Determination of Failure of Treatment of *Plasmodium falciparum* Infection by Using Polymerase Chain Reaction Single-Strand Conformational Polymorphism Fingerprinting

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The inability to distinguish failures of treatment of *Plasmodium falciparum* infection from new infections is an important impediment to the evaluation of antimalarial drugs. On the basis of a pilot study utilizing polymerase chain reaction (PCR) single-strand conformational polymorphism (SSCP) analysis to genotype *P. falciparum* isolates, we sought to confirm that PCR SSCP analysis could reliably distinguish infections for which treatment failed from unrelated infections with a sample size adequate to estimate the accuracy of this technique. PCR SSCP analysis of the MSP-1, MSP-2, and GLURP genes was performed on 72 paired isolates recovered from 36 individuals for whom treatment failed in Thailand. In every case (100% [95% confidence interval (CI), 90%–100%]), the PCR SSCP pattern of the recrudescent isolates matched that of the primary isolate. We determined whether PCR SSCP analysis could separate unrelated infections by comparing each recrudescent isolate with each of the unrelated primary isolates. Of 1,260 comparisons, 1,258 (99.8% [95% CI, 99.4%–100%]) were unique. The results indicate that PCR SSCP analysis can be used to differentiate infections for which treatment failed from reinfections.

A practical method to distinguish failures of antimalarial treatment (recrudescences) from reinfections would represent an important advance. The inability to separate new infections from those for which treatment fails limits the ability to interpret results of antimalarial treatment trials conducted in areas of malaria transmission. The traditional approach has been to prevent reinfection by hospitalizing patients in a malaria-free area for at least 21 days. In many regions and situations, this approach is neither practical nor possible.

Genetic fingerprinting or genotyping of *Plasmodium falciparum* strains may be a solution to the problem of separating true recrudescences from new infections and would facilitate the testing of antimalarial drugs in areas of endemicity. The genes encoding the merozoite surface proteins, MSP-1 [1–4] and MSP-2 [2–5], display extreme size and allelic polymorphism. PCR-based assays to demonstrate size and sequence polymorphism of these genes have been used to genotype *P. falciparum* isolates [4, 6–8]. However, the degree of genetic heterogeneity that exists within these genes is not discernible with allele-specific hybridization or size polymorphism studies alone [1–5, 9].

Single-strand conformational polymorphism (SSCP) analysis is a simple and sensitive technique based on the differential mobility of single-stranded DNA in nondenaturing gels [10]. This technique has the potential to discriminate single base pair differences between DNA fragments and has been used to genotype a variety of microorganisms [11–13]. We recently employed this technique on a single gene (MSP-2) to differentiate 10 paired primary and recrudescent isolates from unrelated *P. falciparum* infections [2]. The objectives of this study were to confirm that PCR SSCP analysis of one or more polymorphic genes could reliably distinguish infections for which treatment failed from unrelated infections with a sample size adequate to precisely estimate the sensitivity and specificity of this technique and to define the field applicability of an SSCP genotyping strategy.

Materials and Methods

Study Population and Specimen Collection

The study population was *P. falciparum*–infected individuals for whom artemisinin derivative treatment regimes failed at the Hospital for Tropical Diseases, Mahidol University, Bangkok, Thailand, between April 1995 and January 1996 [14, 15]. All patients enrolled in these studies were treated in an area.
of Thailand without malaria transmission, thereby excluding reinfection.

Whole blood samples were collected from all study subjects on hospital days 0, 2, 3, and 7 and at the time of treatment failure. Whole blood was collected in EDTA and acid citrate dextrose and frozen at −20°C. Each tube was labeled with the study subjects’ name, hospital number, and date.

**DNA Purification, PCR Amplification, and Analysis**

Genomic DNA was extracted from paired primary and recrudescent whole blood samples (200 µL) with use of Qiagen columns (Qiagen, Chatsworth, CA) as per the manufacturer’s directions. Oligonucleotide primers were synthesized as previously described [2, 8]. Amplification was performed in 1 × PCR buffer (Promega, Madison, WI) with 1.5 mM MgCl₂, 200 mM of each dNTP, 25 pmol of each primer, and 1.25 U of Taq polymerase (Promega) in a total volume of 50 µL. The amplification program consisted of a cycle of preheating to 95°C for 5 minutes followed by 35 cycles at 94°C for 1 minute; annealing at 50°C (MSP-1), 45°C (MSP-2), or 55°C (GLURP) for 1 minute; and extension at 72°C for 2 minutes. PCR products underwent electrophoresis on a 2% agarose gel and were gel-purified with use of Qiagen columns. PCR products of MSP-2 were also digested to completion with Rsal (GIBCO BRL, Gaithersburg, MD). Products were extracted with chloroform, precipitated with ethanol, resuspended in water, and analyzed by electrophoresis on a 4% NuSieve agarose gel (FMC Bioproducts, Rockland, ME) and by SSCP genotyping.

**PCR SSCP Analysis**

PCR products were denatured before analysis by adding 1 µL of 0.5 M NaOH/10 mM EDTA to 100–200 ng of amplified DNA in 9 µL of water followed by heating at 42°C for 5 minutes. Following the addition of load buffer (0.1% xylene cyanol, 0.1% bromophenol blue in formamide), samples were immediately placed on ice and then loaded onto a nondenaturing 10% polyacrylamide minigel (1 mm; Novex, San Diego); the gels were run under constant voltage with 5% glycerol in 0.8× glycerol tolerant buffer (United States Biochemicals, Cleveland) for 2,100 volt hours for MSP-2, 720 volt hours for restriction fragment length polymorphism (RFLP) of MSP-2, 1,056 volt hours for MSP-1, or 2,600 volt hours for GLURP. Lanes containing a 1-kilobase ladder, a 123-bp ladder (GIBCO BRL), and reference strains that had been similarly treated were used to standardize SSCP patterns. The gels were stained with silver (Silverstain Plus Kit, Bio-Rad, Richmond, CA) and dried. Comparisons of primary and recrudescent isolates were performed by running samples side-by-side in the same gel electrophoresis run.

SSCP patterns of DNA fragments were scanned, digitized, and analyzed by using the BioImage System with Whole Band Analysis, version 3 (Millipore, Ann Arbor, MI), and by visual examination. The SSCP pattern of each recrudescent isolate was compared with that of its matched primary isolate and with those of all other primary isolates. Since primary infections are often due to two or more clones of *P. falciparum* [2–4, 7, 9, 16–18], the primary SSCP pattern may have one or more bands than does the recrudescent SSCP pattern. Therefore, our criterion for identity that was that the mobility of each band within the recrudescent SSCP pattern had to match a band within the primary SSCP pattern [2].

Because mobility differs slightly between gels, the BioImage System with Whole Band Analysis was used to standardize across gels. The stringency for identity was set at 2.5%. This stringency setting was determined after assessing interassay variability by repeating amplification and SSCP analysis of the same 10 samples three times. At this setting, the SSCP patterns of the reference, primary, and recrudescent isolates were found to be reproducible (authors’ unpublished data). The BioImage System with Whole Band Analysis aligns the isolates in order of similarity automatically.

**Data Analysis**

Sensitivity was defined as the number of fingerprint matches divided by the number of treatment failures. Specificity was defined as the number of fingerprint nonmatches divided by the number of unrelated infections. As a surrogate estimate of specificity, each recrudescent isolate was compared with the other 35 unrelated primary isolates (1,260 combinations) [4]. The probability of treatment failure given a nonmatching fingerprint is analogous to a positive predictive value and was derived from Bayes’ theorem of conditional probabilities as follows: (sensitivity × prevalence of recrudescent infections)/[(sensitivity × prevalence of recrudescent infections) + [(1 – specificity) × prevalence of reinfections]] [19, 20]. The probability of reinfection given a nonmatching fingerprint is analogous to a negative predictive value and was calculated as follows: (specificity × prevalence of reinfections)/[(specificity × prevalence of reinfections) + [(1 – sensitivity) × prevalence of recrudescent infections]]. In the setting of recurrent parasitemia, the prevalence of reinfections was calculated as follows: 1 − prevalence of treatment failures.

The exact conditional distribution (based on binominal distribution) was used to calculate 95% confidence intervals (StatXact-3, version 3.0.2; Cytel Software Corporation, Cambridge, MA). The multiplication rule of probability theory [21] was utilized to determine the probability that any two unrelated primary isolates would have the same polymorphism at one or more loci. It was assumed that all loci were unlinked (statistically independent) and that the genotypes of our study isolates were representative of the parasite population at large.

**Results**

Sixty patients for whom therapy failed during follow-up in Bangkok (an area without malaria transmission) were identi-
Each recrudescent isolate was compared with the other 35 unre-
gene, and 24 SSCP patterns of the GLURP gene were observed.

MSP-1 block 2 locus, 32 unique SSCP patterns of the MSP-2 Even assuming a worst case scenario for speci®city (i.e.,
thus indicating that these bands likely corresponded to a par-
predictive value and the negative predictive value are affected
the pattern of the primary isolate was initially missing some population (speci®city). The probability of treatment failure
and 2).SSCP analysis is affected by the reinfection rate (prevalence),
chosen for analysis. One isolate could not be ampli®ed. The gous to sensitivity (100% in this study), while the number of
failure between day 7 and day 28) of drug resistance 
the genetic markers used represent highly polymorphic loci. is also affected by the speci®city, and the negative predictive
resulting in index infections with sensitivity (100% in this study), while the number of unrelated infections are analogous to specificity (according to the MSP-1 gene, estimated to be 99.7% in this study). The utility of PCR
SSCP analysis of the GLURP gene or an RFLP of the MSP-2 gene, it was possible to discriminate between all isolate

bands present in the pattern of its corresponding recrudescent isolate. The absent bands were visualized after loading in-
morophism (SSCP) analysis. Genes were ampli®ed from paired isolates

differed; isolates from each patient exhibited an RI type (treatment failure between day 7 and day 28) of drug resistance [22]. The first 37 paired primary and recrudescent isolates identi®ed were chosen for analysis. One isolate could not be ampli®ed. The remaining 36 paired isolates were analyzed by PCR SSCP analysis of a portion of the MSP-1 gene, the MSP-2 gene, an RFLP of the MSP-2 gene, and the GLURP gene (®gures 1 and 2).

In every case (36 [100%] of 36 [95% CI, 90%–100%]), the PCR SSCP pattern of the recrudescent isolates matched or was contained within the pattern of the primary isolate. In one case, the pattern of the primary isolate was initially missing some bands present in the pattern of its corresponding recrudescent isolate. The absent bands were visualized after loading increased amounts of the PCR product from the primary isolate, thus indicating that these bands likely corresponded to a parasite clone present in low numbers in the initial infection.

The genetic markers used represent highly polymorphic loci. For the 36 primary isolates, 34 unique SSCP patterns of the MSP-1 block 2 locus, 32 unique SSCP patterns of the MSP-2 gene, and 24 SSCP patterns of the GLURP gene were observed. Each recrudescent isolate was compared with the other 35 unrelated primary isolates (1,260 combinations) to determine if SSCP analysis could discriminate unrelated infections. In this study, unrelated primary infections were used as surrogates for true reinfections. By using all three genes, two isolate pairs could not be separated (one pair of primary and recrudescent isolates could not be differentiated from another isolate pair). All other isolate pairs (1,258 [99.8%] of 1,260 comparisons [95% CI, 99.4%–100%]) were found to be unique. SSCP analysis of individual genes was able to differentiate recrudescent isolates from unrelated primary isolates as follows: MSP-1 gene, 99.7% (1,256 of 1,260) (®gures 1 and 2); MSP-2 gene, 99.1% (1,248 of 1,260) (®gures 1 and 2); RFLP of MSP-2 gene, 99.5% (1,254 of 1,260) (®gures 1 and 2); and GLURP gene, 97.9% (1,234 of 1,260) (data not shown).

Assuming that the loci are unlinked, the probability of any two unrelated primary isolates having the same polymorphism at both MSP-1 and MSP-2 genes is three in 100,000, and at MSP-1, MSP-2, and GLURP genes, it is five in 10,000,000. Results of SSCP analysis of MSP-1 genes of all 72 isolates are shown in figure 2A. SSCP analysis of additional markers of the isolate pairs that could not be differentiated by the MSP-1 gene or were similar according to the MSP-1 gene are shown in ®gures 2B and 2C. Following analysis of MSP-1 and MSP-2 genes, it was possible to discriminate between all isolate pairs except 4 and 5, which also could not be differentiated by PCR SSCP analysis of the GLURP gene or an RFLP of the MSP-2 gene. These isolate pairs were recovered from two individuals who acquired malaria in the same area and were admitted to the Hospital for Tropical Diseases, Mahidol University, at the same time. Whether these isolates represent unrelated strains with similar alleles (deomes) or related clones is unknown.

The utility of this methodology can be assessed by treating it as if it were a diagnostic test. The number of fingerprint matches divided by the number of treatment failures is analogous to sensitivity (100% in this study), while the number of fingerprint nonmatches divided by the number of unrelated infections is analogous to specificity (according to the MSP-1 gene, estimated to be 99.7% in this study). The utility of PCR SSCP analysis is affected by the reinfection rate (prevalence), the ability of the test to identify treatment failures with a matching fingerprint (sensitivity), and the variability in the genes extracted from P. falciparum isolates recovered from the study population (speci®city). The probability of treatment failure given a matching fingerprint and reinfection given a nonmatching fingerprint is equivalent to the positive predictive value and the negative predictive value, respectively. Both the positive predictive value and the negative predictive value are affected by the reinfection rate; however, the positive predictive value is also affected by the speci®city, and the negative predictive value is affected by the sensitivity.

Even assuming a worst case scenario for speci®city (i.e., reinfection rates of up to 50% and variability within P. falcipa-
rum genes as low as 90%), a matching fingerprint has a chance of >90% of indicating a treatment failure. This probability improves with lower reinfection rates and higher speci®city and is nearly 100% with our observed speci®city at all rates. At reinfection rates of >20%, sensitivity must exceed 97.5%
to exceed 90% probability that a nonmatching fingerprint is a reinfection, while 99.9% specificity will yield >95% probability at reinfection rates as low as 2% (data available from authors).

Discussion

The availability of a practical method to distinguish *P. falciparum* reinfections from recrudescent infections would facilitate antimalarial drug studies in areas with active malaria transmission. In this study, we confirmed that PCR SSCP analysis of polymorphic loci, such as the MSP-1 or MSP-2 gene, is such a method. PCR SSCP analysis of paired primary and recrudescent isolates established that each recrudescent isolate was identical to, or a subpopulation of, its primary isolate. Recrudescent isolates were distinct from all but two of the unrelated primary isolates.

The inability to differentiate new *P. falciparum* infections from recrudescence limits the ability to determine true drug efficacy when treatment studies are performed in areas where malaria is endemic. This concept is illustrated in a recent trial from Africa in which pyronaridine treatment was reported to be 100% efficacious [23]. Because of the inability to differentiate infections for which treatment fails from reinfections, patients were only followed up for 14 days. In contrast, the rates of efficacy for pyronaridine regimens assessed in a malaria-free area of Thailand were only 63% and 88%, and 21 of the 22 recrudescences occurred after day 14 [24, 25]. Therefore, the
actual 28-day RI treatment failure rate in Africa is unknown, and the reported efficacy rate of 100% may be misleading. In studies designed to determine the efficacy of new antimalarial compounds, adequate follow-up time to detect RI treatment failures is necessary. In assessing antimalarial drugs already in use, accurately identifying RI treatment failures is important since these are often the first indicators of emerging drug resistance.

The traditional strategy to separate infections for which treatment fails from reinfections is to hospitalize patients for 21 days and then follow up subjects as outpatients for an additional week. Clearly, this approach has many limitations. It is expensive, subjects often drop out when they feel well, malaria transmission occurs in hospitals in some areas, and 28 days may not be adequate follow-up for treatment with drugs with a long half-life. Two alternative methods have recently been utilized.

Baird and colleagues [27] added a halofantrine treatment arm to estimate the reinfection rate. This strategy is also a potential solution, but it is limited by the number of subjects necessary to precisely estimate the reinfection rate and it assumes that halofantrine is 100% efficacious and that halofantrine does not continue to suppress new infections after treatment. SSCP fingerprinting could be used in conjunction with either of the epidemiological methods described above to determine the true treatment failure rate.

We previously reported the first use of SSCP analysis of a single gene to differentiate 10 infections for which treatment failed from reinfections [2]. The present study was designed to confirm and extend our previous results by analyzing a number of polymorphic markers amplified from more isolates. A large sample of paired primary and recrudescent isolates collected in a malaria-free area was needed to determine the sensitivity (with small confidence intervals) of this technique. We defined sensitivity as the number of recrudescent isolates that matched or were contained within the fingerprints of the primary isolates (36 [100%] of 36 [95% CI, 90%–100%]). Since true reinfections are difficult to identify, a surrogate for specificity was defined as the number of recrudescent isolates that were unique from all unrelated primary isolates (1,258 [99.8%] of 1,260 [95% CI, 99.4%–100%]).

The present study indicates that even in areas with high reinfection rates, when a pair of isolates with matching SSCP fingerprints is recovered from an individual, the probability that this isolate pair represents an infection for which treatment failed will be >90%. The probability that a nonmatching fingerprint represents a reinfection depends on the specificity and the reinfection rate. Because the specificity of PCR SSCP analysis of either the MSP-1 or MSP-2 gene was >99% we believe that analysis of only one of these genes will be sufficient in most cases. Because participants in this study were from a large area of western Thailand and eastern Myanmar, we were concerned that the variability observed might not be representative of that seen for more isolated populations for whom this technique will be used. However, several studies have found marked genetic variability of  P. falciparum isolates, even in isolated regions (reviewed in [28]). Furthermore, in an ongoing analysis of infections in isolated populations, we have observed that almost all isolates are unique by the genetic markers described (authors’ unpublished data). The specificity of PCR SSCP analysis for populations under study can be estimated by comparing isolates recovered from different individuals.

In this study, we observed a high sensitivity; therefore, a nonmatching fingerprint had a high probability of representing a reinfection, even at very low reinfection rates. However, if sensitivity is lower than our estimates, the probability that a nonmatching fingerprint represents a reinfection falls. Our study subjects were largely nonimmune. A study of semiimmune individuals should be performed in a malaria-free area to determine if the high sensitivity that we observed is also present for such populations. This study also used a surrogate for reinfections, and the results ideally should be confirmed for naturally reinfected patients.

Genotyping methods, such as those we describe, also have potential limitations. An individual infected with a given  P. falciparum genotype may be reinfected with the same genotype. If it does occur, genotyping methods may overestimate the treatment failure rate. Additional possible limitations are as follows. PCR-based methods may be limited by the inability to detect mixed infections in which one parasite population greatly exceeds other populations in the analyzed sample because of differences in parasite inhibition, synchronization, or sequestration. Different clones of a primary  P. falciparum isolate may be released from the liver at intervals or may differentially sequester and therefore not be detected in the sample collected on day 0. Because of this concern, we collected blood samples on days 2, 3, and 7.

Despite these theoretical considerations, it is of note that all recrudescent isolates were always apparent in the corresponding primary infection. Last, our analysis was not blinded. To optimize accuracy, we thought that paired isolates should be run side-by-side. SSCP gels were digitized, and matching of band patterns was performed by a software system, thereby decreasing the potential for bias.

In summary, by using PCR SSCP analysis of paired primary and recrudescent isolates, we have demonstrated that recrudescent isolates are identical to, or a subpopulation of, the primary isolates and are distinct from unrelated isolates. Because of its discriminatory power, SSCP analysis of a single polymorphic
gene, such as MSP-1 or MSP-2, is sufficient to distinguish infections for which treatment truly failed from reinfections. This technique is also practical and applicable to drug studies performed in malaria transmission areas. New fingerprinting technologies, such as portable DNA analysis systems and PCR microchips [29], may make this strategy even more feasible in the future.

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