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# Genotyping of *Plasmodium falciparum* isolates by the polymerase chain reaction and potential uses in epidemiological studies

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*The epidemiology of malaria results from the interactions of three gene pools—parasite, human, and mosquito vector—with one another and with their environment. Methods are being developed for characterizing the genetics of human populations at risk and of potential vectors. The characterization of natural populations of Plasmodium and knowledge of their distribution within the human and insect hosts in any given area under study would also greatly enhance understanding of the epidemiology, pathology and biology of this parasite, particularly when combined with simultaneous human and vector studies.*

*This paper describes a polymerase chain reaction (PCR)-based assay which provides a sensitive, reproducible and practical method by which parasite populations within species can be characterized. In order to illustrate the suitability of the PCR assay, four polymorphic domains on the genes of three *P. falciparum* proteins (MSP1 blocks 2 and 4, MSP2, and GLURP) and one largely conserved region (MSP1 block 17) were chosen for amplification by PCR. DNA derived from 15 in-vitro cultured lines of *P. falciparum* (7 of which were cloned) and from blood samples obtained from infected patients in Thailand were used as templates for PCR amplification. The amplification products were analysed by gel electrophoresis for length polymorphisms. Seven allelic variants of GLURP, five of MSP1 block 2, three of MSP1 block 4, and nine of MSP2 were detected. This high degree of polymorphism can be used to characterize the genetic composition of any parasite population, at a given time. The paper discusses the applicability of this type of genotyping to epidemiology and urges the adoption of international standards for its use so that data from different areas and different times can be compared.*

## Introduction

There have been a number of studies on the genetics of human (1, 2) and vector (3, 4) populations exposed to infection by malaria parasites. Comparable studies on the genetics of natural populations of

malaria parasites are relatively few, and no attempt has been made to relate parasite genetics to the genetics of the local human and vector populations in which the parasites occur, and thus to the epidemiology of the disease. Extensive parasitological and clinical observations in endemic areas, and from the application of malaria therapy for the treatment of neurosyphilis, have revealed the existence of numerous biologically distinct strains within each of the four *Plasmodium* species infecting man (5).<sup>a</sup> These four species differ in their clinical, pathological and transmission properties, as well as their responses to drug treatment; strains within species also differ in these characteristics (7–9). Furthermore, the acquisition of clinical and parasitological immunity is markedly species- and strain-specific.

Recent PCR (polymerase chain reaction) analysis has shown that mixed infections are much more common than previously thought (10–12). Limited studies in man (13), and more detailed studies in ani-

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<sup>a</sup> The term "strain" refers to a parasite population whose members share defined characteristics, but which are not necessarily genetically homogeneous (6).

mal models using specific gene probes to estimate the relative numbers of parasite types present daily in mixed infections (14), have shown that the level of parasitaemia and pathology produced by mixed infections may be significantly different from that produced by the same parasites occurring alone. In addition, recent PCR studies in Guinea-Bissau have suggested that the occurrence of a mixed infection, in this case *P. falciparum* and *P. malariae*, may be very highly and unexpectedly topographically localized (15). Clearly, under conditions of natural malaria transmission, the human and mosquito hosts are constantly exposed to heterogeneous parasite populations. Characterizing these populations, describing their dynamics, and defining the factors that govern the interactions of the parasite with its hosts in a given area should greatly facilitate the design of effective malaria control measures. At present, there are no internationally agreed methods whereby this goal can be practically and reproducibly achieved.

The infected cell-agglutination test, described by Eaton, which was the first reliable *in-vitro* method for the differentiation of parasite strains, was used to demonstrate antigenic variation in the malaria parasite (16). The characterization of the highly diverse S-antigens provided the first opportunity to enumerate the different *P. falciparum* strains present in a given endemic region (17). More recently, variant forms of enzymes, antigens, proteins, gene sequences and drug susceptibility have been used to characterize *P. falciparum* isolates and lines (18–21). However, relatively large numbers of parasites are required for these studies and parasites cultured *in vitro* are generally used. It is neither practical nor representative to limit epidemiological studies to cases where sufficient parasite material can be obtained directly from patients for these types of studies, namely from the relatively uncommon cases with heavy infections, or from the placentae from infected mothers. Further disadvantages severely restricting the number of analysable samples are that many parasite isolates fail to thrive in culture and that a large volume of infected blood (5 ml) is required to initiate cultures from field isolates.

Extensive polymorphism has been shown to be present in the genes of a number of malarial proteins (22). The use of the polymerase chain reaction (23), which detects low numbers of parasites in small sample volumes (12, 24–26), overcomes the limitations imposed by *in-vitro* culture and is therefore ideally suited for the detection of genetic variation. Some of the potential of this approach has been demonstrated by the analysis of the genetic polymorphisms exhibited by the two *P. falciparum* merozoite surface proteins (MSP1 and MSP2) (10, 11, 19, 20, 27). We propose that the polymorphisms exhibited

by these, as well as other malaria parasite proteins, can be considered as genetic markers for the genotyping of *P. falciparum* populations present in field samples.

In order to illustrate the resolution of the PCR genotyping assay presented here, four polymorphic regions from three separate and well characterized genes of *P. falciparum* (Fig. 1) were selected as markers for PCR amplification. These were blocks 2 and 4 of MSP1 (28–30), MSP2 (31, 32), and the

Fig. 1. Schematic representation of the genes coding for MSP1, MSP2 and GLURP. All the regions targeted for PCR amplification are shown in black. The position and sequence of the oligonucleotide primer pairs are also given.



repeat region of the glutamate rich protein or GLURP (33). In each case, only one type of variant for these genes is observed in cloned asexual malaria parasites, which are haploid. The four gene segments targeted for amplification are known to be highly polymorphic (22), with the exception of block 17 of MSP2 which was included as an internal control. Two types of polymorphisms, size and sequence, have been described for blocks 2 and 4 in the MSP1 gene (30, 34) and for the repeat region of the MSP2 gene (31, 35), whereas only size variation, due to different number of repeats, has been observed with the GLURP gene (33). In this study allelic variants were analysed only with respect to the size polymorphisms. In view of the high discrimination afforded by the sole use of size polymorphisms, especially following computer analysis, determination of the sequence of variants has not been carried out in this study. We have found length polymorphisms sufficient to define any particular *P. falciparum* isolate and we discuss the potential of this type of analysis for biological and epidemiological investigations.

## Materials and methods

### Parasite source

Parasites cultured *in vitro* from cloned and uncloned lines of *P. falciparum* were obtained from the National Institute for Medical Research, Parasitology Division (London, England), and from the WHO Collaborating Centre on the Biological Characterization of Malaria Parasites, Chulalongkorn University (Bangkok, Thailand). Infected blood samples were collected from patients, with their informed consent, attending the Thamai Hospital (Chantaburi Province, Thailand). Venous blood (2 ml) was obtained by the hospital staff on the day of admission, immediately before or after initiation of treatment. Two patients agreed to provide further daily blood samples while they remained in the hospital. Following discharge (day 4), one patient (denoted U) returned two weeks later for routine examination (R1 sample). Another patient (denoted F) was discharged on day 2, and returned after 14 days for routine examination (R1 sample). He experienced renewed clinical activity a few days later (R2 sample). Blood samples were also taken on each occasion.

### DNA purification

Frozen aliquots (ca. 500  $\mu$ l) from *in vitro* cultures, or the blood samples obtained from patients, were allowed to thaw on ice before mixing with cold PBS (1.3 ml final volume). The parasites and the unlysed erythrocytes were recovered by centrifugation (5 min

at 6000  $\times$  g). The supernate was discarded, the pellet resuspended in 1 ml cold PBS, and saponin added to a final concentration of 0.05%. Immediately after lysis, the parasites, as well as white blood cells, were recovered by centrifugation as above and the pellet was immediately resuspended in 25  $\mu$ l of 4  $\times$  lysis buffer (40 mM Tris pH 8.0 with HCl, 80 mM EDTA pH 8.0, 2% SDS and 2 mg/ml Pronase E). Sterile distilled water was added to a final volume of 100  $\mu$ l and the mixture vortexed before incubation for four hours at 37 °C. Next, 300  $\mu$ l of water was added to the mixture before phenol extraction and ethanol precipitation of the DNA (26, 36). The DNA was dissolved in TE buffer (10 mM Tris pH 8.0 with HCl, 0.1 mM EDTA) so that 1  $\mu$ l of the suspension was equivalent to ca. 5  $\mu$ l of whole blood.

### PCR amplification and analysis

The sequence of the custom-made oligonucleotides we used and their approximate positions on the corresponding genes are given in Fig. 1. The sequences of the oligonucleotides were selected from the UNDP/World Bank/WHO-TDR Malaria Sequence database, which was compiled by Ross Coppel. All PCR reactions were carried out in a total volume of 50  $\mu$ l. Amplification was performed in 50 mM KCl, 10 mM Tris pH 8.3, 2 mM MgCl<sub>2</sub>, 0.1 mg/ml gelatin, 125  $\mu$ M of each dNTP and 1.0 unit of AmpliTaq Polymerase (Perkin Elmer, Birchwood Science Park, Warrington, England). All oligonucleotide primers were used at an individual final concentration of 250 nM, except for the primers specific to GLURP which were used at 200 nM final concentration. The PCR assays were performed using a heating block (PTC-100, MJ Research Inc., Watertown, MA, USA). The amplification programme was as follows. Step 1, 95 °C for 5 min; step 2, annealing at 55 °C for 2 min; step 3, extension at 72 °C for 2 min; step 4, denaturation at 94 °C for 1 min; steps 2-4 were repeated 39 times, then step 2 and step 3 for 5 min. The amplification cycles were terminated by reducing the sample temperature to 20 °C.

PCR products were detected by electrophoresis of 10  $\mu$ l from each reaction on 6% acrylamide gels (29:1 acrylamide:bis-acrylamide), or on 3% (3:1) Nusieve agarose/agarose. Gels were made and run in TBE buffer (100 mM Tris, 100 mM boric acid and 5 mM EDTA); 2  $\mu$ l of 5  $\times$  loading buffer (50 mM Tris pH 8.0, 75 mM EDTA pH 8.0, 0.5% SDS, 10% Ficoll, 30% sucrose and 0.2% bromophenol blue and 0.2% xylene cyanole) were added to each sample prior to electrophoresis. The DNA was stained with ethidium bromide and visualized on an ultraviolet transilluminator. Gel photographs were digitized (Summasketch II, UV Products Ltd.) and analysed using the Molmatch software (University of Glasgow, UV Products Ltd.).

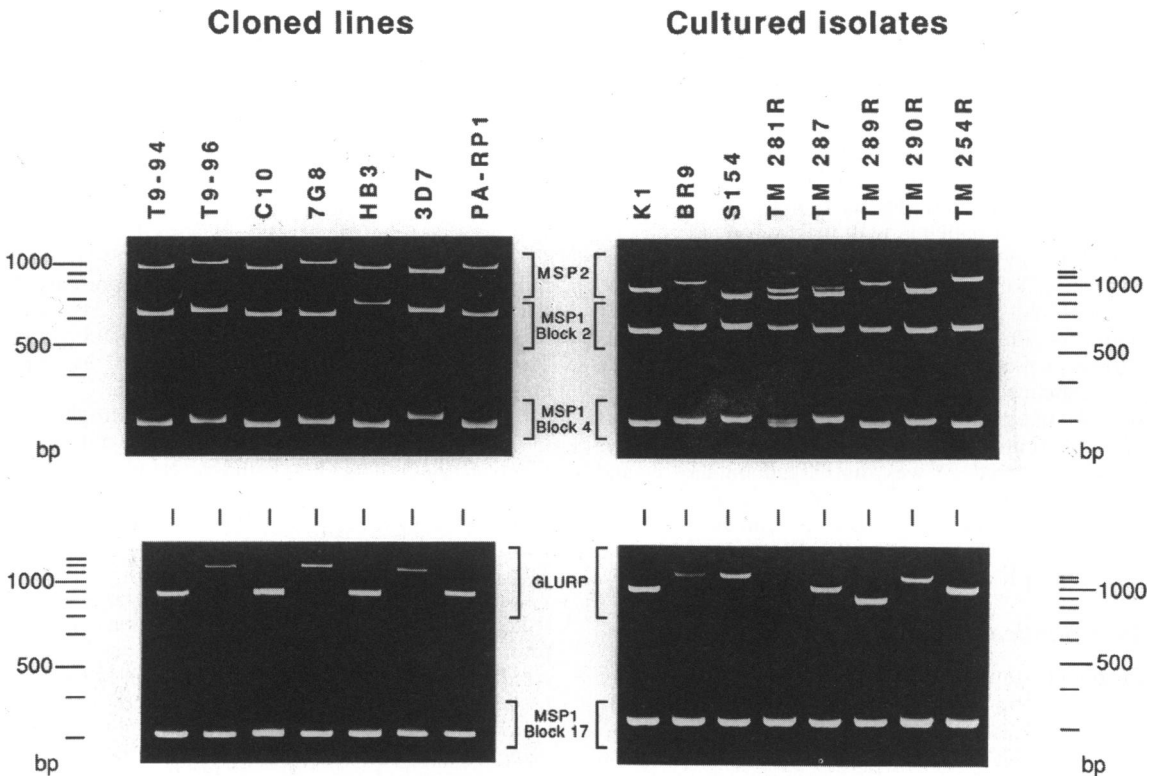
**Results**

DNA obtained from cloned lines of *P. falciparum* was used initially to establish the specificity of the oligonucleotide primers and to optimize the PCR conditions. As predicted, each reaction resulted in the amplification of a single fragment (Fig. 2, left panels). Size variation was observed in all the markers: MSP1 block 2, 500 bp–700 bp; MSP1 block 4, 270 bp–300 bp; MSP2, 800 bp–1100 bp; and GLURP, 800 bp–1200 bp. No size variation was observed for the block 17 of MSP1 (ca. 310 bp), which is considered to have a high degree of conservation. In order to facilitate analysis, the amplification products from blocks 2 and 4 of MSP1 and from MSP2 for each DNA sample were mixed before electrophoresis in the same track. The GLURP and MSP1 block 17 products were similarly mixed. The size of the polymorphic markers remained constant during *in-vitro* culture of cloned parasite lines (data not shown),

and throughout a chronic infection of a cloned *P. falciparum* line in a monkey (G. Mitchell, personal communication). In all cases the size of the PCR products obtained corresponded to that predicted from the published data. In addition, partial sequencing of the PCR products was performed to confirm that each oligonucleotide primer pair amplified the corresponding genetic marker. Using serial dilutions of DNA purified from *P. falciparum* cultures, the sensitivity of detection using the five oligonucleotide primer pairs, was calculated as being in the order of 100 parasite genomes (data not shown). No amplification was observed when the DNA template used was purified from *P. vivax*, *P. malariae*, *P. ovale* or human blood (data not shown).

The profiles of genetic markers of eight different uncloned *in-vitro* cultured Thai isolates are presented in Fig. 2 (right panels). Multiple bands were observed in two cases only. Two MSP2 alleles, indicating at least two different parasite populations,

Fig. 2. Genotyping of *in-vitro* cultured *P. falciparum* parasites. The nature of the PCR amplification products is indicated, as is the origin of the DNA template. Electrophoresis was performed on 6% acrylamide gels in TBE buffer. The DNA size marker is a 100 bp ladder.



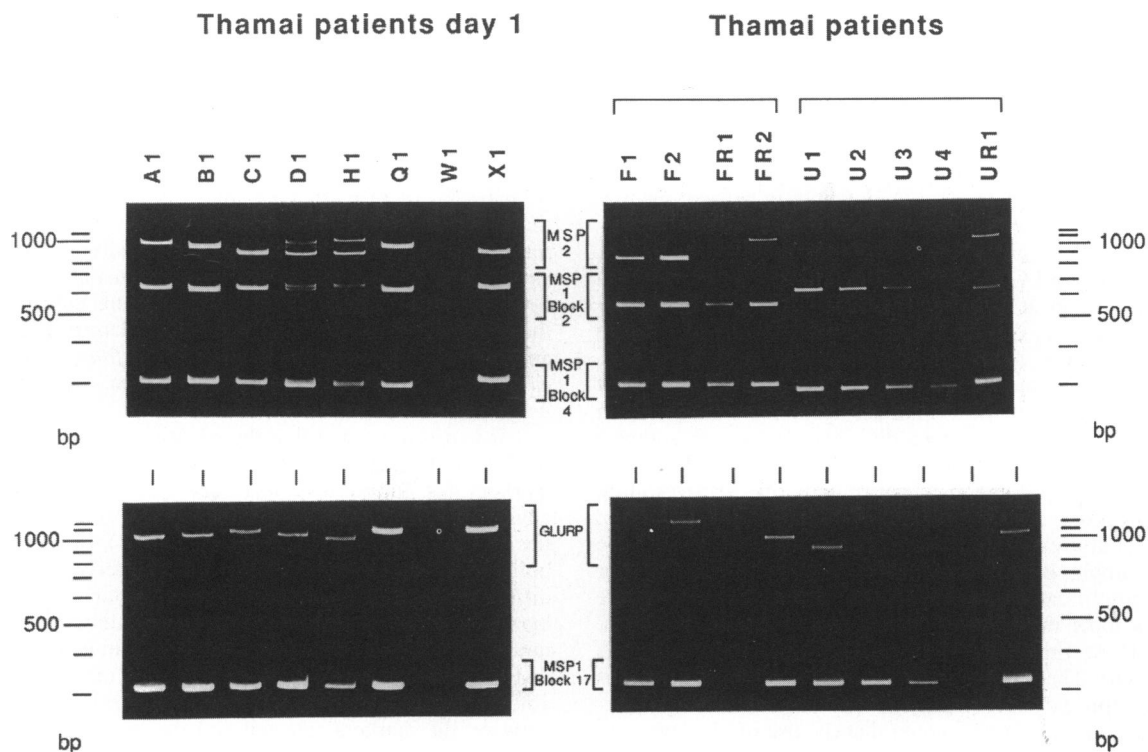
were detected in TM287. In TM281R, four MSP2 alleles and two MSP1 block 4 alleles were observed. Thus, the TM281R culture harboured a minimum of four to eight parasite lines of different genotype. Similarly, multiple alleles were detected in only two samples, D1 and H1, among those obtained from patients at Thamai Hospital on the first day of admission (Fig. 3). In two patients, F and U, samples were taken daily following admission (day 2, 3, etc.), as well as during a routine examination two weeks following discharge (day R1). In one case (patient F) a further sample was obtained when renewed clinical activity was reported (day R2). In both these patients, it was noteworthy that the patterns of PCR products obtained for MSP2 and GLURP from the later samples were different from those observed on the day of admission. With these patients it is not possible to differentiate between reinfection, recrudescence, or drug selection, to account for the changes observed. These results, none the less, underline the

usefulness of this technique when detailed studies are conducted.

### Discussion

Despite extensive observations, many of the factors which govern the epidemiology and pathology of *P. falciparum* and the acquisition of immunity to this parasite in man remain poorly defined. In endemic regions, the age distribution of clinical malaria and of immunity is broadly correlated with the degree of exposure of the population to the parasite, although the occurrence of severe malaria in the more susceptible individuals remains unpredictable. Considerable heterogeneity within the *P. falciparum* parasites present in a given area has been revealed by detailed analysis of infected blood. Frequently two or more parasite lines are found to coexist within one individual (11, 19, 21 and this study) and mixed species infections are frequent (12, 15). It has also been estab-

Fig. 3. Genotyping of *P. falciparum* parasites present in blood samples obtained from patients attending Thamai Hospital. PCR amplification products are labelled as in Fig. 2. Products on the left hand panels are from samples taken from different patients on the day of admission. Products on the right hand panel are from samples taken from two patients F and U on the day of admission (F1 and U1), the second day at the hospital (F2 and U2) and so on. Following discharge these two patients returned to provide further samples (FR1, UR1 and FR2). Electrophoresis was performed on 6% acrylamide gels in TBE buffer. The DNA size marker is a 100 bp ladder.



lished that geographical variation occurs in parasite populations (18). Thus, the diversity of the parasite population, as well as that of the human and mosquito hosts, must be addressed if the mechanisms underlying the pathology of malaria, the acquisition of immunity, the spread of drug resistance, and the conditions of transmission are to be understood.

Parasitological parameters of fundamental importance include (i) the geographic and longitudinal distribution of the different parasite types, (ii) their relationship to disease, pathology and immunity, (iii) their distribution in the various *Anopheles* mosquito hosts, and (iv) the detailed parasitological history of selected individuals of various age groups, socioeconomic activities, and genetic backgrounds. A practical and reproducible method by which parasites can be characterized is central to the performance of these studies. In particular, the typing of parasites from individuals with nonclinical low-level infection, as well as those with clinical malaria, is crucial to the interpretation of epidemiological investigations. PCR methods for parasite characterization are of high sensitivity and specificity and therefore fulfil these criteria.

Efficient genetic typing of parasites requires defined single copy DNA loci which exist as stable polymorphic allelic variants. Analysis of genetic variants of *Plasmodium* species is facilitated by the fact that the parasite is haploid except for the zygote stage. Thus, the detection of two variants of the marker in a given sample will allow subdivision of the parasites present into two distinct populations, the number of subdivisions possible with a particular marker being equal to the total number of allelic variants. The combined analysis of allelic variants from different genetic loci will result in an exponential increase in the resolution of the genotyping method.

In the present study we have targeted the MSP1, MSP2 and GLURP genes because they are well characterized at the molecular level, and because their use clearly illustrates the potentials and pitfalls of the technique. A total of 32 parasite samples were analysed—seven cloned lines, eight *in-vitro* cultured isolates, and the remainder from ten patients from Thamai Hospital including the follow-up samples from patients F and U. It should be emphasized that PCR amplification is not quantitative. Thus, failure to observe a band following the PCR assay does not totally exclude the presence of a variant in the sample under study. Furthermore, competition for amplification between two or more variants in one sample might also result in their unequal detection. These limitations apply to all PCR analyses of this type. The size polymorphisms for each of the regions amplified were computer analysed following digitization. It must be noted that the use of Nusieve aga-

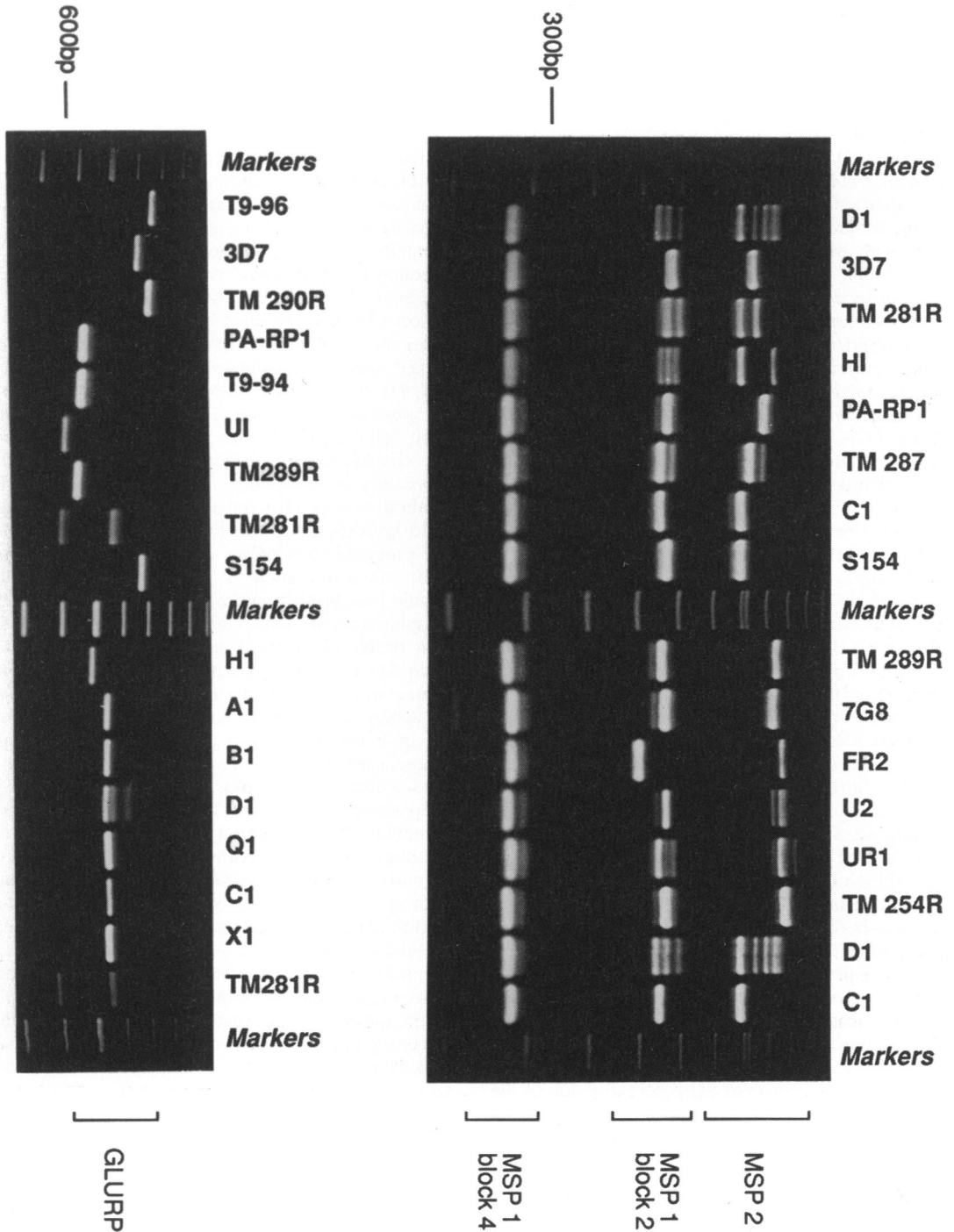
rose (Fig. 4), as compared with polyacrylamide, results both in subtle differences in calculated relative molecular weights and in significantly different patterns for some of the samples (e.g., D1, H1 and TM281R). By use of acrylamide gels, the number of distinguishable size polymorphisms we observed, using the above samples only, was as follows: seven for GLURP, five for block 2 and three for block 4 of MSP1, and nine for MSP2.

Therefore, when genetically homogeneous parasite lines are considered, the number of distinguishable groups into which they can be divided, using only the variants observed so far, is 945 ( $7 \times 5 \times 3 \times 9$ ). For samples in which more than one parasite population exists, the number of possible patterns, and therefore of defined *P. falciparum* infection profiles, that can be detected using the four markers increases substantially. Thus, by calculating the theoretical number of possible combinations of the markers in samples containing one to nine parasite lines, a total of about 14 million different patterns can be obtained using these markers. The resolution of the genotyping assay can be increased further by determining the allelic family to which the block 2 of MSP1 polymorphs belong, namely the MAD20, K1 or RO33 sequence variants. A similar analysis can be employed for the FC27 and Indochina sequence variants of the MSP2 repeat region.

It is interesting to note that the patterns obtained from admission blood samples from different patients, and from the uncloned *in-vitro* cultured parasites, were unique for each of these 18 samples. Conversely, three of the seven cloned lines yielded the same pattern, namely T9-94, C10 and PA-RP1. Whether this increased uniformity is a consequence of selection of a restricted number of parasite lines that thrive under the conditions of *in-vitro* culture, or resulted from undetected laboratory contamination, as suggested by Robson et al. (37), remains a matter for speculation. The results presented here confirm the usefulness of PCR analysis for the identification and monitoring of laboratory strains of *P. falciparum*, as was originally suggested by Wooden and colleagues (38, 39).

It is not suggested that the genetic markers used in this study are the most suitable or only genetic markers for future use. It might indeed be more advantageous to target other polymorphic gene families such as that of the S-antigens (40, 41), or metabolic enzymes provided that the basis underlying the differences in the electrophoretic behaviour of their isozymes proves to be genetical in nature. Other human malaria species could be analysed in a similar fashion once suitable genetic markers become available. It must be emphasized that a causal linkage between the markers selected and the epidemiologi-

Fig. 4. Size polymorphism profiles of some selected samples as resolved by electrophoresis on 3% Nusleve agarose/agarose (3:1) gels in TBE buffer. The DNA size marker is a 100 bp ladder.



cal factor under study is not necessary. The genotyping analysis is intended to act as an indicator of changes in the parasite population, which might serve to establish a correlation with the epidemiological factors under investigation. Should one of the markers either fortuitously or from knowledge and design be directly linked to a specific clinical or biological manifestation of the parasite, it will of course provide an even more powerful tool. Although it was suggested (42) that parasite populations in the field are clonal, field observations combined with mosquito transmission studies (43, 44) indicate that by contrast, the frequency of recombination between different parasite lines is substantial. Even with frequent recombination however, changes in the parasite gene pool of an area will be detected. The value of results obtained by the analysis of samples collected in micro-epidemiological surveys, for instance from the same individual over time, will also remain undiminished. Genotyping of parasites present in mosquitoes collected from endemic areas could help in defining the factors involved in modulating the transmission dynamics of the parasite, and in detecting any differential infectivity for vector subspecies and demes by the various parasite lines. Indeed, the degree of recombination prevalent under differing natural conditions of transmission would be amenable to study.

Studies in which the diversity of *P. falciparum* parasites present in field samples was determined by PCR analysis of polymorphic markers have been carried out by a number of researchers (10, 11, 30, 31, 33–35, 41, 45), and the value of such investigations to advance our knowledge of the parasite's biology have been clearly demonstrated. In this study we show that the analysis of a small number of genetic markers is sufficient to characterize the *P. falciparum* population present in a particular sample. However, the oligonucleotide primers, as well as the protocols for the amplification and detection of the polymorphic markers, differ in different laboratories. Thus, comparison of the data obtained in the different studies is difficult. PCR amplification is logarithmic in nature and thus, minor changes in conditions could result in major alterations to the product. We therefore, propose that a PCR amplification-based method, similar to the one described in this article, be standardized as a universally accepted method for typing *P. falciparum* parasites obtained from field samples. Full realization of the potential value of the genotyping methodology to malaria research can only be achieved if international standards and protocols are adopted.

A detailed discussion of all the relevant factors which affect the outcome of the PCR assay is outside the scope of this article. None the less, standardiza-

tion should include the following interrelated categories, which will be briefly discussed below: (i) The sequence of the oligonucleotide primers, (ii) DNA template preparation, (iii) the PCR buffer composition and the amplification cycling parameters, and (iv) the electrophoretic analysis of the product and data storage.

(i) The specificity and sensitivity of the amplification reaction ultimately depends on the sequence of the oligonucleotide primers. Despite the availability of computer programs which are helpful in the selection of appropriate sequences, the suitability of a particular primer can only be established experimentally. It is necessary to demonstrate that the PCR product is only obtained when *P. falciparum* DNA is present. In particular, no amplification must be observed in the presence of genomic DNA from the other three species of human malaria parasites, since mixed species infections are frequently encountered (12, 15, 26). The size of the PCR product depends on the position of the oligonucleotide primers flanking each polymorphic domain.

(ii) Methods for DNA purification vary from laboratory to laboratory. The choice of a particular method is dependent on the practical constraints of field collection, on the resources available, and on the proposed uses of the samples. The exclusion of PCR inhibitors, such as heparin and haemoglobin, should be a priority in the choice and design of DNA template preparation. A highly practical field collection of blood as dried samples on filter-paper has been devised (39, 45), with the advantage that long-term storage and sample handling post-collection can be achieved at ambient temperatures. Another method in which samples can be collected at ambient temperature but must be processed or stored in the cold within 24 hours has also been described (26). Preparation of the DNA template is traditionally achieved by extraction with organic solvents, although simpler and more rapid methods have been recently devised for use with PCR amplification, namely by boiling enriched parasites in water (44), buffer (26), or in the presence of Chelex, a heavy metal chelator (39, 45). As a result of the sensitivity exhibited by the assay, the problem of contamination is of major concern in PCR studies. Since handling of the material is minimized in the newer methods, it is considered that cross-contamination between samples is less probable. However, in the majority of cases contamination is a consequence of the failure to isolate the PCR reagents from the amplified product. The method used to obtain the DNA template from the samples described in this article, namely phenol/ethanol purification, although considered cumbersome (requiring four centrifugation steps), results in DNA of high purity and stability. No evi-



dence of contamination has been observed in the numerous negative controls included. Furthermore, no cross-contamination could be observed between the samples; as demonstrated by the different patterns of genetic markers for samples processed sequentially, these patterns remained unaltered when the sample order was changed. The template resulting from boiling pelleted parasites in water or buffer is obtained more quickly; however, aliquoting and storage must be performed under sterile conditions since contamination of the resulting solution by microorganisms will result in the degradation of the DNA. The highly practical filter-paper collection and the Chelex extraction method have one disadvantage in that the volume of template that can be used in each PCR assay corresponds to a small volume of whole blood, thus reducing the overall sensitivity of the procedure. Ultimately the rate limiting factor in the genotyping of field samples is not the template preparation step, but the time required for setting up and running the PCR assays, as well as for analysis of the product.

(iii) The composition of the PCR buffer could be of great importance to the efficiency and specificity of the reaction. The different thermostable polymerases available commercially are supplied with equally different reaction buffers. The most influential factors however, are the  $Mg^{2+}$  and oligonucleotide primer concentrations. The optimal annealing temperature during the amplification cycle depends on the length and sequence composition of the primers, and must be derived by trial and error. Each oligonucleotide pair must be optimized with respect to the variables mentioned above. In addition, the specificity and sensitivity of the reaction must be established using a standard dilution of genomic DNA. The optimization of the oligonucleotide primers presented in this article was performed as discussed above, and also included determining the optimum number of cycles during amplification, as well as establishing that only one band is observed irrespective of the original quantity of template DNA. This control is of particular importance since the presence of multiple bands is interpreted as due to the presence of multiple alleles (and therefore, "strains") in the sample. Finally, both sensitivity and specificity can be improved by the use of "nested PCR" amplification (26, 46).

(iv) DNA migration is affected by the DNA sequence and the conditions under which electrophoresis is performed. The electrophoretic matrix must be chosen to provide a level of resolution adequate for differentiation between different variants. Even matrices of equal resolving power, such as acrylamide and Nusieve agarose, can result in different patterns for the same sample. The use of different

electrophoretic buffers, and electrophoresis running conditions (voltage, temperature, presence of ethidium bromide) can also result in alterations of the mobility of the DNA and the resolution of the system. The *P. falciparum* strains used in this study to validate the oligonucleotide primers have been chosen simply because of their availability in the laboratory. The size of the PCR products was estimated by comparison with commercially available molecular size standards. Determination of the polymorphic types can be facilitated, and comparisons between studies rendered more reliable, if the molecular size markers employed are derived from the PCR product of a set of centrally prepared and characterized parasite or DNA template samples. These should include all the variants of the genetic marker under analysis. The homogeneity of the parasites used to make the standard DNA templates is an important criterion for the selection of a particular line as a standard for a particular marker. Once the methodology is standardized, the results are amenable to computerized storage and analysis. Hardware and software for digitizing electrophoretic patterns are available commercially. Comparison of data obtained at different times and from different geographical locations is thereby facilitated. Comparison of data obtained in one study with that derived by the same or different investigators from the same area at a later date or from other regions is highly desirable.

Since sufficient PCR template DNA is relatively easily purified from small aliquots of parasite samples, and is stable during long-term storage, retrospective studies of previously collected samples can be envisaged. Newly discovered markers can be quickly tested with stored material, thus maximizing the usefulness of costly field surveys. The incorporation of the genotype patterns in epidemiological data bases will allow powerful statistical analyses to be performed. It is thus highly desirable that internationally agreed standards and protocols should be designed before unstandardized, and not comparable, studies become widespread.

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## Résumé

### **Le génotypage d'isolats de *Plasmodium falciparum* par amplification génique (PCR) et ses utilisations potentielles dans les études épidémiologiques**

Les quatre espèces de *Plasmodium* qui infectent l'homme diffèrent par leur virulence, leurs caractéristiques de transmission, leur pathologie et leur

pharmacosensibilité. A l'intérieur de chaque espèce, on sait que des isolats différents présentent aussi d'importantes variations de ces propriétés. Les génotypes tant du mammifère hôte que du vecteur présentent une sensibilité et une réponse variables à un parasite donné. De plus, on sait que chez l'homme comme chez l'animal d'expérience, les infections mixtes à *Plasmodium* peuvent soit supprimer soit renforcer le processus infectieux et entraîner une pathologie différente de celle qui aurait été provoquée par les mêmes parasites lors d'une infection monospécifique. Il serait possible d'améliorer nos connaissances de l'épidémiologie du paludisme à *P. falciparum* et des effets des mesures de lutte en analysant les modifications du pool génique des espèces parasitant l'homme et les moustiques. De plus, en associant les mesures épidémiologiques classiques et des techniques moléculaires récentes, il est désormais possible d'étudier simultanément les populations de parasites, de vecteurs et d'hôtes humains.

Un très grand nombre de gènes polymorphiques utilisables comme marqueurs génétiques ont été identifiés chez *P. falciparum*, et trois d'entre eux ont été utilisés dans cette étude pour caractériser les parasites. Quatre domaines polymorphiques, les blocs 2 et 4 du gène MSP1, le gène MSP2 et la région répétitive du gène GLURP, ainsi qu'une région largement conservée du gène MSP1 (bloc 17) ont été sélectionnés pour amplification par PCR (polymerase chain reaction).

L'ADN de 15 lignées de *P. falciparum* cultivées *in vitro* (dont sept clones) et de prélèvements de sang recueillis chez des malades infectés à l'Hôpital de Thamai en Thaïlande, a été utilisé comme étalon pour la PCR. Les produits d'amplification ont été analysés par électrophorèse en gel pour mettre en évidence les polymorphismes de longueur. On a ainsi détecté sept variants alléliques de GLURP, cinq du bloc 2 de MSP1, trois du bloc 4 de MSP1 et neuf de MSP2.

Le degré élevé de polymorphisme observé dans cette étude montre qu'il est possible de définir une population parasitaire en termes génétiques, non seulement chez *P. falciparum*, mais aussi chez les autres espèces parasites de l'homme, à condition d'identifier des gènes polymorphiques appropriés. Les auteurs examinent l'applicabilité du génotypage des parasites, au niveau de l'espèce et de la souche, aux études épidémiologiques. Ils encouragent vivement l'adoption de normes internationales pour cette technique de façon à assurer la comparabilité des résultats en provenance de différentes régions et obtenus à différentes époques.

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