

ALVARO CASTELLANOS M. & CIA.
ABOGADOS
AGENTES DE LA PROPIEDAD INTELECTUAL
Carrera 9 No. 93-09
Bogotá-Colombia

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Jefe de la División de Nuevas Creaciones
Superintendencia de Industria y Comercio
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COMPLEMENTACION DE INFORMACION

Ref.: Expediente No. **01.011.966**

Solicitud de Patente de Invención para:

**"INMUNOENSAYO Y REACTIVO DIAGNOSTICO PARA LA
DETECCION DE LA MALARIA".**

Solicitante: **LG CHEMICAL LTD.**

Yo, **MARGARITA CASTELLANOS ABONDANO**, mayor de edad, identificada y domiciliada como aparece al pie de mi firma, me permito presentar la traducción simple en inglés de las copias certificadas de Corea de los documentos de prioridad No. **2000-7648** de Febrero 17 de 2000, No. **2000-7649** de Febrero 17, 2000, No. **2000-7650** de Febrero 17 de 2000, No. **2000-12172** de Marzo 10 de 2000 y No. **2000-45806** de Agosto 8 de 2000.

Atentamente solicito a Ud. se anexasen dichas traducciones al expediente de la referencia y se continúe con el trámite correspondiente a esta solicitud de Patente de Invención.

Del Señor Jefe,

Santa Fe de Bogotá, D.C.,
XCA/

6 ABR. 2001

Margarita Castellanos
Margarita Castellanos Abondano
C.C. No. 39.779.654 de Saqueen
T.P.A. No. 70819 del Cons. Sup. de la Judicatura
Carrera 9 No. 93 - 09 - Santa Fe de Bogotá, D.C.
Teléfonos : 610 74 80 - 610 74 60
Código No. 08

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ABSTRACT

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The present invention relates to a serological diagnostic method and diagnostic reagent for malaria. Particularly, the present invention relates to a method measuring
5 antibodies against malaria in blood by using Merozoit surface protein(hereinafter refer to "MSP") of *Plasmodium Vivax* of malarial Protozoa as a antigen. Also, the present invention relates to diagnostic reagent for malaria prepared by using the above MSP as antigens. The above serological diagnostic method according to the present invention are very effective in diagnosing malaria of which latent period is long, and
10 the probability of recurrence is high.

- Antígeno + Muestra
⊕ Antigen bonding conjugates (marcado)
que son ab anti-IgM
anti-IgG

REPRESENTATIVE FIGURE

FIGURE 1

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TITLE OF THE INVENTION

DIAGNOSIS FOR MALARIA AND REAGENT THEREFOR

BRIEF DESCRIPTION OF THE DRAWING

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Figure 1 shows the result of measuring IgG and IgM by using malaria-patient' blood plasma(●)and normal people' blood plasma(○)as samples.

DETAILED DESCRIPTION

25 PURPOSE OF THE INVENTION

FIELD OF THE INVENTION AND THE PRIOR ART THEREOF

The present invention relates to a serological diagnostic method and diagnostic reagent for malaria. Particularly, the present invention relates to a method

of measuring antibodies against malaria in blood by using Merozoit surface protein(hereinafter refer to "MSP") of *Plasmodium Vivax* of malarial Protozoa as antigens. Also, the present invention relates to diagnostic reagent for malaria prepared by using the above MSP as antigens.

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Malaria is a disease caused by malarial Protozoa that infects within human erythrocytes and is carried by mosquitoes. Billion people in the world reside in malaria-risk area and over 500 million people become infected by malaria each year. Malaria causes more than 2 million death each year. Malaria widely spreads all over the world, however, in some regions, malarial infection was eradicated or decreased since 1960's due to effective controls. However, recently, incidence of malaria increases all over the world again, due to increase of drug-resistant strain, increase in resistance to insecticide, abnormal weather such as El nino, etc.

Malaria can be categorized into African type, American type and Asian type. Each type differs in geographical distribution as well as characteristics in species and genetic inclination which Protozoa exhibit.

Life cycle of malaria is divided into Schizogony and Sporogony. Schizogony is the life cycle in human host, and Sporogony is the life cycle in mosquito host. Human is infected by sporozoites by the bite of infected mosquitoes. The sporozoites transfer to human liver through blood vessels, and they exist as a dormant state or multiply and develop into schizonts in hepatic cells. After a definite time, the schizonts enter the blood circulation, and multiply in full-scale. When the schizont matures, it ruptures and releases thousands of merozoites into the blood stream. Merozoites invade erythrocytes, and most merozoites go through another round of asexual reproduction, again forming schizonts. Some of the merozoites change into gametocytes. The gametocytes circulate in human bloodstream. When a mosquito bites the infected person, the mosquito sucks up gametocytes along with blood. Then, male gametocyte and female gametocyte in the gut of the mosquito fuse to

form zygote. The zygote, which develops in the gut wall as an oocyte, eventually gives rise to the infective sporozoite, which invades the salivary glands and stomach of the mosquito. The mosquito then can infect another human host.

Antibiotics such as chloroquine and primaquine have been used in treatment
5 of malaria. However, recently, malaria Protozoa have developed drug resistance to such antibiotics. Therefore, acquired drug resistance poses a serious difficulty on treatment of malaria.

A blood smear method has been used in the diagnosis of malaria most frequently. This method is a classical method wherein blood cells infected with
10 malaria Protozoa are stained and the stained sample is then observed through a microscope. However, this method takes a longer time, and technical education of the examiner is required to judge blood cells infected with malaria Protozoa. Recently, a diagnostic reagent detecting malarial antigens has been commercially available. However, the concurrent use of the diagnostic reagent and the blood
15 smear method is recommended because the sensitivity of the diagnostic reagent is low. Another method to examine genes of malaria Protozoa is Polymerase Chain Reaction method. This method has high sensitivity, however, is used only on laboratory scale.

In Korea, malaria has been known as Hakjil. From the 1960s, attempts to
20 eradicate this disease begun, and in the 1970s, cases of malaria were rarely reported. However, after a patient infected with malaria Protozoa was reported near DMZ in 1993, malaria begun to spread rapidly, and it was reported that the number of patients infected with malaria Protozoa was 3,800 in 1998.

Human malaria can be caused by one of four known parasites: *Plasmodium*
25 *vivax*, *Plasmodium falciparum*, *Plasmodium malariae* and *Plasmodium ovale*. Among them, malaria which occurs in Korea is caused by *Plasmodium vivax*. *Plasmodium vivax* is characterized in that diagnosis thereof is difficult because latent period is long, and number of Protozoa in blood is few. The imported diagnostic reagent for

malaria detects malarial antigens such as Lactate Dehydrogenase of malaria. However, the imported diagnostic reagent has a difficulty in diagnosing malaria of Korean type where latent period is long, and numbers of Protozoa and antigen in blood are few, because sensitivity of the diagnostic reagent is only 50%.

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TECHNICAL OBJECT OF THE INVENTION

It is the first object of the present invention to provide a method of measuring antibodies against malaria in blood, in order to diagnose malaria.

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It is the second object of the present invention to provide a diagnostic reagent for malaria, useful in diagnosing malaria.

CONSTITUTION OF THE INVENTION

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To achieve the above objects, the present invention provides a method of measuring antibodies in blood by using MSP that is common to subspecies of malarial Protozoa as a antigen. Also, the present invention relates to diagnostic reagent for malaria, comprising the above MSP.

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The present invention will hereinafter be described in more detail.

The present invention provides a method of measuring antibodies against malaria in blood, in order to diagnose malaria.

There are many subspecies of *Plasmodium Vivax* that generate malaria. These subspecies generate malaria without exception, if a humane body was infected. Because the latent period of malaria generated by the above subspecies is long and the number of Protozoa in blood is few, there are difficulties in diagnosing malaria by detecting antigen. Therefore, in the present invention, malaria is

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diagnosed by measuring antibodies generated in blood by infection of *Plasmodium Vivax*.

First, in the present invention, antigens are prepared and antibodies in blood are measured by antigen-antibody reaction. The above C-terminus of Merozoite Surface Protein of *Plasmodium Vivax* comprises amino acid sequences that are common to subspecies of *Plasmodium Vivax*, and especially comprises a polypeptide consisting of 106 amino acids (referred as "PV200C" hereafter) which exhibit 100% homology among subspecies of *Plasmodium Vivax*, as shown in SEQ. ID NO : 1.

Therefore, a part or whole of Merozoite Surface Protein of *Plasmodium Vivax* are appropriate as a antigen according to the present invention. The C-terminus of Merozoite Surface Protein of *Plasmodium Vivax* is preferable as an antigen according to the present invention, and PV200C polypeptide in the C-terminus of Merozoite Surface Protein of *Plasmodium Vivax* is more preferable as an antigen according to the present invention.

The present invention provides a diagnostic reagent for malaria, comprising a part or whole of Merozoite Surface Protein of *Plasmodium Vivax* as a antigen; labeled antigen conjugates consisting of markers such as horseradish peroxidase and IgM or IgG; and a substrate solution containing a color fixing agent.

A preferable example of the above antigen is a part or whole of PV200C that is common to subspecies of *Plasmodium Vivax*, as shown in SEQ. ID NO : 1. The PV200C polypeptide can be expressed in a large amount from recombinant yeast transformants or E. coli transformants. The present inventors prepared a yeast transformant and E.Coli transformant which express PV200C polypeptide, and deposited them in Korean Collection for Type Cultures of Korea Research Institute of Bioscience and Biotechnology on December 18, 1999 (Deposit No. KCTC 8977P, KCTC 8976P).

The present invention may utilize an enzyme such as horse radish peroxidase(HRP) and alkaline phosphatase or the like as the marker of the labeled antigen conjugate. In the above diagnostic reagent according to the present invention, the labeled antigen conjugate detects existence of malaria-specific antibodies by
5 binding to Fc (fragment crystallization) region of the malaria-specific antibodies in
which there is little variation. }

The substrate solution consists of a buffer solution and a color fixing agent which induces color development when reacted with the marker of the labeled antigen conjugate. The color fixing agent in the substrate solution is decomposed by the
10 marker of the labeled antigen conjugate to induce a color development. Then, existence of malaria-specific antibodies and amount of the antibodies are detected by measuring the absorbance.

The DNA fragment coding PV200C was inserted into a vector to prepare an
15 expression vector. PV200C was expressed from Yeast and E. coli transformed with the expression vector and is purified. Well-plate coated the above PV200C was prepared. When humane plasma was added to the above well-plate, antibodies in the humane plasma were bound to PV200C on the well-plate specifically. Then, the
labeled antigen conjugates were bounded to the above antibodies by recognizing Fc
20 of the antibodies. When the substrate solution was added, the color fixing agent in the substrate solution was decomposed by the marker of the labeled antigen conjugate to induce a color development. Then, existence of malaria-specific antibodies and amount of the antibodies were detected by measuring the absorbance. It was confirmed that IgG, IgM or IgG+IgM reacted with the above antigens in the case that
25 malaria patient' blood plasma sample was added to the antigens. As a result of the above reaction with 20 malaria patient' blood plasma samples, all the samples were judged to be positive. Therefore, it is confirmed that the number of antibodies against the above antigens is large and a diagnosis for malaria is possible by measuring the

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antibodies.

The invention will be further illustrated by the following examples. It will be apparent to those skilled in the art that these examples are given only to illustrate the present invention in more detail, but the invention is not limited to the examples given.

Example 1. Expression and purification of a surface antigen of malaria Protozoa from recombinant yeast

10 Example 1-1. Preparation of recombinant yeast and expression of a surface antigen of malaria Protozoa

The DNA coding PV200C is amplified using cDNA as template, reverstranscribed from RNA purified from malaria-positive blood. The DNA coding PV200C and pYES-2 vectors were cleavaged with restriction enzymes, HindIII and Xho I, respectively. Then, DNA coding PV200C and pYES-2 vector were linked by T4 ligase(Promega) to be a recombinant expression vector that is referred as "pYLJ-MSP". Yeast *Saccharomyces cerevisiae* INVSC1 was transformed with pYLJ-MSP according to Hinnen method(*Proc. Natl. Acad. Sci., U.S.A* 75, 1929-1933, 1978). The yeast transformants were deposited in Korean Collection for Type Cultures of Korea Research Institute of Bioscience and Biotechnology deposited on December 18, 1999 (Deposit No. KCTC 8977P)

The transformants pYLJ-MSP/*S. cerevisiae* INVSC1 were inoculated in YEPD medium containing 2% glucose at 37°C. Then, the culture medium was transferred to YEP medium containing 1% glucose and 1% galactose, respectively, and cultivated. The yeast transformants expressed PV200C polypeptide, exhausting glucose. The expressed PV200C polypeptide was secreted into into culture medium.

Example 1-2. Purification of a surface antigen of malarial Protozoa from yeast transformants.

In order to obtain the PV200C polypeptide secreted into culture medium, the biomass was removed by centrifuging the culture medium that was cultivated for 2 days. Then, only the fluid of culture medium was taken and concentrated by ultrafiltrating apparatus(Amicon, U.S.A.). For easily purifying the concentrated fluid, the surface proteins linked with cationic histidine residues were adsorbed on Probond column(Invitrogen)

The contaminants which were not adsorbed on column were removed by phosphate buffer containing 10mM imidazol, and then the surface proteins adsorbed on column were desorbed and separated by phosphate buffer containing 500mM imidazol. The surface proteins having approximately 90% or more of purity were separated by the above process. Then, the surface proteins having approximately 99% or more of high purity were obtained by concentrating the separated solution and performing Sephacryl S-200 gel filtration chromatography with the concentrate.

Example 2. Expression and purification of a surface antigen of malarial Protozoa from recombinant E, coli.

Example 2-1. Preparation of E, coli transformants and expression of a surface antigen of malarial Protozoa.

The amplified DNA fragments coding MSP PV200C prepared in the same way of Example 1, were purified with phenol/chloroform mixture to produce DNA fragments with high purity. The DNA fragment and vector, pET-19b (Novagen) were cleaved with NdeI and BamHI. Then, the DNA fragment was inserted into pET-19b

to prepare an expression vector, pELK-MSP

E. coli strain BL21(DE3)lysS was transformed with pELK-MSPs by the treatment with CaCl_2 . The transformants were deposited in Korean Collection for Type Cultures of Korea Research Institute of Bioscience and Biotechnology on
5 December 18, 1999 (Deposit No. KCTC 8976P)

The transformants pELK-MSP/strain BL21 (KCTC 8976P) were cultivated for 12 hours in LB medium containing 50 $\mu\text{g/ml}$ of chloramphenicol and 100 $\mu\text{g/ml}$ of ampiciline. Then, 50 ml of the resulting cultures were again inoculated in 1L of LB medium and were cultivated for about 2 hours at 37°C . When absorbance(OD 600)
10 of the cultures became 0.3, IPTG (Isopropyl thio- β -D-galactoside) was added to the medium to a final concentration of 0.2 mM. Then, the cultures were cultivated further for 7 hours.

Example 2-2. Purification of a surface antigen of malarial Protozoa from E. coli
15 **transformants.**

After the cultivation, only the cells were isolated by centrifugation. The obtained cells were suspended in 30 ml of Phosphate Buffered Saline(PBS). Then, E. Coli in the suspension was crushed with Sonicator(Branson, Sonifier 450) and
20 centrifuged at 5000 rpm. Because a part of expressed MSP proteins was in the supernatant and the remainder was in the pellet, both the supernatant and the pellet were completely suspended in 4M urea buffer(pH 7.5) where 4M urea was dissolved in PBS, and then were centrifuged, by which only the supernatant was collected. Because the overexpressed surface protein has His marker consisting of 10 histidines
25 in N-terminus thereof, the protein was adsorbed on Probond column(Invitrogen) for purification. The contaminants which were not adsorbed on column were removed repeatedly with a washing buffer in which 10mM imidazol and 0.1M NaCl were dissolved in PBS. Then, the surface proteins adsorbed on column were desorbed

and separated with an elution buffer in which 500mM imidazol and 0.1M NaCl were dissolved in PBS. It was confirmed that the surface proteins having approximately 90% or more of purity were separated by the above process. Then, it was confirmed that PV200C polypeptide having approximately 99% or more of high purity were
 5 obtained from recombinant E.Coli by concentrating the separated solution and performing Sephacryl S-200 gel filtration chromatography with the concentrate.

10 **Example 3. Preparation of well plate for measuring of the surface antibody**

The solution of recombinant surface antigens of malarial Protozoa prepared in Example 1 or 2 was diluted with 0.1M carbonate buffer (pH 9.5) to 0.5 µg/ml, and 100 µl of the solution was added to each well of 96-well plate for EIA. To adsorb the
 15 surface antigens on the well, the well plate was sealed tightly and left for 18 hours at 4 °C. Then, the surface antigens which were not adsorbed on the well were removed by adding 300 µl of phosphate buffer solution containing 5% general goat serum to each well and leaving it for 18 hours at 4 °C. After the solution that remained in the well was discarded, moisture was removed by leaving the well plate for 1 hour at
 20 ambient temperature. Then, the well plate was transferred to a hermetic container with a dehumidifying agent and stored in a refrigerator at 4 °C.

Example 4. Indirect enzyme immunoassay for antibodies against surface antigen of malaria

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To diagnose malaria by indirect enzyme immunoassay, the solid support prepared by coating 96-well plate(Nunc) with recombinant surface antigens prepared in Example 1 or 2 according to the method of Example 3, and the
labeled antigen conjugate consisting of HRP and anti-human IgM antibody or

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anti-human IgG antibody derived from goat were used.

100 μ l of a sample dilution solution containing 1.15 mg/ml NaOH, 0.2 mg/ml of KH_2PO_4 , 0.2 mg/ml KCl, 8 mg/ml NaCl, 300 μ l/ml bovine serum, 0.2 mg/ml thimerosal and the like was added to each well of 96-well plate(Nunc)
5 coated with recombinant surface antigens. Then, 10 μ l of antibody-negative plasma sample or 10 μ l of antibody-positive plasma sample was added to the wells, and the resulting mixtures were well mixed. The well plate was incubated for 60 minutes in a reactor at 37 °C.

After the reaction terminated, the well plate was washed 5 times with
10 300 μ l of a phosphate buffer solution containing 0.05% Tween 20. Anti-human IgG antibody(Gappel) or anti-human IgM antibody(Bethyl) derived from goat to
which HRP was conjugated was diluted to 1/40,000 with a dilution solution containing 10mM Tris, 0.5M NaCl, 1mM CaCl_2 , 0.5% glycerol, 0.2 ml/ml bovine serum, 100 μ l/ml goat serum, 0.05% Tween 20, 0.2 mg/ml thimerosal and 50
15 μ g/ml phenol red. 100 μ l of the diluted conjugate was added to each well of the 96-well plate and the well plate was incubated for 60 minutes at 37 °C.

After the reaction terminated, the well plate was washed 5 times with 0.05% Tween 20/phosphate buffer solution. 100 μ l of a substrate solution containing 100 μ g/ml tetramethylbenzidine, 0.006% hydrogen peroxide and citrate-phosphate buffer
20 solution(pH 4.5) was added to each well. After color development in a dark place for 30 minutes, 100 μ l of the reaction-stopping solution (2N sulfuric acid solution) was added to each well to terminate the color development reaction. Then, the absorbance was measured at 450 nm (reference wavelength, 650 nm) by using the 96 well plate reader (Molecular Devices, USA).

25 The result of testing 20 malaria-positive sample and 2 malaria-negative sample is shown in Figure 1. In malaria patient' sample, IgG and IgM was detected, and IgG and IgM were detected at high value when IgG and IgM was tested at the same time.

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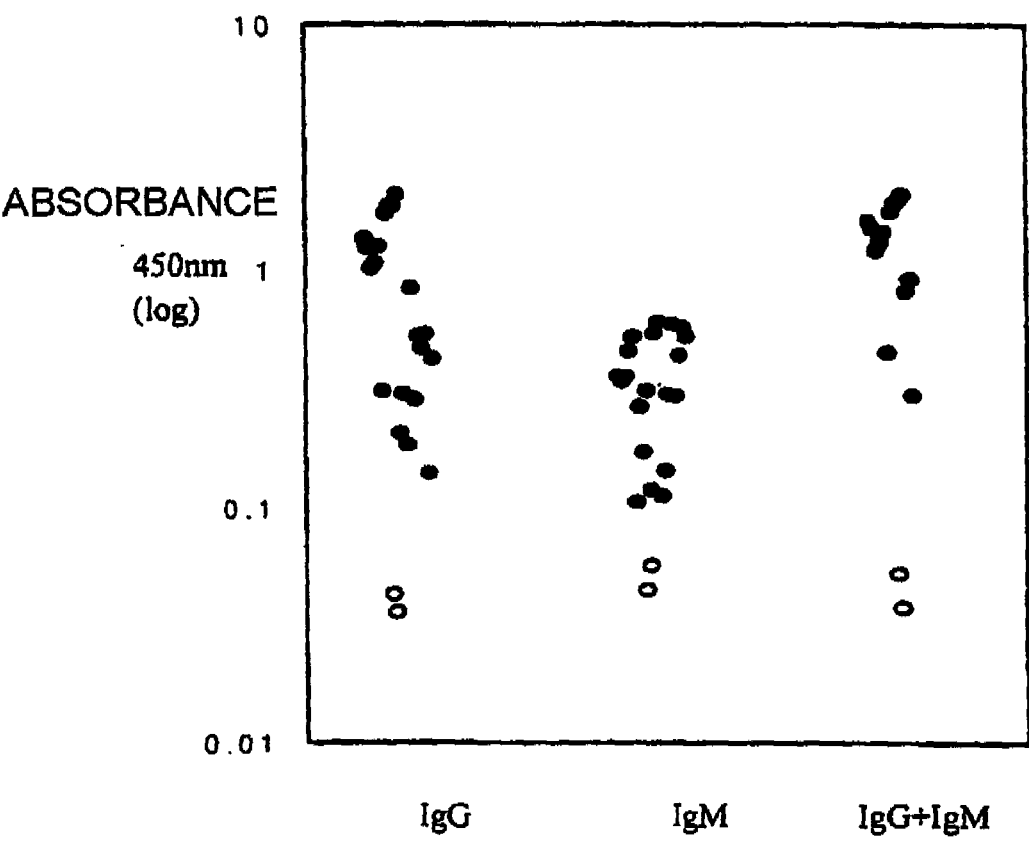
EFFECT OF THE INVENTION

The diagnostic method for malaria that measures antibodies against malaria according to the present invention is more enhanced sensitivity than the prior arts that measure antigens. Particularly, The diagnostic method according to the present invention that measures antibodies against malaria is very effective in diagnosing malaria carrier. Also, the diagnostic method for malaria according to the present invention are very effective in diagnosing malaria of Korean type of which latent period is long, and the probability of recurrence is high.

10 ID NO : 1.

FIGURE

FIGURE 1



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SEQUENCE LIST

SEQ ID NO : 1

Asn Glu Ser Lys Glu Ile Leu Ser Gln Leu Leu Asn Val Gln Thr Gln
Leu Leu Thr Met Ser Ser Glu His Thr Cys Ile Asp Thr Asn Val Pro
5 Asp Asn Ala Ala Cys Tyr Arg Tyr Leu Asp Gly Thr Glu Glu Trp Arg
Cys Leu Leu Thr Phe Lys Glu Glu Gly Gly Lys Cys Val Pro Ala Ser
Asn Val Thr Cys Lys Asp Asn Asn Gly Gly Cys Ala Pro Glu Ala Glu
Cys Lys Met Thr Asp Ser Asn Lys Ile Val Cys Lys Cys Thr Lys Glu
10 Gly Ser Glu Pro Leu Phe Glu Gly Val Phe Cys Ser

ABSTRACT**ABSTRACT**

The present invention relates to a preparation method of the surface protein of malarial Protozoa using Yeast. Preferably, the present invention relates to a preparation method wherein the PV200C gene in C-terminus of Merozoit Surface Protein(MSP), which exhibit 100% homology among subspecies of malarial Protozoa is prepared in form of proteins glycosylated in an active form and only the object proteins are secreted into out of cell and the protein is expressed by utilizing six histidine residues linked with C-terminus of the surface protein and then separated and purified by using a column having an affinity for histidine and gel filtration chromatography. The surface protein PV200C polypeptide prepared from yeast by the preparation method of the present invention has sensitivity and specificity to malarial antigen, and markedly low pseudo-positive signals, and useful in the diagnostic reagent for malaria.

REPRESENTATIVE FIGURE

Figure 1

TITLE OF THE INVENTION

METHOD FOR PRODUCING THE RECOMBINANT MALARIA SURFACE ANTIGEN PROTEIN FROM YEAST

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows a preparation method of expression vector pYLJ-MSP expressing the surface antigen of malarial Protozoa from yeast.

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Figure 2 is a photograph showing a result of electrophoresis of the surface antigen purified by using Probond adsorption resin on SDS-polyacrylamide gel.

Lane 1 is the pellet after centrifugation of the cell culture;

Lane 2 is the supernatant that was concentrated after centrifugation of the cell culture; and

Lane 3 is the concentrated supernatant that was purified on Probond column.

Figure 3 is a photograph showing an assay result of the antigenicity by performing Western Blotting using the serum of the malaria patient.

10 Lane 1 is the cell culture that was concentrated; and

Lane 2 is the purified Merozoite Surface Protein.

DETAILED DESCRIPTION OF THE INVENTION

PURPOSE OF THE INVENTION

15 FIELD OF THE INVENTION AND PRIOR ART THEREOF

The present invention relates to a method for preparing the surface protein of malarial Protozoa using Yeast. Particularly, the present invention relates to a preparation method wherein α -factor leader peptide of yeast is fused with N-terminus of PV200C polypeptide which exist in C-terminus of Merozoite Surface Protein(MSP),
20 which exhibit 100% homology of amino acid sequence among subspecies of *Plasmodium Vivax* and is prepared in form of proteins glycosylated in an active form and only the object proteins are secreted into out of cell.

The present invention relates to a preparation method wherein the protein is expressed by utilizing six histidine residues linked with C-terminus of the surface
25 protein MSP and then separated and purified by using a column having an affinity for histidine and gel filtration chromatography.

Malaria is a disease caused by malarial Protozoa that infects from mosquitoes within human erythrocytes and is carried by mosquitoes. Billion people in the world reside in malaria-risk area and over 500 million people become infected by malaria each year. Malaria causes more than 2 million death each year . Malaria widely
5 spreads all over the world, however, in some regions, malarial infection was eradicated or decreased since 1960's due to effective controls. However, recently, incidence of malaria increases all over the world again, due to increase of drug-resistant strain, increase in resistance to insecticide, abnormal weather such as El nino, etc.

Malaria is a disease caused by malarial Protozoa that infects within human
10 erythrocytes. Life cycle of malaria is divided into Schizogony and Sporogony. Schizogony is the life cycle in human host, and Sporogony is the life cycle in mosquito host. Human is infected by sporozoites by the bite of infected mosquitoes. The sporozoites transfer to human liver through blood vessels, and they exist as a dormant state or multiply and develop into schizonts in hepatic cells. After a definite time, the
15 schizonts enter the blood circulation, and multiply in full-scale. When the schizont matures, it ruptures and releases thousands of merozoites into the blood stream. Merozoites invade erythrocytes, and most merozoites go through another round of asexual reproduction, again forming schizonts. Some of the merozoites change into gametocytes. The gametocytes circulate in human bloodstream. When a mosquito
20 bites the infected person, the mosquito sucks up gametocytes along with blood. Then, male gametocyte and female gametocyte in the gut of the mosquito fuse to form zygote. The zygote, which develops in the gut wall as an oocyte, eventually gives rise to the infective sporozoite, which invades the salivary glands and stomach of the mosquito. The mosquito then can infect another human host.

25 Malaria can be categorized into African type, American type and Asian type. Each type differs in geographical distribution as well as characteristics in species and

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genetic inclination which Protozoa exhibit. Malaria which occurs in Korea is caused by *Plasmodium vivax* (Chom. S.Y., Korean J Parasit., 32(4), 281-284). *Plasmodium vivax* is characterized in that diagnosis thereof is difficult because latent period is long, and number of Protozoa in blood is few. In Korea, malaria has been known as Hakjil.

5 From the 1960s, attempts to eradicate this disease begun, and in the 1970s, cases of malaria were rarely reported. However, after a patient infected with malaria Protozoa was reported near DMZ in 1993, malaria begun to spread rapidly, and it was reported that the number of patients infected with malaria Protozoa was 3,800 in 1998. Though incidence of malaria increases, diagnostic method or reagent for malaria have rarely

10 developed in Korea and the most diagnostic reagent are imported. However, the imported diagnostic reagent for malaria detects malarial antigens such as Lactate Dehydrogenase of malaria. However, the imported diagnostic reagent has a difficulty in diagnosing malaria of Korean type where latent period is long, and numbers of Protozoa and antigen in blood are few, because sensitivity of the diagnostic reagent is

15 only 50%. Therefore, the diagnostic reagent capable of diagnosing malaria of Korean type is required increasingly.

A blood smear method has been used in the diagnosis of malaria most frequently. This method is a classical method wherein blood cells infected with malaria Protozoa are stained and the stained sample is then observed through a

20 microscope. However, this method takes a longer time, and technical education of the examiner is required to judge blood cells infected with malaria Protozoa. Recently, a diagnostic reagent detecting malarial antigens has been commercially available. However, the concurrent use of the diagnostic reagent and the blood smear method is recommended because the sensitivity of the diagnostic reagent is low. Another method

25 to examine genes of malaria Protozoa is Polymerase Chain Reaction method. This method has high sensitivity, however, there is a difficulty in processes thereof.

Therefore, it is not put to practical use and is used only on laboratory scale. Also, such conventional diagnostic methods has a difficulty in diagnosing malaria of Korean type where latent period is long, and number of Protozoa in blood is few.

To solve the above-mentioned problems, the present inventors have developed
5 the method for diagnosing malaria which detects malaria-specific antibodies in blood by using antigens of C-terminus of Merozoite Surface Protein of *Plasmodium vivax* as antigen.

Merozoite Surface Protein is the surface antigen protein which exists specifically in surface of *Plasmodium Vivax*. Especially, PV200C polypeptide
10 encoding C-terminus of MSP consists of 108 amino acids and is known to exhibit 100% homology among subspecies of *Plasmodium Vivax*. Therefore, PV200C polypeptide is useful in diagnosing malaria of Korean type, if it is used in the object antigen protein for diagnosing malaria.

Hitherto studies on expression of malarial antigen itself have been few, and
15 methods for obtaining proteins by overexpressing genes encoding antigen proteins have been developed. Most antigens were expressed after they were fused with proteins such as blood coagulation factor Xa (Ellinger et al., Virol. 180, 81, 1991), protein A of Staphylococcus(Marczinovits et al., J. Biochem., 31, 225, 1993) and β -galactosidase. As a result, expression amount of fused antigens increased. However,
20 because purification process become complex to remove the fused proteins from the antigen protein, it cause the yield of the object protein to decrease and production cost to rise. Also, when they are used as diagnostic reagents, pseudo-positive signals increased due to the proteins that were partners of fusion.

To overcome the above-mentioned problems and develop diagnostic reagent
25 for malaria which is suitable for diagnosing malaria of Korean type, the present inventors have developed a preparation method wherein PV200C polypeptide, which

consist of 108 amino acids which exhibit 100% homology among subspecies of *Plasmodium Vivax* and exist in C-terminus of MSP is cloned from blood of domestic malaria patients, and then is expressed from yeast and purified without cutting. The preparation method of the present invention can express the recombinant polypeptide
5 from yeast and then separate and purify it in a simple method. Such produced antigens are useful in diagnostic reagents as well as vaccine judging malaria patients by detecting antibody against malarial antigens. In addition, such produced antigens can be used as a vaccine.

10 TECHNICAL OBJECT OF THE INVENTION

It is the object of the present invention to provide a preparation method wherein a recombinant expression vector is prepared by means of cloning genes of Merozoite Surface Protein of *Plasmodium vivax*, and is transformed into yeast and then surface protein expressed from the yeast transformant is separated and purified to have a high
15 purity.

It is the object of the present invention to provide a method of producing antigens in a large amount for diagnosing *Plasmodium vivax*, which have high sensitivity and specificity and markedly low pseudo-positive signals by means of the above-mentioned preparation method.

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CONSTRUCTION OF THE INVENTION

To achieve the above objects, the present invention provides an expression vector comprising genes of Merozoite Surface Protein of malarial Protozoa, α -factor leader peptide of yeast, and histidine residues, the transformant transformed with the
25 above expression vector, and the method for preparing MSP by using the above transformant.

The present invention will hereinafter be described in more detail.

The present invention provides the expression vector of fusion protein, pYLJ-MSP comprising α -factor leader peptide of yeast linked with N-terminus of PV200C polypeptide which exist in C-terminus of MSP of malarial Protozoa and six histidine
5 residues linked with C-terminus of PV200C polypeptide, and a yeast transformant pYLJ-MSP/*S. cerevisiae* INVSC1 (Deposit No. KCTC 8977P) transformed with the above expression vector.

The gene of Merozoite Surface Protein of the prevent invention includes a part or whole of the gene, and a more preferable example includes PV200C gene in C-
10 terminus of Merozoite Surface Protein of malarial Protozoa which the sequence of amino acid is known to be constant considerably among various species of *Plasmodium Vivax*. The gene of the prevent invention comprises PV200C gene in C-terminus of the above Merozoite Surface Protein of malarial Protozoa consisting of 108 amino acids which exhibit 100% homology among subspecies of *Plasmodium Vivax*.

15 To clone the PV200C polypeptide, the present inventors purified RNA from malaria-positive blood, and prepared cDNA from the above RNA. Thereafter, the present inventors obtained amplified DNA fragments of PV200C by performing polymerase chain reaction(PCR) by using a pair of primers specific to PV200C and using the above cDNAs as a template.

20 In the meantime, to obtain proteins glycosylated in an active form by making PV200C polypeptide expressed in the yeast to be secreted into out of cell, α -factor leader peptide sequence of yeast was linked with N-terminus of PV200C polypeptide. Amplified DNA fragments of the α -factor leader peptide sequence of yeast could be obtained by performing polymerase chain reaction(PCR) using plasmids containing the
25 α -factor leader peptide sequence of yeast.

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To link the DNA fragments of the α -factor leader peptide of yeast with genes of PV200C polypeptide obtained as described above, an overlapping PCR was performed using the two PCR products as templates. As a result, it was found that the genes consisted of genes encoding 108 amino acids, as shown in SEQ. ID NO : 1. The present inventors obtained pYLJ-MSP which is a recombinant expression vector by inserting fragments of the genes into pYES-2 vector (refer to Figure 3).

Also, the present inventors obtained a transformant pYLJ-MSP/*S. cerevisiae* INVSC1 by transforming a yeast with the recombinant expression vector pYLJ-MSP, and deposited the transformant in Korean Collection for Type Cultures of Korea Research Institute of Bioscience and Biotechnology deposited on December 18, 1999 (Deposit No. KCTC 8977P).

Also, the present invention provides a method for preparing Merozoite Surface Protein of malarial Protozoa by using a yeast transformant.

The PV200C polypeptide expressed from the above transformant was secreted into out of cell by the α -factor leader peptide coupled with N-terminus of PV200C. After secretion, the α -factor leader peptide was removed by peptidase existing in the cell membrane. Accordingly, it was confirmed that only the surface protein was secreted into culture medium.

Because the surface protein expressed from the yeast transformant was secreted into culture medium, the present inventors separated the surface protein by adsorbing the surface protein on Probond column by utilizing six histidine residues linked with C-terminus of the surface protein. Then, Merozoite Surface Protein having 99% or more of high purity was obtained by purifying through a gel filtration chromatography. The separated and purified Merozoite Surface Protein has approximately 18 KDa, and it was confirmed that the expressed surface protein was PV200C polypeptide of malarial

ALVARO CASTELLANOS M. & CIA.
ABOGADOS
AGENTES DE LA PROPIEDAD INTELECTUAL
Carrera 9 No. 93-09
Bogotá-Colombia

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Ministerio de Desarrollo Económico
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COMPLEMENTACION DE INFORMACION

Ref.: Expediente No. 01.011.966

Solicitud de **Patente** de Invención para:

**"INMUNOENSAYO Y REACTIVO DIAGNOSTICO PARA LA
DETECCION DE LA MALARIA".**

Solicitante: **LG CHEMICAL LTD.**

Yo, **MARGARITA CASTELLANOS ABONDANO**, mayor de edad, identificada y domiciliada como aparece al pie de mi firma, me permito presentar la traducción simple en inglés de las copias certificadas de Corea de los documentos de prioridad No. 2000-7648 de Febrero 17 de 2000, No.2000-7649 de Febrero 17,2000, No.2000-7650 de Febrero 17 de 2000, No.2000-12172 de Marzo 10 de 2000 y No. 2000-45806 de Agosto 8 de 2000.

Atentamente solicito a Ud. se anexasen dichas traducciones al expediente de la referencia y se continúe con el trámite correspondiente a esta solicitud de Patente de Invención.

Del Señor Jefe,

Santa Fe de Bogotá, D.C.,
XCA/

6 ABR. 2001

Margarita Castellanos
Margarita Castellanos Abondano
C.C. No. 39.779.654 de Saqueen
T.P.A. No. 70819 del Cons. Sup. de la Judicatura
Carrera 9 No. 93 - 09 - Santa Fe de Bogotá, D.C.
Teléfonos : 610 74 80 - 610 74 60
Código No. 08

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ABSTRACT

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The present invention relates to a serological diagnostic method and diagnostic reagent for malaria. Particularly, the present invention relates to a method measuring
5 antibodies against malaria in blood by using Merozoit surface protein(hereinafter refer to "MSP") of *Plasmodium Vivax* of malarial Protozoa as a antigen. Also, the present invention relates to diagnostic reagent for malaria prepared by using the above MSP as antigens. The above serological diagnostic method according to the present invention are very effective in diagnosing malaria of which latent period is long, and
10 the probability of recurrence is high.

- Antigeno + Muestra
⊕ Antigen binding conjugates (marked)
que son ab anti-IgM
anti-IgG

REPRESENTATIVE FIGURE

FIGURE 1

TITLE OF THE INVENTION

DIAGNOSIS FOR MALARIA AND REAGENT THEREFOR

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows the result of measuring IgG and IgM by using malaria-patient' blood plasma(●)and normal people' blood plasma(○)as samples.

DETAILED DESCRIPTION

PURPOSE OF THE INVENTION

FIELD OF THE INVENTION AND THE PRIOR ART THEREOF

The present invention relates to a serological diagnostic method and diagnostic reagent for malaria. Particularly, the present invention relates to a method

of measuring antibodies against malaria in blood by using Merozoit surface protein(hereinafter refer to "MSP") of *Plasmodium Vivax* of malarial Protozoa as antigens. Also, the present invention relates to diagnostic reagent for malaria prepared by using the above MSP as antigens.

5

Malaria is a disease caused by malarial Protozoa that infects within human erythrocytes and is carried by mosquitoes. Billion people in the world reside in malaria-risk area and over 500 million people become infected by malaria each year. Malaria causes more than 2 million death each year. Malaria widely spreads all over the world, however, in some regions, malarial infection was eradicated or decreased since 1960's due to effective controls. However, recently, incidence of malaria increases all over the world again, due to increase of drug-resistant strain, increase in resistance to insecticide, abnormal weather such as El nino, etc.

Malaria can be categorized into African type, American type and Asian type. Each type differs in geographical distribution as well as characteristics in species and genetic inclination which Protozoa exhibit.

Life cycle of malaria is divided into Schizogony and Sporogony. Schizogony is the life cycle in human host, and Sporogony is the life cycle in mosquito host. Human is infected by sporozoites by the bite of infected mosquitoes. The sporozoites transfer to human liver through blood vessels, and they exist as a dormant state or multiply and develop into schizonts in hepatic cells. After a definite time, the schizonts enter the blood circulation, and multiply in full-scale. When the schizont matures, it ruptures and releases thousands of merozoites into the blood stream. Merozoites invade erythrocytes, and most merozoites go through another round of asexual reproduction, again forming schizonts. Some of the merozoites change into gametocytes. The gametocytes circulate in human bloodstream. When a mosquito bites the infected person, the mosquito sucks up gametocytes along with blood. Then, male gametocyte and female gametocyte in the gut of the mosquito fuse to

form zygote. The zygote, which develops in the gut wall as an oocyte, eventually gives rise to the infective sporozoite, which invades the salivary glands and stomach of the mosquito. The mosquito then can infect another human host.

Antibiotics such as chloroquine and primaquine have been used in treatment
5 of malaria. However, recently, malaria Protozoa have developed drug resistance to such antibiotics. Therefore, acquired drug resistance poses a serious difficulty on treatment of malaria.

A blood smear method has been used in the diagnosis of malaria most frequently. This method is a classical method wherein blood cells infected with
10 malaria Protozoa are stained and the stained sample is then observed through a microscope. However, this method takes a longer time, and technical education of the examiner is required to judge blood cells infected with malaria Protozoa. Recently, a diagnostic reagent detecting malarial antigens has been commercially available. However, the concurrent use of the diagnostic reagent and the blood
15 smear method is recommended because the sensitivity of the diagnostic reagent is low. Another method to examine genes of malaria Protozoa is Polymerase Chain Reaction method. This method has high sensitivity, however, is used only on laboratory scale.

In Korea, malaria has been known as Hakjil. From the 1960s, attempts to
20 eradicate this disease begun, and in the 1970s, cases of malaria were rarely reported. However, after a patient infected with malaria Protozoa was reported near DMZ in 1993, malaria begun to spread rapidly, and it was reported that the number of patients infected with malaria Protozoa was 3,800 in 1998.

Human malaria can be caused by one of four known parasites: *Plasmodium*
25 *vivax*, *Plasmodium falciparum*, *Plasmodium malariae* and *Plasmodium ovale*. Among them, malaria which occurs in Korea is caused by *Plasmodium vivax*. *Plasmodium vivax* is characterized in that diagnosis thereof is difficult because latent period is long, and number of Protozoa in blood is few. The imported diagnostic reagent for

malaria detects malarial antigens such as Lactate Dehydrogenase of malaria. However, the imported diagnostic reagent has a difficulty in diagnosing malaria of Korean type where latent period is long, and numbers of Protozoa and antigen in blood are few, because sensitivity of the diagnostic reagent is only 50%.

5

TECHNICAL OBJECT OF THE INVENTION

It is the first object of the present invention to provide a method of measuring antibodies against malaria in blood, in order to diagnose malaria.

10 It is the second object of the present invention to provide a diagnostic reagent for malaria, useful in diagnosing malaria.

CONSTITUTION OF THE INVENTION

15 To achieve the above objects, the present invention provides a method of measuring antibodies in blood by using MSP that is common to subspecies of malarial Protozoa as a antigen. Also, the present invention relates to diagnostic reagent for malaria, comprising the above MSP.

20 The present invention will hereinafter be described in more detail.

The present invention provides a method of measuring antibodies against malaria in blood, in order to diagnose malaria.

There are many subspecies of *Plasmodium Vivax* that generate malaria.
25 These subspecies generate malaria without exception, if a humane body was infected. Because the latent period of malaria generated by the above subspecies is long and the number of Protozoa in blood is few, there are difficulties in diagnosing malaria by detecting antigen. Therefore, in the present invention, malaria is

diagnosed by measuring antibodies generated in blood by infection of *Plasmodium Vivax*.

First, in the present invention, antigens are prepared and antibodies in blood are measured by antigen-antibody reaction. The above C-terminus of Merozoite Surface Protein of *Plasmodium Vivax* comprises amino acid sequences that are common to subspecies of *Plasmodium Vivax*, and especially comprises a polypeptide consisting of 108 amino acids (referred as "PV200C" hereafter) which exhibit 100% homology among subspecies of *Plasmodium Vivax*, as shown in SEQ. ID NO : 1.

Therefore, a part or whole of Merozoite Surface Protein of *Plasmodium Vivax* are appropriate as a antigen according to the present invention. The C-terminus of Merozoite Surface Protein of *Plasmodium Vivax* is preferable as an antigen according to the present invention, and PV200C polypeptide in the C-terminus of Merozoite Surface Protein of *Plasmodium Vivax* is more preferable as an antigen according to the present invention.

15

The present invention provides a diagnostic reagent for malaria, comprising a part or whole of Merozoite Surface Protein of *Plasmodium Vivax* as a antigen; labeled antigen conjugates consisting of markers such as horseradish peroxidase and IgM or IgG; and a substrate solution containing a color fixing agent.

A preferable example of the above antigen is a part or whole of PV200C that is common to subspecies of *Plasmodium Vivax*, as shown in SEQ. ID NO : 1. The PV200C polypeptide can be expressed in a large amount from recombinant yeast transformants or E. coli transformants. The present inventors prepared a yeast transformant and E.Coli transformant which express PV200C polypeptide, and deposited them in Korean Collection for Type Cultures of Korea Research Institute of Bioscience and Biotechnology on December 18, 1999 (Deposit No. KCTC 8977P, KCTC 8976P).

25

The present invention may utilize an enzyme such as horse radish peroxidase(HRP) and alkaline phosphatase or the like as the marker of the labeled antigen conjugate. In the above diagnostic reagent according to the present invention, the labeled antigen conjugate detects existence of malaria-specific antibodies by
5 binding to Fc (fragment crystallization) region of the malaria-specific antibodies in
which there is little variation.

The substrate solution consists of a buffer solution and a color fixing agent which induces color development when reacted with the marker of the labeled antigen conjugate. The color fixing agent in the substrate solution is decomposed by the
10 marker of the labeled antigen conjugate to induce a color development. Then, existence of malaria-specific antibodies and amount of the antibodies are detected by measuring the absorbance.

The DNA fragment coding PV200C was inserted into a vector to prepare an
15 expression vector. PV200C was expressed from Yeast and E. coli transformed with the expression vector and is purified. Well-plate coated the above PV200C was prepared. When humane plasma was added to the above well-plate, antibodies in the humane plasma were bound to PV200C on the well-plate specifically. Then, the
labeled antigen conjugates were bounded to the above antibodies by recognizing Fc
20 of the antibodies. When the substrate solution was added, the color fixing agent in the substrate solution was decomposed by the marker of the labeled antigen conjugate to induce a color development. Then, existence of malaria-specific antibodies and amount of the antibodies were detected by measuring the absorbance. It was confirmed that IgG, IgM or IgG+IgM reacted with the above antigens in the case that
25 malaria patient' blood plasma sample was added to the antigens. As a result of the above reaction with 20 malaria patient' blood plasma samples, all the samples were judged to be positive. Therefore, it is confirmed that the number of antibodies against the above antigens is large and a diagnosis for malaria is possible by measuring the

antibodies.

The invention will be further illustrated by the following examples. It will be apparent to those skilled in the art that these examples are given only to illustrate the present invention in more detail, but the invention is not limited to the examples given.

Example 1. Expression and purification of a surface antigen of malaria Protozoa from recombinant yeast

10 Example 1-1. Preparation of recombinant yeast and expression of a surface antigen of malaria Protozoa

The DNA coding PV200C is amplified using cDNA as template, reverstranscribed from RNA purified from malaria-positive blood. The DNA coding PV200C and pYES-2 vectors were cleavaged with restriction enzymes, HindIII and Xho I, respectively. Then, DNA coding PV200C and pYES-2 vector were linked by T4 ligase(Promega) to be a recombinant expression vector that is referred as "pYLJ-MSP". Yeast *Saccharomyces cerevisiae* INVSC1 was transformed with pYLJ-MSP according to Hinnen method(*Proc. Natl. Acad. Sci., U.S.A* 75, 1929-1933, 1978). The yeast transformants were deposited in Korean Collection for Type Cultures of Korea Research Institute of Bioscience and Biotechnology deposited on December 18, 1999 (Deposit No. KCTC 8977P)

The transformants pYLJ-MSP/*S. cerevisiae* INVSC1 were inoculated in YEPD medium containing 2% glucose at 37°C. Then, the culture medium was transferred to YEP medium containing 1% glucose and 1% galactose, respectively, and cultivated. The yeast transformants expressed PV200C polypeptide, exhausting glucose. The expressed PV200C polypeptide was secreted into into culture medium.

Example 1-2. Purification of a surface antigen of malarial Protozoa from yeast transformants.

In order to obtain the PV200C polypeptide secreted into culture medium, the biomass was removed by centrifuging the culture medium that was cultivated for 2 days. Then, only the fluid of culture medium was taken and concentrated by ultrafiltrating apparatus(Amicon, U.S.A.). For easily purifying the concentrated fluid, the surface proteins linked with cationic histidine residues were adsorbed on Probond column(Invitrogen)

The contaminants which were not adsorbed on column were removed by phosphate buffer containing 10mM imidazol, and then the surface proteins adsorbed on column were desorbed and separated by phosphate buffer containing 500mM imidazol. The surface proteins having approximately 90% or more of purity were separated by the above process. Then, the surface proteins having approximately 99% or more of high purity were obtained by concentrating the separated solution and performing Sephacryl S-200 gel filtration chromatography with the concentrate.

Example 2. Expression and purification of a surface antigen of malarial Protozoa from recombinant E, coli.

Example 2-1. Preparation of E, coli transformants and expression of a surface antigen of malarial Protozoa.

The amplified DNA fragments coding MSP PV200C prepared in the same way of Example 1, were purified with phenol/chloroform mixture to produce DNA fragments with high purity. The DNA fragment and vector, pET-19b (Novagen) were cleavaged with NdeI and BamHI. Then, the DNA fragment was inserted into pET-19b

to prepare an expression vector, pELK-MSP

E. coli strain BL21(DE3)lysS was transformed with pELK-MSPs by the treatment with CaCl_2 . The transformants were deposited in Korean Collection for Type Cultures of Korea Research Institute of Bioscience and Biotechnology on
5 December 18, 1999 (Deposit No. KCTC 8976P)

The transformants pELK-MSP/strain BL21 (KCTC 8976P) were cultivated for 12 hours in LB medium containing 50 $\mu\text{g/ml}$ of chloramphenicol and 100 $\mu\text{g/ml}$ of ampicilline. Then, 50 ml of the resulting cultures were again inoculated in 1L of LB medium and were cultivated for about 2 hours at 37°C. When absorbance(OD 600)
10 of the cultures became 0.3, IPTG (Isopropyl thio- β -D-galactoside) was added to the medium to a final concentration of 0.2 mM. Then, the cultures were cultivated further for 7 hours.

**Example 2-2. Purification of a surface antigen of malarial Protozoa from E, coli
15 transformants.**

After the cultivation, only the cells were isolated by centrifugation. The obtained cells were suspended in 30 ml of Phosphate Buffered Saline(PBS). Then, E. Coli in the suspension was crushed with Sonicator(Branson, Sonifier 450) and
20 centrifuged at 5000 rpm. Because a part of expressed MSP proteins was in the supernatant and the remainder was in the pellet, both the supernatant and the pellet were completely suspended in 4M urea buffer(pH 7.5) where 4M urea was dissolved in PBS, and then were centrifuged, by which only the supernatant was collected. Because the overexpressed surface protein has His marker consisting of 10 histidines
25 in N-terminus thereof, the protein was adsorbed on Probond column(Invitrogen) for purification. The contaminants which were not adsorbed on column were removed repeatedly with a washing buffer in which 10mM imidazol and 0.1M NaCl were dissolved in PBS. Then, the surface proteins adsorbed on column were desorbed

and separated with an elution buffer in which 500mM imidazol and 0.1M NaCl were dissolved in PBS. It was confirmed that the surface proteins having approximately 90% or more of purity were separated by the above process. Then, it was confirmed that PV200C polypeptide having approximately 99% or more of high purity were obtained from recombinant E.Coli by concentrating the separated solution and performing Sephacryl S-200 gel filtration chromatography with the concentrate.

10 **Example 3. Preparation of well plate for measuring of the surface antibody**

The solution of recombinant surface antigens of malarial Protozoa prepared in Example 1 or 2 was diluted with 0.1M carbonate buffer (pH 9.5) to 0.5 µg/ml, and 100 µl of the solution was added to each well of 96-well plate for EIA. To adsorb the surface antigens on the well, the well plate was sealed tightly and left for 18 hours at 4 °C. Then, the surface antigens which were not adsorbed on the well were removed by adding 300 µl of phosphate buffer solution containing 5% general goat serum to each well and leaving it for 18 hours at 4 °C. After the solution that remained in the well was discarded, moisture was removed by leaving the well plate for 1 hour at ambient temperature. Then, the well plate was transferred to a hermetic container with a dehumidifying agent and stored in a refrigerator at 4 °C.

Example 4. Indirect enzyme immunoassay for antibodies against surface antigen of malaria

25

To diagnose malaria by indirect enzyme immunoassay, the solid support prepared by coating 96-well plate(Nunc) with recombinant surface antigens prepared in Example 1 or 2 according to the method of Example 3, and the labeled antigen conjugate consisting of HRP and anti-human IgM antibody or

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anti-human IgG antibody derived from goat were used.

100 μ l of a sample dilution solution containing 1.15 mg/ml NaOH, 0.2 mg/ml of KH_2PO_4 , 0.2 mg/ml KCl, 8 mg/ml NaCl, 300 μ l/ml bovine serum, 0.2 mg/ml thimerosal and the like was added to each well of 96-well plate(Nunc)
5 coated with recombinant surface antigens. Then, 10 μ l of antibody-negative plasma sample or 10 μ l of antibody-positive plasma sample was added to the wells, and the resulting mixtures were well mixed. The well plate was incubated for 60 minutes in a reactor at 37 °C.

After the reaction terminated, the well plate was washed 5 times with
10 300 μ l of a phosphate buffer solution containing 0.05% Tween 20. Anti-human IgG antibody(Gappel) or anti-human IgM antibody(Bethyl) derived from goat to which HRP was conjugated was diluted to 1/40,000 with a dilution solution containing 10mM Tris, 0.5M NaCl, 1mM CaCl_2 , 0.5% glycerol, 0.2 ml/ml bovine serum, 100 μ l/ml goat serum, 0.05% Tween 20, 0.2 mg/ml thimerosal and 50
15 μ g/ml phenol red. 100 μ l of the diluted conjugate was added to each well of the 96-well plate and the well plate was incubated for 60 minutes at 37 °C.

After the reaction terminated, the well plate was washed 5 times with 0.05% Tween 20/phosphate buffer solution. 100 μ l of a substrate solution containing 100 μ g/ml tetramethylbenzidine, 0.006% hydrogen peroxide and citrate-phosphate buffer
20 solution(pH 4.5) was added to each well. After color development in a dark place for 30 minutes, 100 μ l of the reaction-stopping solution (2N sulfuric acid solution) was added to each well to terminate the color development reaction. Then, the absorbance was measured at 450 nm (reference wavelength, 650 nm) by using the 96 well plate reader (Molecular Devices, USA).

25 The result of testing 20 malaria-positive sample and 2 malaria-negative sample is shown in Figure 1. In malaria patient' sample, IgG and IgM was detected, and IgG and IgM were detected at high value when IgG and IgM was tested at the same time.

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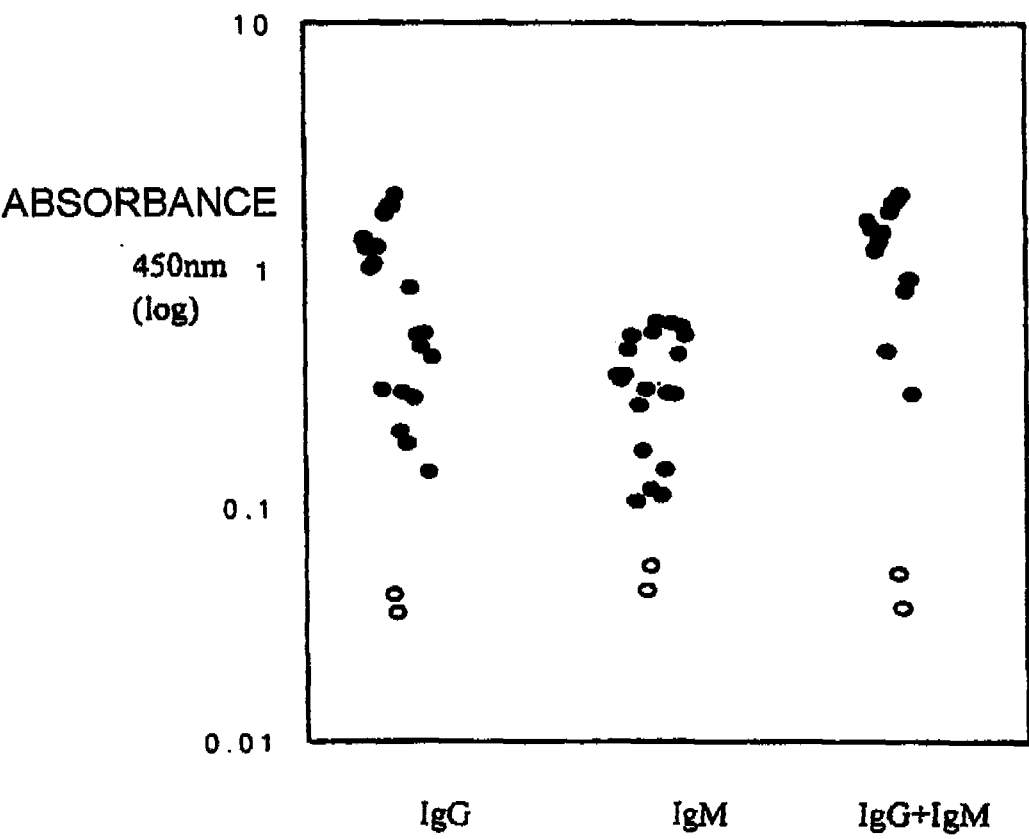
EFFECT OF THE INVENTION

The diagnostic method for malaria that measures antibodies against malaria according to the present invention is more enhanced sensitivity than the prior arts that measure antigens. Particularly, The diagnostic method according to the present invention that measures antibodies against malaria is very effective in diagnosing malaria carrier. Also, the diagnostic method for malaria according to the present invention are very effective in diagnosing malaria of Korean type of which latent period is long, and the probability of recurrence is high.

10 ID NO : 1.

FIGURE

FIGURE 1



398 437

SEQUENCE LIST

SEQ ID NO : 1

5 Asn Glu Ser Lys Glu Ile Leu Ser Gln Leu Leu Asn Val Gln Thr Gln
Leu Leu Thr Met Ser Ser Glu His Thr Cys Ile Asp Thr Asn Val Pro
Asp Asn Ala Ala Cys Tyr Arg Tyr Leu Asp Gly Thr Glu Glu Trp Arg
Cys Leu Leu Thr Phe Lys Glu Glu Gly Gly Lys Cys Val Pro Ala Ser
Asn Val Thr Cys Lys Asp Asn Asn Gly Gly Cys Ala Pro Glu Ala Glu
Cys Lys Met Thr Asp Ser Asn Lys Ile Val Cys Lys Cys Thr Lys Glu
10 Gly Ser Glu Pro Leu Phe Glu Gly Val Phe Cys Ser

ABSTRACT**ABSTRACT**

The present invention relates to a preparation method of the surface protein of malarial Protozoa using Yeast. Preferably, the present invention relates to a preparation method wherein the PV200C gene in C-terminus of Merozoit Surface Protein(MSP), which exhibit 100% homology among subspecies of malarial Protozoa is prepared in form of proteins glycosylated in an active form and only the object proteins are secreted into out of cell and the protein is expressed by utilizing six histidine residues linked with C-terminus of the surface protein and then separated and purified by using a column having an affinity for histidine and gel filtration chromatography. The surface protein PV200C polypeptide prepared from yeast by the preparation method of the present invention has sensitivity and specificity to malarial antigen, and markedly low pseudo-positive signals, and useful in the diagnostic reagent for malaria.

REPRESENTATIVE FIGURE

Figure 1

TITLE OF THE INVENTION

METHOD FOR PRODUCING THE RECOMBINANT MALARIA SURFACE ANTIGEN PROTEIN FROM YEAST

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows a preparation method of expression vector pYLJ-MSP expressing the surface antigen of malarial Protozoa from yeast.

Figure 2 is a photograph showing a result of electrophoresis of the surface antigen purified by using Probond adsorption resin on SDS-polyacrylamide gel.

Lane 1 is the pellet after centrifugation of the cell culture;

Lane 2 is the supernatant that was concentrated after centrifugation of the cell culture; and

Lane 3 is the concentrated supernatant that was purified on Probond column.

Figure 3 is a photograph showing an assay result of the antigenicity by performing Western Blotting using the serum of the malaria patient.

Lane 1 is the cell culture that was concentrated; and

Lane 2 is the purified Merozoite Surface Protein.

DETAILED DESCRIPTION OF THE INVENTION

PURPOSE OF THE INVENTION

FIELD OF THE INVENTION AND PRIOR ART THEREOF

The present invention relates to a method for preparing the surface protein of malarial Protozoa using Yeast. Particularly, the present invention relates to a preparation method wherein α -factor leader peptide of yeast is fused with N-terminus of PV200C polypeptide which exist in C-terminus of Merozoite Surface Protein(MSP), which exhibit 100% homology of amino acid sequence among subspecies of *Plasmodium Vivax* and is prepared in form of proteins glycosylated in an active form and only the object proteins are secreted into out of cell.

The present invention relates to a preparation method wherein the protein is expressed by utilizing six histidine residues linked with C-terminus of the surface protein MSP and then separated and purified by using a column having an affinity for histidine and gel filtration chromatography.

Malaria is a disease caused by malarial Protozoa that infects from mosquitoes within human erythrocytes and is carried by mosquitoes. Billion people in the world reside in malaria-risk area and over 500 million people become infected by malaria each year. Malaria causes more than 2 million death each year . Malaria widely spreads all over the world, however, in some regions, malarial infection was eradicated or decreased since 1960's due to effective controls. However, recently, incidence of malaria increases all over the world again, due to increase of drug-resistant strain, increase in resistance to insecticide, abnormal weather such as El nino, etc.

Malaria is a disease caused by malarial Protozoa that infects within human erythrocytes. Life cycle of malaria is divided into Schizogony and Sporogony. Schizogony is the life cycle in human host, and Sporogony is the life cycle in mosquito host. Human is infected by sporozoites by the bite of infected mosquitoes. The sporozoites transfer to human liver through blood vessels, and they exist as a dormant state or multiply and develop into schizonts in hepatic cells. After a definite time, the schizonts enter the blood circulation, and multiply in full-scale. When the schizont matures, it ruptures and releases thousands of merozoites into the blood stream. Merozoites invade erythrocytes, and most merozoites go through another round of asexual reproduction, again forming schizonts. Some of the merozoites change into gametocytes. The gametocytes circulate in human bloodstream. When a mosquito bites the infected person, the mosquito sucks up gametocytes along with blood. Then, male gametocyte and female gametocyte in the gut of the mosquito fuse to form zygote. The zygote, which develops in the gut wall as an oocyte, eventually gives rise to the infective sporozoite, which invades the salivary glands and stomach of the mosquito. The mosquito then can infect another human host.

Malaria can be categorized into African type, American type and Asian type. Each type differs in geographical distribution as well as characteristics in species and

genetic inclination which Protozoa exhibit. Malaria which occurs in Korea is caused by *Plasmodium vivax* (Chom. S.Y., Korean J Parasit., 32(4), 281-284). *Plasmodium vivax* is characterized in that diagnosis thereof is difficult because latent period is long, and number of Protozoa in blood is few. In Korea, malaria has been known as Hakjil.

5 From the 1960s, attempts to eradicate this disease begun, and in the 1970s, cases of malaria were rarely reported. However, after a patient infected with malaria Protozoa was reported near DMZ in 1993, malaria begun to spread rapidly, and it was reported that the number of patients infected with malaria Protozoa was 3,800 in 1998. Though incidence of malaria increases, diagnostic method or reagent for malaria have rarely

10 developed in Korea and the most diagnostic reagent are imported. However, the imported diagnostic reagent for malaria detects malarial antigens such as Lactate Dehydrogenase of malaria. However, the imported diagnostic reagent has a difficulty in diagnosing malaria of Korean type where latent period is long, and numbers of Protozoa and antigen in blood are few, because sensitivity of the diagnostic reagent is

15 only 50%. Therefore, the diagnostic reagent capable of diagnosing malaria of Korean type is required increasingly.

A blood smear method has been used in the diagnosis of malaria most frequently. This method is a classical method wherein blood cells infected with malaria Protozoa are stained and the stained sample is then observed through a

20 microscope. However, this method takes a longer time, and technical education of the examiner is required to judge blood cells infected with malaria Protozoa. Recently, a diagnostic reagent detecting malarial antigens has been commercially available. However, the concurrent use of the diagnostic reagent and the blood smear method is recommended because the sensitivity of the diagnostic reagent is low. Another method

25 to examine genes of malaria Protozoa is Polymerase Chain Reaction method. This method has high sensitivity, however, there is a difficulty in processes thereof.

Therefore, it is not put to practical use and is used only on laboratory scale. Also, such conventional diagnostic methods has a difficulty in diagnosing malaria of Korean type where latent period is long, and number of Protozoa in blood is few.

To solve the above-mentioned problems, the present inventors have developed
5 the method for diagnosing malaria which detects malaria-specific antibodies in blood by using antigens of C-terminus of Merozoite Surface Protein of *Plasmodium vivax* as antigen.

Merozoite Surface Protein is the surface antigen protein which exists specifically in surface of *Plasmodium Vivax*. Especially, PV200C polypeptide
10 encoding C-terminus of MSP consists of 108 amino acids and is known to exhibit 100% homology among subspecies of *Plasmodium Vivax*. Therefore, PV200C polypeptide is useful in diagnosing malaria of Korean type, if it is used in the object antigen protein for diagnosing malaria.

Hitherto studies on expression of malarial antigen itself have been few, and
15 methods for obtaining proteins by overexpressing genes encoding antigen proteins have been developed. Most antigens were expressed after they were fused with proteins such as blood coagulation factor Xa (Ellinger et al., Virol. 180, 81, 1991), protein A of Staphylococcus(Marczinovits et al., J. Biochem., 31, 225, 1993) and β -galactosidase. As a result, expression amount of fused antigens increased. However,
20 because purification process become complex to remove the fused proteins from the antigen protein, it cause the yield of the object protein to decrease and production cost to rise. Also, when they are used as diagnostic reagents, pseudo-positive signals increased due to the proteins that were partners of fusion.

To overcome the above-mentioned problems and develop diagnostic reagent
25 for malaria which is suitable for diagnosing malaria of Korean type, the present inventors have developed a preparation method wherein PV200C polypeptide, which

consist of 108 amino acids which exhibit 100% homology among subspecies of *Plasmodium Vivax* and exist in C-terminus of MSP is cloned from blood of domestic malaria patients, and then is expressed from yeast and purified without cutting. The preparation method of the present invention can express the recombinant polypeptide
5 from yeast and then separate and purify it in a simple method. Such produced antigens are useful in diagnostic reagents as well as vaccine judging malaria patients by detecting antibody against malarial antigens. In addition, such produced antigens can be used as a vaccine.

10 TECHNICAL OBJECT OF THE INVENTION

It is the object of the present invention to provide a preparation method wherein a recombinant expression vector is prepared by means of cloning genes of Merozoite Surface Protein of *Plasmodium vivax*, and is transformed into yeast and then surface protein expressed from the yeast transformant is separated and purified to have a high
15 purity.

It is the object of the present invention to provide a method of producing antigens in a large amount for diagnosing *Plasmodium vivax*, which have high sensitivity and specificity and markedly low pseudo-positive signals by means of the above-mentioned preparation method.

20

CONSTRUCTION OF THE INVENTION

To achieve the above objects, the present invention provides an expression vector comprising genes of Merozoite Surface Protein of malarial Protozoa, α -factor leader peptide of yeast, and histidine residues, the transformant transformed with the
25 above expression vector, and the method for preparing MSP by using the above transformant.

The present invention will hereinafter be described in more detail.

The present invention provides the expression vector of fusion protein, pYLJ-MSP comprising α -factor leader peptide of yeast linked with N-terminus of PV200C polypeptide which exist in C-terminus of MSP of malarial Protozoa and six histidine
5 residues linked with C-terminus of PV200C polypeptide, and a yeast transformant pYLJ-MSP/*S. cerevisiae* INVSC1 (Deposit No. KCTC 8977P) transformed with the above expression vector.

The gene of Merozoite Surface Protein of the prevent invention includes a part or whole of the gene, and a more preferable example includes PV200C gene in C-
10 terminus of Merozoite Surface Protein of malarial Protozoa which the sequence of amino acid is known to be constant considerably among various species of *Plasmodium Vivax*. The gene of the prevent invention comprises PV200C gene in C-terminus of the above Merozoite Surface Protein of malarial Protozoa consisting of 108 amino acids which exhibit 100% homology among subspecies of *Plasmodium Vivax*.

15 To clone the PV200C polypeptide, the present inventors purified RNA from malaria-positive blood, and prepared cDNA from the above RNA. Thereafter, the present inventors obtained amplified DNA fragments of PV200C by performing polymerase chain reaction(PCR) by using a pair of primers specific to PV200C and using the above cDNAs as a template.

20 In the meantime, to obtain proteins glycosylated in an active form by making PV200C polypeptide expressed in the yeast to be secreted into out of cell, α -factor leader peptide sequence of yeast was linked with N-terminus of PV200C polypeptide. Amplified DNA fragments of the α -factor leader peptide sequence of yeast could be obtained by performing polymerase chain reaction(PCR) using plasmids containing the
25 α -factor leader peptide sequence of yeast.

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To link the DNA fragments of the α -factor leader peptide of yeast with genes of PV200C polypeptide obtained as described above, an overlapping PCR was performed using the two PCR products as templates. As a result, it was found that the genes consisted of genes encoding 108 amino acids, as shown in SEQ. ID NO : 1. The present inventors obtained pYLJ-MSP which is a recombinant expression vector by inserting fragments of the genes into pYES-2 vector (refer to Figure 3).

Also, the present inventors obtained a transformant pYLJ-MSP/*S. cerevisiae* INVSC1 by transforming a yeast with the recombinant expression vector pYLJ-MSP, and deposited the transformant in Korean Collection for Type Cultures of Korea Research Institute of Bioscience and Biotechnology deposited on December 18, 1999 (Deposit No. KCTC 8977P).

Also, the present invention provides a method for preparing Merozoite Surface Protein of malarial Protozoa by using a yeast transformant.

The PV200C polypeptide expressed from the above transformant was secreted into out of cell by the α -factor leader peptide coupled with N-terminus of PV200C. After secretion, the α -factor leader peptide was removed by peptidase existing in the cell membrane. Accordingly, it was confirmed that only the surface protein was secreted into culture medium.

Because the surface protein expressed from the yeast transformant was secreted into culture medium, the present inventors separated the surface protein by adsorbing the surface protein on Probond column by utilizing six histidine residues linked with C-terminus of the surface protein. Then, Merozoite Surface Protein having 99% or more of high purity was obtained by purifying through a gel filtration chromatography. The separated and purified Merozoite Surface Protein has approximately 18 KDa, and it was confirmed that the expressed surface protein was PV200C polypeptide of malarial

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Protozoa by analysis of amino acid sequences in N-terminus of the surface protein.

In the meantime, Western Blotting was performed with the serum of malaria patients to assay antigenicity of the surface protein separated and purified from the yeast transformant. As a result, only MSP PV200C polypeptide having 18 KDa was
5 detected. Therefore, it was proved that the protein separated and purified from the yeast transformant according to the preparation method of the present invention was an antigen having high sensitivity and specificity.

As the above-described, when the surface protein of malarial Protozoa expressed from recombinant yeast transformant by the preparation method of the
10 prevent invention is separated and purified by using a column having an affinity for histidine and gel filtration chromatography, we can obtain the surface proteins of high purity which have high sensitivity and specificity to antibody and markedly low pseudo-positive signals. Therefore, the proteins can be used in a diagnostic reagent for diagnosing malaria of Korean type as well as a vaccine.

15 The invention will be further illustrated by the following examples. It will be apparent to those skilled in the art that these examples are given only to illustrate the present invention in more detail, but the invention is not limited to the examples given.

Example 1. Preparation of cDNA from malaria-positive blood

20 TRI reagent TM (Sigma Co.) was used to purify RNA from malaria-positive blood. The purification method is the following method provided by Sigma Co. A mixture of 0.1 ml of TRI reagent TM and 0.1 ml of malaria-positive blood was left for 5 minutes at ambient temperature. 20 µl of BCP was mixed with the mixture, and the resulting mixture was left for 5 minutes at ambient temperature and then was
25 centrifuged for 15 minutes at 12,000 rpm and 4 . As a result of centrifugation, three

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layers were formed. Only the upper layer in which RNAs were contained was transferred to a new tube, and then 50 μ l of isopropanol was added to the tube. The tube was left for 5 minutes at ambient temperature. The resulting mixture was centrifuged for 10 minutes at 12,000 rpm and 4 . The supernatant was discarded. The
5 pellet was washed with 75% ethanol and dissolved in 20 μ l of deionized distilled water, which was used to prepare cDNA.

9 μ l of the above-purified RNA and 1 μ l of N6 random primer (Genotech) were mixed and reacted for 5 minutes at 65 and then transferred to ice for cooling. 4 μ l of RT buffer, 2 μ l of 0.1M DTT, 1 μ l of 10mM dNTP, 1 μ l of Reverse
10 Transcriptase(Gibco Co.), 1 μ l of RNase inhibitor(Promega) and 1 μ l of deionized distilled water were added to the above mixture, and the resulting mixture was reacted for 1 hour at 42 and then reacted for 10 minutes at 70 to prepare cDNA.

Example 2. preparation of recombinant plasmids comprising genes
15 **encoding PV200C.**

Polymerase chain reaction was performed using cDNAs prepared in Example 1 as a template and a pair of primers, SEQ. ID. NO:1 and SEQ. ID. NO:3, as follows.

The reaction mixture containing 5 μ l of PCR buffer, 5 μ l of 2.5 mM dNTP, 1 μ l of sense primer, 1 μ l of anti-sense primer, 2 μ l of template DNA and 35 μ l of deionized
20 distilled water was reacted for 30 seconds at 94 . Thereafter, 1 μ l of Vent polymerase(BioLab) was added to the mixture. Then, the reaction having the following cycle was repeated 36 times:

Denaturation 94 , 30 sec

Primer annealing 55 , 30 sec

H09 44

Extension 72 , 30 sec

Again, polymerase chain reaction was performed using the above-amplified DNAs as a template and a pair of primers, SEQ. ID. NO:2 and SEQ. ID. NO:3. The PCR was performed in the same condition as the above condition. The amplified DNAs were confirmed through electrophoresis in 1% agarose gel. The amplified DNAs(referred as "Pv200-ct657" hereafter) and pBluscript KS(+)(Stratagene) vector were cleaved with restriction enzyme, EcoR V, and Pv200-ct657 was inserted into pBluscript KS(+) vector by using T4 DNA ligase(Promega). As a result, recombinant plasmids(referred as "pBC-Pv200-ct657" hereafter) were produced. Then, E. coli strain JM 109 was transformed with pBC-Pv200-ct657.

Example 3. preparation of expression vector pYLJ-MSP

Polymerase chain reaction was performed using pBC-Pv200-ct657 prepared in Example 2 as a template and a pair of primers, SEQ. ID. NO:4 and SEQ. ID. NO:5. The PCR was performed in the same condition as the above reaction condition. The amplified DNAs(referred as "Pv200-19" hereafter) were confirmed through electrophoresis in 1% agarose gel.

In order to obtain α -factor leader peptide sequence of yeast to be linked with N-terminus of PV200C polypeptide, PCR was performed using the α -IFN pYLBC(deposit No. KCTC 0051BP) comprising the sequence encoding α -factor leader peptide of yeast as a template and a pair of primers, SEQ. ID. NO:6 and SEQ. ID. NO:7. The PCR was performed in the same condition as the above reaction condition. The amplified DNAs(referred as " α -leader of yeast" hereafter) were confirmed through electrophoresis in 1% agarose gel.

To link the above-amplified Pv200-19 with α -factor leader peptide sequence of

yeast, an overlapping PCR was performed using the above two PCR products as templates and a pair of primers, SEQ. ID. NO:5 and SEQ. ID. NO:6. A reaction mixture containing 5 μ l of PCR buffer, 5 μ l of 2.5 mM dNTP, 1 μ l of sense primer, 1 μ l of anti-sense primer, 1 μ l of PCR products of Pv200-19, 1 μ l of PCR products of α -factor leader peptide of yeast and 35 μ l of deionized distilled water, was reacted for 30 seconds at 94 . Thereafter, 1 μ l of Vent polymerase(BioLab) was added to the mixture. Then, the reaction having the following cycle was repeated 36 times:

Denaturation 94 , 30 sec

Primer annealing 55 , 30 sec

10 Extension 72 , 30 sec

The amplified DNAs(referred as " α -Pv200-19" hereafter) were confirmed through electrophoresis in 1% agarose gel.

The above-amplified α -Pv200-19 and pYES-2 vectors were cleavaged with restriction enzymes, HindIII and Xho I, respectively. Then, α -Pv200-19 and pYES-2
15 vector were linked by T4 ligase to be a recombinant expression vector that is referred as "pYLJ-MSP"(Refer to Figure 3). As a result of analyzing α -Pv200-19 sequence in pYLJ-MSP, it was confirmed that α -Pv200-19 consisted of the genes encoding 108 amino acids as shown SEQ. ID. NO:8. Yeast *Saccharomyces cerevisiae* INVSC1 was transformed with pYLJ-MSP according to Hinnen method(*Proc. Natl. Acad. Sci.*,
20 U.S.A 75, 1929-1933, 1978), and then the yeast transformants were cultivated overnight in minimal medium lacking uracil. The yeast transformants were deposited in Korean Collection for Type Cultures of Korea Research Institute of Bioscience and Biotechnology deposited on December 18, 1999 (Deposit No. KCTC 8977P)

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Example 4. expression and purification of MSP from yeast

In order to produce the surface protein MSP from yeast, the transformants pYLJ-MSP/*S. cerevisiae* INVSC1 were inoculated in 100 ml of YEPD medium containing 2% glucose and cultivated overnight. Then, the culture medium was transferred to 2L of YEP medium containing 1% glucose and 1% galactose, respectively, and cultivated for 72 hours at 30 . The yeast transformants expressed PV200C polypeptide, exhausting glucose. The expressed PV200C polypeptide was secreted into out of cell by the α -factor leader peptide linked with N-terminus of PV200C polypeptide. Thereafter, the α -factor leader peptide was removed by 10 peptidase existing in cell membrane. Accordingly, only the PV200C polypeptide was secreted into culture medium.

In order to obtain the PV200C polypeptide secreted into culture medium, the biomass was removed by centrifuging the culture medium that was cultivated for 2 days. Then, only the fluid of culture medium was taken and concentrated by 15 ultrafiltrating apparatus(Amicon, U.S.A.). For easily purifying the concentrated fluid, the surface proteins linked with cationic histidine residues were adsorbed on Probond column(Invitrogen) to which anionic nickels were coupled by using six histidine residues linked with C-terminus of the above-amplified Pv200-19. The contaminants which were not adsorbed on column were removed by phosphate buffer containing 20 10mM imidazol, and then the surface proteins adsorbed on column were desorbed and separated by 500 mM imidazol and phosphate buffer. As shown in Figure 2, the surface proteins having approximately 90% or more of purity were separated by the process. Then, the surface proteins having approximately 99% or more of high purity were obtained by concentrating the separated solution and performing Sephacryl S-200 25 gel filtration chromatography with the concentrate.

4/12 477

Example 5. assay of antigenicity of the surface protein of malarial

Protozoa purified from yeast

The surface protein PV200C polypeptide obtained in Example 4 was analyzed with 12% SDS-PAGE. As a result, it was detected in 18 kDa position, and it was identified as the object protein of the present invention by sequencing amino acids of N-terminus thereof.

In addition, Western Blotting was performed with the serum of the malaria patient to assay antigenicity of the surface protein of malarial Protozoa purified from yeast. Western Blotting was performed according to the method suggested by Towbin et al. (*Proc. Natl. Acad. Sci., USA*, 76, 4350-4354, 1979). After the electrophoresed gel was immersed in a buffer solution containing 25 mM Tris buffer, 190 mM glycine(pH 8.3) and 20% methanol for 30 minutes, the protein was transferred to nitrocellulose membrane by electrophoresis using the same buffer. Then, the protein-bound nitrocellulose membrane was incubated for 1 hour at ambient temperature in a phosphate buffer(pH 7.4) containing 3% Skim milk. The serum of the malaria patient diluted to 1/100 was added to the membrane and was incubated for 1 hour at ambient temperature. Thereafter, the nitrocellulose membrane was washed 5 times at 5 minute-intervals with 0.05% Tween 20/phosphate buffer solution, and then the second antibody(Vector Labs) to which HRP was conjugated was diluted to 1/2000, added to the membrane and incubated for 1 hour at ambient temperature. After the incubation, the nitrocellulose membrane was washed with the above solution, and then both 4-chloro-naphthol and hydrogen peroxide were added to the membrane to induce color development. As a result, it was confirmed that only the PV200C polypeptide was detected at 18 kDa position. Therefore, it was confirmed that the surface protein PV200C polypeptide expressed and purified by the preparation method of the present

4/13 4/13

invention was an antigen having high specificity.

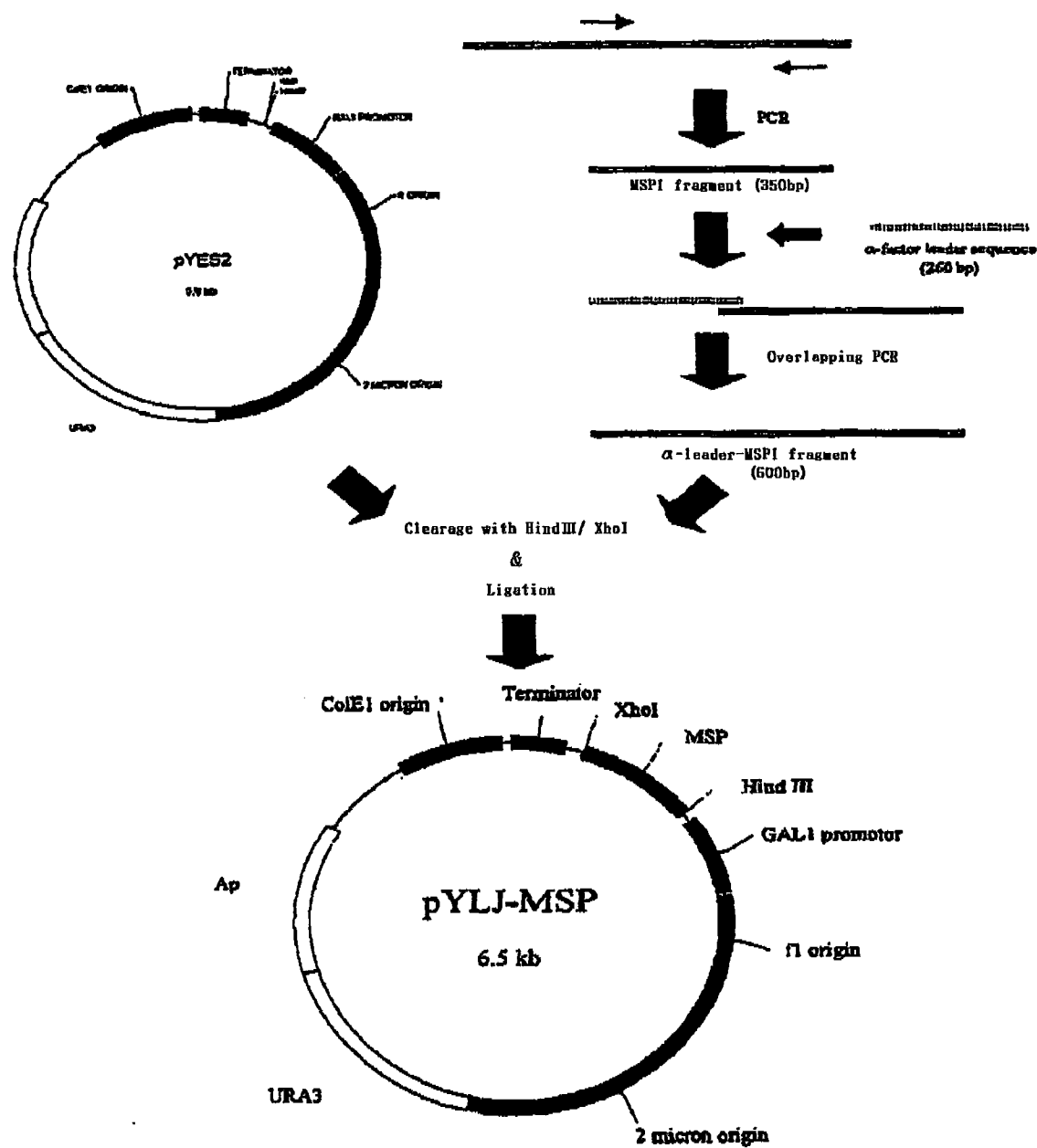
EFFECTS OF THE INVENTION

The present invention relates to a preparation method wherein α -factor leader
5 peptide of yeast is fused with N-terminus of PV200C polypeptide in C-terminus of
Merozoit Surface Protein of malarial Protozoa and is prepared in form of proteins
glycosylated in an active form and only the object proteins are secreted into out of cell
and the protein is expressed by utilizing six histidine residues linked with C-terminus
of the surface protein and then the protein bound to histidine residue is separated and
10 purified by using a column having an affinity for histidine and gel filtration
chromatography. In accordance with the preparation method of the present invention,
by expressing the surface protein of malarial Protozoa expressed from yeast in an
active form by gene recombinant technique, the surface protein with high purity can be
produced more easily and rapidly than the prior art method does. The surface protein
15 purified by the preparation method of the present invention has high sensitivity and
specificity to antibody, and useful in the diagnostic reagent for malaria and vaccine.

414 419

FIGURE

FIGURE 1



415 420

FIGURE 2

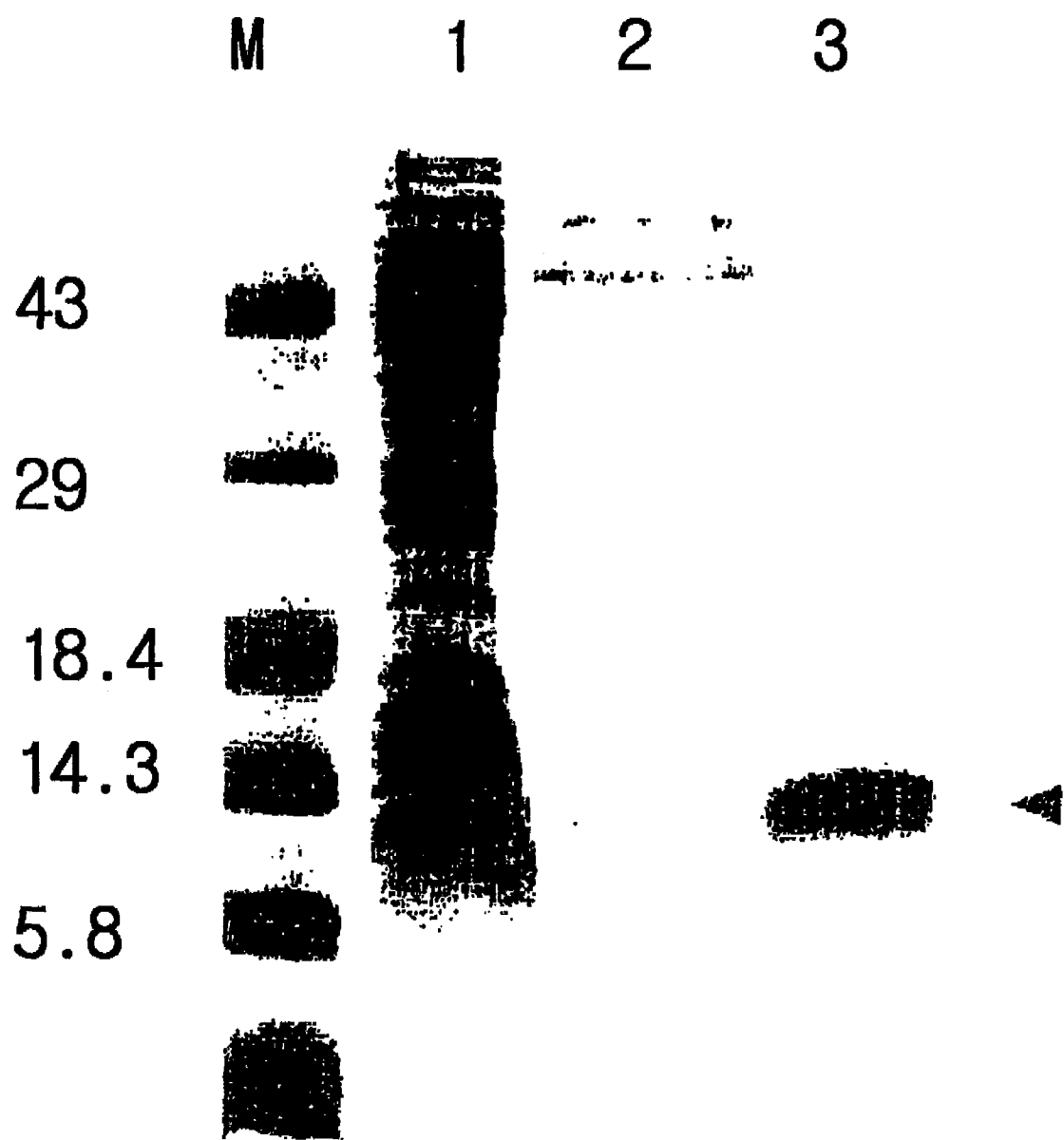


FIGURE 3

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M

4

5

43

29

18.4

14.3

5.8

417 422

SEQUENCE LIST

SEQ ID NO.:1

aaaaaagtcg acgccaaaaa ggccgagctg g

5 SEQ ID NO.:2

aaaaaagtcg acgagaagta cctcccgttc c

SEQ ID NO.:3

aaaaaaatcg atttaaagct ccatgcacag g

10

SEQ ID NO.:4

tctctagata agagaaacga gtccaaggaa

SEQ ID NO.:5

15 aaactcgagt cagtgggtggt ggtgggtggtg gctacagaaa actcc

SEQ ID NO.:6

aaaaagctta tgagatttcc ttca

20 SEQ ID NO.:7

tctcttatct agagataccc

SEQ ID NO.:8

Asn Glu Ser Lys Glu Ile Leu Ser Gln Leu Leu Asn Val Gln Thr Gln
 25 Leu Leu Thr Met Ser Ser Glu His Thr Cys Ile Asp Thr Asn Val Pro
 Asp Asn Ala Ala Cys Tyr Arg Tyr Leu Asp Gly Thr Glu Glu Trp Arg
 Cys Leu Leu Thr Phe Lys Glu Glu Gly Gly Lys Cys Val Pro Ala Ser
 Asn Val Thr Cys Lys Asp Asn Asn Gly Gly Cys Ala Pro Glu Ala Glu
 Cys Lys Met Thr Asp Ser Asn Lys Ile Val Cys Lys Cys Thr Lys Glu
 30 Gly Ser Glu Pro Leu Phe Glu Gly Val Phe Cys Ser

ABSTRACT**ABSTRACT**

The present invention relates to a preparation method of the surface protein of malarial Protozoa using E.Coli. Preferably, the present invention relates to a preparation method wherein PV200C polypeptide in C-terminus of Merozoit Surface Protein, which exhibit 100% homology among subspecies of malarial Protozoa is expressed in form of having the histidine marker linked by using recombinant vector which express the above-mentioned polypeptide, and its E.coil transformant and then separated and purified the surface protein PV200C polypeptide by using a column having an affinity for histidine and gel filtration chromatography. The surface protein PV200C polypeptide prepared from E.coli by the preparation method of the present invention can be produce in a large amount and has high purity, sensitivity and specificity, and markedly low pseudo-positive signals, and useful in the diagnostic reagent for malaria.

15

REPRESENTATIVE FIGURE

Figure 1

TITLE OF THE INVENTION

METHOD FOR PRODUCING THE RECOMBINANT MALARIA
SURFACE PROTEIN FROM E.COLI

BRIEF DESCRIPTION OF THE DRAWING

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Figure 1 shows a preparation method of expression vector pELK-MSP expressing PV200C polypeptide present in C-terminus of Merozoite Surface Protein of malarial Protozoa from E.Coli.

5 Figure 2 is a photograph showing a result of electrophoresis of PV200C polypeptide purified by using histidine affinity resin on SDS-PAGE.

M is a size marker;

Lane 1 is the cell culture in which the expression was induced;

Lane 2 is the cell culture in which the expression was not induced; and

10 Lane 3 is the protein that was purified on a column.

Figure 3 is a photograph showing a result of Western Blotting of PV200C polypeptide using the antibodies of the malaria patient.

M is a size marker;

15 Lane 4 is the cell culture in which the expression was induced; and

Lane 5 is the purified Merozoite Surface Protein.

DETAILED DESCRIPTION OF THE INVENTION

OBJECT OF THE INVENTION

20 FIELD OF THE INVENTION AND PRIOR ART THEREOF

The present invention relates to a method for preparing Merozoite Surface Protein(MSP) of malarial Protozoa. Particularly, the present invention relates to an

expression vector comprising genes of PV200C , histidine marker, and T7 promotor; the transformant transformed with the above expression vector; and a method for obtaining malarial antigens by using the above expression vector.

Malaria is a disease caused by malarial Protozoa that infects within human erythrocytes and is carried by mosquitoes. One sixth(billion) of people in the world reside in malaria-risk area and over 500 million people become infected by malaria each year. Malaria causes more than 2 million death each year. Malaria widely spreads all over the world, however, in some regions, malarial infection was eradicated or decreased since 1960's due to effective controls. However, recently, incidence of malaria increases all over the world again, due to increase of drug-resistant strain, increase in resistance to insecticide, abnormal weather such as El nino, etc.

Malaria can be categorized into African type, American type and Asian type depending on geographical distribution as well as characteristics in species and genetic inclination which Protozoa exhibit. Human malaria can be caused by one of four known parasites: *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium malariae* and *Plasmodium ovale*.

Malaria is caused by malarial Protozoa that infects within human erythrocytes and is carried by mosquitoes. Life cycle of malaria is divided into Schizogony and Sporogony. Schizogony is the life cycle in human host, and Sporogony is the life cycle in mosquito host. Human is infected by sporozoites by the bite of infected mosquitoes. The sporozoites transfer to human liver through

blood vessels, and they exist as a dormant state or multiply and develop into schizonts in hepatic cells. After a definite time, the schizonts enter the blood circulation, and multiply in full-scale. When the schizont matures, it ruptures and releases thousands of merozoites into the blood stream. Merozoites invade erythrocytes, and most merozoites go through another round of asexual reproduction, again forming schizonts. Some of the merozoites change into gametocytes. The gametocytes circulate in human bloodstream. When a mosquito bites the infected person, the mosquito sucks up gametocytes along with blood. Then, male gametocyte and female gametocyte in the gut of the mosquito fuse to form zygote. The zygote, which develops in the gut wall as an oocyte, eventually gives rise to the infective sporozoite, which invades the salivary glands and stomach of the mosquito. The mosquito then can infect another human host.

Among them, malaria which occurs in Korea is caused by *Plasmodium vivax* (Chom. S.Y., Korean J Parasit., 32(4), 281-284). *Plasmodium vivax* is characterized in that diagnosis thereof is difficult because latent period is long, and number of Protozoa in blood is few. In the 1970s, cases of malaria were rarely reported. However, in 1993, malaria begun to spread rapidly, and it was reported that the number of patients infected with malaria Protozoa was 3,800 in 1998. Though incidence of malaria increases, the diagnostic method or reagent for malaria were rarely developed in Korea and the most diagnostic reagent are imported. The imported diagnostic reagent for malaria detects malarial antigens such as Lactate Dehydrogenase of malaria. However, sensitivity of the imported diagnostic reagent

to malaria of Korean type where the number of antigen are few is only 50%. Therefore, the diagnostic reagent capable of diagnosing malaria of Korean type is required increasingly.

A blood smear method wherein blood cells infected with malaria Protozoa are stained and the stained sample is then observed through a microscope has been used for diagnosing malaria most frequently. However, this method takes a longer time, and technical education of the examiner is required to judge blood cells infected with malaria Protozoa. Recently, to solve the above-mentioned problems, a diagnostic reagent detecting malarial antigens has been commercially available. However, the concurrent use of the diagnostic reagent and the blood smear method is recommended because the sensitivity of the diagnostic reagent is low. Another method to examine genes of malaria Protozoa is Polymerase Chain Reaction method. This method has high sensitivity, however, there is a difficulty in processes thereof. Therefore, it is not put to practical use and is used only on laboratory scale. Also, such conventional diagnostic methods has a difficulty in diagnosing malaria of Korean type where latent period is long, and number of Protozoa in blood is few.

To solve the above-mentioned problems, the present inventors have developed the diagnostic method for malaria by detecting antibodies in blood of malaria patients using antigens of C-terminus of specific Merozoite Surface Protein of *Plasmodium Vivax*.

Merozoite Surface Protein is surface protein which exist specifically in the

surface of *Plasmodium Vivax* and PV200C polypeptide coding the C-terminus of MSP consists of 108 amino acids which exhibit 100% homology among subspecies of *Plasmodium Vivax*. Accordingly, PV200C polypeptide is very useful for diagnosing malaria of Korean type, if it is used in the object antigen protein for

5 diagnosing malaria.

Hitherto studies on expression of malarial antigen itself have been few, and methods for obtaining proteins by overexpressing genes encoding antigen proteins have been developed. Most antigens were expressed after they were fused with proteins such as blood coagulation factor Xa (Ellinger et al., Virol. 180, 81, 1991),

10 protein A of Staphylococcus (Marczinovits et al., J. Biochem., 31, 225, 1993) and β -galactosidase. As a result, expression amount of fused antigens increased. However, when they are used as diagnostic reagents, pseudo-positive signals increased due to the proteins that were partners of fusion. Although there are attempts to solve the above problem by means of cutting the fused proteins (Chang

15 et al., Biotechnology 3, 985-990, 1985 ; Ellinger et al., Virology, 180, 811-813, 1991), it is not effective in additional decrease of pseudo-positive signals, and the yield decreases and the preparation cost increases because of complex cutting and purification processes.

To solve the above-mentioned problems, the present inventors optimized the

20 expression condition of polypeptide PV200C in C-terminus of specific Merozoite Surface Protein of *Plasmodium Vivax*, malarial Protozoa of malaria of Korean type in E.coli. The preparation method of the present invention can produce antigens

which is highly pure in a large amount and it was confirmed that the anitgens are very sensitive to antibody.

TECHNICAL OBJECT OF THE INVENTION

5 It is object of the present invention to provide a method wherein a recombinant expression vector is prepared by means of cloning genes of Merozoite Surface Protein of *Plasmodium vivax* and is transformed into E.coli, and which produce antigens in a large amount for diagnosing malaria of Korean type which have high sensitivity and specificity and markedly low pseudo-positive signals by
10 using the above transformed E.coli.

CONSTRUCTION OF THE INVENTION

To solve the above-mentioned object, the present invention relates to an expression vector comprising genes of Merozoite Surface Protein of malarial
15 Protozoa(*Plasmodium Vivax*), histidine marker, and T7 promotor; the transformant transformed with the above expression vector; and a method for preparing Merozoite Surface Protein by using the above expression vector.

The present invention will hereinafter be described in more detail.

The present invention relates to an expression vector comprising genes of
20 Merozoite Surface Protein, histidine marker, and T7 promotor.

The genes of Merozoite Surface Protein of the present invention may comprise a part or whole of genes of Merozoite Surface Protein. They may

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preferably comprise the genes of PV200C consisting of the genes encoding 108 amino acids which exhibit 100% homology among subspecies of *Plasmodium Vivax*, as shown in SEQ. ID NO : 1. In a preferable embodiment, the present invention utilizes genes having a size of approximately 330 bp, which are obtained
5 by polymerase chain reaction(PCR) from blood of malaria patients. It is apparent to those skilled in the art that size of genes to be inserted into adventitious gene insertion section of the expression vector, base pair sequence and the like may be varied by prior art techniques.

To obtain the expression vector which can produce the PV200C polypeptide
10 in a large amount from E.Coli, pELK-MSP is prepared by cloning genes of PV200C in downstream of T7 promotor of pET-19b. (Refer to Figure 1) Because there are lacZ genes in pET-19b that is a starting vector, when isopropyl thio- β -D-galactoside(IPTG) is added, IPTG acts as an expression inducer and induce the expression of lacZ. Therefore, the expression of PV200C from T7 promotor
15 becomes induced.

Further, the present invention provides E.Coli transformed with the pELK-MSP. The E.Coli can be prepared by introducing pELK-MSP into E.Coli strain, BL21(DE3)lysS. The introduction method may be a prior art method such as heat shock method and electroporation. Besides BL21(DE3)lysS, various E.Coli strains
20 for transformation can be used. E.Coli/BL21(DE3)lysS/pELK-MSP was deposited in Korean Collection for Type Cultures of Korea Research Institute of Bioscience and Biotechnology deposited on December 18, 1999 (Deposit No. KCTC 8976P).

The present invention provides the preparation method of malarial antigens wherein MSP PV200C protein is expressed in large amount from the above E.coli transformants and then separated and purified.

Preferably, the purification of PV200C protein may use chromatography 5 having an affinity for histidine and gel filtration chromatography. Because 10 histidine residues are expressed in a state where they are linked with N-terminus of PV200C polypeptide expressed by the expression vector, the PV200C polypeptide expressed by the expression vector is firstly purified by histidine affinity resin which is easy in separation and purification by binding of histidine residue. After 10 the separated protein is concentrated, the protein is secondly purified by continuously performing gel filtration chromatography. It was confirmed that antigens of malarial Protozoa having 99% or more of high purity can be obtained by the above method.

The PV200C protein obtained by the above purification method consists of 15 108 amino acids, has a size of 15 KDa and polypeptides that are not severed. Antigenicity and sensitivity of the above antigen were examined using the serum of malaria patient. As a result, it was confirmed by Western Blotting that the malaria patient had antibodies against PV200C and the sensitivity thereof was very high.

The surface protein purified by the preparation method of the present 20 invention has high sensitivity and specificity to antibody as well as high purity. Also, the surface protein purified by the preparation method of the present invention has markedly low pseudo-positive signals, and can be used in malaria antigen for

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diagnosing malaria.

The invention will be further illustrated by the following examples. It will be apparent to those skilled in the art that these examples are given only to illustrate the present invention in more detail, but the invention is not limited to the examples
5 given.

Example 1. Preparation of cDNA from malaria-positive blood.

TRI reagent TM(Sigma Co.) was used to purify RNA from malaria-positive blood. The purification method is the following method provided by Sigma Co. A
10 mixture of 0.1 ml of TRI reagent TM(Sigma Co., Cat. No. T9424) and 0.1 ml of malaria-positive blood was left for 5 minutes at ambient temperature. 20 μ l of BCP was mixed with the mixture, and the resulting mixture was left for 5 minutes at ambient temperature and then was centrifuged for 15 minutes at 12,000 rpm and 4 . As a result of centrifugation, three layers were formed. Only the top layer in which
15 RNAs were contained was transferred to a new tube, and then 50 μ l of isopropanol was added to the tube. The tube was left for 5 minutes at ambient temperature. The resulting mixture was centrifuged for 10 minutes at 12,000 rpm and 4 . The supernatant was discarded. The pellet was washed with 75% ethanol and dissolved in 20 μ l of deionized distilled water treated with DEPC(Diethyl pyrocarbonate),
20 which was used to prepare cDNA.

9 μ l of the above-purified RNA and 1 μ l of N6 random primer (Genotech)

were mixed and reacted for 5 minutes at 65 and then transferred to ice. 4 μ l of PCR buffer, 2 μ l of 0.1M DTT, 1 μ l of 10mM dNTP, 1 μ l of Reverse Transcriptase(Gibco Co.), 1 μ l of RNase inhibitor(Promega) and 1 μ l of deionized distilled water were added to the above mixture, and the resulting mixture was 5 reacted for 1 hour at 42 and then reacted for 10 minutes at 70 to prepare cDNA.

Example 2. preparation of recombinant plasmids comprising genes encoding PV200C

Polymerase chain reaction was performed using cDNAs prepared in Example 10 1 as a template and a pair of primers, sense primer and anti-sense primer, having SEQ. ID. NO:2 and SEQ. ID. NO:3, respectively. The reaction mixture containing 5 μ l of PCR buffer, 5 μ l of 2.5 mM dNTP, 1 μ l of sense primer, 1 μ l of anti-sense primer, 2 μ l of template of cDNA and 35 μ l of deionized distilled water was reacted for 30 seconds at 94 . Thereafter, 1 μ l of Vent polymerase(BioLab) was added to 15 the mixture. Then, the reaction having the following cycle was repeated 36 times:

Denaturation 94 , 30 sec

Primer annealing 55 , 30 sec

Extension 72 , 30 sec.

Again, polymerase chain reaction was performed using the above-amplified 20 DNAs as a template and a pair of primers, sense primer and anti-sense primer, having SEQ. ID. NO:3 and SEQ. ID. NO:4, respectively. The PCR was performed

in the same condition as the above condition. It was confirmed that the amplified DNA had a size of approximately 657 bp through electrophoresis in 1% agarose gel. The amplified DNAs(referred as "Pv200-ct657" hereafter) and pBluscript KS(+)(Stratagene) vector were cleavaged with restriction enzyme, EcoR V, and 5 Pv200-ct657 was inserted into pBluscript KS(+) vector by using T4 DNA ligase(Promega). As a result, recombinant plasmids(referred as "pBC-Pv200-ct657" hereafter) were produced. Then, E. coli strain JM 109 was transformed with pBC-Pv200-ct657 for storage.

10 Example 3. Preparation of expression vector pELK-MSP

Polymerase chain reaction was performed using pBC-Pv200-ct657 prepared in Example 2 as a template and a pair of primers, sense primer and anti-sense primer, having SEQ. ID. NO:5 and SEQ. ID. NO:6, respectively. It was confirmed that the amplified DNA fragment had a size of approximately 330 bp through 15 electrophoresis in 1% agarose gel. The amplified DNA fragments were purified with phenol/chloroform mixture to produce DNA fragments with high purity. The DNA fragment and vector, pET-19b (Novagen) were cleavaged with NdeI and BamHI. Then, the DNA fragment was inserted into pET-19b to prepare an expression vector, pELK-MSP having a size of approximately 6.0 kb (Refer to 20 Figure 2).

E. coli strain BL21(DE3)lysS was transformed with pELK-MSPs by the treatment with CaCl_2 . The transformants were deposited in Korean Collection for

column were removed repeatedly with a washing buffer in which 10mM imidazol and 0.1M NaCl were dissolved in PBS. Then, the surface proteins adsorbed on column were desorbed and separated with an elution buffer in which 1mM imidazol and 0.1M NaCl were dissolved in PBS. As shown in Figure 2, it was confirmed by 5 15% SDS PAGE that the surface proteins having approximately 90% or more of purity were separated by the process.

Then, it was confirmed that PV200C polypeptide having approximately 99% or more of high purity were obtained from recombinant E.Coli by concentrating the separated solution and performing Sephacryl S-200 (Pharmacia Co., Sweden) gel 10 filtration chromatography with the concentrate.

Example 5. assay of antigenicity of the surface protein of malarial Protozoa purified from E. coli

Western Blotting(Towbin et al, *Proc. Natl. Acad. Sci.* 76, 4350-4354, 1979) 15 was performed with the serum of the malaria patient to assay antigenicity of the MSP PV200C prepared in Example 4. The PV200C prepared in Example 4 was loaded into 15% SDS-PAGE gel with a concentration of 5 μ g per well and electrophoresed. After the electrophoresed gel was immersed in a transfer buffer solution containing 25mM Tris-HCl, 190 mM glycine (pH 8.3) and 20% methanol 20 for 30 minutes, the protein was transferred to nitrocellulose membrane(Hafer Transphor Power Lid, Pharmacia Co.). Then, the protein-bound nitrocellulose membrane was blocked for 1 hour at ambient temperature in a blocking buffer(3%

Skim milk /PBS, pH 7.4). The serum of the malaria patient diluted to 1/100 was added to the membrane and was incubated for 1 hour at ambient temperature. Thereafter, the nitrocellulose membrane was washed 5 times at 5 minute-intervals with a washing buffer solution(0.05% Tween/PBS). The second antibody (Vector 5 Labs, U.S.A.) to which HRP was conjugated was diluted to 1/2000, added to the membrane and incubated for 1 hour at ambient temperature. After the incubation, the nitrocellulose membrane was washed with the washing buffer solution, and then both 4-chloro-naphthol and hydrogen peroxide were added to the membrane to induce color development.

10 As a result, it was confirmed that only the surface protein PV200C polypeptide was blotted at 15 kDa position. Therefore, it was confirmed that the surface protein PV200C polypeptide expressed and purified by the preparation method of the present invention was an antigen having high specificity. (Refer to Figure 3)

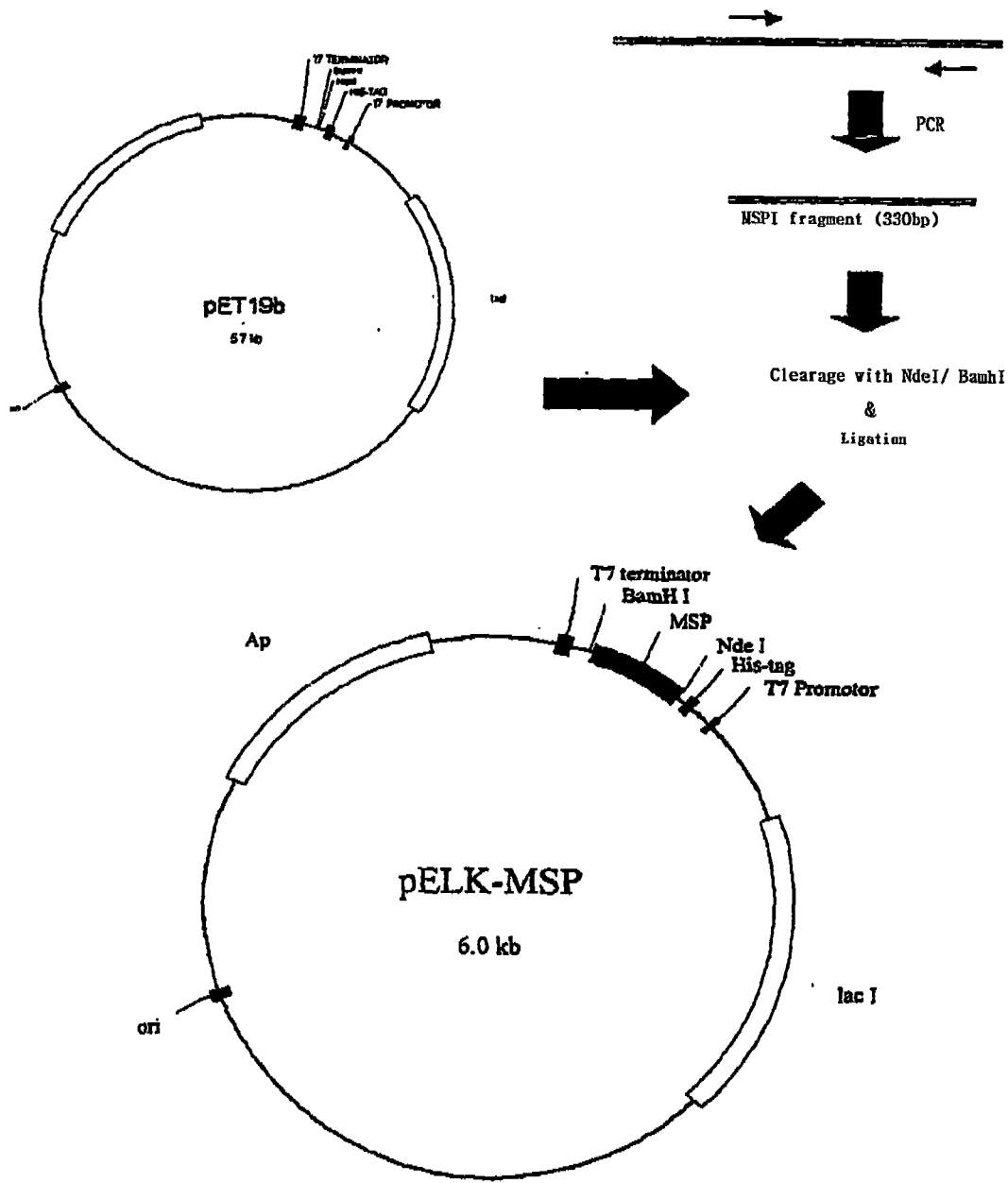
15 EFFECTS OF THE INVENTION

In accordance with the preparation method of the present invention, there is provided a preparation method wherein PV200C polypeptide of MSP of malarial Protozoa can be produced more easily and rapidly than the prior art method does. The surface protein of malarial Protozoa purified by the preparation method of the 20 present invention has high purity, sensitivity and specificity, and useful in the diagnostic reagent for malaria.

133 438

FIGURE

FIGURE 1



435 470

FIGURE 3

M

4

5

43

29

18.4

14.3

5.8

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SEQUENCE LIST

SEQ ID NO.:1

Asn Glu Ser Lys Glu Ile Leu Ser Gln Leu Leu Asn Val Gln Thr Gln
5 Leu Leu Thr Met Ser Ser Glu His Thr Cys Ile Asp Thr Asn Val Pro
Asp Asn Ala Ala Cys Tyr Arg Tyr Leu Asp Gly Thr Glu Glu Trp Arg
Cys Leu Leu Thr Phe Lys Glu Glu Gly Gly Lys Cys Val Pro Ala Ser
Asn Val Thr Cys Lys Asp Asn Asn Gly Gly Cys Ala Pro Glu Ala Glu
Cys Lys Met Thr Asp Ser Asn Lys Ile Val Cys Lys Cys Thr Lys Glu
10 Gly Ser Glu Pro Leu Phe Glu Gly Val Phe Cys Ser

SEQ ID NO.:2

aaaaaagtcg acgccaaaaa ggccgagctg g

15 SEQ ID NO.:3

aaaaaagtcg acgagaagta cctcccgttc c

SEQ ID NO.:4

aaaaaaatcg atttaaagct ccatgcacag g

20

SEQ ID NO.:5

ggcccatatg aacgagtcca aggaaatatt atcc

SEQ ID NO.:6

25 ggacggatcc tcattagcta cagaaaactc cctcaaagag

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ABSTRACT**ABSTRACT**

The present invention relates to an enzyme immunoassay and diagnostic reagent for malaria. More preferably, the present invention relates to an enzyme immunoassay and diagnostic reagent comprising Merozoite Surface Protein as antigen for malaria which detect malaria-specific antibodies in serum sample by using Merozoite Surface Protein of *Plasmodium vivax* as antigen. The present invention comprises an indirect enzyme immunoassay using anti-human IgG antibody and/or anti-human IgM antibody, and an antigen sandwich enzyme immunoassay using a part or whole of malarial Merozoite Surface Protein. The immunoassays detecting malaria-specific antibodies in serum according to the present invention have high specificity and sensitivity, and are useful in diagnosing a type of malaria where latent period is long and number of Protozoa in blood is few.

TITLE OF THE INVENTION

15 ENZYME IMMUNOASSAY FOR MALAIA AND REAGENT USED THEREFOR

DETAILED DESCRIPTION OF THE INVENTON**OBJECT OF THE INVENTON****FIELD OF THE INVENTION AND ITS PRIOR ART**

The present invention relates to an enzyme immunoassay and diagnostic reagent for malaria. Particularly, the present invention relates to an enzyme immunoassay for malaria which detects malaria-specific antibodies in plasma by using antigens of Merozoite Surface Protein(MSP) of *Plasmodium vivax* of malarial Protozoa and diagnostic reagent for malaria prepared by using antigen of the above surface protein.

25 Malaria is a disease caused by malarial Protozoa that infects within human erythrocytes and is carried by mosquitoes. Billion people in the world reside in

2nd label antigen conjugate
L7 2do Anticuerpo
anti-IgM
IgG.

malaria-risk area and over 500 million people become infected by malaria each year. Malaria causes more than 2 million death each year. Malaria widely spreads all over the world, however, in some regions, malarial infection was eradicated or decreased since 1960's due to effective controls. However, recently, incidence of malaria
5 increases all over the world again, due to increase of drug-resistant strain, increase in resistance to insecticide, abnormal weather such as El nino, etc.

Malaria can be categorized into African type, American type and Asian type. Each type differs in geographical distribution as well as characteristics in species and genetic inclination which Protozoa exhibit.

10 Life cycle of malaria is divided into Schizogony and Sporogony. Schizogony is the life cycle in human host, and Sporogony is the life cycle in mosquito host. Human is infected by sporozoites by the bite of infected mosquitoes. The sporozoites transfer to human liver through blood vessels, and they exist as a dormant state or multiply and develop into schizonts in hepatic cells. After a definite time, the schizonts enter the
15 blood circulation, and multiply in full-scale. When the schizont matures, it ruptures and releases thousands of merozoites into the blood stream. Merozoites invade erythrocytes, and most merozoites go through another round of asexual reproduction, again forming schizonts. Some of the merozoites change into gametocytes. The gametocytes circulate in human bloodstream. When a mosquito bites the infected
20 person, the mosquito sucks up gametocytes along with blood. Then, male gametocyte and female gametocyte in the gut of the mosquito fuse to form zygote. The zygote, which develops in the gut wall as an oocyte, eventually gives rise to the infective sporozoite, which invades the salivary glands and stomach of the mosquito. The mosquito then can infect another human host.

25 Antibiotics such as chloroquine and primaquine have been used in treatment of malaria. However, recently, malaria Protozoa have developed drug resistance to such

antibiotics. Therefore, acquired drug resistance poses a serious difficulty on treatment of malaria.

A blood smear method has been used in the diagnosis of malaria most frequently. This method is a classical method wherein blood cells infected with malaria Protozoa are stained and the stained sample is then observed through a microscope. However, this method takes a longer time, and technical education of the examiner is required to judge blood cells infected with malaria Protozoa. Recently, a diagnostic reagent detecting malarial antigens has been commercially available. However, the concurrent use of the diagnostic reagent and the blood smear method is recommended because the sensitivity of the diagnostic reagent is low. Another method to examine genes of malaria Protozoa is Polymerase Chain Reaction method. This method has high sensitivity, however, there is used only on laboratory scale.

In Korea, malaria has been known as Hakjil. From the 1960s, attempts to eradicate this disease begun, and in the 1970s, cases of malaria were rarely reported. However, after a patient infected with malaria Protozoa was reported near DMZ in 1993, malaria begun to spread rapidly, and it was reported that the number of patients infected with malaria Protozoa was 3,800 in 1998.

Human malaria can be caused by one of four known parasites: *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium malariae* and *Plasmodium ovale*. Among them, malaria which occurs in Korea is caused by *Plasmodium vivax* (Chom. S.Y., Korean J Parasit., 32(4), 281-284). *Plasmodium vivax* is characterized in that diagnosis thereof is difficult because latent period is long, and number of Protozoa in blood is few. The imported diagnostic reagent for malaria detects malarial antigens such as Lactate Dehydrogenase of malaria. However, the imported diagnostic reagent has a difficulty in diagnosing malaria of Korean type where latent period is long, and numbers of Protozoa and antigen in blood are few, because sensitivity of the diagnostic

reagent is only 50%.

The present inventors have developed an enzyme immunoassay for malaria by detecting malaria-specific antibodies in blood of malaria patients. More particularly, the present invention provides an indirect enzyme immunoassay using anti-human IgG 5 antibody or anti-human IgM antibody, and an antigen sandwich enzyme immunoassay using a part or whole of malarial Merozoite Surface Protein. The immunoassays for malaria of the present invention are more sensitive than the prior art immunoassay detecting specific antibodies, and can diagnose malarial carriers as well as malaria patients, and are useful in diagnosing malaria of Korean type where latent period is 10 long and numbers of Protozoa and antigen in blood are few.

TECHNICAL OBJECT OF THE INVENTION

It is the object of the present invention to provide an enzyme immunoassay for malaria which detects malaria-specific antibodies in blood by using antigens of surface protein of malarial Protozoa.

15 Particularly, it is object of the present invention to provide an indirect enzyme immunoassay using anti-human IgG antibody and/or anti-human IgM antibody.

It is object of the present invention to provide an antigen sandwich enzyme immunoassay using a part or whole of malarial Merozoite Surface Protein.

Further, it is object of the present invention to provide the diagnostic reagent 20 for malaria having enhanced sensitivity and specificity by using antigens of a part or whole of Merozoite Surface Protein of malarial Protozoa.

CONSTRUCTION OF THE INVENTION

To solve the above-mentioned problems, the present invention provides an enzyme immunoassay for malaria which detects malaria-specific antibodies in blood 25 by using antigens of surface protein of malarial Protozoa.

Particularly, the present invention provides an indirect enzyme immunoassay

2141 4/16

using anti-human IgG antibody and/or anti-human IgM antibody.

Also, the present invention provides an antigen sandwich enzyme immunoassay using a part or whole of malarial Merozoite Surface Protein.

The present invention provides antigens of PV200C polypeptide in C-terminus
5 of Merozoite Surface Protein of malarial Protozoa.

Also, the present invention provides the recombinant transformant expressing said surface antigen protein.

Further, the present invention provides a diagnostic reagent for malaria comprising the surface antigens produced from the recombinant transformant, a solid
10 support coated with said surface antigens, the second labeled antigen conjugates combined with enzyme markers bound to said surface antigen, and a substrate solution containing a color fixing agent.

The present invention will hereinafter be described in more detail.

The invention provides antigens of surface protein of malarial Protozoa in order
15 to detect malaria-specific antibodies in blood.

The invention provides antigens of C-terminus of Merozoite Surface Protein of malarial Protozoa in order to detect malaria-specific antibodies in blood using antigen-antibody reaction. The above C-terminus comprises amino acid sequences that are common to subspecies of *Plasmodium Vivax*, and especially comprises a polypeptide
20 consisting of 108 amino acids (referred as "PV200C" hereafter) which exhibit 100% homology among subspecies of *Plasmodium Vivax*, as shown in SEQ. ID NO : 1.

Therefore, the present invention uses antigens of a part or whole of Merozoite Surface Protein of malarial Protozoa. Preferably, the present invention uses antigens of C-terminus of Merozoite Surface Protein of malarial Protozoa. More preferably, the
25 present invention uses antigens of PV200C polypeptide in C-terminus of Merozoite Surface Protein of malarial Protozoa. The above protein can be expressed in a large

amount from recombinant transformants. The present inventors prepared a yeast transformant and E.Coli transformant which express PV200C polypeptide, and deposited them in Korean Collection for Type Cultures of Korea Research Institute of Bioscience and Biotechnology on December 18, 1999 (Deposit No. KCTC 8977P, 5 KCTC 8976P). The prevent invention provides PV200C, surface antigen of malarial Protozoa having 99% or more of high purity expressed by the above recombinant transformant and then separated and purified as antigens of immunoassay and diagnostic reagent for malaria.

The present invention provides an indirect enzyme immunoassay for malaria 10 using a solid support coated with surface antigens of malarial Protozoa.

The present invention provides a 96-well plate coated with surface antigen of malarial Protozoa separated and purified from transformant as a solid support for diagnosing malaria. In order to detect the surface antigen coated in well plate, the above immunoassay uses ~~the second anti-human IgG antibody and/or the second anti-~~ 15 human IgM antibody derived from goat ~~combined with horse radish peroxidase(HRP)~~ as the second labeled antigen conjugate.

Indirect enzyme immunoassay for diagnosing malaria comprises (1) pouring plasma sample into each well of the well plate coated with recombinant surface antigen; (2) adding the second ~~labeled~~ antigen conjugate consisting of HRP and the 20 ~~IgG and IgM derived from goat to combine the second labeled antigen conjugate with~~ the antibody bound to the surface antigen; (3) washing the well plate; (4) adding a substrate solution consisting of a buffer solution and a color fixing agent to induce color development; and (5) measuring the absorbance by using a 96-well plate reader.

In the above indirect enzyme immunoassay for malaria, malaria-specific 25 antibodies in blood of the patient are combined with the surface antigens of malarial Protozoa immobilized on the solid support and then HRP-anti-human IgG antibody or

HRP-anti-human IgM antibody which is the second labeled antigen conjugate is combined with the antigen-antibody complex. Thereafter, a color fixing agent that is included in the substrate solution added is decomposed by HRP of the second labeled antigen conjugate to induce a color development, and then absorbance is measured by
5 using a 96-well plate reader to detect existence of malaria-specific antibodies and amount of the antibodies.

In the above indirect immunoassay, malaria-positive samples(plasma of the malaria patients) and malaria-negative samples(plasma of normal people) were diagnosed. As a result, the above method exhibited approximately 90% of sensitivity
10 and 95.8% of specificity.

The present invention provides an antigen sandwich enzyme immunoassay for malaria using HRP-surface antigen conjugates.

The present invention provides HRP-surface antigen conjugates.

Particularly, the present invention provides HRP-surface antigen conjugates
15 prepared by mixing surface antigen of malarial Protozoa separated and purified from recombinant transformant with HRP reaction solution oxidized. An antigen sandwich enzyme immunoassay using HRP-surface antigen conjugate comprises (1) pouring plasma sample into each well of the well plate coated with recombinant surface antigen; (2) adding HRP-surface antigen conjugate to combine HRP-surface antigen
20 conjugate with the antibody bound to the surface antigen; (3) washing the well plate; (4) adding a substrate solution consisting of a buffer solution and a color fixing agent to induce color development; and (5) measuring the absorbance by using a 96-well plate reader.

In the malarial antigen sandwich enzyme immunoassay of the prevent
25 invention, malaria-specific antibodies in blood of the patient are combined with the surface antigens of malarial Protozoa immobilized on the solid support and then HRP-

44/4 999

anti-human IgG antibody or HRP-anti-human IgM antibody which is the second labeled antigen conjugate is combined with the antigen-antibody complex. Thereafter, a color fixing agent that is included in the substrate solution added is decomposed by HRP of the second labeled antigen conjugate to induce a color development, and then

5 absorbance is measured by using a 96-well plate reader to detect existence of malaria-specific antibodies and amount of the antibodies. The above indirect enzyme immunoassay indirectly detects malaria-specific antibodies in blood of the patient, by using anti-human IgG antibody and/or anti-human IgM antibody derived from goat as an antigen of the second labeled antigen conjugate. In the meantime, the above antigen

10 sandwich enzyme immunoassay can detect all malaria-specific antibodies in blood of the patient, by using a surface antigen itself derived from malarial Protozoa as an antigen of the labeled antigen conjugate. Therefore, the above antigen sandwich enzyme immunoassay is an immunoassay having enhanced sensitivity and specificity, which can diagnose malaria patients in the latent period as well as malaria patients.

15 In the above indirect enzyme immunoassay, malaria-positive samples(plasma of the malaria patients) and malaria-negative samples(plasma of normal people) were diagnosed. As a result, the above method exhibited approximately 98.5% of sensitivity and 99.6% of specificity.

The enzyme immunoassay for malaria of the present invention provides the

20 antigen protein expressed from the recombinant yeast or E.Coli which is transformed with the expression vector prepared by insertion of genes encoding antigen proteins of *Plasmodium vivax*, and purified and a solid support coated with said antigen protein. When the blood sample of man who is suspected to be a malaria patient is poured into the solid support, malaria-specific antibodies in blood become specifically combined

25 with the antigens immobilized on the solid support. After the second labeled antigen conjugate is added to this, the second labeled antigen conjugate becomes combined

with malaria-specific antibodies by recognizing Fc (fragment crystallization) region of the malaria-specific antibodies combined with the antigens immobilized on the solid support. Then, the marker of the second labeled antigen conjugate decomposes the color fixing agent in the substrate solution to induce a color development. The indirect
5 enzyme immunoassay of the present invention detects malaria-specific antibodies bound to the antigen by using HRP-anti IgG or HRP-anti IgM conjugate using anti-human IgG antibody and/or anti-human IgM antibody wherein anti-human IgG antibody and/or HRP-anti-human IgM antibody is/are derived from goat. Also, the above antigen sandwich enzyme immunoassay of the present invention detects malaria-
10 specific antibodies combined with the antigen by using HRP-surface antigen conjugate using a part or whole of surface protein of Malaria Protozoa. In above two methods, plasma of the malaria patients and plasma of normal people were diagnosed for malaria. As a result, both methods exhibited more than 90 % of sensitivity and specificity. Especially, the antigen sandwich immunoassay of the present invention
15 exhibited markedly enhanced sensitivity and specificity because it detects all malaria-specific antibodies in blood.

The present invention provides a diagnostic reagent for malaria having enhanced sensitivity and specificity, comprising an antigen using the surface protein of malarial Protozoa; a solid support coated with surface antigens of malarial Protozoa;
20 the second labeled antigen conjugates combined with the marker; and a substrate solution containing a color fixing agent.

Preferably, in the above diagnostic reagents of the present invention, an antigen includes a part or whole of Merozoite Surface Protein of malarial Protozoa, and a preferable example includes a part or whole of PV200C polypeptide of specific surface
25 protein of *Plasmodium Vivax*, which exist commonly among subspecies of *Plasmodium Vivax*, as shown in SEQ. ID NO : 1.

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The marker of the second labeled antigen conjugate for the diagnostic reagent of the present invention includes, but is not limited to, for example, an enzyme such as horse radish peroxidase(HRP) and alkaline phosphatase, colloidal gold, fluorescent materials, dyes or the like. In the above diagnostic reagent of the present invention, the
5 second labeled antigen conjugate detects existence of malaria-specific antibodies by binding to Fc (fragment crystallization) region of the malaria-specific antibodies in which there is little variation. Preferably, As the second labeled antigen conjugate, the present invention provides HRP-anti-human IgG antibody and/or HRP-anti-human IgM antibody wherein anti-human IgG antibody and/or HRP-anti-human IgM antibody
10 is/are derived from goat. Also, as a preferable labeled antigen conjugate, the present invention provides HRP-surface antigen conjugate wherein the surface antigen is a part or whole of surface protein of Malaria Protozoa.

Finally, the diagnostic reagent of the present invention comprises a substrate solution inducing color development when reacted with the marker of the second
15 labeled antigen conjugate. Preferably, the substrate solution consists of a buffer solution and a color fixing agent, tetramethylbenzidine which induces color development when reacted with the marker of the labeled antigen conjugate. The color fixing agent such as tetramethylbenzidine in the substrate solution is decomposed by HRP which is a preferable marker of the second labeled antigen conjugate to induce
20 color development. Then, existence of the IgM and amount of the IgM are detected by measuring the absorbance.

The invention will be further illustrated by the following examples. It will be apparent to those skilled in the art that these examples are given only to illustrate the present invention in more detail, but the invention is not limited to the examples given.

11/17 452

Example 1. Expression and purification of surface antigen of malarial Protozoa from the yeast transformant

(Step 1) Preparation of the yeast transformant and expression of surface antigen of malarial Protozoa

5 The above-amplified α -Pv200-19 and pYES-2 vectors were cleavaged with restriction enzymes, HindIII and Xho I, respectively. Then, α -Pv200-19 and pYES-2 vector were linked by T4 ligase to be a recombinant expression vector that is referred as "pYLJ-MSP". Yeast *Saccharomyces cerevisiae* INVSC1 was transformed with pYLJ-MSP according to Hinnen method(*Proc. Natl. Acad. Sci.*, U.S.A 75, 1929-1933, 10 1978), and then the yeast transformants pYLJ-MSP/*S. cerevisiae* INVSC1 were obtained. The yeast transformants were deposited in Korean Collection for Type Cultures of Korea Research Institute of Bioscience and Biotechnology deposited on December 18, 1999 (Deposit No. KCTC 8977P)

In order to produce the surface protein MSP from yeast, the transformants 15 pYLJ-MSP/*S. cerevisiae* INVSC1 were inoculated in 100 ml of YEPD medium containing 2% glucose and cultivated at 37 overnight. Then, the culture medium was transferred to 2L of YEP medium containing 1% glucose and 1% galactose, respectively, and cultivated for 72 hours at 30 . The yeast transformants expressed PV200C polypeptide, exhausting glucose. The expressed PV200C polypeptide was 20 secreted into out of cell by the α -factor leader peptide linked with N-terminus of PV200C polypeptide. Thereafter, the α -factor leader peptide was removed by peptidase existing in cell membrane. Accordingly, only the desired protein was secreted into culture medium.

(Step 2) Purification of surface antigen expressed from the yeast transformant

25 In order to obtain the PV200C polypeptide secreted into culture medium, the

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biomass was removed by centrifuging the culture medium that was cultivated for 2 days. Then, only the fluid of culture medium was taken and concentrated by ultrafiltrating apparatus(Amicon, U.S.A.). For easily purifying the concentrated fluid, the surface proteins linked with cationic histidine residues were adsorbed on Probond
5 column(Invitrogen) to which anionic nickels were coupled by using six histidine residues linked with C-terminus of the above-amplified Pv200 expressed from the recombinant yeast. The contaminants which were not adsorbed on column were removed by phosphate buffer containing 10mM imidazol, and then the surface proteins adsorbed on column were desorbed and separated by 500 mM imidazol and phosphate
10 buffer. The surface proteins having approximately 90% or more of purity were separated by the process. The surface proteins having approximately 99% or more of high purity were obtained by concentrating the separated solution and performing Sephacryl S-200 gel filtration chromatography with the concentrate.

The prevent invention used the surface antigen of malarial gametocyte having
15 high purity separated and purified from yeast transformant as antigens of immunoassay and diagnostic reagent for malaria.

Example 2. Expression and purification of surface antigen of malarial Protozoa from the E.coli transformant

20 (Step 1) Preparation of the E.coli transformant and expression of surface antigen of malarial Protozoa

The amplified DNA fragments were purified with phenol/chloroform mixture to produce DNA fragments with high purity. The DNA fragment and vector, pET-19b (Novagen) were cleavaged with NdeI and BamHI and were ligased with T4 ligase and
25 then recombinant expressiong vector, pELK-MSP were obtained. E. coli strain

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BL21(DE3)lysS was transformed with pELK-MSPs by the treatment with CaCl_2 . The E.coli transformants pELK-MSP/BL21 were obtained. The transformants were deposited in Korean Collection for Type Cultures of Korea Research Institute of Bioscience and Biotechnology deposited on December 18, 1999 (Deposit No. KCTC 5 8976P)

The transformants pELK-MSP/strain BL21 (KCTC 8976P) were cultivated for 12 hours in LB medium containing 50 $\mu\text{g/ml}$ of chloramphenicol and 100 $\mu\text{g/ml}$ of ampiciline. Then, 50 ml of the resulting cultures were again inoculated in 1L of LB medium and were cultivated for about 2 hours at 37 . When absorbance(OD 600) of 10 the cultures became 0.3, IPTG (Isopropyl β -D-Thiogalactopyranoside) was added to the medium to a final concentration of 0.2 mM. Then, the cultures were cultivated further for 7 hours.

(Step 2) Purification of surface antigen expressed from the E.coli transformant

After the cultivation, only the cells were isolated by centrifugation. The 15 obtained cells were suspended in 30 ml of Phosphate Buffered Saline(PBS). Then, E. Coli in the suspension was crushed with Sonicator(Branson, Sonifier 450) and centrifuged at 5000 rpm. Because a part of expressed MSP proteins was in the supernatant and the remainder was in the pellet, both the supernatant and the pellet were completely suspended in 4M urea buffer(pH 7.5) where 4M urea was dissolved in 20 PBS, and then were centrifuged, by which only the supernatant was collected.

Because the overexpressed surface protein has His marker consisting of 10 histidines in N-terminus thereof, the protein was adsorbed on Probond column(Invitrogen) for purification. The contaminants which were not adsorbed on column were removed repeatedly with a washing buffer in which 10mM imidazol was 25 dissolved in PBS. Then, the surface proteins adsorbed on column were desorbed and

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separated with an elution buffer in which 500 mM imidazol was dissolved in PBS. It was confirmed that the surface proteins having approximately 90% or more of purity were separated by the process. Then, it was confirmed that PV200C polypeptide having approximately 99% or more of high purity were obtained from recombinant
5 E.Coli by concentrating the separated solution and performing Sephacryl S-200 (Pharmacia Co., Sweden) gel filtration chromatography with the concentrate.

The prevent invention used PV200C polypeptide of MSP of malarial gametocyte having high purity separated and purified from E.coli transformant as antigens of immunoassay and diagnostic reagent for malaria.

10 **Example 3. Preparation of well plate coated with the surface antigens of malarial Protozoa**

To provide a solid support to be used for the diagnosis of malaria, the solution of recombinant surface antigens of malarial Protozoa prepared in Example 1 or 2 was diluted with 0.1M carbonate buffer (pH 9.5) to 0.5 µg/ml, and 100 µl of the solution
15 was added to each well of 96-well plate. To adsorb the surface antigens on the well, the well plate was sealed tightly and left for 18 hours at 4 . Then, the surface antigens which were not adsorbed on the well were removed by adding 300 µl of phosphate buffer solution containing 5% general goat serum to each well and leaving it for 18 hours at 4 . After the solution that remained in the well was discarded, moisture was
20 removed by leaving the well plate for 1 hour at ambient temperature. Then, the well plate was transferred to a hermetic container with a dehumidifying agent and stored in a refrigerator at 4 .

In the embodiment of the present invention, the above-prepared well plate coated with the surface antigens of malarial Protozoa was used as a solid support in a
25 diagnostic reagent and immunoassay for malaria.

2/51 456

Example 4. Diagnosis of malaria by indirect enzyme immunoassay

To diagnose malaria by indirect enzyme immunoassay, the solid support prepared by coating 96-well plate(Nunc) with recombinant surface antigens prepared in Example 1 or 2 according to the method of Example 3, and the
5 labeled antigen conjugate consisting of HRP and anti-human IgM antibody or anti-human IgG antibody derived from goat were used.

100 μ l of a sample dilution solution containing 1.15 mg/ml NaOH, 0.2 mg/ml of KH_2PO_4 , 0.2 mg/ml KCl, 8 mg/ml NaCl, 300 μ l/ml bovine serum, 0.2 mg/ml thimerosal and the like was added to each well of 96-well plate(Nunc)
10 coated with recombinant surface antigens. Then, 10 μ l of antibody-negative plasma sample or 10 μ l of antibody-positive plasma sample was added to the wells, and the resulting mixtures were well mixed. The well plate was incubated for 60 minutes in a reactor at 37 . After the reaction terminated, the well plate was washed 5 times with 300 μ l of a phosphate buffer solution containing 0.05%
15 Tween 20. The second anti-human IgG antibody(Cappel) or the second anti-human IgM antibody(Bethyl) derived from goat to which HRP was conjugated was diluted to 1/40,000 with a dilution solution containing 10mM Tris, 0.5M NaCl, 1mM CaCl_2 , 0.5% glycerol, 0.2 ml/ml bovine serum, 100 μ l/ml goat serum, 0.05% Tween 20, 0.2 mg/ml thimerosal and 50 μ g/ml phenol red. 100 μ l
20 of the second diluted enzyme conjugate was added to each well of the 96-well plate and the well plate was incubated for 60 minutes at 37 . After the reaction terminated, the well plate was washed 5 times with 0.05% Tween 20/phosphate buffer solution. 100 μ l of a substrate solution containing 100 μ g/ml tetramethylbenzidine, 0.006% hydrogen peroxide and citrate-phosphate buffer
25 solution(pH 4.5) was added to each well. After color development in a dark place

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for 30 minutes, 100 μ l of the reaction-stopping solution (2N sulfuric acid solution) was added to each well to terminate the color development reaction. Then, the absorbance was measured at 450 nm (reference wavelength, 650 nm) by using the 96 well plate reader (Molecular Devices, USA).

5 Tetramethylbenzidine that is a color fixing agent in the substrate solution was decomposed by HRP conjugated to anti-human IgG antibody or anti-human IgM antibody, to induce the color development. Then, existence of malaria-specific antibodies and amount of the antibodies were detected by measuring the absorbance.

10 According to the above method, 20 malaria-positive samples(plasma of the malaria patients) and 98 malaria-negative samples(plasma of normal people) were diagnosed for malaria. When positive cut-off value was determined by adding 0.5 to the mean of the negative samples, according to the indirect enzyme immunoassay using the recombinant surface antigens of malarial Protozoa according to the present
15 invention, 18 out of the 20 malaria patients' samples were judged to be positive and 46 out of 48 normal people's samples were judged to be negative. Therefore, the indirect enzyme immunoassay of the present invention exhibited 90% of sensitivity and 95.8% of specificity.

20 **Example 5. Preparation of the surface antigen of malarial Protozoa conjugated to HRP**

The labeled antigen conjugates to be used for antigen sandwich enzyme immunoassay were prepared.

The recombinant surface antigens of malarial Protozoa prepared in Example 1
25 or 2 was dialyzed for 1 day at 4 against 1 L of 0.01 M sodium carbonate buffer

solution (pH 9.6). The buffer solution was exchanged 3 times during the dialysis. Also, 5 mg of HRP was dissolved in 0.5 ml of distilled water in a tube, and then 100 μ l of 42 mg/ml NaIO₄ was added to the HRP solution. After the tube was wrapped with foil, it was shaken for 30 minutes at ambient temperature for the oxidation reaction. After 5 HRP was oxidized enough, 60 μ l of 1M glycerol was added to the reaction solution. Thereafter, the tube wrapped with foil was shaken for 30 minutes at ambient temperature to terminate the oxidation reaction.

For a conjugation reaction of HRP and the surface antigen, salts were removed from the HRP reaction solution by PD10 column (Pharmacia) saturated with 0.01 M 10 sodium carbonate buffer (pH 9.6). The above solution of the surface antigens was added to the oxidized HRP reaction solution. Then, the tube was wrapped with foil and shaken overnight at ambient temperature to prepare conjugates of HRPs and the surface antigens. After the conjugation reaction of HRP and the surface antigen was terminated, in order to stabilize the HRP-surface antigen conjugates, 40.8 μ l of 4 15 mg/ml NaBH₄ solution was added to the tube, and the tube wrapped with foil was shaken at 4 °C for 2 hours for the reaction.

To remove HRPs in the reaction solution which were not conjugated, the surface proteins conjugated to HRPs were adsorbed on Probond column that was equilibrated with phosphate buffer solution by utilizing histidine residues linked with C-terminus of 20 the surface protein. Then, HRPs which were not conjugated were removed by phosphate buffer solution. The HRP-surface antigen conjugates adsorbed on Probond column were desorbed by phosphate buffer solution containing 1M imidazol. The conjugates were collected. The protein concentration of the collected HRP-surface antigen conjugates was determined by using commercially available BSA protein 25 quantitative test kit. The sample was stored in a refrigerator after addition of bovine

454 459

serum albumin (BSA) of final concentration of 1 %. The above-prepared HRP-surface antigen conjugates were used as labeled antigen conjugates in an antigen sandwich enzyme immunoassay and diagnostic reagent for malaria.

5 **Example 6. Diagnosis of malaria by antigen sandwich enzyme immunoassay using HRP-surface antigen conjugates**

Antigen sandwich enzyme immunoassay for antibody of surface antigen of malarial was performed by using the HRP-surface antigen conjugates prepared by combining HRP with recombinant surface antigen as prepared in Example 5.

- 10 30 μ l of the solution of the HRP-surface antigen conjugates was added to each well of 96-well plate(Nunc) coated with recombinant surface antigens. Then, 100 μ l of malaria-positive samples(plasmas of malaria patients) or 100 μ l of malaria-negative samples(plasmas of normal people) was added to the wells, and the resulting mixtures were well mixed. The well plate was incubated for 90 minutes in a reactor at 37 .
- 15 After the reaction terminated, the well plate was washed 5 times with each 300 μ l of a phosphate buffer solution containing Tween 20. 100 μ l of a substrate solution containing 100 μ g/ml tetramethylbenzidine, 0.006% hydrogen peroxide and citrate-phosphate buffer solution(pH 4.5) was added to each well of the well plate. After color development of the plate in a dark place for 30 minutes, 100 μ l of the reaction-stopping
- 20 solution (2N sulfuric acid solution) was added to each well to terminate the color development reaction. Then, the absorbance was measured at 450 nm (reference wavelength, 650 nm) by using the 96 well plate reader (Molecular Devices, USA). Tetramethylbenzidine that is a color fixing agent in the substrate solution was decomposed by HRP conjugated to the surface antigen, to induce the color
- 25 development. Then, existence of malaria-specific antibodies and amount of the

antibodies were detected by measuring the absorbance.

According to the above method, 202 malaria-positive samples(plasmas of malaria patients) and 400 malaria-negative samples(plasmas of normal people) were diagnosed for malaria. When positive cut-off value was determined by adding 0.5 to the mean of the negative samples, according to the antigen sandwich enzyme immunoassay using the HRP-surface antigen conjugates according to the present invention, 398 out of the 400 malaria patients' samples were judged to be positive and 199 out of 202 normal people's samples were judged to be negative. Therefore, the antigen sandwich enzyme immunoassay of the present invention exhibited 98.5% of sensitivity and 99.6% of specificity.

An indirect enzyme immunoassay using anti-human IgG antibody and/or anti-human IgM antibody derived from goat, and an antigen sandwich enzyme immunoassay using HRP-surface antigen conjugates detect malarial specific-antibody in blood. Therefore, they are immunoassays having enhanced sensitivity and specificity, which can diagnose malarial carriers as well as malarial patients.

EFFECTS OF THE INVENTION

An indirect enzyme immunoassay using anti-human IgG antibody and/or anti-human IgM antibody, and an antigen sandwich enzyme immunoassay using HRP-surface antigen conjugates detect malarial specific-antibody. Therefore, they can diagnose malarial carriers as well as malarial patients. Also, they can simplify the immunoassay because of reacting plasma sample and antigen conjugates at the same time. A diagnostic reagent for malaria comprising antigens of the surface protein of malarial Protozoa, a solid support coated with said surface antigens, the second labeled antigen conjugates, and a substrate solution containing a color fixing agent can detect existence of malaria-specific antibodies and amount of the antibodies more simply and

2156 7/10

exactly.

Therefore, an enzyme immunoassay and diagnostic reagent for malaria. according to the present invention are useful in diagnosing malaria of Korean type where latent period is long and numbers of Protozoa and antigen in blood are few and 5 the possibility of recurrence is high.

SEQUENCE LIST

SEQ ID NO.:1

Asn Glu Ser Lys Glu Ile Leu Ser Gln Leu Leu Asn Val Gln Thr Gln
Leu Leu Thr Met Ser Ser Glu His Thr Cys Ile Asp Thr Asn Val Pro
10 Asp Asn Ala Ala Cys Tyr Arg Tyr Leu Asp Gly Thr Glu Glu Trp Arg
Cys Leu Leu Thr Phe Lys Glu Glu Gly Gly Lys Cys Val Pro Ala Ser
Asn Val Thr Cys Lys Asp Asn Asn Gly Gly Cys Ala Pro Glu Ala Glu
Cys Lys Met Thr Asp Ser Asn Lys Ile Val Cys Lys Cys Thr Lys Glu
Gly Ser Glu Pro Leu Phe Glu Gly Val Phe Cys Ser

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459**ABSTRACT****ABSTRACT**

The present invention relates to an immunoassay, diagnostic reagent and diagnostic method for malaria by using antigens of malarial Protozoa. Particularly, the present invention relates to an immunoassay, diagnostic reagent and diagnostic method for malaria which detect malaria Protozoa antigen-specific IgM in blood. The immunoassay, diagnostic reagent and diagnostic method detecting malaria-specific IgM in blood according to the present invention can distinguish malaria patients from normal people who completely recover from malaria, and makes an early diagnosis of malaria patient possible.

REPRESENTATIVE FIGURE

Figure 1

KEY WORD

Malaria, Diagnostic Reagent, malaria-specific IgM

TITLE OF THE INVENTION

IMMUNOASSAY, A DIAGNOSTIC REAGENT AND A DIAGNOSTIC METHOD FOR MALARIA BY DETECTION OF MALARIA-SPECIFIC IgM

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a graph showing a result of diagnosing 216 malaria-positive samples and 353 malaria-negative samples for malaria, according to malaria-specific IgM capture enzyme immunoassay.

Figure 2 is a graph showing results of diagnosing 75 malaria-positive samples and 92 malaria-negative samples for malaria, according to malaria-specific IgM capture enzyme immunoassay and indirect enzyme immunoassay.

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Figure 3 is a graph showing results of diagnosing 129 malaria-negative samples classified by age for malaria, according to malaria-specific IgM capture enzyme immunoassay and antigen sandwich enzyme immunoassay.

5 DETAILED DESCRIPTION

PURPOSE OF THE INVENTION

FIELD OF THE INVENTION AND THE PRIOR ART THEREOF

The present invention relates to an immunoassay, diagnostic reagent and
10 diagnostic method for malaria. Particularly, the present invention relates to an immunoassay, diagnostic reagent and diagnostic method for malaria which detects IgM against antigens of malarial Protozoa.

Malaria is a disease caused by malarial Protozoa that infects within human
15 erythrocytes and is carried by mosquitoes. Billion people in the world reside in malaria-risk area and over 500 million people become infected by malaria each year. Malaria causes more than 2 million death each year. Malaria widely spreads all over the world, however, in some regions, malarial infection was eradicated or decreased since 1960's due to effective controls. However, recently, incidence of malaria
20 increases all over the world again, due to increase of drug-resistant strain, increase in resistance to insecticide, abnormal weather such as El nino, etc.

Malaria can be categorized into African type, American type and Asian type. Each type differs in geographical distribution as well as characteristics in species and genetic inclination which Protozoa exhibit.

25 Life cycle of malaria is divided into Schizogony and Sporogony. Schizogony is the life cycle in human host, and Sporogony is the life cycle in mosquito host. Human is infected by sporozoites by the bite of infected mosquitoes. The sporozoites transfer to human liver through blood vessels, and they exist as a dormant state or

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multiply and develop into schizonts in hepatic cells. After a definite time, the schizonts enter the blood circulation, and multiply in full-scale. When the schizont matures, it ruptures and releases thousands of merozoites into the blood stream. Merozoites invade erythrocytes, and most merozoites go through another round of asexual reproduction, again forming schizonts. Some of the merozoites change into gametocytes. The gametocytes circulate in human bloodstream. When a mosquito bites the infected person, the mosquito sucks up gametocytes along with blood. Then, male gametocyte and female gametocyte in the gut of the mosquito fuse to form zygote. The zygote, which develops in the gut wall as an oocyte, eventually gives rise to the infective sporozoite, which invades the salivary glands and stomach of the mosquito. The mosquito then can infect another human host.

Antibiotics such as chloroquine and primaquine have been used in treatment of malaria. However, recently, malaria Protozoa have developed drug resistance to such antibiotics. Therefore, acquired drug resistance poses a serious difficulty on treatment of malaria.

A blood smear method has been used in the diagnosis of malaria most frequently. This method is a classical method wherein blood cells infected with malaria Protozoa are stained and the stained sample is then observed through a microscope. However, this method takes a longer time, and technical education of the examiner is required to judge blood cells infected with malaria Protozoa. Recently, a diagnostic reagent detecting malarial antigens has been commercially available. However, the concurrent use of the diagnostic reagent and the blood smear method is recommended because the sensitivity of the diagnostic reagent is low. Another method to examine genes of malaria Protozoa is Polymerase Chain Reaction method. This method has high sensitivity, however, is used only on laboratory scale.

In Korea, malaria has been known as Hakjil. From the 1960s, attempts to eradicate this disease begun, and in the 1970s, cases of malaria were rarely

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reported. However, after a patient infected with malaria Protozoa was reported near DMZ in 1993, malaria begun to spread rapidly, and it was reported that the number of patients infected with malaria Protozoa was 3,800 in 1998.

Human malaria can be caused by one of four known parasites: *Plasmodium*
5 *vivax*, *Plasmodium falciparum*, *Plasmodium malariae* and *Plasmodium ovale*. Among
them, malaria which occurs in Korea is caused by *Plasmodium vivax*. *Plasmodium*
vivax is characterized in that diagnosis thereof is difficult because latent period is
long, and number of Protozoa in blood is few. The imported diagnostic reagent for
malaria detects malarial antigens such as Lactate Dehydrogenase of malaria.
10 However, the imported diagnostic reagent has a difficulty in diagnosing malaria of
Korean type where latent period is long, and numbers of Protozoa and antigen in
blood are few, because sensitivity of the diagnostic reagent is only 50%.

TECHNICAL OBJECT OF THE INVENTION

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It is the first object of the present invention to provide an immunoassay for
malaria which detects malaria-specific IgM in blood by using antigens of malarial
Protozoa.

It is the second object of the present invention to provide an IgM capture
20 immunoassay for malaria which detects malaria-specific IgM in blood

It is the third object of the present invention to provide a diagnostic reagent for
malaria with elevated specificity and sensitivity which comprises antigens of malaria
Protozoa.

It is the forth object of the present invention to provide a diagnostic method for
25 malaria which detects malaria-specific IgM in blood by using antigens of malaria
Protozoa.

CONSTITUTION OF THE INVENTION

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To achieve the above-mentioned objects, the present inventors have developed an immunoassay for malaria which detects only malaria-specific IgM in blood of malaria patients among antibodies against malarial Protozoa. The present
5 inventors have developed a diagnostic method for malaria by detecting antibodies for malaria. This method can distinguish with high sensitivity malaria patients from normal people who completely recover from malaria. However, among antibodies against malaria, IgG can last for several years or several tens of years after experience of malaria. Therefore, a method of examining antibodies including IgG
10 against malaria in the area where malaria was prevalent for a long time results in many pseudo-positive signals, which can cause confusion. In the antigen sandwich immunoassay of the present invention, few malarial antibody-positive responses have been found in teens to thirties, however, 5% or more of malarial antibody-positive responses have been found in forties or more, in case of normal people
15 uninfected with malaria. That is, relatively high IgG pseudo-positive signals occur in forties or more. This is because probability that people over forties were exposed to mosquitoes infected with malaria before 1960s is high. Similarly, in the area where malaria is prevalent, malaria-specific IgGs remain in the body of man who completely recovers from malaria, and relatively high pseudo-positive signals appear in the assay
20 for antibody to malaria. Therefore, effectiveness of the assay for antibody against malaria to diagnose malaria will decrease. Accordingly, for diagnosing malaria in people over forties or the area where malaria is prevalent, a method of examining IgM of which titer disappears with time after the treatment of malaria will be more effective than a method of examining IgG of which titer remains even after the treatment of
25 malaria.

The present invention provides an immunoassay for malaria which detects malaria-specific IgM in blood by using antigens of malarial Protozoa. As a embodiments, the present invention provides an IgM capture immunoassay for

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malaria that uses anti-human IgM antibody on a solid support. The immunoassay for malaria according to the present invention is more sensitive than the prior art immunoassay detecting specific antigens, and makes an early diagnosis of malaria patient possible. Also, the immunoassay for malaria according to the present invention can distinguish malaria patients from normal people who completely recover from malaria.

The present invention provides an immunoassay for malaria detecting malaria-specific IgM in blood by using antigens of malarial Protozoa. Preferably, the present invention provides an IgM capture immunoassay detecting malaria-specific IgM, and more preferably, the present invention provides an IgM capture enzyme immunoassay. Also, preferably, the present invention provides an immunoassay detecting malaria-specific IgM in blood by using anti-human IgM antibody.

An IgM capture immunoassay for malaria detecting malaria-specific IgM in blood according to the present invention, comprises (i) immobilizing anti-human IgM antibodies on a solid support; (ii) pouring blood serum or plasma sample to combine malaria-specific IgM with the anti-human IgM antibodies immobilized on the solid support; (iii) adding labeled antigen conjugates to combine the labeled antigen conjugates with the malaria-specific IgM bound to the anti-human IgM antibodies immobilized on the solid support; and (iv) analyzing by using the markers of the labeled antigen conjugates bound to the malaria-specific IgM.

The present invention provides a diagnostic reagent for malaria detecting malaria-specific IgM in blood, comprising antigens of malarial Protozoa. Preferably, the present invention provides a diagnostic reagent for malaria, comprising a solid support coated with anti-human IgM antibodies; labeled antigen conjugates; and a substrate solution containing a color fixing agent. As a preferable example of the above-mentioned labeled antigen conjugates, there is a labeled enzyme bounded to malaria surface antigen prepared from recombinant transformants. Also, the present invention provides a diagnostic method for malaria which detects malaria-specific IgM

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in blood by using antigens of malaria Protozoa.

The present invention will hereinafter be described in more detail.

The present invention provides an immunoassay for malaria detecting
5 malaria-specific IgM in blood by using antigens of malarial Protozoa,

In the above immunoassay, a surface antigen of malarial Protozoa may be used as the antigen of malarial Protozoa. Also, in the above immunoassay, a recombinant antigen may be used as the antigen of malarial Protozoa.

A preferable example of the surface antigen of malarial Protozoa includes a
10 part or whole of Merozoite Surface Protein of *Plasmodium Vivax*, and a more preferable example includes C-terminus of Merozoite Surface Protein of *Plasmodium Vivax*. The above C-terminus of Merozoite Surface Protein of *Plasmodium Vivax* comprises amino acid sequences that are common to subspecies of *Plasmodium Vivax*, and especially comprises a polypeptide consisting of 108 amino acids (referred
15 as "PV200C" hereafter) which exhibit 100% homology among subspecies of *Plasmodium Vivax*, as shown in SEQ. ID NO : 1. A preferable example of the C-terminus of Merozoite Surface Protein of *Plasmodium Vivax* includes a part or whole of PV200C polypeptide in the C-terminus of Merozoite Surface Protein of *Plasmodium Vivax*. Also, a preferable example of amino acid sequences of the PV200C
20 polypeptide includes amino acid sequences as shown in SEQ. ID NO : 1.

The PV200C polypeptide can be expressed in a large amount from recombinant transformants. The present inventors prepared a yeast transformant and E.Coli transformant which express PV200C polypeptide, and deposited them in Korean Collection for Type Cultures of Korea Research Institute of Bioscience and
25 Biotechnology on December 18, 1999 (Deposit No. KCTC 8977P, KCTC 8976P). The present invention provides an immunoassay and diagnostic reagent for malaria which detecting malaria-specific antibodies by using the above PV200C polypeptide purified with 99% purity and expressed from recombinant transformants.

The present invention provides an immunoassay for malaria detecting malaria-specific IgM in blood by using antigens of malarial Protozoa. Preferably, the present invention provides an IgM capture immunoassay detecting malaria-specific IgM, and more preferably, the present invention provides an IgM capture enzyme immunoassay. Also, preferably, the present invention provides an immunoassay detecting malaria-specific IgM in blood by using anti-human IgM antibody.

The present invention provides an immunoassay for malaria detecting malaria-specific IgM in blood by using antigens of malarial Protozoa. Preferably, the present invention provides an IgM capture immunoassay detecting malaria-specific IgM in blood by using antigens of malarial Protozoa, and more preferably, the present invention provides an IgM capture enzyme immunoassay detecting malaria-specific IgM in blood by using antigens of malarial Protozoa. Also, preferably, the present invention provides an immunoassay detecting malaria-specific IgM in blood by using antigens of malarial Protozoa and anti-human IgM antibody.

The present invention provides a 96-well plate coated with anti-human IgM antibody derived from goat as a preferable solid support for diagnosing malaria. Also, the present invention provides a labeled antigen conjugate consisting of a marker and an antigen of malarial Protozoa to detect malaria-specific IgM. The labeled antigen conjugate of the present invention consists of a marker and an antigen, and the antigen maybe preferably a recombinant surface antigen of malarial Protozoa purified from yeast or E.Coli transformant to detect malaria-specific IgM. The marker of the labeled antigen conjugate includes, but is not limited to, for example, an enzyme such as horse radish peroxidase(HRP) and alkaline phosphatase, colloidal gold, fluorescent materials, dyes or the like. Preferably, the marker of the labeled antigen conjugate is an enzyme, and more preferably, horse radish peroxidase(HRP).

A preferable example of IgM capture immunoassay for malaria detecting malaria-specific IgM in blood according to the present invention, comprises (i) immobilizing anti-human IgM antibodies on a solid support; (ii) pouring blood serum or

plasma sample to combine malaria-specific IgM with the anti-human IgM antibodies immobilized on the solid support; (iii) adding labeled antigen conjugates consisting of markers and antigens of malarial Protozoa to combine the labeled antigen conjugates with the malaria-specific IgM; and (iv) analyzing by using the markers of the labeled
5 antigen conjugates bound to the malaria-specific IgM.

Preferably, in the above IgM capture immunoassay, the present invention may utilize a surface antigen of malarial Protozoa as the antigen of the labeled antigen conjugate in the step (iii). A preferable example of the surface antigen includes a part or whole of Merozoite Surface Protein of *Plasmodium Vivax*.

10 A more preferable example of IgM capture immunoassay for malaria detecting malaria-specific IgM in blood according to the present invention, comprises (1) pouring blood serum or plasma sample into each well of the well plate coated with anti-human IgM antibody derived from goat; (2) washing the well plate; (3) adding the labeled antigen conjugate consisting of HRP and the recombinant surface antigen to
15 combine the labeled antigen conjugate with the IgM bound to the anti-human IgM antibody; (4) washing the well plate; (5) adding a substrate solution consisting of a buffer solution and a color fixing agent to induce color development; and (6) measuring the absorbance by using a 96-well plate reader.

As a preferable embodiment, the immunoassay for malaria of the present
20 invention provides a solid support coated with anti-human IgM antibody derived from goat. Also, as a preferable embodiment, the immunoassay for malaria of the present invention provides a labeled antigen conjugate wherein an antigen protein is bound to HRP as described in the example 1 below. The antigen protein is expressed from the recombinant yeast or E.Coli which is transformed with the expression vector prepared
25 by insertion of genes encoding antigen proteins of *Plasmodium vivax*, and purified.

When the blood sample of man who is suspected to be a malaria patient is poured into the solid support coated with anti-human IgM antibody derived from goat,

human IgM in blood becomes combined with the anti-human IgM antibody immobilized on the solid support. After the labeled antigen conjugate consisting of HRP and surface antigen of malarial Protozoa is added to the solid support, the conjugate becomes combined with IgM of the malaria patient among IgMs bound to
5 the anti-human IgM antibodies immobilized on the solid support. The HRP of the conjugate bounded to IgM of the malaria patient decomposes the color fixing agent in the substrate solution to induce color development. Finally, the immunoassay for malaria of the present invention diagnoses the infection of malaria by detecting existence of the IgM in blood and amount of the IgM by measuring the absorbance.

10 In the above immunoassay of the present invention, plasmas of the malaria patients and plasmas of normal people were diagnosed for malaria. As a result, the above method exhibited more than 98% of sensitivity and more than 99% of specificity. Particularly, all the plasmas of normal people in forties or more who were infected with malaria in the past and are suspected to have IgG against malaria were
15 diagnosed to be negative. The immunoassay detecting malaria-specific IgM according to the present invention has more improved specificity than the above antigen sandwich enzyme immunoassay does, and much more improved sensitivity and specificity than the above indirect enzyme immunoassay does.

Also, the present invention provides a diagnostic reagent for malaria detecting
20 malaria-specific IgM in blood, comprising antigens of malarial Protozoa.

Anti-human IgM antibody for the diagnostic reagent of the present invention includes, but is not limited to, for example, anti-human IgM antibody derived from goat. Preferably, the anti-human IgM antibody is specific to mu chain of IgM, purified by affinity between antigen and antibody, and does not cross-react with IgG or other
25 immunoglobulin.

The present invention provides a diagnostic reagent for malaria detecting malaria-specific IgM in blood, comprising antigens of malarial Protozoa. Preferably, the present invention provides a diagnostic reagent for malaria detecting malaria-

467 472

specific IgM in blood, comprising surface antigens of malarial Protozoa. In the above diagnostic reagent according to the present invention, a preferable example of the surface antigen of malarial Protozoa includes a part or whole of Merozoite Surface Protein of *Plasmodium Vivax*, and a more preferable example includes C-terminus of Merozoite Surface Protein of *Plasmodium Vivax*. A preferable example of the C-terminus of Merozoite Surface Protein of *Plasmodium Vivax* includes a part or whole of PV200C polypeptide in the C-terminus of Merozoite Surface Protein of *Plasmodium Vivax*. Also, a preferable example of amino acid sequences of the PV200C polypeptide includes amino acid sequences as shown in SEQ. ID NO : 1.

Also preferably, the present invention provides a diagnostic reagent for malaria detecting malaria-specific IgM in blood, comprising a solid support coated with anti-human IgM antibodies; labeled antigen conjugates; and a substrate solution containing a color fixing agent. Preferably, in the above diagnostic reagent, the present invention may utilize a surface antigen of malarial Protozoa as the antigen of the labeled antigen conjugate. A preferable example of the surface antigen of malarial Protozoa includes a part or whole of Merozoite Surface Protein of *Plasmodium Vivax*, and a more preferable example includes C-terminus of Merozoite Surface Protein of *Plasmodium Vivax*.

The marker of the labeled antigen conjugate for the diagnostic reagent of the present invention includes, but is not limited to, for example, an enzyme such as horse radish peroxidase(HRP) and alkaline phosphatase, colloidal gold, fluorescent materials, dyes or the like. Preferably, the marker of the labeled antigen conjugate is horse radish peroxidase(HRP).

Also, the diagnostic reagent of the present invention comprises a substrate solution inducing color development when reacted with the marker of the labeled antigen conjugate. Preferably, the substrate solution consists of a buffer solution and a color fixing agent which induces color development when reacted with the marker of the labeled antigen conjugate. A preferable example of the color fixing agent includes

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tetramethylbenzidine and the like. The color fixing agent such as tetramethylbenzidine in the substrate solution is decomposed by HRP which is a preferable marker of the labeled antigen conjugate to induce color development. Then, existence of the IgM and amount of the IgM are detected by measuring the
5 absorbance.

Also, The present invention provides a diagnostic method for malaria comprising detection of malaria-specific IgM in blood, using antigens of malarial Protozoa. In the above diagnostic method, preferably The present invention provides a diagnostic method for malaria comprising detection of malaria-specific IgM in blood,
10 using anti-human IgM antibody. In the above diagnostic method according to the present invention, a example of the antigen of malarial Protozoa includes a surface antigen of malarial Protozoa, and a preferable example includes a part or whole of Merozoite Surface Protein of *Plasmodium Vivax*, and a more preferable example includes C-terminus of Merozoite Surface Protein of *Plasmodium Vivax*. A preferable
15 example of the C-terminus of Merozoite Surface Protein of *Plasmodium Vivax* includes a part or whole of PV200C polypeptide in the C-terminus of Merozoite Surface Protein of *Plasmodium Vivax*. Also, a preferable example of amino acid sequences of the PV200C polypeptide includes amino acid sequences as shown in SEQ. ID NO : 1.

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The invention will be further illustrated by the following examples. It will be apparent to those skilled in the art that these examples are given only to illustrate the present invention in more detail, but the invention is not limited to the examples given.

25 **Example 1. Expression and purification of a surface antigen of malarial Protozoa from yeast transformants**

Example 1-1. Preparation of yeast transformants and expression of a surface antigen of malarial Protozoa

The DNA coding PV200C is amplified using cDNA as template
5 reverstranscribed from RNA purified from malaria-positive blood. The DNA coding
PV200C and pYES-2 vectors were cleavaged with restriction enzymes, HindIII and
Xho I, respectively. Then, DNA coding PV200C and pYES-2 vector were linked by T4
ligase(Promega) to be a recombinant expression vector that is referred as "pYLJ-
MSP". Yeast *Saccharomyces cerevisiae* INVSC1 was transformed with pYLJ-MSP
10 according to Hinnen method(*Proc. Natl. Acad. Sci.*, U.S.A 75, 1929-1933, 1978). The
yeast transformants were deposited in Korean Collection for Type Cultures of Korea
Research Institute of Bioscience and Biotechnology deposited on December 18, 1999
(Deposit No. KCTC 8977P)

The transformants pYLJ-MSP/*S. cerevisiae* INVSC1 were inoculated in YEPD
15 medium containing 2% glucose at 37°. Then, the culture medium was transferred to
YEP medium containing 1% glucose and 1% galactose, respectively, and cultivated.
The yeast transformants expressed PV200C polypeptide, exhausting glucose. The
expressed PV200C polypeptide was secreted into out of cell by the α -factor leader
peptide linked with N-terminus of PV200C polypeptide. Thereafter, the α -factor
20 leader peptide was removed by peptidase existing in cell membrane. Accordingly,
only the PV200C polypeptide was secreted into culture medium.

Example 1-2. Purification of a surface antigen of malarial Protozoa from yeast transformants.

In order to obtain the PV200C polypeptide secreted into culture medium, the
25 biomass was removed by centrifuging the culture medium that was cultivated for 2
days. Then, only the fluid of culture medium was taken and concentrated by
ultrafiltrating apparatus(Amicon, U.S.A.). For easily purifying the concentrated fluid,
the surface proteins linked with cationic histidine residues were adsorbed on Probond

470-775

column(Invitrogen) to which anionic nickels were coupled by using six histidine residues linked with C-terminus of the above-amplified Pv200-19.

The contaminants which were not adsorbed on column were removed by phosphate buffer containing 10mM imidazol, and then the surface proteins adsorbed
5 on column were desorbed and separated by 500 mM imidazol and phosphate buffer. The surface proteins having approximately 90% or more of purity were separated by the above process. Then, the surface proteins having approximately 99% or more of high purity were obtained by concentrating the separated solution and performing Sephacryl S-200 gel filtration chromatography with the concentrate.

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Example 2. Expression and purification of a surface antigen of malarial Protozoa from E, coli transformants.

**Example 2-1. Preparation of E, coli transformants and expression of a surface
15 antigen of malarial Protozoa.**

The amplified DNA fragments coding MSP PV200C prepared in the same way of Example 1-1, were purified with phenol/chloroform mixture to produce DNA fragments with high purity. The DNA fragment and vector, pET-19b (Novagen) were
20 cleavaged with NdeI and BamHI. Then, the DNA fragment was inserted into pET-19b to prepare an expression vector, pELK-MSP

E. coli strain BL21(DE3)lysS was transformed with pELK-MSPs by the treatment with CaCl₂. The transformants were deposited in Korean Collection for Type Cultures of Korea Research Institute of Bioscience and Biotechnology on
25 December 18, 1999 (Deposit No. KCTC 8976P)

The transformants pELK-MSP/strain BL21 (KCTC 8976P) were cultivated for 12 hours in LB medium containing 50 µg/ml of chloramphenicol and 100 µg/ml of

amphotericin. Then, 50 ml of the resulting cultures were again inoculated in 1L of LB medium and were cultivated for about 2 hours at 37°C. When absorbance(OD 600) of the cultures became 0.3, IPTG (Isopropyl thio-β-D-galactoside) was added to the medium to a final concentration of 0.2 mM. Then, the cultures were cultivated further
5 for 7 hours.

Example 2-2. Purification of a surface antigen of malarial Protozoa from E. coli transformants.

After the cultivation, only the cells were isolated by centrifugation. The
10 obtained cells were suspended in 30 ml of Phosphate Buffered Saline(PBS). Then, E. Coli in the suspension was crushed with Sonicator(Branson, Sonifier 450) and centrifuged at 5000 rpm. Because a part of expressed MSP proteins was in the supernatant and the remainder was in the pellet, both the supernatant and the pellet were completely suspended in 4M urea buffer(pH 7.5) where 4M urea was dissolved
15 in PBS, and then were centrifuged, by which only the supernatant was collected.

Because the overexpressed surface protein has His marker consisting of 10 histidines in N-terminus thereof, the protein was adsorbed on histidine affinity column(Invitrogen) for purification. The contaminants which were not adsorbed on column were removed repeatedly with a washing buffer in which 10mM imidazole and
20 0.1M NaCl were dissolved in PBS. Then, the surface proteins adsorbed on column were desorbed and separated with an elution buffer in which 500mM imidazole and 0.1M NaCl were dissolved in PBS. It was confirmed that the surface proteins having approximately 90% or more of purity were separated by the above process.

Then, it was confirmed that PV200C polypeptide having approximately 99% or
25 more of high purity were obtained from recombinant E.Coli by concentrating the separated solution and performing Sephacryl S-200 gel filtration chromatography with the concentrate.

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Example 3. Diagnosis of malaria by IgM capture enzyme immunoassay

To diagnose malaria by IgM capture enzyme immunoassay, a solid support coated with anti-human IgM antibodies derived from goat, and the labeled antigen conjugates consisting of HRP and recombinant surface antigens
5 purified from yeast or E.Coli transformant were used.

The plate was prepared as follows: anti-human IgM antibodies (anti-human IgM mu chain-specific, affinity purified, Bethyl Co., USA) derived from goat were diluted with phosphate buffer solution (pH 7.0) to 1~10 µg/ml and then 100~200 µl of the mixture was added to each well of the polystyrene plate. The
10 well plate was left for approximately 12~18 hours at ambient temperature, and the solution that remained in the well was removed by suction. 250 µl of 0.1% gelatin or 0.1% casein dissolved in phosphate buffer solution was added to each well, and the well plate was left for 2 hours at ambient temperature. Then, the solution that remained in the well was removed by suction. The well plate was
15 dried for 18 hours in a cold room and used.

100 µl of a sample dilution solution that is a phosphate buffer solution containing 0.1% bovine serum albumin was added to each well of the well plate coated with anti-human IgM antibodies derived from goat. Then, 2~20 µl of antibody-negative plasma sample or 2~20 µl of antibody-positive plasma sample
20 was added to the wells, and the resulting mixtures were well mixed. The well plate was incubated for 60 minutes in a reactor at 37 °C. After the reaction terminated, the well plate was washed 5 times with 300 µl of a phosphate buffer solution containing 0.05% Tween 20.

The surface antigens of malarial Protozoa purified from yeast or E.Coli
25 transformant which were prepared in Example 1 or 2 were conjugated with HRPs according to the following method.

The recombinant surface antigens of malarial Protozoa prepared in Example 1 or 2 was dialyzed for 1 day at 4 °C against 1 L of 0.01 M sodium carbonate buffer

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solution (pH 9.6). The buffer solution was exchanged 3 times during the dialysis. Also, 5 mg of HRP was dissolved in 0.5 ml of distilled water in a tube, and then 100 μ l of 42 mg/ml NaIO_4 was added to the HRP solution. After the tube was wrapped with foil, it was shaken for 30 minutes at ambient temperature for the oxidation reaction. After 5 HRP was oxidized enough, 60 μ l of 1M glycerol was added to the reaction solution. Thereafter, the tube wrapped with foil was shaken for 30 minutes at ambient temperature to terminate the oxidation reaction.

For a conjugation reaction of HRP and the surface antigen, salts were removed from the HRP reaction solution by PD10 column (Pharmacia) saturated with 10 0.01 M sodium carbonate buffer (pH 9.6). The above solution of the surface antigens was added to the oxidized HRP reaction solution. Then, the tube was wrapped with foil and shaken overnight at ambient temperature to prepare conjugates of HRPs and the surface antigens. After the conjugation reaction of HRP and the surface antigen was terminated, in order to stabilize the HRP-surface antigen conjugates, 40.8 μ l of 4 15 mg/ml NaBH_4 solution was added to the tube, and the tube wrapped with foil was shaken at 4 $^{\circ}\text{C}$ for 2 hours for the reaction. To remove HRPs in the reaction solution which were not conjugated, the surface proteins conjugated to HRPs were adsorbed on Probond column that was equilibrated with phosphate buffer solution by utilizing histidine residues linked with C-terminus of the surface protein. Then, HRPs which 20 were not conjugated were removed by phosphate buffer solution. The HRP-surface antigen conjugates adsorbed on Probond column were desorbed by phosphate buffer solution containing 1M imidazole. The conjugates were collected. The protein concentration of the collected HRP-surface antigen conjugates was determined by using commercially available BSA protein quantitative test kit. The sample was 25 stored in a refrigerator after addition of bovine serum albumin (BSA) of final concentration of 1 %.

The above-prepared conjugates consisting of HRPs and the surface antigens were diluted to 1:100 to 1:1,000 with phosphate buffer solution (pH 7.0)

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containing 1% BSA and 0.05% Tween 20. Then, 100 μ l of the solution of the HRP-surface antigen conjugates was added to each well of the well plate. The well plate was incubated for 30 minutes at 37 $^{\circ}$ C. After the reaction terminated, the well plate was washed 5 times with phosphate buffer solution containing 0.05% Tween 20. 100 μ l of a substrate solution containing 100 μ g/ml tetramethylbenzidine, 0.006% hydrogen peroxide and citrate-phosphate buffer solution (pH 4.5) was added to each well of the plate. After color development in a dark place for 30 minutes, 100 μ l of the reaction-stopping solution (1N sulfuric acid solution) was added to each well to terminate the color development reaction. Then, the absorbance was measured at 450 nm (reference wavelength, 650 nm) by using the 96 well plate reader (Molecular Devices, USA). Tetramethylbenzidine that is a color fixing agent in the substrate solution was decomposed by HRP of the labeled antigen conjugate, to induce the color development. Then, existence of malaria-specific IgM and amount of the IgM were detected by measuring the absorbance.

According to the above method, 216 malaria-positive samples (plasmas of malaria patients) and 353 malaria-negative samples (plasmas of normal people) were diagnosed for malaria. When positive cut-off value was determined by adding 0.05 to the mean of the negative samples, according to IgM capture enzyme immunoassay using the recombinant surface antigen of malarial Protozoa according to the present invention, 212 out of 216 malaria patients' samples were judged to be positive and 351 of 353 normal people's samples were judged to be negative. Therefore, the IgM capture enzyme immunoassay of the present invention exhibited 98.1% of sensitivity and 99.4% of specificity (Refer to Figure 1).

Example 4. Comparison of IgM capture enzyme immunoassay with indirect enzyme immunoassay

In the IgM capture enzyme immunoassay, a solid support coated with anti-human IgM antibodies derived from goat, and the labeled antigen conjugates consisting of HRPs and the recombinant surface antigens purified from yeast or E.Coli transformant were used. The IgM capture enzyme immunoassay was performed according to the method of Example 3.

Also, the indirect enzyme immunoassay was performed according to the following method for comparison with IgM capture enzyme immunoassay.

The solution of recombinant surface antigens of malarial Protozoa prepared in Example 1 or 2 was diluted with 0.1M carbonate buffer (pH 9.5) to 0.5 µg/ml, and 100 µl of the solution was added to each well of 96-well plate. To adsorb the surface antigens on the well, the well plate was sealed tightly and left for 18 hours at 4 °C. Then, the surface antigens which were not adsorbed on the well were removed by adding 300 µl of phosphate buffer solution containing 5% general goat serum to each well and leaving it for 18 hours at 4 °C. After the solution that remained in the well was discarded, moisture was removed by leaving the well plate for 1 hour at ambient temperature. Then, the well plate was transferred to a hermetic container with a dehumidifying agent and stored in a refrigerator at 4 °C.

100 µl of a sample dilution solution containing 1.15 mg/ml NaOH, 0.2 mg/ml of KH₂PO₄, 0.2 mg/ml KCl, 8 mg/ml NaCl, 300 µl/ml bovine serum, 0.2 mg/ml thimerosal and the like was added to each well of 96-well plate(Nunc) coated with recombinant surface antigens according to the above method. Then, 10 µl of antibody-negative plasma sample or 10 µl of antibody-positive plasma sample was added to the wells, and the resulting mixtures were well mixed. The well plate was incubated for 60 minutes in a reactor at 37 °C. After the reaction terminated, the well plate was washed 5 times with 300 µl of a phosphate buffer solution containing 0.05% Tween 20. Anti-human IgG antibody(Cappel) or anti-human IgM antibody(Bethyl) derived from goat to which HRP was conjugated was diluted to 1/40,000 with a dilution solution containing 10mM Tris, 0.5M NaCl, 1mM CaCl₂, 0.5% glycerol, 0.2 ml/ml bovine serum, 100 µl/ml

goat serum, 0.05% Tween 20, 0.2 mg/ml thimerosal and 50 µg/ml phenol red. 100 µl of the diluted conjugate was added to each well of the 96-well plate and the well plate was incubated for 60 minutes at 37 °C. After the reaction terminated, the well plate was washed 5 times with 0.05% Tween 20/phosphate buffer solution. 100 µl of a substrate solution containing 100 µg/ml tetramethylbenzidine, 0.006% hydrogen peroxide and citrate-phosphate buffer solution (pH 4.5) was added to each well. After color development in a dark place for 30 minutes, 100 µl of the reaction-stopping solution (2N sulfuric acid solution) was added to each well to terminate the color development reaction. Then, the absorbance was measured at 450 nm (reference wavelength, 650 nm) by using the 96 well plate reader (Molecular Devices, USA). Tetramethylbenzidine that is a color fixing agent in the substrate solution was decomposed by HRP conjugated to anti-human IgG antibody or anti-human IgM antibody, to induce the color development. Then, existence of malaria-specific antibodies and amount of the antibodies were detected by measuring the absorbance.

To compare the IgM capture enzyme immunoassay with the indirect enzyme immunoassay, 75 malaria-positive samples (plasmas of malaria patients) and 92 malaria-negative samples (plasmas of normal people) were diagnosed for malaria. When positive cut-off value was determined by adding 0.05 to the mean of the negative samples, according to the IgM capture enzyme immunoassay using the recombinant surface antigens of malarial Protozoa according to the present invention, 73 out of 75 malaria patients' samples were judged to be positive and 92 of 92 normal people's samples were judged to be negative. Therefore, the IgM capture enzyme immunoassay of the present invention exhibited 97.3% of sensitivity and 100% of specificity.

In the meantime, when positive cut-off value was determined by adding 0.2 to the mean of the negative samples, according to the indirect enzyme immunoassay of the present invention, 59 out of 75 malaria patients' samples were judged to be

positive and 85 of 92 normal people's samples were judged to be negative. Therefore, the indirect enzyme immunoassay of the present invention exhibited 78.6% of sensitivity and 92.4% of specificity. (Refer to Figure 2).

5 Example 5. Comparison of IgM capture enzyme immunoassay with antigen sandwich enzyme immunoassay in normal people classified by age

In the IgM capture enzyme immunoassay, a solid support coated with anti-human IgM antibodies derived from goat, and the labeled antigen conjugates
10 consisting of HRPs and the recombinant surface antigens purified from yeast or E.Coli transformant were used. The IgM capture enzyme immunoassay was performed according to the method of Example 3.

Also, the antigen sandwich enzyme immunoassay was performed according to the following method for comparison with IgM capture enzyme immunoassay.

15 30 μ l of the solution of the HRP-surface antigen conjugates was added to each well of 96-well plate(Nunc) coated with recombinant surface antigens according to the method of Example 4. Then, 100 μ l of malaria-positive samples(plasmas of malaria patients) or 100 μ l of malaria-negative samples(plasmas of normal people) was added to the wells, and the resulting mixtures were well mixed. The well plate was
20 incubated for 90 minutes in a reactor at 37 °C. After the reaction terminated, the well plate was washed 5 times with each 300 μ l of a phosphate buffer solution containing Tween 20. 100 μ l of a substrate solution containing 100 μ g/ml tetramethylbenzidine, 0.006% hydrogen peroxide and citrate-phosphate buffer solution(pH 4.5) was added to each well of the well plate. After color development of the plate in a dark place for
25 30 minutes, 100 μ l of the reaction-stopping solution (2N sulfuric acid solution) was added to each well to terminate the color development reaction. Then, the absorbance was measured at 450 nm (reference wavelength, 650 nm) by using the 96 well plate reader (Molecular Devices, USA). Tetramethylbenzidine that is a color

fixing agent in the substrate solution was decomposed by HRP conjugated to the surface antigen, to induce the color development. Then, existence of malaria-specific antibodies and amount of the antibodies were detected by measuring the absorbance.

5

To compare the IgM capture enzyme immunoassay with the antigen sandwich enzyme immunoassay, plasma samples of 129 normal people were diagnosed for malaria. The 129 normal people consisted of 26 people in teen, 43 people in twenties, 33 in thirties and 27 in forties.

10 When positive cut-off value was determined by adding 0.05 to the mean of the negative samples, according to the IgM capture enzyme immunoassay using the recombinant surface antigens of malarial Protozoa according to the present invention, 129 of 129 normal people's samples were judged to be negative. Therefore, the IgM capture enzyme immunoassay of the present
15 invention exhibited 100% of specificity.

In the meantime, when positive cut-off value was determined by adding 0.05 to the mean of the negative samples, according to the antigen sandwich enzyme immunoassay of the present invention, 127 of 129 normal people's samples were judged to be negative. Therefore, the antigen sandwich enzyme
20 immunoassay of the present invention exhibited 98.4% of specificity. Especially, when plasma samples of normal people in thirties or less who were born after the prevalence period of malaria were diagnosed for malaria, the antigen sandwich enzyme immunoassay of the present invention exhibited 100% of specificity. However, when plasma samples of normal people in forties
25 who were born in the prevalence period of malaria were diagnosed for malaria, 2 of 27 normal people's samples were judged to be positive and the antigen sandwich enzyme immunoassay of the present invention exhibited 92.6% of specificity. Therefore, the specificity of the antigen sandwich enzyme

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immunoassay of the present invention decreased in specific ages (Refer to Figure 3).

EFFECT OF THE INVENTION

The immunoassay, diagnostic reagent and diagnostic method for malaria
5 detecting malaria-specific IgM in blood by using antigens of malarial Protozoa according to the present invention can distinguish malaria patients from normal people who completely recover from malaria, because they detect only malaria-specific IgM in blood. Therefore, malaria can be diagnosed effectively in older people and in the area where malaria was prevalent for a long time.

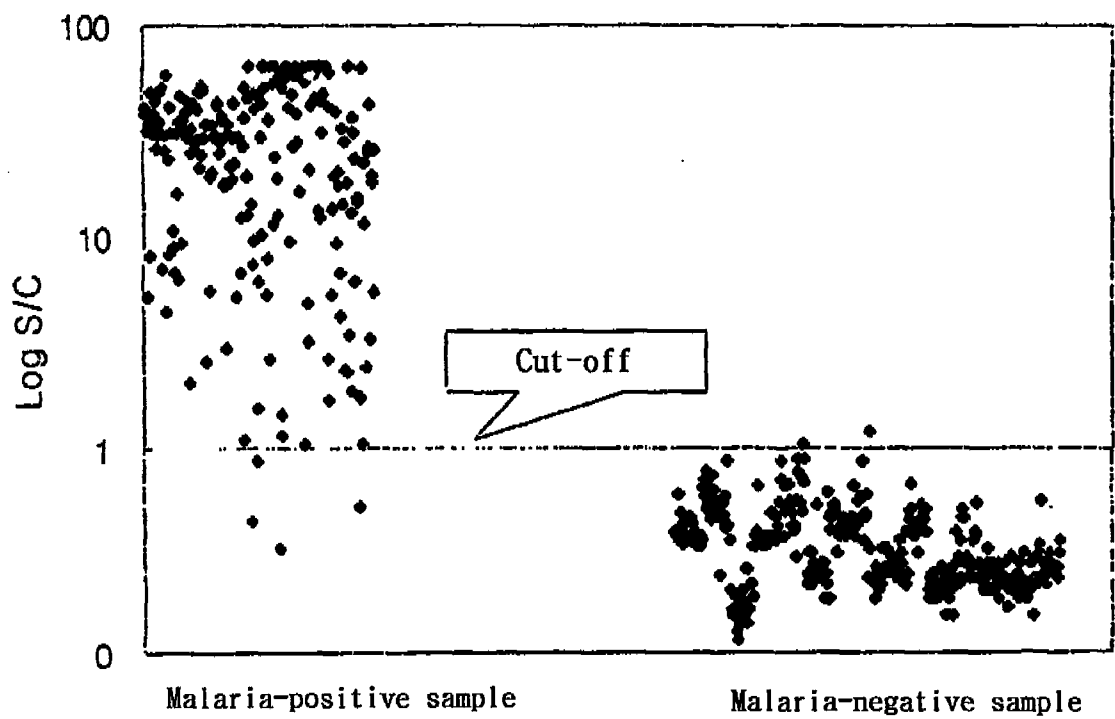
10 Also, the immunoassay, diagnostic reagent and diagnostic method for malaria detecting malaria-specific IgM in blood according to the present invention are very effective in diagnosing malaria where latent period is long, numbers of Protozoa and the antigen in blood are few, and the probability of recurrence is high, because they can diagnose malaria in a latent period. Further, the immunoassay, diagnostic
15 reagent and diagnostic method for malaria detecting malaria-specific IgM in blood according to the present invention are useful in an early diagnosis of the malaria patient. In addition, the immunoassay and diagnostic reagent for malaria detecting malaria-specific IgM in blood according to the present invention exhibited more enhanced specificity and sensitivity than the indirect enzyme immunoassay and
20 diagnostic reagent in the above examples, and more enhanced specificity than the antigen sandwich enzyme immunoassay and diagnostic reagent in the above examples.

The invention will be further illustrated by the following examples. It will be apparent to those skilled in the art that these examples are given only to illustrate the
25 present invention in more detail, but the invention is not limited to the examples given.

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FIGURES

FIGURE 1



S/C : Specimen absorbance/cut-off ratio

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FIGURE2

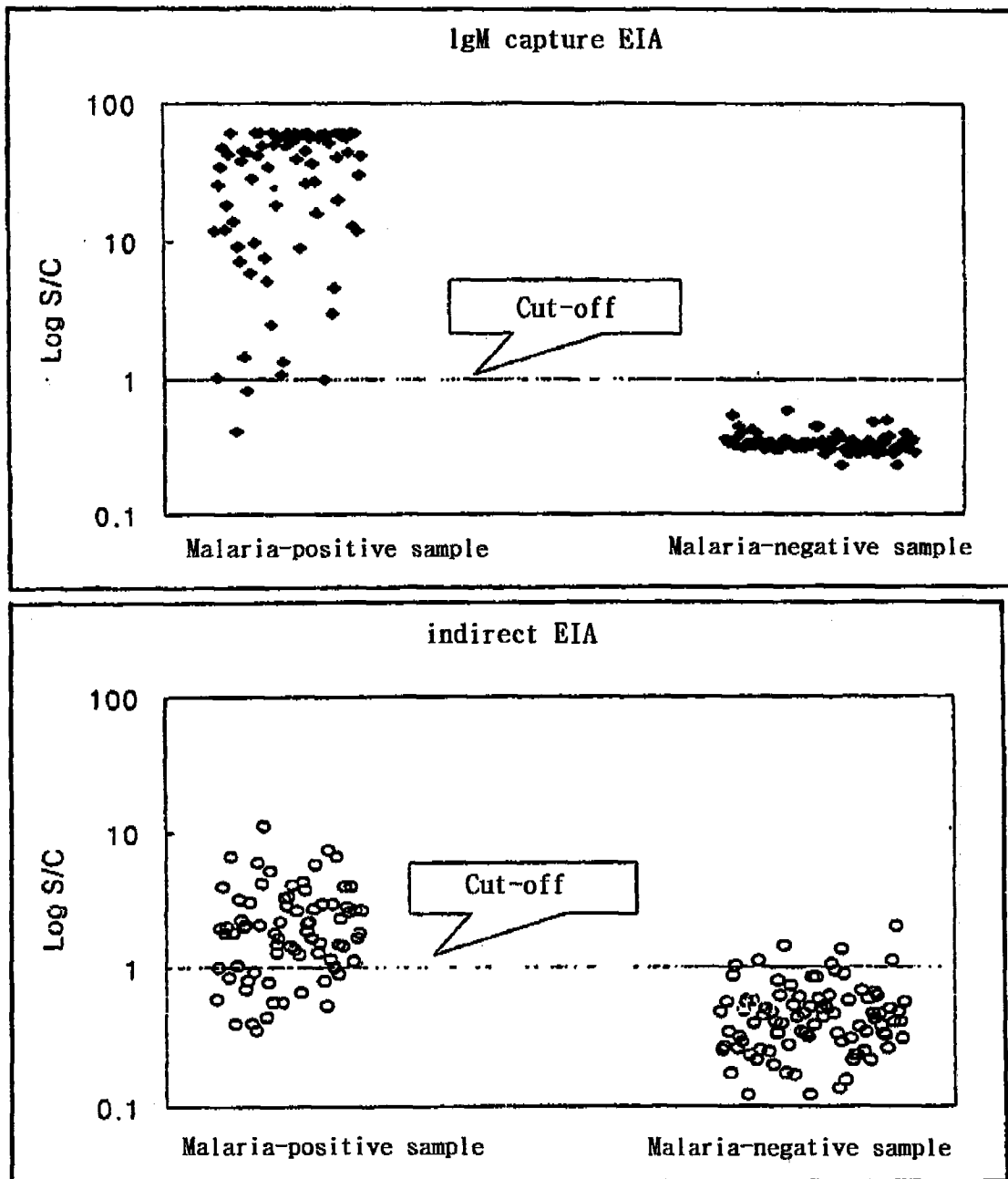
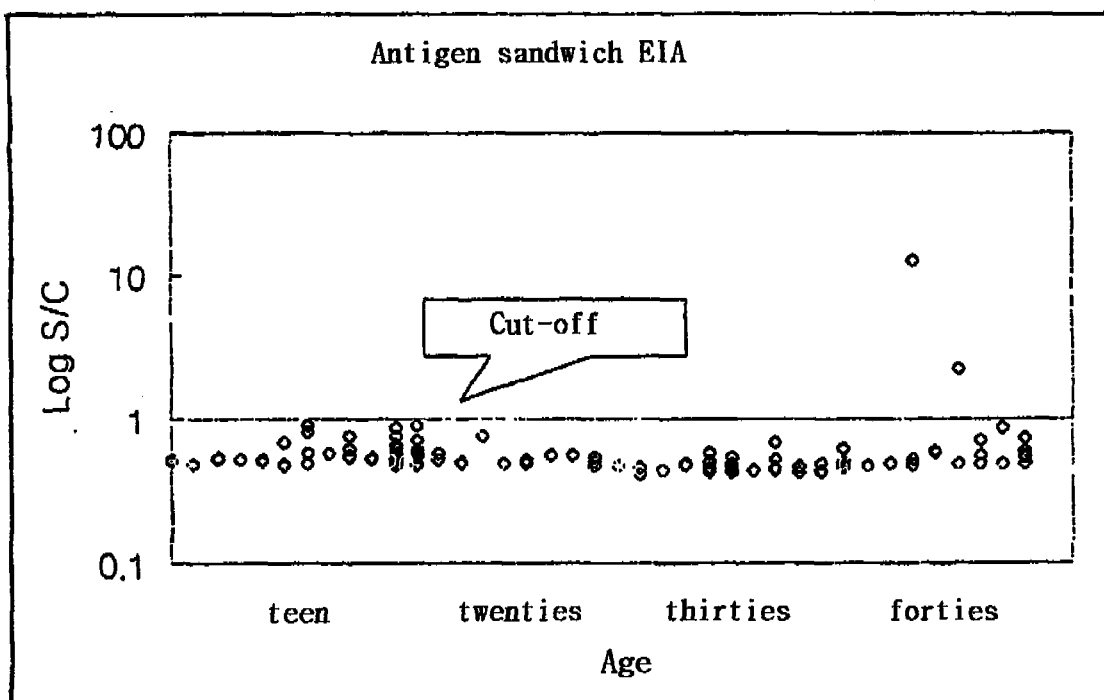
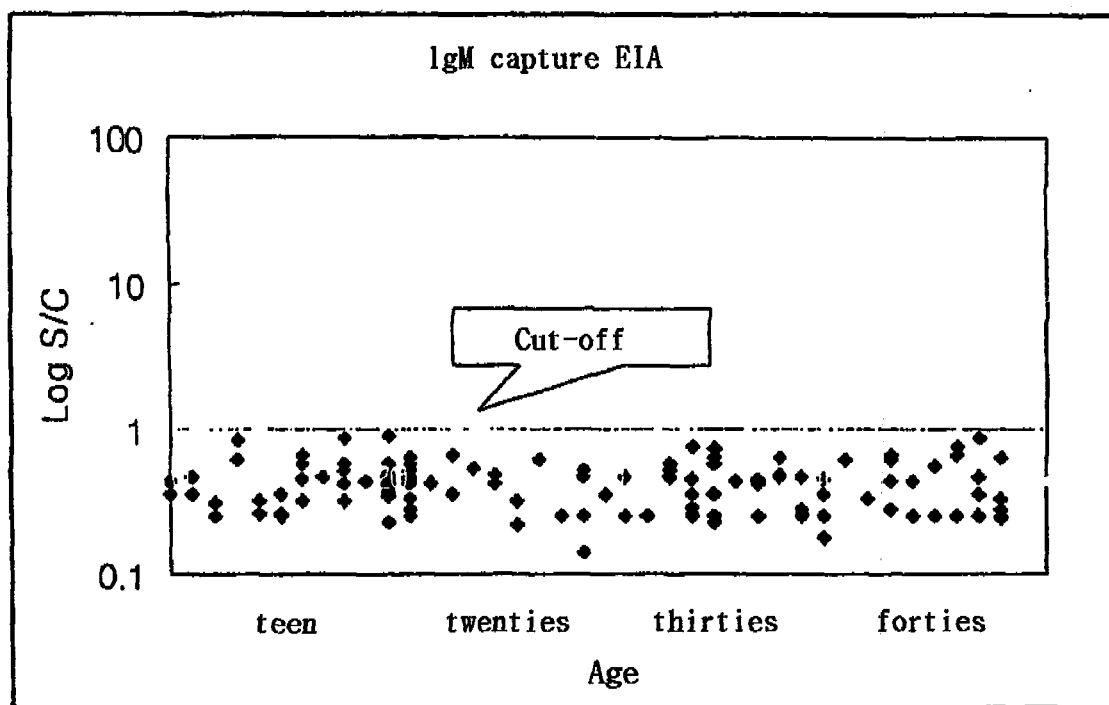
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FIGURE 3

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Asn Glu Ser Lys Glu Ile Leu Ser Gln Leu Leu Asn Val Gln Thr Gln
 Leu Leu Thr Met Ser Ser Glu His Thr Cys Ile Asp Thr Asn Val Pro
 Asp Asn Ala Ala Cys Tyr Arg Tyr Leu Asp Gly Thr Glu Glu Trp Arg
 Cys Leu Leu Thr Phe Lys Glu Glu Gly Gly Lys Cys Val Pro Ala Ser
 5 Asn Val Thr Cys Lys Asp Asn Asn Gly Gly Cys Ala Pro Glu Ala Glu
 Cys Lys Met Thr Asp Ser Asn Lys Ile Val Cys Lys Cys Thr Lys Glu
 Gly Ser Glu Pro Leu Phe Glu Gly Val Phe Cys Ser