Chula Med J Vol. 57 No. 3 May - June 2013

Genotyping of *Plasmodium falciparum* by allele-specific amplification of the merozoite surface protein-1 locus

Wathiporn Saichanapun* Sunee Seethamchai**
Taweesak Tia* Chaturong Putaporntip*

Saichanapun W, Seethamchai S, Tia T, Putaporntip C. Genotyping of *Plasmodium falciparum* by allele-specific amplification of the merozoite surface protein-1 locus. Chula Med J 2013 May – Jun; 57(3): 305 - 20

Problem/background : *Allele-specific amplification is an important tool for a large scale study of geographic distribution of pathogens harboring polymorphic genes. In this study, we have developed a simple method to differentiate strains of Plasmodium falciparum based on polymorphic block 2 located at the 5' portion of the merozoite surface protein-1 gene (PfMsp-1). Block 2 of PfMsp-1, a strong candidate for asexual blood-stage vaccine, contains 3 basic sequence types with MAD20 type, K1 type and RO33 type as representative alleles.*

Objective : *To develop a polymerase chain reaction (PCR) method capable of detecting PfMsp-1 genotypes.*

Design : *Experimental study.*

Setting : *Department of Parasitology, Faculty of Medicine, Chulalongkorn University.*

Materials and Methods : *The method deployed semi-nested PCR targeting allele-specific sequences of each representative type of blocks 2 of PfMsp-1. The performance of the method was evaluated with 50 P. falciparum blood samples collected from patients in Chanthaburi, Tak and Yala provinces.*

* Molecular Biology of Malaria and Opportunistic Parasites Research Unit, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand

** Faculty of Science, Naresuan University, Phitsanulok 65000, Thailand

Results : *The method is sensitive to detect as few as 5 parasites in the samples whereas specific alleles of block 2 could be unequivocally determined. Minor parasite populations in the isolates containing multiple clone infections were detected by the method. Of the 50 P. falciparum isolates examined, MAD20, K1 and RO33 allelic types were identified in 28, 14 and 8 samples by the PCR genotyping method, respectively. Multiple clone infections were observed in 4 of these isolates.*

Conclusion : *The PCR genotyping method developed in this study is applicable for large-scale population genetic analysis of P. falciparum population based on the PfMsp-1 locus.*

Keywords : *Malaria, Plasmodium falciparum, merozoite surface protein 1, allele-specific amplification, genotyping.*

Reprint request: Putaporntip C. Molecular Biology of Malaria and Opportunistic Parasites Research Unit, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand.

Received for publication. May 1, 2012.

วฒิภรณ์ สายชนะพันธ์, สุนีย์ สีธรรมใจ, ทวีศักดิ์ แซ่เตีย, จตุรงค์ พุทธพรทิพย์. การตรวจสอบจีโนไทป์ของเชื้อพลาสโมเดียม ฟัลซิปารั่มโดยการเพิ่มปริมาณอัลลีลที่จำเพาะของยีนสำหรับโปรตีนบน ผิวเมอร์โรซอยต์ชนิดที่หนึ่ง. จุฬาลงกรณ์เวชสาร 2556 พ.ค. – มิ.ย.; 57(3): 305 – 20

เหตุผลของการทำวิจัย : *การเพิ่มปริมาณดีเอ็นเอของอัลลีลที่จำเพาะเป็นเทคนิคที่สำคัญสำหรับการวิจัยที่ใช้จำนวนตัวอย่างเชื้อก่อโรคจำนวนมาก เพื่อศึกษาการแพร่กระจายทางภูมิศาสตร์ของยีนทีมีภาวะหลายรูปแบบ ในการศึกษาครั้งนี้คณะผู้วิจัยได้พัฒนาวิธีการที่สะดวกสำหรับการแยกสายพันธุ์ของเชื้อพลาสโมเดียมฟาลซิปารั่มโดยการใช้บริเวณ block 2 ทางด้านปลาย 5' ของยีนที่สร้างโปรตีนบนผิวเมอร์โรซอยต์ชนิดที่หนึ่งหรือยีน PfMsp-1 ซึ่ง block 2 ของยีน PfMsp-1 เป็นโปรตีนองค์ประกอบที่สำคัญของวัคซีนป้องกันมาลาเรียระยะที่อยู่ในเม็ดเลือดแดง โดยบริเวณ block 2 นี้มีรูปแบบของอัลลีลพื้นฐาน 3 รูปแบบคืออัลลีลแบบ MAD20 K1 และ RO33*

วัตถุประสงค์ : *เพื่อพัฒนาวิธีการเพิ่มปริมาณดีเอ็นเอด้วยปฏิกิริยาลูกโซ่โพลีเมอเรสหรือพีซีอาร์ สำหรับการตรวจสอบจีโนไทป์ของยีน PfMsp-1*

รูปแบบการวิจัย : *การศึกษาเชิงพรรณนา*

สถานที่ทำการศึกษา : *ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย*

ตัวอย่างและวิธีการศึกษา : *คณะผู้วิจัยได้พัฒนาวิธีการใช้เทคนิค semi-nested-พีซีอาร์ เพื่อเพิ่มจำนวนยีนเป้าหมายโดยใช้ไพรเมอร์ที่จำเพาะต่อรูปแบบของอัลลีลแต่ละแบบในบริเวณ block 2 ของยีน PfMsp-1 โดยทำการวิเคราะห์ตัวอย่างเลือดผู้ป่วยที่ติดเชื้อมาลาเรียชนิดพลาสโมเดียมฟาลซิปารั่มทั้งหมด 50 ตัวอย่างที่เก็บจากจังหวัดจันทบุรี จังหวัดตาก และจังหวัดยะลา*

ผลการศึกษา : *เทคนิคนี้มีความไวในการตรวจสอบสูงสามารถตรวจได้ แม้มีปริมาณเชื้อมาลาเรียต่ำเพียง 5 ตัวต่อตัวอย่าง นอกจากนี้ยังสามารถตรวจสอบจีโนไทป์ที่แตกต่างกันในตัวอย่างเดียวกันได้ ผลการตรวจสอบจีโนไทป์ของตัวอย่างเชื้อพลาสโมเดียมฟาลซิปารั่ม ทั้งหมด 50 ตัวอย่าง พบว่าสามารถตรวจพบจีโนไทป์ชนิด MAD20 จำนวน 28 ตัวอย่าง ชนิด K1 จำนวน 14 ตัวอย่าง และชนิด RO33 จำนวน 8 ตัวอย่าง นอกจากนี้ยังตรวจพบเชื้อที่มีการปะปนกันของจีโนไทป์ที่แตกต่างกันใน 4 ตัวอย่าง*

สรุป : *วิธีการตรวจสอบจีโนไทป์ด้วยวิธีพีซีอาร์ที่ได้พัฒนาขึ้นในการศึกษาครั้งนี้เป็นประโยชน์อย่างมากสำหรับการวิเคราะห์สายพันธุ์ของเชื้อพลาสโมเดียมฟาลซิปารั่มโดยใช้ยีน PfMsp-1 เป็นยีนเป้าหมาย*

คำสำคัญ : *มาลาเรีย, พลาสโมเดียมฟาลซิปารั่ม, โปรตีนบนผิวเมอร์โรซอยต์ชนิดที่หนึ่ง, การเพิ่มปริมาณดีเอ็นเอของอัลลีลที่จำเพาะ, การตรวจสอบจีโนไทป์.*

Approximately, 300 million people are infected by malaria each year. Of these, nearly one million cases die of the disease as a consequence of complicated and severe infections. Among the 5 species of *Plasmodium* that cause human malaria, *Plasmodium falciparum* is the most prevalent and most pernicious. Attempts to control and eradicate malaria have posed several obstacles mainly due to the emergence and wide spread occurrence of antimalarial drug resistant parasite strains and the presence of insecticide resistant mosquito vectors. Therefore, development of a malaria vaccine is an essential task for malaria control. Several malaria vaccine candidates have been identified aiming at either destroying various stages of the parasites or inhibition of parasite entry into the host cells that is a crucial step in parasite survival. However, malaria parasites in outbred population usually harbor diverse genetic background, resulting in antigenic diversity among several malaria vaccine candidates, an issue that could compromise an effective vaccine development. Undoubtedly, knowledge on parasite population genetic structure and dynamics of parasite spread is fundamental to malaria control measures because effectiveness of a malaria vaccine can be variable depending on parasite strains circulating in an endemic area whereas early identification of drug resistant parasite strains emerging in a community will be more feasible to control or eradicate than long-term widespread occurrence of drug resistant parasites. Therefore, a practical genotyping method for *P. falciparum* is essential to enhance malaria control program.

A number of polymorphic genetic markers derived from both coding and non-coding loci have been identified in *Plasmodium falciparum* that can be applied for population genetic analysis. [1 - 3] However, analysis using coding loci, especially genes encoding proteins capable of inducing protective immune responses or involved in drug actions, has additional implications for public health in terms of vaccine development and anti-malarial drug use.

One of the attractive targets for vaccine development against *P. falciparum* infections is a polymorphic molecule expressed on the surface of merozoite, designated merozoite surface protein 1 (PfMsp-1). Both *in vitro* and *in vivo* studies have revealed that antibodies to PfMSP1 can inhibit parasite growth and confer protection in immunization trials in animals. [4] Sequence comparison of *PfMsp-1* from a number of parasite isolates encompassing a 5 kb coding region has shown that the protein is encoded by a single copy gene, containing 5 conserved, 5 semi-conserved and 7 variable blocks. [5 - 7] Variation in PfMsp-1 is basically dimorphic, i.e. one or the other nucleotide exists at the positions where substitutions occur. Exception to the dimorphic variation of the gene is the presence of 3 major allelic types in block 2 characterized by K1 type, MAD20 type and RO33 type. Both K1 and MAD20 types possess tripeptide repeats with sequence and size polymorphism among isolates whereas a limited sequence variation has been detected in RO33 type which lacks apparent repeats. [8,9] Extensive sequence variation in *PfMsp-1* has arisen from intragenic recombination between dimorphic parental alleles in anopheline mosquito vectors. [10] Therefore, polymorphism in *PfMsp-1* is an attractive target for *P. falciparum* identification and tracking.

Although a genotyping method based on *PfMsp-1* by nested polymerase chain reaction (PCR) can differentiate *P. falciparum* strains, large scale application can be compromised by time and budget required. [3] Meanwhile, mixed infections of different *PfMsp-1* within isolates are prevalent in endemic areas and cannot be entirely determined by direct sequencing *per se*. Here we develop a rapid genotyping method based on available sequences of *PfMsp-1* by nested PCR targeting the polymorphic loci located in blocks 2 of *PfMsp-1*. The method can differentiate each of 3 major types of block 2 of PfMsp-1 whereas variation in the number of repeats in MAD20 type and K1 type further provides fine resolution of subtypes of alleles in the 2 major types. The method developed herein is applicable to isolates with mixed infections of different *PfMsp-1* alleles and has practical implication for large - scale field studies of *P. falciparum* populations.

Materials and Methods

Parasite samples

From 2009 to 2010, finger pricked blood samples (~0.1 ml) were collected from *P. falciparum*-infected patients attending malaria clinics in Tak Province (*n* = 21), Chantaburi Province (*n* = 19) and Yala Province (*n* = 10). Diagnosis of *P. falciparum* infection was based on both microscopic examination of Giemsa stained thin and thick blood films. All of these patients had febrile symptoms during blood sample collection.

DNA extraction

DNA was extracted from each blood sample using the QIAGEN DNA minikit (Hilden, Germany) following the manufacturer's protocol. After purification, these DNA samples were stored at -30°C until use.

Nested PCR for malaria diagnosis

DNA from each blood sample was subject to nested PCR targeting small subunit ribosomal RNA of 5 human malaria species using primers and amplification condition as previously described. [11,12]

Allele-specific genotyping method

The PCR primers were derived from *PfMsp-1* sequences available in the GenBank™ database (accession numbers AB276005, X05624 and X03371). Structure of *PfMsp-1* and the location of primers are shown in Figure 1. DNA fragments encompassing conserved blocks 1 and 5 were amplified by PCR using primers common to MAD20 type, K1 type and RO33 type: FMSP1B1F (5'-CACAATGTGTAACACATGAAAG-3') and FMSP1 B5R (5'-CAAGTGGATCAGTAAATAAACTATC-3'). The thermal cycler profile for primary PCR included a pre-denaturation at 94°C for 1 min; 35 cycles of denaturation at of 96°C for 30 sec; primer-template annealing at 55°C for 30 sec; primer extension at 61°C for 1.30 min, and post amplification extension at 72°C for 5 min in PCR System 9700 thermocycler (Applied Biosystem GeneAmp®, PE Biosystems, Foster City, CA). Amplification was done in 25 μl reaction mixture containing 1 μL of *P. vivax* DNA, 2.5 mM each deoxynucleoside triphosphate, 2.5 μl of 10X PCR buffer, 0.4 units of Ex*Taq* DNA Polymerase (Takara, Seta, Japan) and 0.25 μM of each primers. Amplified products generated from primary PCR reaction were used as templates for determination of

alleles of block 2 in 3 separate amplification reactions in secondary (semi-nested) PCR. Primers for semi-nested PCR included FMSP1B2R (5'-CCATCAATTA AATATTTGAAACC-3') and one of the following primers: (1) FMAD20F (5'-GAACAGCTGTTACAAC TAGTACAC-3') specific to MAD20 type, (2) FK1F (5'-GAAATTACTACAAAAGGTGCAAGTG-3') specific to K1 type and (3) FRO33F (5'-GGAGCAAATACTC AAGTTGTTGC-3') specific to RO33 type. A semi-nested PCR contained 25 μl of reaction mixtures using primers specific to each major type of block 2 of *PfMsp-1*. The thermal cycler profile for semi-nested PCR was essentially the same as that for primary PCR except using 25 cycles of amplification and polymerization for 1 min. Semi-nested PCR products were analyzed by electrophoresis in 3 % agarose gels (agarose for 150-1,500 bp, Nacalai Tesque, Japan), stained with eithidium bromide and visualized under UV transilluminator.

Specificity

Positive controls were 3 *P. falciparum*-positive samples (isolates CT114-MAD20 type, CT312-K1 type, YL1345-RO33 type) whose block 2 sequences of *PfMsp-1* were previously determined by sequencing. These positive controls were used as single templates or as various combinations of different templates. Potential cross amplification of the semi-nested PCR developed in this study was determined by using known DNA samples containing *P. vivax* (*n* = 3), *P. ovale* (*n* = 1), *P. malariae* (*n*=1) and *P. knowlesi* (*n* = 1). Distilled water was used as negative control.

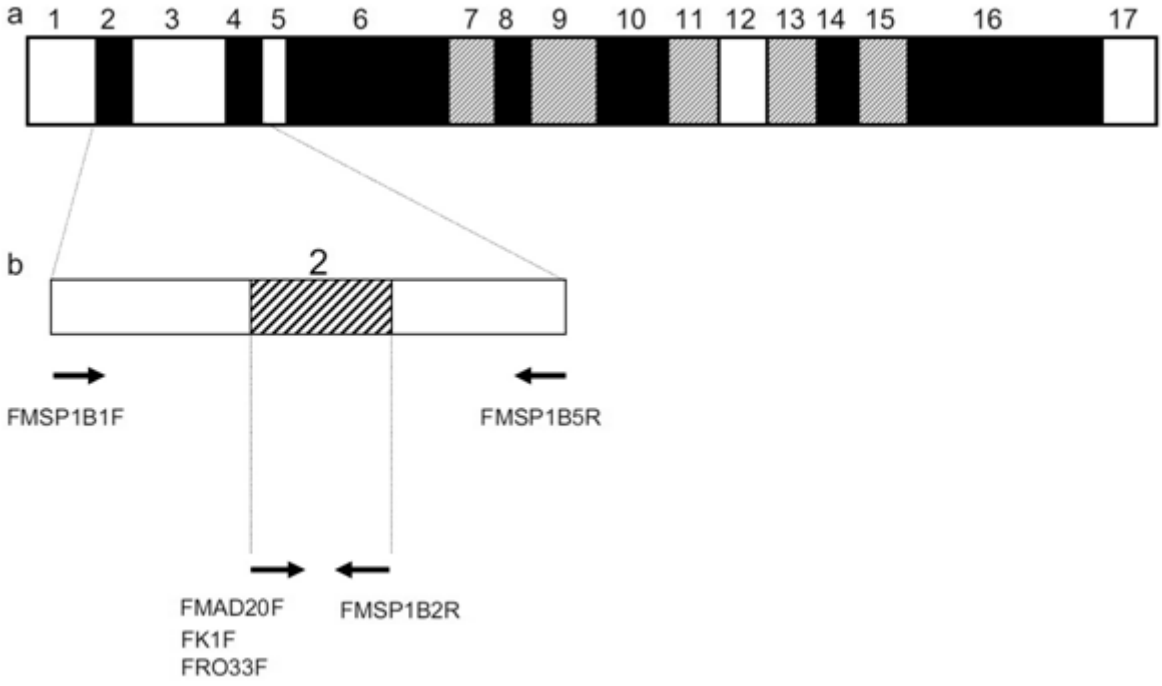

Figure 1. (a) Structure of the *Plasmodium falciparum* merozoite surface protein-1 (PfMsp-1). Conserved semiconserved and variable blocks are shown in unfilled, upward diagonal and filled boxes, respectively. Blocks are numbered above the scheme. (b) Target fragment for PCR amplification. Arrows underneath indicate locations and directions of primers (FMSP1B1F, FMSP1B5R, FMAD20F, FK1F, FRO33F and FMSP1B2R) for genotyping of block 2.

Sensitivity

To test whether each pair of allele-specific semi-nested PCR primers of block 2 yields comparable sensitivity, the minimum limit of DNA templates for PCR detection of *PfMsp-1* was semi-quantitatively determined. First the DNA fragments encompassing conserved blocks 1 and 5 (1041 - 1059 bp) were amplified using primers FMSP1B1F and FMSP1B5R. Because DNA sample isolated from malaria-infected patients contained both human and parasite DNA, estimation of *P. falciparum* DNA was indirectly inferred from the amount of PCR amplified product and the length of the amplified target DNA. The amount of PCR product was quantified by using NanoDrop apparatus according to the manufacturer's instruction (Thermo Fisher Scientific, Delaware, USA). After the copy number of the target DNA was calculated, the PCR product was diluted to obtain 10^3, 10^2, 10, 7, 5, 1 and 0.5 copies of *PfMsp-1*. Sensitivity of detection of each major allelic type of block 2 was determined by semi-nested PCR using specific semi-nested PCR primers. The detection limit was obtained when the minimum dilution of DNA template gave reproducible positive results in 3 independent PCR reactions.

Ethical aspects

The ethical approval of this study has been obtained by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University (IRB No. 365/53).

Results

Specificity Genotyping Method

Amplification of *PfMsp-1* encompassing block 2 of *PfMsp-1* using allele-specific primers has generated 316, 320 and 308 kb for MAD20 type, K1 type and RO33 type, respectively, when positive controls (samples with known genotypes of block 2) were used as templates. No cross amplification between these 3 major types was observed when non-matched primers of block 2 were used. For example, primers FMAD20F and FMSP1B2R gave PCR products of expected size when isolate CT114 expressing MAD20 type of block 2 was used as template whereas no PCR product was obtained using different combinations of primers (FK1F and FMSP1B2R, and FRO33F and FMSP1B2R). To test if these primers retained specificity when isolates contained multiple-clone infections, we artificially mixed DNA samples from isolates with known different *PfMsp-1* sequences of one or another of MAD20, K1 (isolate CT312) or RO33 (isolate YL1345) types. These combinations of 2 or more different types of block 2 of PfMSP1were used as templates for subsequent PCR typing. Template containing genomic DNA from MAD20 type plus K1 type gave successful amplifications using primers FMAD20F and FMSP1B2R. On the other hand, no amplification was detected for reactions containing primers FK1F and FMSP1B2R or FRO33F and FMSP1B2R. When triple genotypes of block 2 were artificially applied to the same tube, positive results were consistently and correctly obtained, showing perfect concordant results between specific pairs of primers and the corresponding block 2 genotypes present in the samples (Figure 2). This indicates that primers of block 2 of *PfMsp-1* gave high accuracy of amplification regardless of the number of mixed genotypes in the samples. No amplification was observed when control water or non-*P. falciparum* DNA were used as templates.

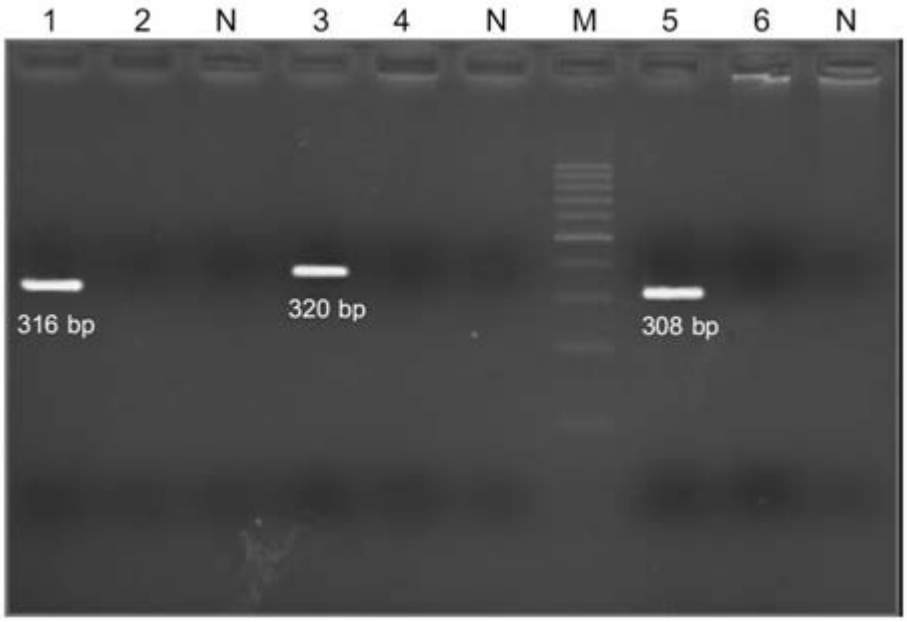

Figure 2. Specificity of the semi-nested PCR assay for genotyping of block 2 of *PfMsp-1*. Agarose gelectrophoresis shows PCR products amplified by using primers FMAD20F and FMSP1B2R (lanes 1, 2 and 3), FK1F and FMSP1B2R (lanes 4, 5, and 6), and FRO33F and FMSP1B2R (lanes 7, 8 and 9). DNA templates are: lane 1 = MAD20 + K1 + RO33; lane 2 = K1 + RO33; lane 3 = K1 + MAD20 + RO33; lane 4 = MAD20 + RO33; lane 5 = RO33 + MAD20 + K1; and lane 6 = MAD20 + K1. M, 100 bp ladder marker; N, negative control.

Sensitivity of Genotyping Method

The PCR products amplified by primers FMSP1B1F and FMSP1B5R were semi-quantified and the copy numbers of *PfMsp-1* could be inferred. The minimum copy number of *PfMsp-1* in the templates that gave positive PCR results by using internal primers FMAD20F and FMSP1B2R, FK1F and FMSP1B2R, and FRO33F and FMSP1B2R were 5 copies of target DNA. All of these estimations were repeated 3 times independently and consistent results were obtained, indicating high sensitivity of the procedure. Table 1 summarizes the sensitivity of the genotyping method by different combinations of pairs of primers. We further determined whether the sensitivity of each pair of primers can be altered by isolates with multiple-clone infections. Three isolates (CT114, CT312 and YL1345) were artificially mixed using substantial difference in copy numbers of these templates to test whether competitive amplification occurs under this condition. Figure 3 depicts results from this evaluation showing that regardless of the quantity of each genotype in the template mixtures, all pairs of primers could detect distinct block 2 genotypes with comparable efficacy.

Subgenotyping of PfMsp-1 block 2

MAD20 type and K1 type can be subdivided into minor allelic types based on variation of tandem repeat units in each type. Using 3% agarose gel electrophoresis, It was possible to demonstrate that amplifications by PCR with primers FMAD20F and FMSP1B2R or primers FK1F and FMSP1B2R generated positive results with size variation of the amplified products whereas primers FRO33F and FMSP1B2R gave positive band with no size variation (Figure 4).

Table 1. Sensitivity of PCR amplification of *PfMsp-1* block 2 using different pairs of primers.

Forward primer	Reverse primer	Allelic type	Size of PCR product (bp)	Sensitivity*
FMAD20F	FMSP1B2R	MAD20	316	5 copies
FK1F	FMSP1B2R	K1	320	5 copies
FRO33F	FMSP1B2R	RO33	308	5 copies

* Miminum number of *PfMsp-1* copies in the templates for PCR that can be amplified by each pair of primers.

Genotyping of clinical isolates

It is important that all of these primers should be applicable to characterize natural isolates. Analysis of 50 *P. falciparum* clinical isolates identified 11 genotypes of block 2. Of these, genotypes 3 and 11, characterized by MAD20 type (320 bp) and RO33 type (310 bp) predominated (16.4%), respectively. Four isolates (CTRB23, TSY2772, TSY2660 and TSY3130) contained different block 2 genotypes, i.e. MAD20 type mixed with K1 type. One isolate (CT341) had coexistence of K1 type and RO33 type. In total 55 genotypes of block 2 of *PfMsp-1* were detected in 50 clinical isolates (Table 2). Subgenotyping of isolates expressing MAD20 type and K1 type harboring size variation ranging from 250 to 360 bp and 300 to 350 bp, respectively, could be identified by 3 % agarose gel electrophoresis whereas no size variation (310 bp) occurred in all isolates having RO33 type.

Discussion

We have developed a simplified method for strain differentiation of *P. falciparum* based on allelic polymorphism in block 2 of *PfMsp-1*. One of the advantages of using polymorphic loci of *PfMsp-1* block 2 as a gene target is the possibility to identify 3 major genotypes including MAD20 type, K1 type and RO33 type as well as subgenotypes of MAD20 type and K1 type based on variation in the number of repeating units, resulting in length polymorphism of the PCR products. All primers have been designed to possess similar annealing temperature so that a single amplification condition is applicable to all pairs of primers; thereby simultaneous genotyping of these 3 major types could be performed in a single thermocycler albeit 3 reaction tubes are required for each sample. Therefore, the method is rapid, reproducible and easy to perform. Although *PfMsp-1* is a single copy gene per haploid genome, as few as 5 copies of the gene that are equivalent to DNA from 5 *P. falciparum* parasites or ~0.0001% parasitemia can be identified by semi-nested PCR indicating high sensitivity of the method.

Potential artefactual results from in vitro recombination have been demonstrated when allelic genes were amplified by PCR.[13] However, our *PfMsp-1* genotyping method is unlikely compromised by such phenomenon because we used allele-specific primers for PCR. Furthermore, the target gene fragment did not span a long fragment, precluding the possibility to obtain false amplification of *in vitro* recombinant products. Results of genotyping using artificial

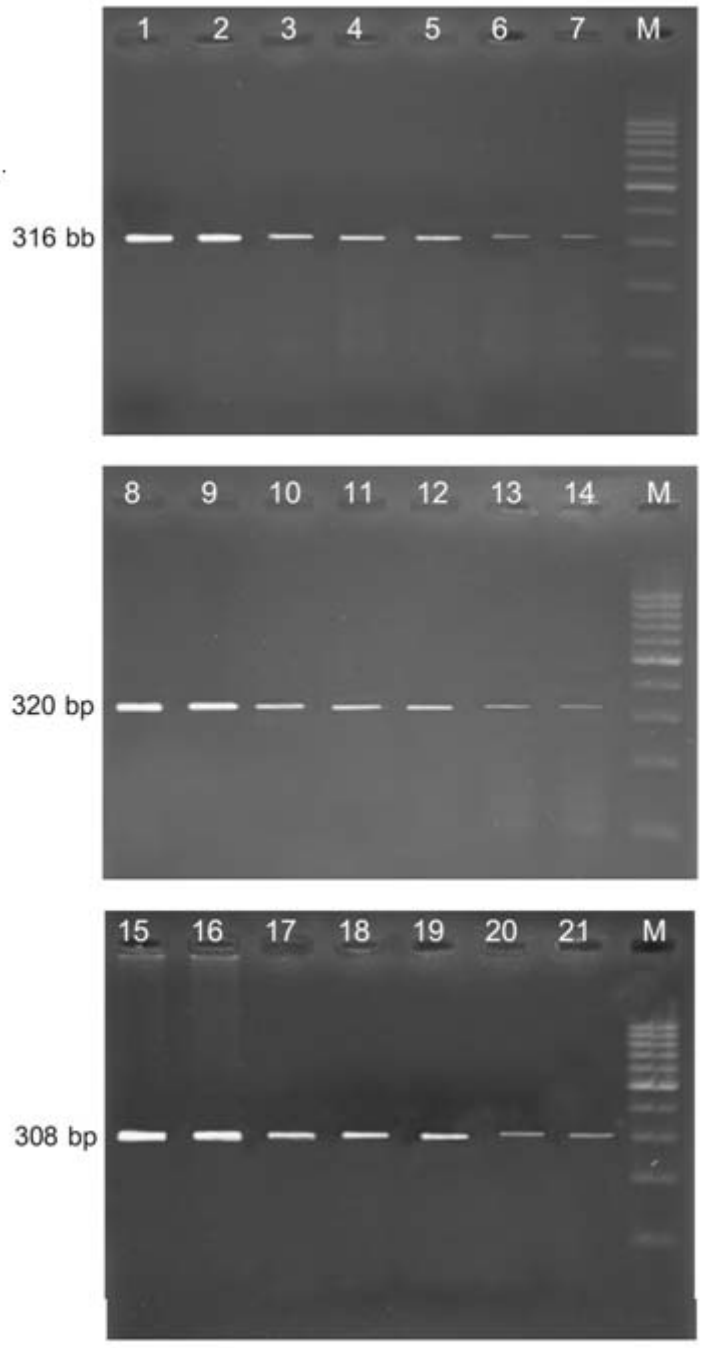

Figure 3. Sensitivity and specificity of semi-nested PCR assay for genotyping of block 2 of *PfMsp-1*. Agarose gel electrophoresis shows PCR products amplified by using primers FMAD20F and FMSP1B2R (lanes 1-7), FK1F and FMSP1B2R (lanes 8-14), and FRO33F and FMSP1B2R (lanes 15-21). DNA templates are: lanes 1-21 = MAD20 + K1 + RO33. Copy numbers of *PfMsp-1* in templates corresponding to respective isolates are 5/5/100 (lanes 7, 14 and 21), 5/100/1000 (lanes 6, 13 and 20), 100/100/10 (lanes 5, 12 and 19), 100/100/100 (lanes 4, 11 and 18), 100/1000/1000 (lanes 3, 10 and 17), 1000/1000/100 (lanes 2, 9 and 16), and 1000/10/10 (lanes 1, 8 and 15). M, 100-bp ladder marker.

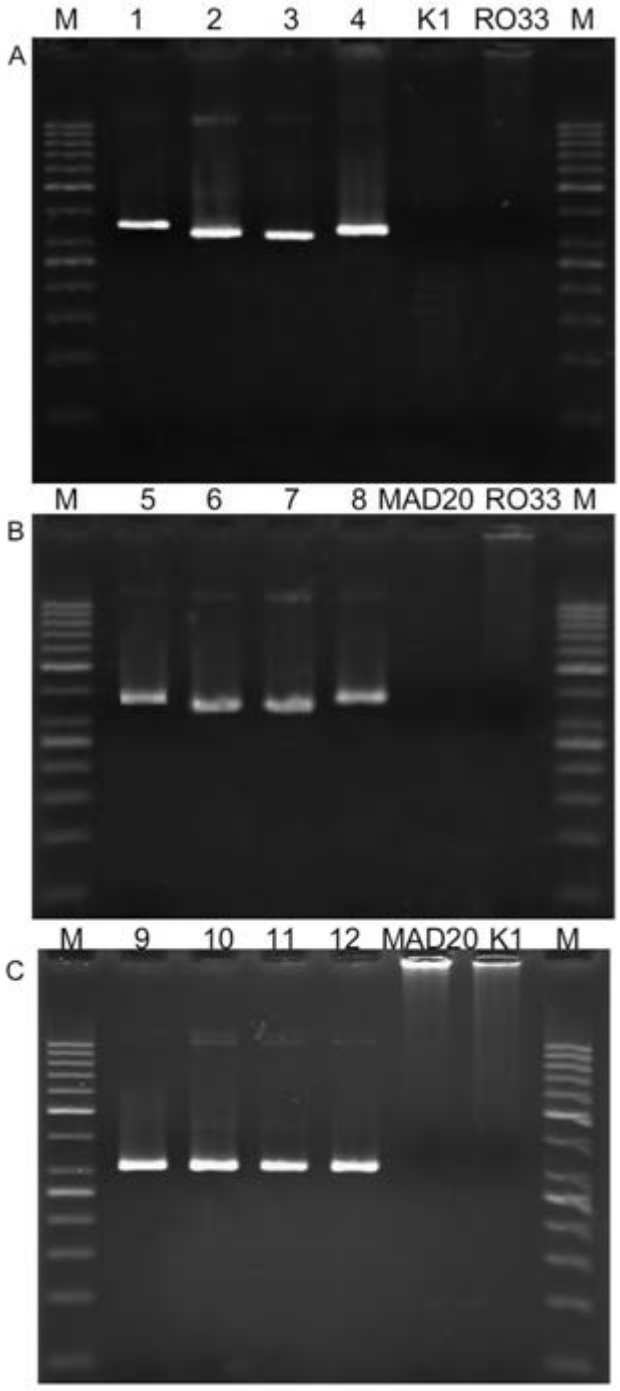

Figure 4. Genotyping of block 2 of *PfMsp-1*. Agarose gel electrophoresis shows PCR products amplified by using primers FMAD20F and FMSP1B2R (**A**), FK1F and FMSP1B2R (**B**), and FRO33F and FMSP1B2R (**C**). DNA templates are: lanes 1- 4 = 4 clinical isolates with MAD20 type; lanes 5 - 8 = 4 clinical isolates with K1 type ; lanes 9 -12 = 4 clinical isolates with RO33 type. M, 100-bp ladder marker. MAD20, K1 and RO33 are representative genotypes of control *P. falciparum* DNA.

Table 2. Distribution of *PfMsp-1* genotypes of block 2 among 50 clinical isolates.

Genotype	Major Type	Size (bp)	Isolate	Frequency
1	MAD20	270	CTRB23, CTRA66	2
2	MAD20	300	CT338, TSY3179, TSY2772, TSY2844 TSY2647, TSY266	6
3	MAD20	320	CT18, YL1816, TSY2947, TSY2961, TSY2965 TSY2973, TSY2974, TSY3130, TSY3136	9
4	MAD20	310	CT446, TSY2970, TSY2639, TSY2643	4
5	MAD20	330	CT229, TSY2976, TSY2977, TSY2638	4
6	MAD20	360	CTRC44, CTRA75, CTRB17	3
7	K1	310	CT312, CT344, TSY3172, CTRB23	4
8	K1	320	CT339, CT341, CT476, CT213, CTRA49 CTRC41, TSY2978, TSY2660	8
9	K1	330	CT450, CT223, TSY2634, TSY3130	4
10	K1	350	YL1957, TSY2772	2
11	RO33	310	YL1350, YL1352, YL1353, YL1364, YL1813 YL1817, YL1355, YL1359, CT341	9
			Tota	55

* Underlined isolates contained multiple genotypes

mixtures of known numbers of different PfMsp-1 alleles as templates for PCR also confirmed the fidelity of the procedure and sensitivity of detecting minor populations of *P. falciparum* containing distinct genotypes. Previous studies on diversity in *PfMsp-1* and *PfMsp-2* of *P. falciparum* have revealed a high prevalence of multi-clone infections among natural isolates from Brazil, Thailand and Vietnam.[8, 14, 15] Likewise, results from *PfMsp-1* genotyping of 50 field isolates using the semi-nested PCR method described herein provide further supportive evidence that it is suitable for field application.

Several lines of evidences indicate that PfMsp-1 is a leading asexual blood stage vaccine candidate for *P. falciparum*. Immunization trials in *Aotus* monkeys using merozoite-derived Msp-1 conferred complete protection against subsequent blood stage challenge with homologous strain of *P. falciparum*[16] whereas partial protection was obtained when challenge with parasite expressing different alleles of Msp-1.[17] An *in vitro* study has shown that antibodies against block 2 of PfMsp-1 could inhibit erythrocyte invasion by merozoites in allele-specific fashion.[18] The strength of immunological recognition of antibodies to specific alleles of block 2 also depends on both the tripeptide repeat sequence and the number of repeating units.[18] An integrated serological and molecular population genetics approach has purported that block 2 of PfMsp-1 is a target of allele-specific protective immunity upon natural infection.[19] Taken together, protective immunity against *P. falciparum* is partly induced by specific epitopes in

block 2 of PfMsp-1 that are subject to sequence and size variation. Importantly, block 2 of PfMsp-1 exhibits extensive antigenic diversity among natural parasite isolates.[8, 20, 21] The distribution of the 3 major allelic types of block 2 substantially differs from one malaria endemic areas to another.[8, 9] Therefore, a rationale design of a malaria vaccine derived from block 2 of PfMsp-1 requires knowledge on allelic distribution of this antigenic target within *P. falciparum* population circulating in each endemic area and the promising effective vaccine may need to incorporate appropriate epitopes in a subunit vaccine that exist in parasite population. Undoubtedly, the block 2 of *PfMsp-1* genotyping method developed in this study has practical implication for malaria vaccine program.

Recent resurgence of malaria in several endemic areas has caused significant impacts on public health and disease control.[22 - 24] Undoubtedly, tracing the populations of malaria parasites will provide some clues to the understanding of disease transmission. The extent of allelic diversity and multiplicity of infections in term of *PfMsp-1* correlate with endemicity of malaria and intensity of transmission in the mosquito vectors.[3, 14, 15] Meanwhile, during an outbreak or in isolated geographic areas, populations of malaria may not be panmictic; rather clonally expanding.[13, 23] Therefore, exploitation of proper genetic markers is an essential tool for population genetic analysis as a basis for malaria control.

Conclusion

The PCR genotyping method developed in this study can efficiently differentiate *P. falciparum* genotypes. At least 11 different genotypes of *P. falciparum* were identified among 50 clinical isolates in Thailand whereas multiple clone infections were detected by the method. These support the usefulness of the technique for genetic analysis in large-scale population.

Acknowledgements

We are grateful to all patients who participated in this study. This study was supported by the Graduate School Thesis Grant (academic year 2011).

References

1. Anderson TJ, Su XZ, Bockarie M, Lagog M, Day KP. Twelve microsatellite markers for characterization of *Plasmodium falciparum* from finger-prick blood samples. Parasitology 1999 Aug; 119(Pt 2): 113 - 25
2. Viriyakosol S, Siripoon N, Petcharapirat C, Petcharapirat P, Jarra W, Thaithong S, Brown KN, Snounou G. Genotyping of *Plasmodium falciparum* isolates by the polymerase chain reaction and potential uses in epidemiological studies. Bull World Health Organ 1995; 73(1): 85 - 95
3. Kaneko O, Kimura M, Kawamoto F, Ferreira F, Tanabe K. *Plasmodium falciparum*: allelic variation in the merozoite surface protein 1 gene in wild isolates from southern Vietnam. Exp Parasitol 1997 May; 86(1): 45 - 57
4. Holder AA. The precursor to major merozoite surface antigens: structure and role in Immunity. Prog Allergy 1988; 41: 72 - 97
5. Tanabe K, Mackay M, Goman M, Scaife JG. Allelic dimorphism in a surface antigen

gene of malaria parasite *Plasmodium falciparum*. J Mol Biol 1987 May 20; 195(2): 273 - 87

6. Miller LH, Roberts T, Shahabuddin M, McCutchan TF. Analysis of sequence diversity in the *Plasmodium falciparum* merozoite surface protein-1 (MSP-1). Mol Biochem Parasitol 1993 May; 59 (1): 1 - 14
7. Certa U, Rotmann D, Matile H, Reber-Liske R. A naturally occurring gene encoding the major surface antigen precursor p190 of *Plasmodium falciparum* lacks tripeptide repeats. EMBO J 1987 Dec; 6 (13): 4137– 42
8. Jongwutiwes S, Tanabe K, Nakazawa S, Yanagi T, Kanbara H. Sequence variation in the tripeptide repeats and T cell epitopes in P190 (MSA-1) of *Plasmodium falciparum* from field isolates. Mol Biochem Parasitol 1992 Mar; 51(1): 81-9
9. Jiang G, Daubenberger C, Huber W, Matile H, Tanner M, Pluschke G. Sequence diversity of the merozoite surface protein 1 of *Plasmodium falciparum* in clinical isolates from the Kilombero District, Tanzania. Acta Trop 2000 Jan 5; 74(1): 51 - 61
10. Kerr PJ, Ranford-Cartwright LC, Walliker D. Proof of intragenic recombination in *Plasmodium falciparum*. Mol Biochem Parasitol 1994 Aug; 66(2): 241-8
11. Putaporntip C, Hongsrimuang T, Seethamchai S, Kobasa T, Limkittikul K, Cui L, Jongwutiwes S. Differential prevalence of *Plasmodium* infections and cryptic *Plasmodium knowlesi* malaria in humans in Thailand. J Infect Dis 2009 Apr; 199(8):1143 - 50
12. Jongwutiwes S, Buppan P, Kosuvin R, Seethamchai S, Pattanawong U, Sirichaisinthop J, Putaporntip C. *Plasmodium knowlesi* Malaria in humans and macaques, Thailand. Emerg Infect Dis 2011 Oct; 17(10): 1799 - 806
13. Tanabe K, Sakihama N, Farnert A, Rooth I, Bjorkman A, Walliker D, Ranford-Cartwright L. In vitro recombination during PCR of *Plasmodium falciparum* DNA: a potential pitfall in molecular population genetic analysis. Mol Biochem Parasitol 2002 Jul; 122(2): 211 - 6
14. Sakihama N, Kimura M, Hirayama K, Kanda T, Na-Bangchang K, Jongwutiwes S, Conway D, Tanabe K. Allelic recombination and linkage disequilibrium within Msp-1 of *Plasmodium falciparum*, the malignant human malaria parasite. Gene 1999 Apr; 230(1): 47 - 54
15. Tanabe K, Sakihama N, Nakamura Y, Kaneko O, Kimura M, Ferreira MU, Hirayama K. Selection and genetic drift of polymorphisms within the merozoite surface protein-1 gene of *Plasmodium falciparum*. Gene 2000 Jan; 241(2): 325 - 31
16. Siddiqui WA, Tam LQ, Kramer KJ, Hui GS, Case SE, Yamaga KM, Chang SP, Chan EB, Kan SC. Merozoite surface coat precursor protein completely protects Aotus monkeys against *Plasmodium falciparum* malaria. Proc Natl Acad Sci USA 1987 May; 84 (9): 3014 - 8
17. Siddiqui WA, Taylor DW, Kan SC, Kramer K, Richmond-Crum S. Partial protection of *Plasmodium falciparum*-vaccinated Aotus

trivirgatus against a challenge of a heterologous strain. Am J Trop Med Hyg 1978 Nov; 27 (6): 1277 - 8

18. Locher CP, Tam LQ, Chang SP, McBride JS, Siddiqui WA. *Plasmodium falciparum*: gp195 tripeptide repeat-specific monoclonal antibody inhibits parasite growth in vitro. Exp Parasitol 1996 Oct; 84(1): 74 - 83
19. Conway DJ, Cavanagh DR, Tanabe K, Roper C, Mikes ZS, Sakihama N, Bojang KA, Oduola AM, Kremsner PG, Arnot DE, et al. A principal target of human immunity to malaria identified by molecular population genetic and immunological analyses. Nat Med 2000 Jun; 6(6): 689 - 92
20. Atroosh WM, Al-Mekhlafi HM, Mahdy MA, Saif-Ali R, Al-Mekhlafi AM, Surin J. Genetic diversity of *Plasmodium falciparum* isolates from Pahang, Malaysia based on MSP-1 and MSP-2 genes. Parasit Vectors 2011 Dec; 4: 233
21. Bharti PK, Shukla MM, Sharma YD, Singh N. Genetic diversity in the block 2 region of the merozoite surface protein-1 of *Plasmodium falciparum* in central India. Malar J 2012 Mar; 11(1): 78
22. Sleigh AC, Liu XL, Jackson S, Li P, Shang LY. Resurgence of vivax malaria in Henan Province, China. Bull World Health Organ 1998; 76(3): 265 – 70
23. Lim CS, Kim SH, Kwon SI, Song JW, Song KJ, Lee KN. Analysis of *Plasmodium vivax* merozoite surface protein-1 gene sequences from resurgent Korean isolates. Am J Trop Med Hyg 2000 Feb; 62 (2): 261- 5
24. Hay SI, Cox J, Rogers DJ, Randolph SE, Stern DI, Shanks GD, Myers MF, Snow RW. Climate change and the resurgence of malaria in the East African highlands. Nature 2002 Feb; 415(6874): 905 - 9