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A PCR Method for Molecular Epidemiology of *Plasmodium falciparum Msp-1*

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Merozoite surface protein-1 (MSP-1) of *Plasmodium falciparum* is a strong malaria vaccine candidate. However, MSP-1 exhibits extensive antigenic polymorphism, an issue which may compromise the development of effective vaccine based on this molecule. Since polymorphic nature of MSP-1 has not been fully understood in endemic areas of malaria, variation of the MSP-1 gene (*Msp-1*) must be studied in detail in natural parasite populations. Here, a PCR-based method for determination of *P. falciparum Msp-1* haplotype is described, which can detect up to 24 different haplotypes per infected person. The method can be applied to various purposes of molecular epidemiology of not only *Msp-1* haplotype but the genetic structure of *P. falciparum* populations.

Keywords : *Plasmodium falciparum*, Merozoite surface protein-1 (MSP-1), Polymorphism, Molecular epidemiology, PCR

INTRODUCTION

Merozoite surface protein-1 (MSP-1) of *Plasmodium falciparum* is a strong malaria vaccine candidate [10, 12, 18]. Immunization with affinity-purified MSP-1 or recombinant MSP-1 can protect monkeys from infection of *P. falciparum* [25, 33] and antibodies against MSP-1 inhibit the growth of the parasite *in vitro* [3-4, 7-8, 19, 30]. Importantly, high levels of antibodies to MSP-1 are reported to correlate with protection from clinical malaria [14-15, 31-32]. However, the protein exhibits extensive antigenic polymorphism among parasite strains/isolates [26]. Since antigenic polymorphism is one of key issues in developing effective vaccines, the extent and mode of polymorphism of the MSP-1 gene (*Msp-1*) and a mechanism for generating the genetic diversity of *Msp-1* must be studied in detail. Limited studies with culture-adapted parasite strains have revealed a general feature of the polymorphism displayed by *Msp-1* [34]: variation in the gene is not widely polymorphic but basically dimorphic in several variable blocks that are interspersed with conserved or semiconserved blocks. Intragenic recombination can account for the major variability of *Msp-1* [27, 34]. However, many questions regarding allelic variation of *Msp-1* in natural parasite populations still remain to be answered; for example, difference in the prevalence of *Msp-1* in different geographic areas, longitudinal changes in frequency distribution of *Msp-1* in light of the host immune response, and frequency of heterozygosity in oocysts, in which meiotic recombination takes place. To investigate these issues it seems necessary to develop a method suitable for monitoring allelic variation of *Msp-1* in a large number of field samples. For this purpose, we have developed to a PCR-based method to detect 24 different *Msp-1* haplotypes with particular reference on intragenic recombination. Here, strategy of the method and its potential applications to molecular epidemiology of *P. falciparum* will be described.

METHOD

1. Structure of *P. falciparum Msp-1*

Details of the method for determination

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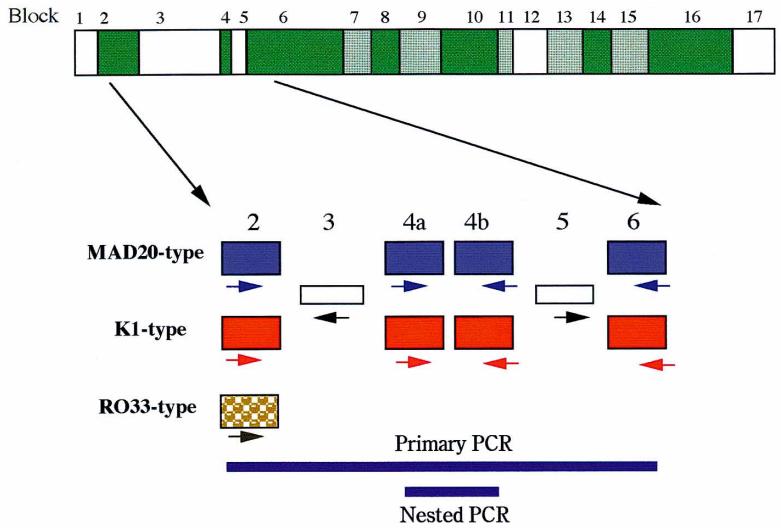

Fig. 1 Structure of the *P. falciparum* merozoite surface protein-1 gene (*Msp-1*) and strategy for determination of *Msp-1* haplotypes. Upper bar represents the structure of Msp-1 consisting of five conserved blocks (open bar), in which sequences are conserved among parasite strains/isolates, interspersed with seven variable (green bar) and five semi-conserved (hatched green bar) blocks. Strategy for *Msp-1* haplotype determination is illustrated below (see text for details).

of *Msp-1* haplotype have been described elsewhere [22]. Here, strategy of the method will be outlined with the guidance of the structure of *Msp-1* (Fig. 1). There are several features in the variation in *Msp-1* among parasite strains/isolates [34]: first, *Msp-1* consists of five conserved blocks which are interspersed with several semi-conserved or variable blocks. Second, variation in variable blocks is not widely polymorphic but basically dimorphic, represented by the two prototype alleles, K1 allele and MAD20 allele. Sequence variations within the same allelic type is nearly identical but drastically differ between the two major allelic types. The exception is polymorphic block 2, in which three major allelic types occur, depending on the presence or absence of 9-bp repeats and the number and type of the repeating units [6, 24, 29]. Third, intragenic recombination between two parental alleles generates new alleles in the progeny, which has been demonstrated by experimental cross [23]. The potential recombinations sites are confined to conserved blocks 3 and 5 and a short conserved stretch in variable block 4, which is therefore divided into subblocks 4a and 4b [9, 21]. (Another potential recombination sites occur in conserved blocks 1 and 17. However, they are not considered here, because recombination wherein does not create re-shuffling of variable blocks.) No evidence of recombination between blocks 6 to 16 has been reported to date [20, 29, 35].

2. Rationale of looking at recombination in *Msp-1*

Although limited variations occur in conserved blocks and variable blocks within the same allelic types (*i.e.*, K1-allelic type or MAD20-allelic type) recombination in blocks 3, 4 and 5 can account for the major variability of *Msp-1* if found among parasite strains/isolates. Since *Msp-1* is a single copy gene, each parasite possesses only one allelic type in each variable block. Therefore, it is possible to detect major allelic variations of *Msp-1* by focusing on linkage of allelic types in variable blocks 2, 4a, 4b and 6. Based on this rationale, a PCR method has been developed, which determines allelic types in blocks 2 and 6 at first by primary PCR and then blocks 4a and 4b by nested PCR using the primary PCR product. Linkage of allelic type in each variable block is thus successfully determined. Therefore, up to 24 different *Msp-1* haplotypes can be distinguished by focussing on recombination by this PCR method.

Several polymorphic genes of *P. falci-*

parum, including *Msp-1*, *Msp-2*, *Glurp* (glutamate-rich protein gene) and *Csp* (circumsporozoite protein gene) have been used as markers to study the genetic structure of natural parasite populations [1-2, 16]. Differences in allelic types as well as the number of repeat sequences in *Msp-1* and *Msp-2* can be detected by PCR, followed by hybridization using allelic type-specific oligonucleotides. *Msp-1* block 2 and *Msp-2* polymorphic block are typed in this way and size difference of more than 15 bp can be detected in 2-3% agarose gel. However, in isolates, in which multiple parasite genotypes infections are found, genotypes having the same number of repeats cannot be distinguished by the method. Thus, determination of a single polymorphic block has limitations of detecting the number of alleles in multiple infections. Indeed, it is common to find different number of alleles for *Msp-1* and *Msp-2* in the same isolate. Size polymorphism is also used for *Glurp* and *Csp*. In the case of *Csp*, extensive polymorphism concentrates primarily in the two major T-cell epitope regions, Th2R and Th3R [11, 13], in which variations are detected by sequencing. However, sequencing is not only relatively time-consuming for processing a large number of field samples but also has sometimes a potential risk. The reason for it stems from that direct sequencing of templates with mixed parasite genotypes infections often yields unclear sequences at positions of nucleotide changes and that subcloning into plasmid vector when employed prior to sequencing often leads to artifact recombination in *E. coli* between plasmids containing different alleles.

3. PCR-based determination of *Msp-1* haplotype

Based on the above mentioned rationale, PCR primers specific to allelic type sequences as well as inter-allele conserved primers were designed (Fig. 1) [22]: K1-allelic type specific forward primer, K2F, MAD20-allelic type specific forward primer, M2F, and RO33-allelic type specific forward primer, R2F, in block 2; inter-allele conserved reverse primer, C3R, in block 3. Likewise, allelic type specific primers in blocks 4a (K4F and M4F), 4b (K4R and M4R), and 6 (K6R and M6R), and a conserved primer, C5F, in block 5 were designed. *Msp-1* haplotype is determined by the following three steps: at the first step, parasite DNA template is subjected to 5 different amplification reactions; that is, three for determination of allelic type in blocks 2 using primers either of K2F, M2F or R2F and C3R, and the remaining two for allelic type in block 6 using primers either of K6R or M6R and C5F. The PCR conditions have been described elsewhere [22]. Positive signals at predicted sizes in agarose gel are photographed after electrophoresis. At the second step, amplification of blocks 2 to 6 is performed using relevant primers determined at the first step: for example, if an isolate gave a positive signal for MAD20 allelic type in block 2 and for K1 allelic type in block 6, primers M2F and K6R are used to amplify blocks 2 to 6. If an isolates had more than one allelic types in blocks 2 and/or 5, multiple amplification reactions are performed in different microtubes using respective primers. At the third step, amplified products are subjected to nested PCR: the product is placed into four different reactions having a set of primers K4F and K4R, K4F and M4R, M4F and K4R, and M4F and M4R. An association (*i.e.* linkage) of allelic types in blocks 2, 4a, 4b and 6 (*Msp-1* haplotype) is thus determined and assigned into either of 24 possible *Msp-1* haplotypes as shown in Fig. 2.

Instead of individual determinations of allelic types in blocks 2 and 6, six different reactions using either of K2F, M2F or R2F and either of K6R or M6R can be directly performed. The procedure for nested PCR to determine allelic types in blocks 4a and 4b is the same as described above. *Msp-1* haplotype determination with this direct procedure yielded results mostly identical (> 90%) to the individual typing procedure for a test batch of isolates (not shown).

APPLICATION

The PCR method can be applied to study various aspects of molecular epidemiology of *P. falciparum*. Frequency distribution of *Msp-1* haplotype at different occasions suggests the genetic structure of *P. falciparum* in an endemic area. Difference in the frequency distribution among different geographic areas would reveal a spatial difference of *P. falciparum* populations. If such spatial difference occurs, then a question should arise

concerning a mechanism(s) for genetic differentiation of parasite populations in different areas. Since MSP-1 is a target of the host immune response, it is of interest to see if immunological pressures to the parasite select particular *Msp-1* haplotype at the population level. Both cross-sectional and longitudinal surveys are required to discuss this issue. Difference in the number and frequency distribution of *Msp-1* haplotype at different age groups or individuals with or without clinical malaria may suggest the presence of such immune pressures. Comparison between asymptomatic (immune) and symptomatic (nonimmune) individuals would be of particular interest. In nonimmunes, the mean number of *Msp-1* haplotypes per person is expected to be higher than in immunes if *Msp-1* haplotype-specific immunity is present. In southern Vietnam, where malaria transmission is low, stable pattern for frequency distribution of *Msp-1* haplotype has been observed [17]. This may suggest that changes in *P. falciparum* population would be slow, if any, in the area. Apart from immunological selection, genetic drift can be considered as another mechanism for differentiation of the parasite population.

The mean number of *Msp-1* haplotypes per infected person reflects the intensity of malaria transmission. In high transmission areas the mean number would be higher than in low transmission areas. This is

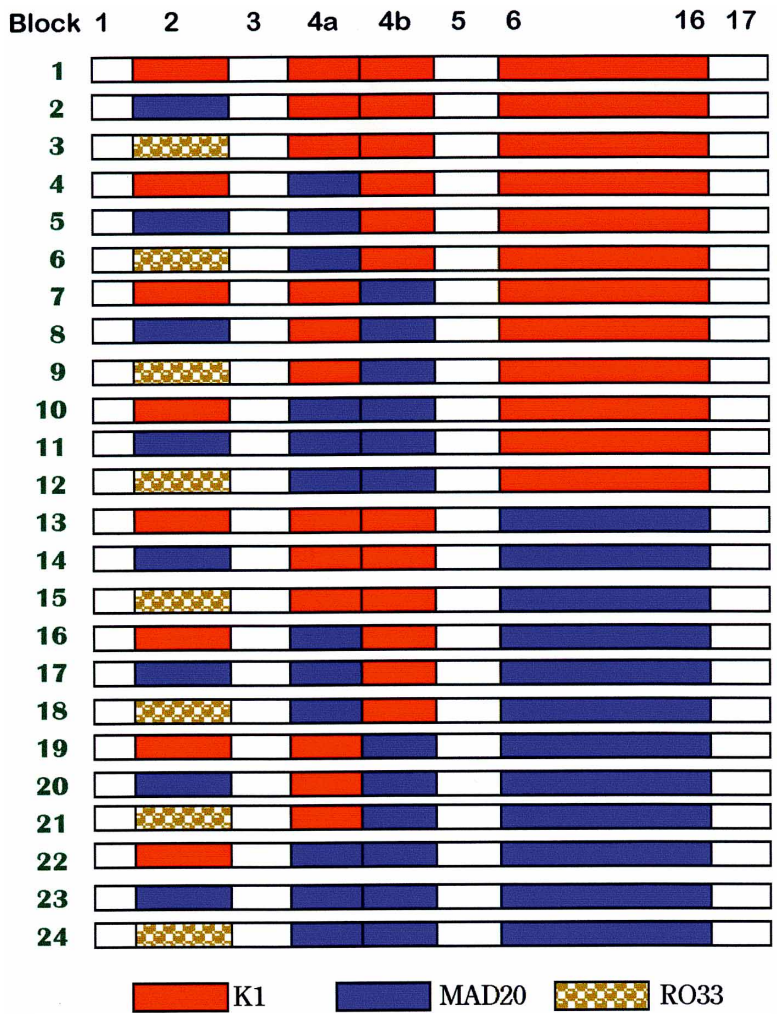

Fig. 2 Scheme for 24 different *Msp-1* haplotypes determined by a PCR-based method focussing on allelic recombination in the gene.

because inoculations of new *Msp-1* haplotypes would be brought by repeated mosquito-bitings. In the case of analysis of 108 southern Vietnam filed isolates, we detected the mean number of *Msp-1* haplotypes of 1.8, and one isolate had as many as 7 different haplotypes [22]. Since malaria prevalence in southern Vietnam is mesoendemic, the mean number would be higher in hyper- to holo-endemic areas. Gene flow can be monitored by analyzing the total number of distinct *Msp-1* haplotyes out of 24 possible haplotypes. Gene flow from other populations would alter frequency distribution of *Msp-1* haplotypes in a given population. In this context, it is of interest to see the total number and frequency distribution in areas in the rim of malaria prevalence or a malaria-virgin area, where *P. falciparum* has been introduced recently.

As stated before, allelic recombination can account for the major variability of *Msp-1*. Thus, estimation of the extent of recombination is of vital importance. Since our PCR method has the rationale to detect allelic recombination within *Msp-1*, it is potentially useful for analyzing the extent of recombination in *Msp-1*. For recombination to occur, heterologous gametes having different *Msp-1* haplotypes must be ingested by an mosquito upon feeding, because meiotic recombination takes place only at the oocyst stage. This issue can be tested by analyzing heterozygosity of *Msp-1* haplotypes in oocysts from wild caught mosquitoes [1, 28], although such sampling of oocysts is extremely difficult. The recombination rate can be assumed by examining linkage disequilibrium between polymorphic loci in *Msp-1* at the blood stage in natural populations. In an area of high transmission intensity, linkage disequilibrium would not be expected to occur in non-immune populations. If linkage disequilibrium was seen in immune populations, any selection in favor of particular *Msp-1* haplotypes can be discussed. Cavanagh et al. have [5] shown concordant appearance of antibodies specific to allelic type in block 2 and the presence of parasite genotypes showing the same allelic type. If balancing selection was operating, such allelic type-specific antibodies might select particular *Msp-1* haplotypes.

The PCR method can also be applied to monitor the prevalence of *in vivo* drug resistance of *P. falciparum* in local areas. Facing the world-wide spread of chloroquine resistance of *P. falciparum*, reliable method for assessing drug resistance *in vivo* is urgently needed. Re-appearance or continuous presence of parasite after drug administration has been used as an index for the *in vivo* resistance. However, in malaria fields it is common to see that patients have multiple parasite genotypes infections, in which some parasite are resistant but others susceptible. Therefore, clear distinction of parasite populations in terms of drug resistance would be important to assess the prevalence of *in vivo* drug resistance. Our PCR method can be used for this purpose. Furthermore, new infections may frequently occur in a high transmission area and thus it may be difficult, without genotyping, to distinguish persisting drug resistant parasites from those introduced by mosquito bites after chemotherapy.

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