Berkala Ilmu Kedokteran
Vol. 34, No. 2, 2002

Amplification and cloning of block 2 of the gene encoding Merozoite Surface Protein-1 (MSP-1) of *Plasmodium falciparum* isolated from Kokap, Yogyakarta

Elsa Herdiana[1], Supargiyono[1], Widya Asmara[2]
[1] Department of Parasitology, Faculty of Medicine, Gadjah Mada University,
[2] Department of Microbiology, Faculty of Veterinary Medicine, Gadjah Mada University

ABSTRACT

Elsa Herdiana, Supargiyono, Widya Asmara - ***Amplification and cloning of block 2 of the gene encoding Merozoite Surface Protein-1 (MSP-1) of Plasmodium falciparum isolated from Kokap, Yogyakarta***

Background: Falciparum malaria remains a major public health problem in tropical and subtropical countries including Indonesia. The disease is caused by *Plasmodium falciparum* and spread by *Anopheline* mosquitoes. The widespread of *Plasmodium* which are resistant to antimalarial drugs and *Anopheline* mosquitoes which are resistant to insecticides have urged to look for alternative solutions including the development of protective vaccines. Several vaccine candidates have been studied, one of them is Merozoite Surface Protein-1 (MSP-1) which is expressed on the surface of merozoite. It was shown that this protein induces protective immune responses. Variation on the gene encoding MSP-1 of *Plasmodium falciparum* has been well documented but such data from Indonesia population have never been studied.
Objective: The aim of this study is to amplify and clone MSP-1 gene of *P. falciparum* isolated from Kokap, Yogyakarta.
Methods: Block 2 of the gene encoding MSP-1 was amplified by Polymerase Chain Reaction (PCR) and the PCR amplification products were cloned using p*GEM*® -T vector and transformed into *Escherichia coli* JM 109.
Result: From 19 PCR results, 3 were cloned and 10 colonies were picked up. Nine of 10 showed the MSP-1 gene insertion by PCR method.
Conclusion: Block 2 of the gene encoding MSP-1 of *P. falciparum* isolated from Kokap, Yogyakarta was successfully amplified by PCR method. This study resulted in 9 recombinant plasmids which contained MSP-1 gene as the outcome of cloning and transformation into *E.coli*.

Key words: *P. falciparum* - MSP-1 - PCR - Amplification - Cloning

ABSTRAK

Elsa Herdiana, Supargiyono, Widya Asmara - ***Amplifikasi dan kloning gen penyandi Merozoite Surface Protein (MSP-1), blok 2 P. falciparum isolat Kokap Yogyakarta***

Latar belakang penelitian: Malaria falciparum masih merupakan masalah kesehatan yang perlu mendapat perhatian di negara subtropis dan tropis termasuk Indonesia. Penyebab penyakit ini adalah *Plasmodium falciparum* yang ditularkan dari satu orang ke orang lain melalui gigitan nyamuk *Anopheles* betina sebagai vektor. Semakin meluasnya kasus resistensi *P. falciparum* terhadap obat antimalaria dan resistensi vektor nyamuk terhadap insektisida memerlukan pemecahan alternatif berupa pemberian vaksin untuk pencegahan malaria. Sampai sekarang beberapa kandidat vaksin sudah banyak diteliti, di antaranya adalah Merozoit

Elsa Herdiana, Supargiyono, Bagian Parasitologi, Fakultas Kedokteran Universitas Gadjah Mada, Yogyakarta, Indonesia. Widya Asmara, Bagian Mikrobiologi, Fakultas Kedokteran Hewan Universitas Gadjah Mada, Yogyakarta

Surface Protein-1 (MSP-1), yang diekspresikan pada permukaan merozoit. Protein ini terbukti dapat menginduksi respon imun yang protektif. Sampai kini isolasi MSP-1 dari isolat lokal (Indonesia) belum pernah dilakukan, diamplifikasi dan diklon untuk dipelajari dalam rangka penyediaan vaksin yang imunogenik.
Tujuan: Mengamplifikasi dan mengklon gen penyandi *MSP-1 P. falciparum* isolat lokal (Kokap).
Bahan dan cara penelitian: Amplifikasi gen penyandi *MSP-1* blok 2 dilakukan dengan teknik *Polymerase Chain Reaction* (PCR) dengan desain primer yang sudah dipublikasi. Klonasi dilakukan dengan menginsersikan gen MSP-1 hasil amplifikasi pada vektor p*GEM*® -T dan kemudian ditransformasikan dalam *Escherichia coli* JM109.
Hasil: Dari 19 sampel yang berhasil diamplifikasi, 3 diklon dan diambil 10 koloni bakteri *E.coli* hasil transformasi untuk diperiksa berhasil tidaknya insersi gen MSP-1. Sembilan dari 10 koloni menunjukkan adanya insersi gen *MSP-1 P. falciparum*.
Simpulan: Gen penyandi MSP-1 blok 2 *P. falciparum* isolat Kokap, Yogyakarta, telah berhasil diamplifikasi dengan metode *PCR*. Penelitian ini menghasilkan 9 plasmid rekombinan sebagai hasil klonasi dan transformasi ke dalam *E.coli*.

(B.I.Ked. Vol. 34, No.2: 69-75, 2002)

INTRODUCTION

Malaria is still a serious disease in some countries. It is caused by protozoa of the genus *Plasmodium* that live intracellular and within the erythrocyte. There are 4 human *Plasmodia: Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium falciparum*. Among them, *P. falciparum* is the most pathogenic one. It causes disease with various manifestations from asymptomatic to severe complicated ones[1]. Half of the world's population lives in endemic areas for malaria; and 2-3 million people are killed annually. Most of malaria cases in Indonesia are caused by *P.vivax* and *P. falciparum*. In Yogyakarta Province, almost all malaria cases happen in Kulon Progo and caused by *P. falciparum*, *P. vivax* and mixed i.e 40%, 59,7% and 0,3% respectively. Kokap was one of the endemic area with High Case Incidence (HCI) status since 1998[2].

The wide spread of *Plasmodium* which are resistant to antimalarial drugs and anopheles mosquitoes which are resistant to insecticides have urged on the seek of alternative solution including the development of protective vaccines[3]. Antigens of the invasive blood stage of the parasite, merozoite, has been proposed as vaccine candidate[4]. Merozoite Surface Protein-1 (MSP-1, also referred to as P195, PMMSA, or MSA-1) is one of the most studied of all malaria proteins. MSP-1 is processed at the end of schizogony just prior to the release of merozoite from the mature schizont. Its presence on the merozoit surface makes it could be recognized easily by the immune system[5]. Immunization of monkeys and mice with purified MSP-1 of *P. falciparum* has resulted in such protection[6].

This gene has an exon, and divided into 17 distinct blocks defined by their content of conserved, semi conserved or variable sequences[4]. Block 2 is a region that contains a set of degenerate tripeptide sequence repeats and although the repeats in MSP-1 form a relatively small part of the polipeptide chain, this region is the most variable part of the polipeptide chain[7]. The variability of this antigen among different parasite isolates could be an obstacle to develop an effective vaccine. However, it could be partly solved by producing local vaccine against local isolates with the assumption that the extent of polymorphism among local isolates is limited. So far, gene encoding MSP-1 epitope has been studied on *P. falciparum* isolates from different geographical regions, but has not been conducted yet on *P. falciparum* isolates in Indonesia. Cloning MSP-1 gene, block 2, is one of the means of amplifying a gene and preparing it for subsequent sequencing. Therefore, the objectives of this study are: 1.To amplify block 2 of MSP-1 gene of *P. falciparum* from local isolates (Kokap), 2.To provide recombinant clones containing block 2 of MSP-1 gene of *P. falciparum* from local isolates (Kokap)

MATERIAL AND METHODS

Material for DNA works were phenol, chloroform, isoamylalkohol, reverse and forward primers, dNTP, *Taq Polymerase* (Promega), PCR buffer and MgCl2..p*GEM*® -T was used as cloning

vector and *E.coli* JM109 was used as the cloning host.

Procedures

1. Subjects

Blood samples for plasmodium isolation were taken from patients with clinical symptoms of malaria who visited Public Health Center in Kokap, an endemic area with High Incidence Case (>5‰), in Yogyakarta Province, from August to October 1999. Age, sex, address and occupation of each patient were recorded.

2. Sample collection and identification

Duplicate blood samples were obtained by venous puncture from each patient and collected into 1.5 ml microfuge tube containing 15% Acid Citrate Dextrose (ACD) anticoagulant. A thick and thin smear microscope slides were prepared for each sample and stained with 10% Giemsa. Positive sample for *P. falciparum* infection was identified microscopically searching for ring or gametocyte stages characteristic for *P. falciparum*. Blood samples were kept in -20°C until processed further. Positive blood samples for *P. falciparum* were then used for isolation of plasmodial DNA.

3. Extraction of plasmodial DNA

P. falciparum genomic DNA from 0.1-1.5 ml blood sample was extracted using phenol: chloroform method. The extraction result, the DNA pellet, was resuspended in 50 ml Tris ED buffer pH 8.0 and kept at -20°C.

4. Amplification of gene encoding B cell epitope of MSP-1 protein

A gene encoding B cell epitope of MSP-1 from *P. falciparum* was isolated through in vitro amplification of this gene with PCR technique. A pair of primers: forward and reverse were designed to prime the synthesis of gene encoding B cell epitop of MSP-1.

Primer : Forwards 5' : cacatgaaagttatcaag-aacttgtc 3'
Reverse 5' : gtacgtctaatcatttg-cacg 3'

Amplification reactions were carried out in a volume of 50 µl under optimized PCR condition in thermocycler (Hybaid) program for denaturation step at 94°C. PCR mixture of 50 µl was prepared in 0.5 ml microfuge tube by adding 5 µl 10X PCR buffer, 1.50 µl of 50 mM MgCl2, 1.0 µl of 10 mM dNTPs (dATP, dTTP, dCTP & dGTP), 0.50 µl of 40 pmoles of each primer, 1.5 µl of Taq DNA Polymerase (1 u/µl) and variables volumes of template depend on the degree of parasitemia. The sterile destilled water was then added to a final volume of 50 µl. The tube containing PCR mixture was then run in thermocycler programmed as mentioned above. After completion of PCR, an aliquot of PCR product was analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining. Picture of gels was taken under ultra violet light and the position of bands were compared to DNA bands of the marker. PCR products which demonstrated specific bands for MSP-1 were used for cloning.

5. Cloning procedure

After isolated using PCR, the MSP-1 (PCR product) was cloned into p*GEM*® -T vector with this following procedures:

a. Purification of PCR product

PCR products that showed specific band size for MSP-1 (600 base pairs) were considered for purification. PCR product was purified with Wizard's DNA preps Purification System Kit (Promega).

b. Ligation procedure

Amplified DNA fragment with single deoxyadenosin 3' overhang was ligated to p*GEM*® -T vector, which possesses single deoxythimidin 3' overhang, prepared by Promega. These single 3'-T overhangs at the insertion site greatly improve the eficiency of PCR product ligation into the plasmid[8]. Optimization of insert DNA to the vector DNA was performed according the formula and ranges of optimization obtained from Promega Technical Manual. Ligation reaction mixture for this study was: 1 µl T4 DNA ligase 10x buffer, 1 µl p*GEM*® -T vector (50ng/µl), 5 µl PCR product of MSP-1, 2 µl ddH_2O. For positive control:

1 µl T4 DNA ligase, 1µl p*GEM*® -T vector, 2 µl control insert DNA and for background: 1 µl T4 DNA ligase 10x buffer, 1 µl p*GEM*® -T vector (50 ng/µl), T4 DNA ligase (3 weiss units/µl), 1 µl ddH_2O. This mixture was mixed by pipetting and incubated overnight at 40°C and transformed into competent *E.coli* JM 109 cells.

c. Transformation procedure

The competent *E.coli* JM 109 cells were transformed by heat shock technique with ligated DNA prepared above[9]. Positive and background controls were included in transformation reactions. A hundred microliters of the above competent *E.coli* JM 109 was put into each of the three tubes and incubating in ice for 20 minutes. The tube containing the mixture was then transferred to 42°C water bath and incubated for 3 minutes, then incubated on ice for 15 minutes. Five hundred microliters of LB medium was added to the above-incubated mixture and incubated in 37°C water bath for an hour. The transformation reaction was then spread out on color indicator LB plate containing ampicillin, IPTG, and X-gall. Triple plates were prepared for each reaction mixture by spreading out 150 ml, 200 ml, 250 ml reaction mixture to each plate. The next day plates were screened for blue white transformant colonies and stored at 4°C. White colonies were considered for further analysis of insert DNA.

d. Analysis for insert DNA

To determine the presence of insert, the plasmid DNA was extracted from each clone by Promega Wizard Plus Plasmid DNA Purification System, base on DNA denaturation in alkalic pH. Soluble DNA plasmid found at supernatant will be tied by resin and was eluted by low ionic solution like TE buffer/water. The result of recombinant plasmid isolation was amplified by PCR using a pair of specific primers for MSP-1.

RESULT AND DISCUSION

PCR is an in vitro DNA amplification method which can amplify one single copy DNA in an exponential rate to be 1.1 x 10^{6-8} copies[10]. Thirty samples were collected from Kokap from August to October 1999. Nineteen of them showed *P. falciparum* positive under microscopic examination of stained thin blood smear and all of them also demonstrated positive for MSP-1 gene at approximately 600 bp (FIGURE 1). Despite the fact that only 19 samples were positive, all samples were isolated and amplified. In the beginning the standard condition of PCR as recommended by Wooden[11]. The PCR products visualized in agarose gel 1.5% by ultra violet light and showed 19 samples were positive. However, non-specific band also appeared in each positive sample. Therefore, annealing temperature was increased to 53°C and

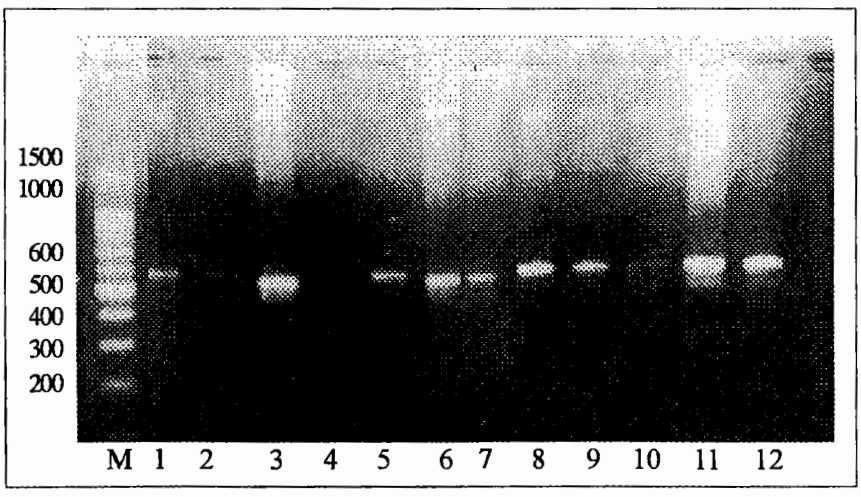

FIGURE 1. Agarose gel electrophoresis sample of 12 PCR products of gene encoding MSP-1 on *P. falciparum* from Kokap isolates. M=DNA marker 100bp ladder, 1.K31; 2.K32; 3.K33; 4.K35; 5.K36; 6.K37; 7.K38; 8.K45; 9.K46, 10.K47; 11.K48; 12.K49

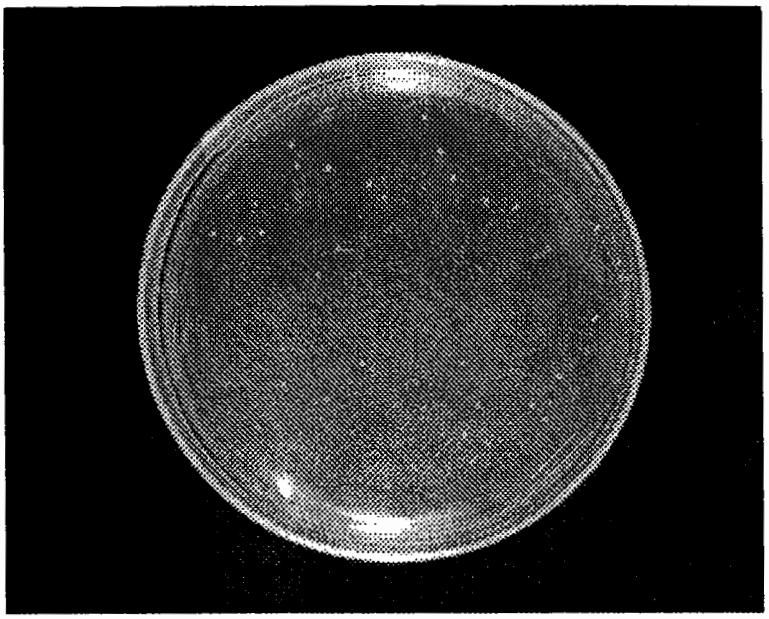

FIGURE 2. Colonies of transformed *E.coli* JM109 (200ul volume of transforman cell) containing MSP- 1 gene of *P. falciparum* of Kokap isolates; plating on LB plate containing X-gal, IPTG & ampicillin. B=Blue colony, W=White colony.

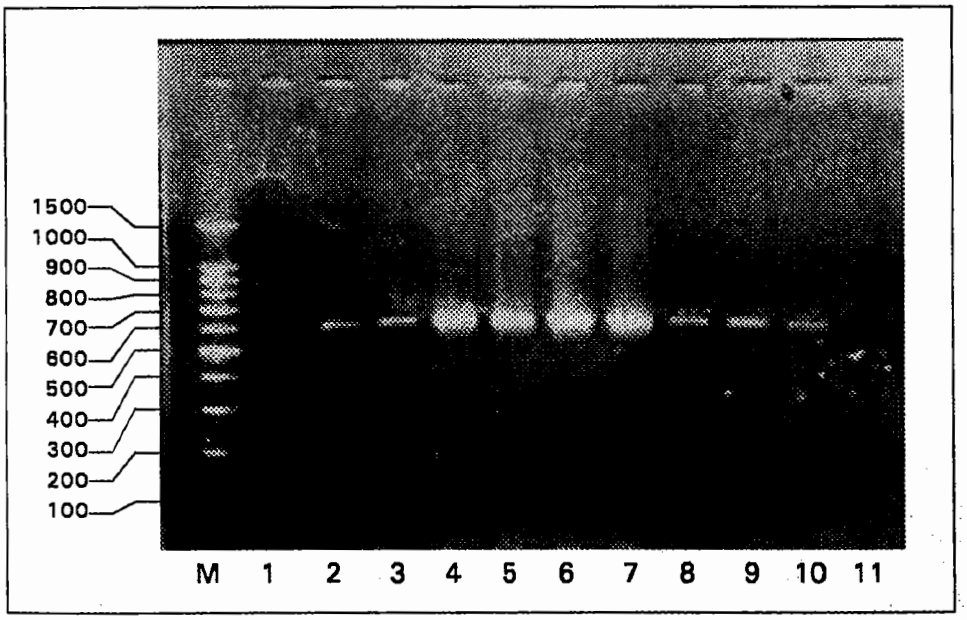

FIGURE 3. Agarose gel electrophoresis of PCR product of recombinant plasmid DNA inserted with MSP-1 *P. falciparum* of Kokap isolates. M=marker DNA 100 bp ladder; 1.K45.1; 2.K45.2; 3.K45.3; 4.K48.1; 5.K48.2; 6.K48.3; 7.K48.4; 8.K49.1; 9.K49.2; 10.K49.3, 11.Blue colony (control). All recombinant plasmid (9) contained MSP-1 gene insert, except K45.1 & blue colony.

amplification condition become: predenaturation 95°C, 5', denaturation 95°C, 30", annealing 53°C, 30", extension 72°C, 2'30", for 35 cycles and extension 72°C, 5'. Even though there is still possibility for the microscopic negative samples to be positive with PCR amplification because it can detect the lower parasitemia (0,0001%)[12] but in this study they were all negative. Heme and other constituents of blood and serum have been shown to inhibit activity of the thermostable DNA Polymerase used in the PCR reaction. Therefore another DNA isolation procedure such as Chelex-100 is preferable[13].

To know whether each sample contains one allele or more, the fragment should be sequenced. For the purpose of sequencing the PCR product should be cloned into a sequencing plasmid and to select whether or not the plasmid contains an insert, blue and white color screening process based on insertional-inactivation of the a-peptide was used[12]. The cloning result as observed by color screening for isolates from Kokap did show white and blue colonies as follow; for DNA control transforman cells 200 µl, 150 cell volume demonstrated 18 white colonies and 0 blue colony, 26 white colonies and 0 blue colony respectively. Result of DNA K45 transformation 250 µl, 200 µl, 150 µl, cell volume were 5 white colonies, 15 blue colonies; 5 white colonies, 20 blue colonies; 5 white colonies, 2 blue colonies respectively. Result of DNA K48 transformation 250 µl, 200 µl, 150 µl, were 10 white colonies, 7 blue colonies; 20 white colonies, 1 blue colony; 9 white colonies, 6 blue colonies (FIGURE 2). Result of DNA 49 transformation 250 µl, 200 µl, 150 µl cell volume were 50 white colonies, 4 blue colonies; 47 white colonies, 6 blue colonies; 33 white colonies, 1 blue colony respectively.

The transformation experiment using positive control did show white and blue colonies approximately as it was expected, more white colonies and reasonable number of blue colonies. Although the transformation of K45 produce lower amount of white colonies comparing to that of DNA control insert it was still more than 15%, the minimal percentage expected[12].

For each isolate, 3 white colonies (recombinant plasmid) were picked up and growth in LB medium with ampicillin. One blue was used as control. DNA recombinant plasmid were isolated and purified by Wizard Purification Kit (Promega). The purification results of the above colonies were further analyzed by PCR amplification method using the same MSP-1 primers. The assumption was that the white colonies carrying the DNA insert. Eight from nine white colonies were consistent with of color screening on the indicator plates (FIGURE 3).

Therefore, it was possible to say that MSP-1 gene block 2 was most likely cloned into p*GEM*® -T vector and transformed into *E.coli* JM 109. However, K45.1 showed an inconsistent result with that of color screening of plates. Instead of the MSP-1, secondary products or primers might be cloned in the p-*GEM*T vector. To avoid this problem, when the amplification resulted in multiple bands or there was a large amount of dimmer, it was suggested to excise the fragment of interest from agarose gel directly before it was going to be purified[14].

To identify the variability of block 2 of the gene encoding MSP-1 among the Kokap isolates, PCR fragment products of MSP-1 of the cloning mentioned before should be sequenced

CONCLUSION

1. Block 2 of the gene encoding epitop MSP-1 *P. falciparum* from Kokap isolate was successfully amplified by optimized PCR conditions and specific primers for MSP-1 gene designed based on published sequence.
2. Block 2 of the gene encoding of MSP-1, *P. falciparum* was cloned into p*GEM*® -T vector and transformed into *E.coli*. There were 9 recombinant plasmids containing MSP-1 gene: K45.2, K45.3, K48.1, K48.2, K48.3, K48.4, K49.1, K49.2, K49.3.

REFERENCES

1. Phillip RE, Gilles HM. Malaria. Medicine Int.1988; 52: 2220-5.
2. Kandepkes Kabupaten Kulon Progo. Profil Kesehatan Kabupaten Kulon Progo, Kulon Progo.1996.
3. Simmonds RS, Shearer MH, Kennedy RC. DNA vaccine from principle to practise. Parasitol. Today.1997; 13 (9): 328-31.

4. Cavanagh DR, Elhassan IM, Roper C, Robinson VJ, Holder AA, Theodor TG,et al. A longitudinal study of type specific antibody responses to *P. falciparum* merozoit surface protein in an area of unstable malaria in Sudan. J. Immunol.1998; 161:347-59.
5. Kemp DJ, Coppel RL, Anders RF. Repetitive proteins and genes of malaria. Ann.Rev.Microbiol. 1987; 57(12): 3765-9.
6. Miller LH, Roberts T, Shahabuddin M, McCuthan TF. Analysis of sequence diversity in the Plasmodium falciparum Merozoit Surface Protein-1 (MSP-1).Mol. Biochem. Parasitol.1993; 59:1-14.
7. Tanabe K, MacKay M, Goman M, Scarfe JG. Allelic dimorphism in a surface antigen of the malaria parasite Plasmodium falciparum. J.Mol. Biol.1987; 195: 273-87.
8. Mezci LM & Storts DR. PCR technology: current innovations, Griffin HG & Griffin AM. 1994 ed CRC Press, Boca Raton FC.
9. Sambrook J, Fritcsh EF, Maniatis T. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press. 1988.
10. Sathastvan. PCR and Cloning of DNA into expression vector Molecular Biology Protocols. 2001 (cited 2003 May, 1). Available from URL:http//www.esb.utexas.edu/sata/bio331/protocols/pdf.
11. Wooden J, Kyes S, Sibley CH. PCR and strain identification in Plasmodium falciparum. Parasitol. Today.1993; 9(8): 303-5.
12. Hyde JE. Molecular Parasitology.1st ed. Open University Press. 1990.
13. Doyle K. Protocols & Application Guide.3rd ed Promega Corporation. 1996.
14. Jacqui Robles & Mary Doers. P*GEM*®-T vector systems troubleshooting guide. 1998. (cited 2003 May, 1) Available from URL: http//www.promega.com/pnotes/45/2159e.html