Co.) and then transformed into *E. coli* INVαF' according to the procedures of Invitrogen Co.

DNA sequencing and analysis

The CS protein gene sequence of the Korean isolate was determined using an ABI PRISM^{TM} dye terminator cycle sequencing ready reaction kit FS (Perkin Elmer Co.) according to the supplier's manual. Computer analysis of the sequence information for sequence alignment was performed with DNASIS from Hitachi Co. USA, respectively (24).

Construction of CS protein expression vector

For the expression of the CS protein in *E. coli*, the CS protein gene fragment was amplified from the pCR2.1^{TM} cloning vector containing the PCR product of the CS protein gene. PCR amplification was performed with the primers, Fex1 and Rex1. Fex1 is homologous to the sequence coding for the signal peptide and the first 4 amino acids of the mature protein as predicted by sequence analysis. The complement Rex1 is homologous to the region 21 bases from the 3' region to the stop codon (1). Therefore these primers amplify the whole gene corresponding to the mature CS protein. The PCR product was treated with *Bam*HI and *Hin*dIII, and integrated into the cleavage sites for the restriction enzymes *Bam*HI and *Hin*dIII of pQE30 (Qiagen Co.). The resulting plasmid was subsequently used for the expression of the CS sequence-$(His)_6$ fusion protein in *E. coli* M15 cells harboring the recombinant plasmid pCS210 (Fig. 3). The transformants were confirmed by *Eco*RI/*Hin*dIII digestion and PCR with the same primer set as described above.

Expression and purification of recombinant CS protein

Previously described methods were used for expression of the *E. coli* derived CS protein (16, 27). Purification of the CS fusion proteins was carried out using immobilized metal ion affinity chromatography (27). Purification was done under native and denaturing conditions according to supplier's instructions (Qiagen Co.)

SDS-PAGE analysis

Proteins in each purification step were analysed on 4-20% Tris-Glycine SDS-PAGE (Novex Co.) at 30 mA for 90 min. The molecular weight standards were phosphorylase B (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa) (Bio-Rad Co.).

Western blot analysis

Recombinant CS protein was separated on 4-20% Tris-Glycine SDS-PAGE gels and then transferred to a nitrocellulose membrane (29). After the transfer, the membrane was cut into strips and blocked for non-specific binding with 3% skim milk for 12 h at 4°C and then washed in phosphate buffered saline (PBS) with 0.05% Tween 20 for 3 × 10 min. The strips were allowed to react with primary antibody (diluted 1:100, v/v) for 4 h, and then washed using the same procedure described above. After adding diluted secondary antibody (1:1,000, v/v), peroxidase conjugated anti-human IgG from goat (Sigma Co.) was incubated for 3 h at room temperature. For color development, 0.2% diaminobenzidine and 0.02% H_2O_2/PBS, was applied to each well.

Enzyme-linked immunosorbent assay

For the *P. vivax* ELISA, 96-well Immunon plates (Dynatech Co.) were coated with recombinant CS protein as antigen, blocked with 1% BSA, and incubated with sera. Bound immunoglobulin was detected using peroxidase labeled anti-human IgG (gamma) and ABTS (2, 2-azino-di-[3-ethylbenzthiazoline sulfonate]) as substrate.

Results

Construction of a pCSHW

Amplified CS protein gene by PCR from genomic DNA was analysed on a 1.5% agarose gel (Fig. 1). The amplification of the CS protein gene yielded an approximately 1,300 bp DNA fragment, respec-

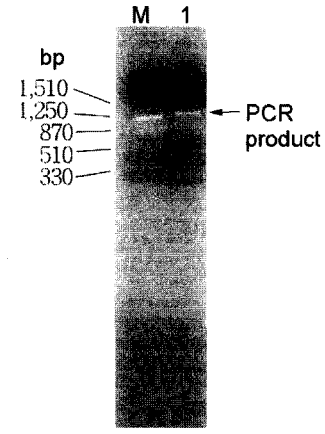

Fig. 1. Agarose gel electrophoresis of the PCR products of CS protein gene from *Plasmodium vivax* Korean isolate. Lane M, marker KBII.

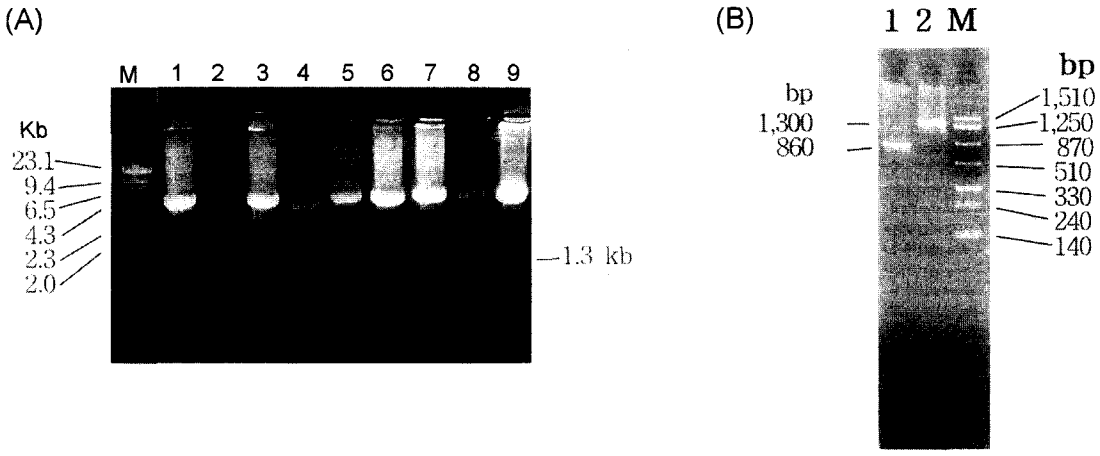

Fig. 2. (A) The detection of cloned pCR™2.1 vector containing the PCR product of CS protein gene by digestion with *Eco*RI. Lanes 1-9, PCR product inserted clones, Lane M, lambda/*Hin*dIII DNA size marker. (B) The confirmation of cloned pCR™ 2.1 containing the PCR product of the CS protein gene by nested PCR. Lane 1, second PCR product with Fex2/Rex2 primers; Lane 2, first PCR product with Fex1/Rex1 primers; Lane M, marker KBII.

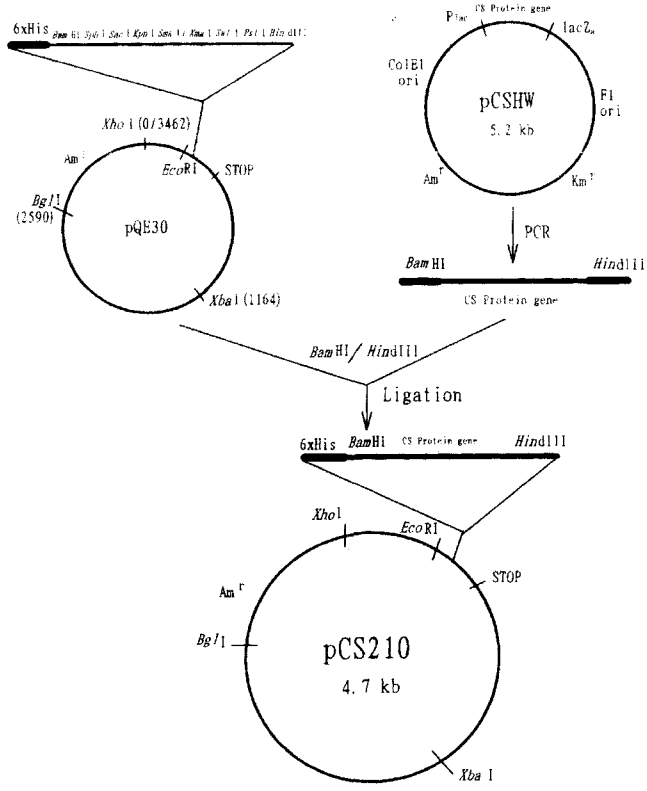

Fig. 3. Strategy for the construction of recombinant plasmid (pCS210) containing CS protein gene.

tively, after purifying the PCR product ligated with a pCR 2.1™ cloning vector (3.9 kb). The transformants confirmed the PCR insert by *Eco*RI digestion (Fig. 2, A) and by nested PCR (Fig. 2, B). The plasmid containing the PCR product was named pCSHW (Fig. 3, 5.2 kb).

DNA sequencing

As the results of DNA sequencing, the cloned CS gene was shown to be 1,123 bp except the signal peptide, and consisted of 374 amino acids similar to the NK (North Korean) strain (Fig. 4). As Anort *et al.* (1988) described the CS protein gene sequence of the NK strain, the Korean isolate also has two copies of a repeat sequence Ala-Gly-Gly-Asn (AGGN) at the end of a 3'-terminal repeat. This Ala-Gly-Gly-Asn repeat is a common feature in the strain-specific tandem repeats of *P. cynomolgi* CS proteins that are closely related to the *P. vivax* and *P. knowesi* CS proteins. In fact, the tandem repeats of NIH and Mulligan strains of *P. cynomolgi* maintain 44 repeats of the sequence Ala-Gly-Gly-Asn. Before the Ala-Gly-Gly-Asn repeat of Korean isolate CS protein gene is a sequence of 9 amino acids, Asn-Ala-Ala-Asn-Lys-Lys-Ala-Glu-Asp (NAANKKAED) which is similar to the NK strain. In addition, it has 20 nonapeptide repeats between region I and II. The amino acid sequences of non-

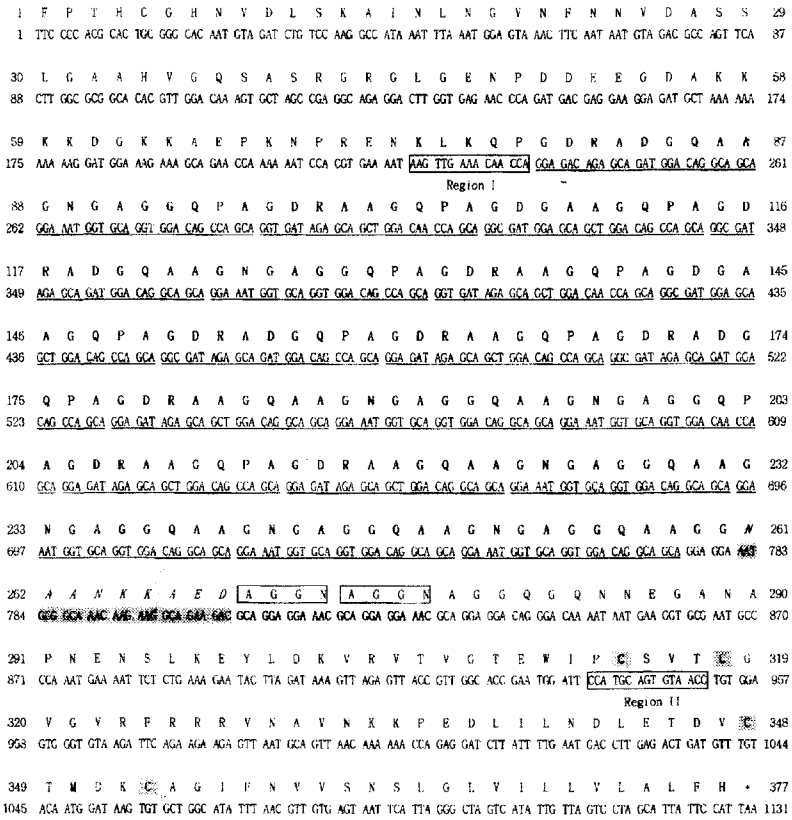

Fig. 4. The nucleotide sequence and deduced amino acid sequence of the CS protein of *P. vivax* from Korean isolate.

Table 1. The comparison of amino acid sequences and their positions of nonapeptide repeat in CS protein between Korean isolate and NK strain

Amino acid sequence	Korean isolate	NK strain
GDRADGQAA	1, 5	3
GNGAGGQPA	2, 6, **14**	4, **14**
GDRAAGQPA	3, 7, **15**	5, 8, 10, **15**, 16, 17, 18
GDGAAGQPA	4, 8, 10	6
GDRADGQPA	9, 11	1, 2, 7, **9, 11**
GDRAAGQAA	**12**, 16	**12**, 19
GNGAGGQAA	**13**, 17, 18, 19, **20**	**13, 20**

apeptide repeats and their positions are shown in Table 1, 7 nonapeptides have exactly the same positions and amino acid sequences compared with the NK strain. The DNA sequence of Korean isolate has shown 89.4% homology with the NK strain, 88.6% with the CH-4 strain, 84.3% with the SOL1 strain, and 64.3% with the Ph-46 strain. Compared with the NK strain, various amino acid variations were observed in the repeat region of the Korean isolate (Fig. 5).

Expression of CS protein in *E. coli*

To generate the expression plasmids, the CS protein gene was amplified from pCSHW by PCR, and subcloned into expression vector pQE30 by digestion with *Bam*HI and *Hind*III as shown in Fig. 3. The resultant plasmid pCS210 contained the CS protein gene fused to the $(His)_6$-tag. The recombinant plasmid pCS210 was transferred into *E. coli* M15. 1 mM IPTG was added to cultures of *E. coli* M15 (pCS210) logarithmically grown on LB plus ampicillin and kanamycin liquid to induce the CS protein. A new band was shown after the addition of IPTG to *E. coli* M15 (pCS210) (Fig. 6). The molecular weight of purified CS protein (about 65 kDa) under native condition is approximately twice the expected molecular weight (38.1 kDa) of the CS protein open reading frame (Fig. 7). However, the CS protein purified under denaturing

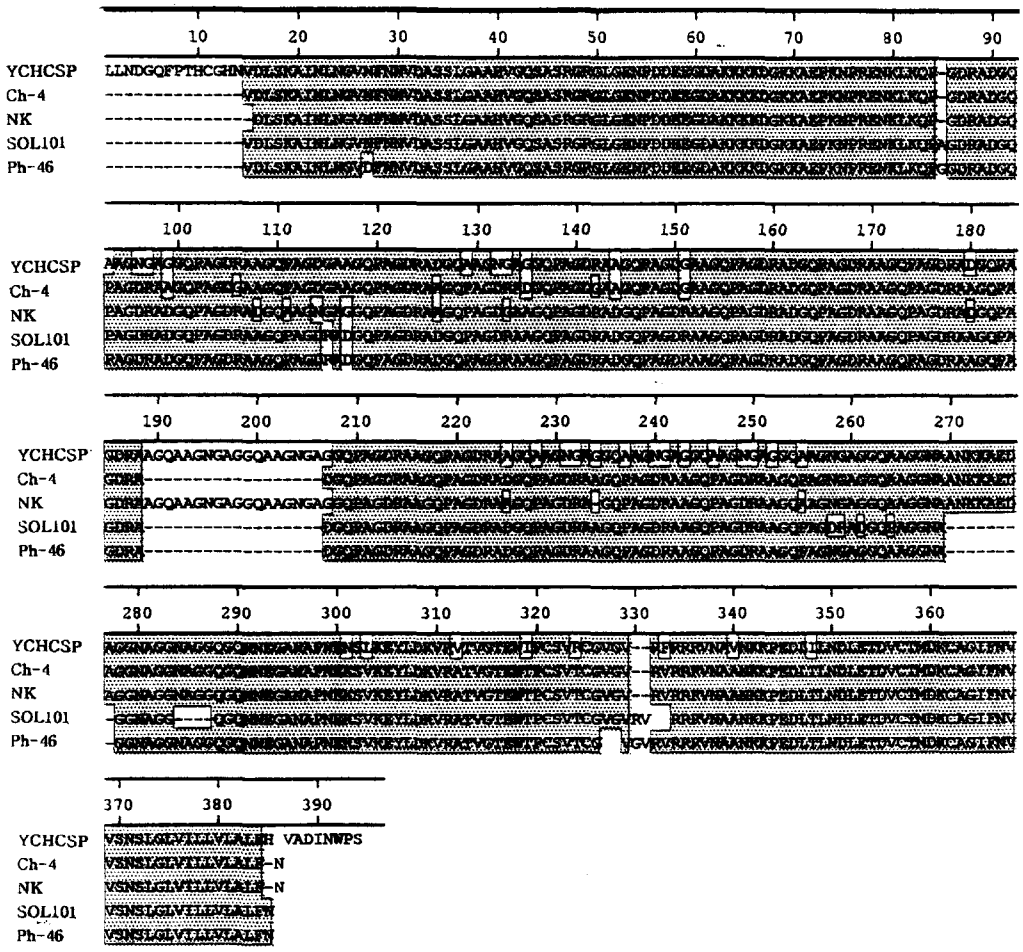

Fig. 5. Comparisons of amino acid sequences of several strains of *P. vivax* CS protein. Korean isolate (YCHCSP), NK (accession; M20670), Ch-4 (accession; U08978), SOL101 (accession; U089831), and Ph-46 (accession; U08980) strains. All amino acid sequences were obtained from GeneBank BLAST (http://WWW.ncbi.nlm.nih.gov).

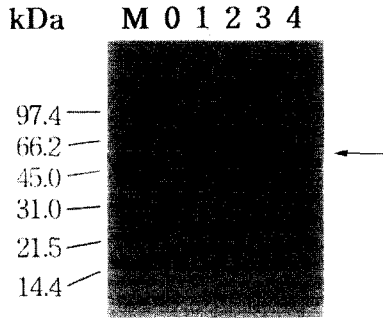

Fig. 6. Time course of expression of 6x His tagged CS protein. The proteins were collected 0, 1, 2, 3, and 4 h after addition of 1 mM IPTG.

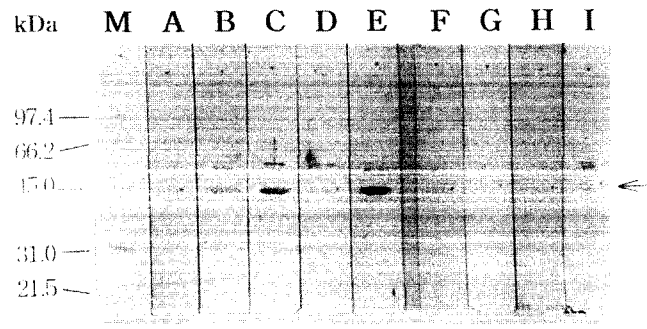

Fig. 8. Western blot analysis of malaria patients and normal persons. A: Sera of malaria patient collected 15 days after onset, B: 26 days after onset, C: 30 days after onset, D: 7 days after onset, E: 25 days after onset, F-I: normal persons.

condition (about 44 kDa) has similar molecular weight to that of the deduced CS protein (Fig. 7B). This may be due to four cysteines on the 3' end that are thought to contribute to two disulfide bonds (Fig. 4).

Antigenicity of recombinant CS protein

The serum of malaria patients specifically recognized recombinant CS protein (Fig. 8). However, the serum of normal persons and a patient who fell ill after a long incubation period did not react with the recombinant CS protein (Fig. 9, A). The intensity of reactions depended upon the duration after onset of symptoms, respectively. In ELISA, the intensity of the reactions depended upon the concentrations of purified recombinant CS protein (Fig. 9A). This recombinant CS protein reacted only with the antisera of *P. vivax* PV210 and with the mixed infection of *P. vivax* PV210 and *P. falciparum* (Fig. 9B).

Discussion

There has been confusion about the origin of vivax malaria when it re-emerged in 1993 (7). The cause of malaria transmission is important to determine because malaria is a contagious disease. In the beginning of malaria transmission, the composition of malaria patients was mainly army soldiers who served or had served near the demilitarized zone (DMZ) and inhabitants who live near the DMZ (18). Most scientists agree with the view that vivax malaria might come from North Korea based on the epidemiological analysis of the geographical distribution of the patients. However, there is no direct knowledge about it because malaria prevalence in North Korea is unknown at present. We determined genetic analysis of the vivax malaria which is prevalent in the northern part of Kyonggi-do, South

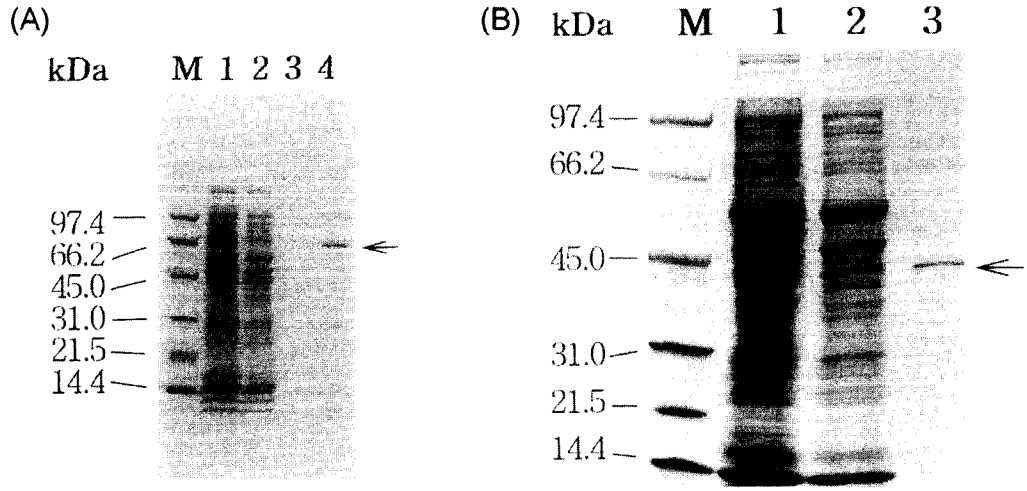

Fig. 7. (A) SDS-PAGE patterns of CS proteins purified under native conditons using Ni-NTA agarose resin. Lane 1, crude cell lysate; Lane 2, flow-through; Lane 3, wash with 20 mM imidazole; Lane 4, elute with 250 mM imidazole. (B) SDS-PAGE patterns of CS protein purified under denaturation condition using Ni-NTA agarose resin. Lane 1, crude cell lysate; Lane 2, flow-through; Lane 3, elute with buffer C (pH 6.3) containing 100 mM EDTA.

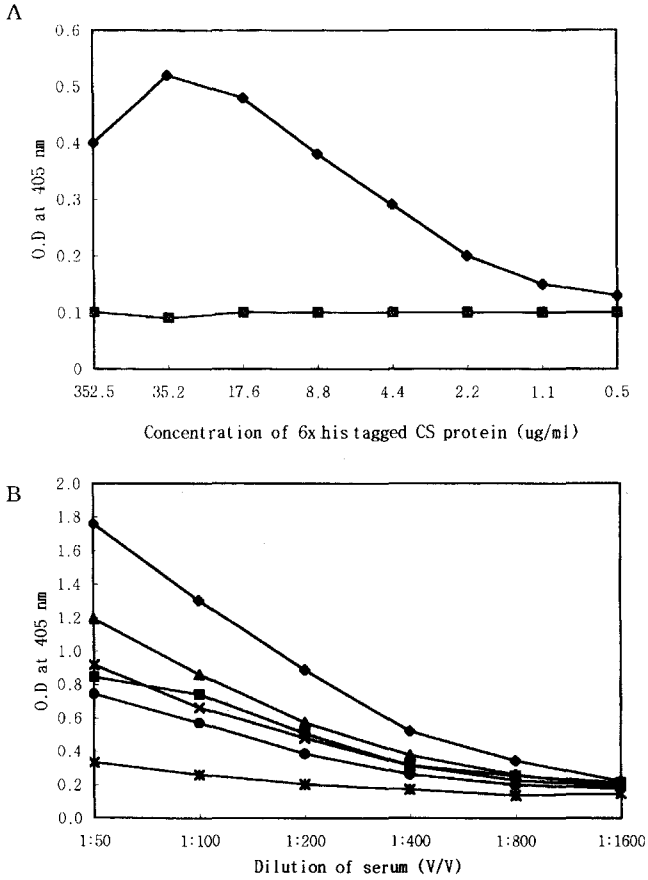

Fig. 9. (A) The antigenicity of 6x his tagged CS protein according to its various concentrations. ◆; malaria patient infected by *P. vivax* PV210, ■; malaria patient with long incubation period. (B) The antigenicity according to various serum dilution. ◆, ■, ▲, X; malaria patients infected by *P. vivax*, ● ; malaria patient mixed infection by *P. vivax* PV210 and *P. falciparum*, *; malaria patient infected by *P. falciparum*.

Korea. We analysed the CS protein gene which was well studied in many countries and their geographical variations according to the origin of its countries. The possible origin of vivax malaria which is prevalent in South Korea could be determined if the CS protein gene of the isolate was compared with those of other countries. Thus, we collected malaria parasites from a 16-year old student living in Yonchon-gun, Kyonggi-do, in 1996. He has not traveled abroad and has lived there from birth until that time therefore, the patient was regarded as an indigenous case. The results of DNA sequencing of the CS protein gene suggest high homology with the North Korean strain and has a lot of common features which can be seen in other strains of *P. vivax*. The amino acid sequence of the isolate was somewhat different from the North Korean strain in repeat region, its order and composition of the 9 amino acid repeat (Table 1). It is well known that the repeat region has B-cell epitopes, thus if an immunological diagnosis method using this protein was introduced in Korea, its amino acid sequence variation of the CS protein differs from other strains.

Until now, the indirect immunofluorescent assay technique has been used in Korea NIH as an immunological diagnosis method in addition to microscopic diagnosis. Although it has high specificity and sensitivity, it is difficult to compare its data with those of other laboratories because the methods of antigen preparation were not the same in each case and the interpretation of results was highly subjective. In order to avoid any errors reulting from the above technique, we decided to use ELISA as an immunological method. The CS protein gene, represented in Fig. 4, was cloned into expression vector (pQE30, Qiagen Co.) and transfomed into *E.coli* M15. The CS protein was induced with 1 mM IPTG, expressed and purified with an affinity column. The molecular weight of purified CS protein is approximately 44 kDa (monomeric form), and 65 kDa (dimeric form). Through Western blot analysis with this antigen, the positive serum could be differentiated from the negative serum. Also in ELISA, the CS protein did not react with *P. falciparum*, due to its high specificity for *P. vivax* patients or with the sera of the vivax malaria patient who has a long incubation period. This might be due to the half life of immunogloblin G against the CS protein. Considering about 70% of malaria patients fall ill after long incubation periods, the purified CS protein might be a good antigen to detect asymptomatic persons who will progress into patients or just remain as the only source of infection. This will be explicitly discussed in another paper.

According to the aforementioned data, we could temporarily conclude that the vivax malaria has come from North Korea based on the DNA sequence and the amino acid sequence of the CS protein gene. The purified CS protein may play an important role to estimate the transmission rate of certain areas and periods, to detect asymptomatic patients, and to prevent the source of infection in Korea.

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