MAJOR ARTICLE

Relapses of *Plasmodium vivax* Infection Usually Result from Activation of Heterologous Hypnozoites

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(See the article by Chen et al., on pages 934–41, and the editorial commentary by Collins, on pages 919–20.)

Background. Relapses originating from hypnozoites are characteristic of *Plasmodium vivax* infections. Thus, reappearance of parasitemia after treatment can result from relapse, recrudescence, or reinfection. It has been assumed that parasites causing relapse would be a subset of the parasites that caused the primary infection.

Methods. Paired samples were collected before initiation of antimalarial treatment and at recurrence of parasitemia from 149 patients with vivax malaria in Thailand ($n = 36$), where reinfection could be excluded, and during field studies in Myanmar ($n = 75$) and India ($n = 38$).

Results. Combined genetic data from 2 genotyping approaches showed that novel *P. vivax* populations were present in the majority of patients with recurrent infection (107 [72%] of 149 patients overall [78% of patients in Thailand, 75% of patients in Myanmar {Burma}, and 63% of patients in India]). In 61% of the Thai and Burmese patients and in 55% of the Indian patients, the recurrent infections contained none of the parasite genotypes that caused the acute infection.

Conclusions. The *P. vivax* populations emerging from hypnozoites commonly differ from the populations that caused the acute episode. Activation of heterologous hypnozoite populations is the most common cause of first relapse in patients with vivax malaria.

Plasmodium vivax is the second most prevalent malaria parasite in the world, infecting >75 million people each year, predominantly in Asia and in Central and South America [1, 2]. *P. vivax* and *Plasmodium ovale* differ from *Plasmodium falciparum* in that they have a hypnozoite stage, which persists in the liver and causes relapses after clearance of the acute blood-stage infection. *P. vivax* from tropical regions is characterized by multiple relapses with short latent periods (3–5 weeks), whereas parasites from temperate regions usually have long incubation and latent periods (5–10 months) and are less commonly associated with relapses. The biology of relapses is poorly understood.

Resistance to chloroquine among *P. vivax* has emerged in Oceania, Southeast Asia, and, more recently, in Central and South America [3–8]. Pyrimethamine-sulfadoxine resistance is more widespread [9–11]. Assessment of *P. vivax* treatment responses is essential to monitor the emergence of resistance. For *P. falciparum* malaria, parasite genotyping to distinguish homologous parasites (suggesting treatment failure) from heterologous parasites (suggesting a newly acquired infection) in recurrent infections is needed to interpret the findings of drug efficacy trials conducted within areas of endemicity [12–15]. Interpretation of recurrences of *P.*

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vivax malaria in drug efficacy trials is complicated by relapses, which result from reactivation of hypnozoites. If the parasites causing the relapse arise from a dormant subset of the inoculated sporozoites that caused the primary infection, then genotyping cannot distinguish relapse from recrudescence. Comparison of *P. vivax* parasites associated with relapses and those associated with the respective primary infections has been reported previously in a small number of patients: 6 patients in Canada who had acquired the infection in different areas of endemicity [16], 10 patients in Brazil [17], and 5 patients in Thailand [18]. Parasite diversity was assessed using molecular markers in the first 2 studies [19, 20] (*pvcs* and *pvmsp1,* and *pvmsp1* alone, respectively), whereas a panel of monoclonal antibodies was used in the third study. Although it was concluded that parasites associated with primary infection and those associated with relapse are usually of a similar genetic composition, evidence for novel genotypes in *P. vivax* associated with relapses was obtained for 24% of the patients (1 of 6 patients in Canada [16], 2 of 10 patients in Brazil [17], and 2 of 5 patients in Thailand [18]).

The objective of the present study was to characterize large numbers ($n = 149$) of paired primary and recurrent *P. vivax* samples collected in drug efficacy trials, to determine the association between primary and relapse infections. The samples were collected in 3 distinct areas of endemicity: 36 samples in Thailand, 75 in Myanmar, and 38 in India. In all studies, efficacious asexual-stage treatment was given, although there are no recent studies from the study areas in India and Myanmar to confirm full susceptibility to chloroquine. In Thailand and Myanmar, no radical treatment (i.e., primaquine) was given. In Thailand, reinfection could be excluded because there was no reexposure after treatment, whereas, in Myanmar, reinfection could not be excluded, although the intensity of transmission was low and recurrences were likely to be relapses. In India, recurrent episodes were often observed many months after treatment and could have been either relapses or newly acquired infections. Two types of genetic markers were used for genotyping: 2 polymorphic *P. vivax* genes [19, 20] that were the focus of polymerase chain reaction (PCR) restriction fragment–length polymorphism (RFLP) analysis and microsatellite loci present in the genome of *P. vivax* [21, 22].

MATERIALS AND METHODS

Study Sites and Collection of Blood Samples

Fully informed, written consent to undergo blood sampling was obtained from each subject. Samples were collected in prospective studies to assess drug efficacy, each of which was approved by the respective scientific and ethics committees. Sites where malaria transmission was low or absent were chosen, to reduce the possibility that reinfection could be a confounding factor. The intensity of transmission of *P. vivax* in Dawei, Myanmar, and in periurban Calcutta, India, has not been assessed formally recently; however, on the basis of a combination of incidence, prevalence, and clinical-spectrum data, the intensity of transmission is thought to be low (close to 1 infectious bite per person per year). There is no malaria transmission in or near Bangkok.

Thailand. Blood samples were collected from adult male patients who, between 1992 and 1998, presented to the Bangkok Hospital for Tropical Diseases with acute symptomatic *P. vivax* malaria. Most patients came from the western border of Thailand, where recent large studies have confirmed full susceptibility to chloroquine in *P. vivax* and a relapse rate of 50%, with a mean interval of 3 weeks to the first relapse if rapidly eliminated antimalarials are given [9, 23]. Chloroquine suppresses the first relapse but not the second relapse; therefore, after chloroquine treatment, recurrences of parasitemia in Thailand occur ~6 weeks after treatment [9, 23]. Patients were randomly allocated to receive different antimalarial treatment regimens (table 1). The patients were kept under daily parasitological and clinical observation for 28 days. Blood samples were collected at admission, before and after the start of treatment, and at recurrence of *P. vivax* parasitemia. The samples were frozen and stored at −30°C or −70°C until DNA extraction. Two characteristics of this study are especially useful for later interpretation of the results from studies conducted in areas of endemicity. First, because the study was conducted in a hospital setting in Bangkok, where there is no *P. vivax* transmission, reinfection can therefore be excluded. Second, detailed information on the efficacies of antimalarial treatment with and without primaquine treatment (which ensures radical cure) suggested that recrudescence was very unlikely [9, 24]. Therefore, these sample pairs allowed us to examine the genotypes of parasites causing relapse infections.

Myanmar. Over a 5-month period (December 2002 through April 2003), 75 patients who were infected with *P. vivax* were recruited from Dawei in Myanmar. Malaria is perennial, with low-intensity transmission (entomological inoculation rate, ~1 infectious bite per person per year). Most cases of malaria were seen during the rainy season, which peaks from June to August. The majority of cases (>80%) occurred in children >5 years of age and in adults. All patients received standard chloroquine treatment (10/10/5 mg base/kg divided over 3 days) (table 1). Only the first dose was observed. Blood samples obtained by fingerprick were collected from patients before treatment and at recurrence of vivax parasitemias after treatment. Patients were monitored until day 28 after treatment. Hence, recrudescence, relapse, or newly acquired infections were all possible. For 9 patients, recurrent parasitemia was recorded either early (between day 14 and 19 after treatment) or late (between day 43 and 49 after treatment), whereas, for the remainder of the

Table 1. Antimalarial drugs administered in the 3 studies.

Study site, drug	Treatment regimen	Patients, no.
Thailand		
Quinine sulfate	10 mg of salt/kg 3 times daily for 7 days	3
Halofantrine	8 mg base/kg 3 times in 1 day	1
Artesunate	3.3 mg/kg (adult dose, 200 mg) on the first day and then 1.65 mg/kg (adult dose, 100 mg daily) for 4 additional days	30
Artemether	2.7 mg/kg (adult dose, 160 mg) on the first day and then 1.3 mg/kg daily (adult dose, 80 mg daily) for 4 additional days	2
Myanmar, chloroquine	Chloroquine (10/10/5 mg base/kg) divided over 3 days and artesunate (2 mg/kg/day) for 7 days in case of failure	75
India		
Chloroquine	10 mg of base/kg, followed 6 h later by 5 mg of base/kg, and then by 2 doses of 5 mg/kg every 24 h	12
Chloroquine and primaquine		
For 5 days	Same dose of chloroquine as that used for chloroquine-only regimen and primaquine (adult dose, 15 mg of base daily) for 5 days	12
For 14 days	Same dose of chloroquine as that used for chloroquine-only regimen, followed by primaquine (adult dose, 15 mg of base daily) for 14 days	14

patients, recurrent parasitemia was recorded between days 21 and 30 after treatment.

India. The study involved 155 men with *P. vivax* malaria in Calcutta, India. Thirty-eight patients were randomly allocated to 3 oral treatment groups: chloroquine alone or 2 regimens involving chloroquine and primaquine (table 1). Patients were excluded from the study if they had glucose-6-phosphate dehydrogenase deficiency. All patients were monitored weekly until day 28 after treatment and then monthly thereafter for 18 months. In Calcutta, *P. vivax* is endemic, although transmission intensity is low. Therefore, in this study, relapse, recrudescence, and new infections were also all possible. The time to reappearance was longer (mean, 130 days; median, 77 days; range, 45−441 days) than that noted in Thailand or Myanmar.

In Thailand, Myanmar, and India, resistance to the drugs used for treatment was unlikely. Therefore, although some recrudescences resulting from inadequate drug absorption or unusual disposition kinetics may have occurred, most recurrent infections were probably either relapses or, possibly, newly acquired infections; in Bangkok, such infections must have been relapses.

DNA Template Preparation

DNA was purified from blood samples by use of the commercially available DNA Blood Kit (Qiagen). Dry blood spots were collected from Myanmar and India and were purified using the QIAamp DNA kit (Qiagen). The final volume of the DNA solution used as a template for the amplification reactions was 2 μL, which is equivalent to either 2 μL of whole blood (frozen whole-blood samples collected from Thai patients) or 0.3 μL of whole-blood spot (dry blood spots collected from patients in Myanmar and India). Confirmation of the microscopic diagnosis of *P. vivax* infection and testing for the presence of other *Plasmodium* species involved the use of a PCR-based protocol described elsewhere [25].

Genotyping of *P. vivax*

We amplified 2 polymorphic antigenic markers: the *pvcs* gene and the central fragment (F2) of the *pvmsp1* gene. Primers, amplification conditions, and digestion reactions for the 2 loci have been described elsewhere [19].

P. vivax genome sequence data for the Salvador I strain were obtained from The Institute for Genomic Research (TIGR) through the *P. vivax* Genome Project Web site [26]. Tandem repeats were identified using the Tandem Repeats Finder program [27, 28]. Microsatellite primers were designed using the primer3 program [29], and all forward primers were labeled with fluorescent dye. The sequences and the dyes used for labeling the different loci are presented in table 2.

A seminested PCR approach was adopted for all the fragments. All amplification reactions were performed in a total volume of 10 μL and in the presence of 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 250 nmol/L of each oligonucleotide primer, 2.5 mmol/L $MgCl_2$, 125 μmol/L of each of the 4 deoxynucleoside triphosphates, and 0.4 U of TaKaRa polymerase (TaKaRa BIO). Primary amplification reactions were initiated with 2 μL of the template genomic DNA prepared from the blood samples, and 1 μL of the product of these reactions was used to initiate the secondary amplification reactions. Forward and reverse oligonucleotides were used in the primary reaction, whereas internal and reverse oligonucleotides were used in the secondary reaction. The cycling parameters for PCR were as follows: initial denaturation for 5 min at 95°C preceded annealing performed for 30 s at 52°C, extension performed for

Table 2. Primers used for microsatellite genotyping of *Plasmodium vivax* parasites.

TIGR ID	Repeat unit, bp	Motif	Cycles, no. Nested 1	Cycles, no. Nested 2	Primer	Sequence (5′→3′)	Size,[a] bp	Labeled
14.297	3	AAG	25	25	Forward	TGACATCTTTCAAATATTCCTTT	200	
					Reverse	TGAAAAATGTTCCGCTACTT		
					Internal	TACACCCTTTAGGTCCTCGT		PET
1.501	7	GGTGAGA	25	20	Forward	TCCTGTAACTCCTGCTCTGT	120	
					Reverse	CTTACTTCTACGTGCCCACT		
					Internal	AATTGTAGTTCAGCCCATTG		6FAM
3.502	8	AACGGATG	25	25	Forward	CCATGGACAACGGGTTAG	168	
					Reverse	TCCTACTCAGGGGGAATACT		
					Internal	GTGGACCGATGGACCTAT		PET

NOTE. See the "Genotyping of *P. vivax*" subsection of Materials and Methods for information on polymerase chain reaction protocols. 6FAM, 6-carboxyfluoroscein; ID, identification; PET, proprietary fluorochrome dye; TIGR, The Institute for Genomic Research.

[a] No. of base pairs of these loci in the Salvador 1 genome sequence.

30 s at 72°C, and denaturation performed for 30 s at 94°C. After a final annealing step was performed, followed by 2 min of extension, the reaction was stopped. PCR products were stored at 4°C until analysis.

We measured variation in microsatellite allele size by use of an ABI 3100 genetic analyzer (PE Applied Biosystems) and comparison with a size standard (GeneScan 500 LIZ; Applied Biosystems). We used GeneScan and Genotyper software (Applied Biosystems) to measure allele length and to quantify peak heights. Multiple alleles per locus were scored if they were >33% of the height of the predominant alleles present.

We initially selected 6 loci that provided very clean amplification, with no stutter peaks, on the basis of amplification of 6 samples from Thailand. These loci were 9.264, 14.297, 13.239, 1.501, 3.502, and 3.503. Loci were named using their chromosomal location, followed by a numerical identifier. Two of these loci showed low polymorphism with high-frequency common alleles. These loci were dropped because they were uninformative. One other locus (TIGR identification [ID] 9.264) was also eliminated, because strong stutter peaks were observed in some samples, generating spurious data. The set of 3 loci (TIGR IDs 1.501, 3.502, and 14.297) was used for analysis of all paired samples.

We included multiple, randomly distributed, negative control samples (human DNA or no template) in each amplification run. A subset of the samples ($n = 10$) were analyzed in triplicate to confirm the consistency of the results obtained. All pairs of primers were tested for specificity by use of genomic DNA from *P. falciparum* or humans. Serial dilution of genomic DNA was performed to test the sensitivity of primers.

Comparison of Acute Infection and Recurrence Genotypes

We compared the genotypes of pre- and post-treatment samples for 36 paired samples from Thailand, 75 pairs from Myanmar, and 38 pairs from India. On the basis of analysis of these paired samples, we classified the parasite populations as being genet-

Table 3. Overall classification of the genotyped paired samples.

Country	Days to recurrence, median (range)	All	Different[a]	Related[b]	Novel[c]
Myanmar	28.0 (14–49)	75	46 (61)	29 (39)	55 (73)
Thailand	21.0 (13–28)[d]	36	22 (61)	14 (39)	28 (78)
India	76.5 (45–441)	38	21 (55)	17 (45)	24 (63)
All	…	149	89 (60)	60 (40)	107 (72)

NOTE. Data are no. (%) of paired samples, unless otherwise indicated.

[a] None of the alleles detected in pretreatment samples was observed in the posttreatment samples for any 1 of the 5 markers used.

[b] For all 5 markers, at least 1 of the alleles detected in pretreatment samples was also observed in the posttreatment samples.

[c] The no. of the samples classified as "related" but for which ⩾1 of the microsatellite loci provided evidence that ⩾1 of the alleles detected in the recurrent episode sample were not present in the admission sample, added to the no. of samples classified as "different."

[d] The day of recurrence was not recorded for 4 of the 36 Thai patients, although a blood sample was collected.

Table 4. Patterns of variation in the 3 microsatellite chromosome loci used.

Study site	Paired samples, no.	Alleles at each locus, no.			Frequency of the most common alleles, %			1 H_e			Maximum probability[a]
		1.501	3.502	14.297	1.501	3.502	14.297	1.501	3.502	14.297	
Myanmar	75	10	10	6	30	28	34	0.14	0.17	0.23	0.005
Thailand	36	11	13	9	22	17	25	0.12	0.12	0.17	0.002
India	38	14	7	7	25	30	30	0.08	0.18	0.19	0.002

NOTE. The Institute for Genomic Research identification is used to identify each locus. Variation in the 3 markers was assessed by counting the nos. of different alleles present in each location and by measuring expected heterozygosity (H_e; the chance of drawing 2 different alleles from a population sample) by use of the formula $H_e = n/(n-1)(1-\Sigma p_i^2)$, where p is the frequency of the ith allele and n is the sample size.

[a] Maximum probability of 2 parasite clones carrying the same microsatellite alleles by chance, assuming random association between alleles at each locus.

ically related or different, by use of the following criteria for each locus.

Related parasite population. All or a subset of the alleles detected in pretreatment samples were also observed in the posttreatment samples. For "related" samples, the microsatellite genotyping data were further used to identify patients for whom there was also evidence of a novel genotype(s) in the recurrent sample not found among genotypes identified in the corresponding admission sample.

Different parasite populations. For any one locus, none of the alleles detected in pretreatment samples were observed in the posttreatment samples. If a paired sample was classified as "different" for any one of the markers considered (antigen genes or microsatellite loci), then the 2 parasite populations were considered to be genetically different.

RESULTS

Informative PCR-RFLP analysis was conducted for 131 of the 149 paired samples. For 50 (38%) of these 131 samples (42% of samples from Myanmar, 33% of samples from Thailand, and 34% of samples from India), the parasite populations in the samples obtained at admission and those obtained at recurrence were genetically distinct. The *pvmsp1* locus alone was informative in 35 of this subset of 50 samples, and it was found to be more informative than *pvcs*. Analysis of microsatellite loci revealed a higher proportion of paired samples (50% [i.e., 75 of 149 paired samples]) that were classified as being genetically distinct (56% of samples from Thailand, 47% of samples from Myanmar, and 53% of samples from India). The 3 microsatellite markers contributed equally to this classification. When the results obtained independently by use of each method (microsatellite genotyping and PCR-RFLP analysis) were compared, the classifications were found to be concordant for 90 (69%) of the 131 pairs analyzed (54 pairs were classified as related, and 36 were classified as different). Microsatellite analysis allowed an additional 27 paired samples to be distinguished that differed in parasite genotype composition, and PCR-RFLP analysis allowed another 14 paired samples to be distinguished (8 samples through *pvmsp1* alone).

We combined results from the 2 genotyping approaches (table 3). Overall, 89 (60%) of 149 paired samples were genetically distinct from each other (61% of samples from Thailand, 61% of samples from Myanmar, and 55% of samples from India). Microsatellite genotyping revealed that, in some cases, new parasite genotypes appeared in the infection causing recurrence together with the genotype causing the primary infection. Therefore, at recurrence, novel *P. vivax* populations appeared in 107 (72%) of 149 patients (78% of those from Thailand, 73% of those from Myanmar, and 63% of those from India).

The overall diversity of *P. vivax* was determined, on the basis of microsatellite genotyping, for the samples obtained from the 3 sites (table 4). Despite low transmission intensity, mixed genotype infections were common (table 5). The arithmetic mean MOI (minimum number of different parasite genotypes pres-

Table 5. Genetic complexity of *Plasmodium vivax* populations present in the paired samples.

Study site	Mean MOI for samples (% of samples or patients with single genotype)			Mean MOI for samples with novel genotypes[a]
	At admission	At recurrence	All	
Myanmar	1.87 (35)	1.55 (51)	2.63 (43)	0.77
Thailand	1.69 (47)	1.53 (56)	2.67 (51)	0.97
India	1.11 (89)	1.24 (79)	1.84 (84)	0.74

NOTE. MOI, minimum no. of different parasite genotypes present.

[a] Observed in samples obtained at recurrence.

ent) in primary infections was similar in Thailand and Myanmar (mean MOI, 1.69 and 1.87, respectively) and was higher than that noted for samples collected from India (mean MOI, 1.11). A similar pattern was observed for the samples obtained at recurrence of infection (mean MOI: 1.53 for samples from Thailand, 1.55 for samples from Myanmar, and 1.24 for samples from India). The proportion of samples with single genotypes noted at admission and at recurrence of infection did not differ significantly at the 3 study sites. On average, 0.97, 0.77, and 0.74 new genotypes were detected in the samples obtained at recurrence of infection in patients in Thailand, Myanmar, and India, respectively. When these results are combined, the majority of patients appear to have carried multiple genotypes during the observation period. In Thailand, 49% of patients carried >1 genotype (mean MOI, 2.67); in Myanmar, 57% carried >1 genotype (mean MOI, 2.63); and in India, 16% carried >1 genotype (mean MOI, 1.84). In India, where patients were followed for >1 year, median times to reappearance tended to be shorter for sample pairs carrying the same allele at all 3 loci (median time to reappearance, 64 days) than in pairs with different alleles at $\geq$1 of the loci (median time to reappearance, 80 days); however, this difference was not statistically significant ($P = .14$, Mann-Whitney U test). Median times to reappearance were similar for patients treated with chloroquine alone (91 days) or with chloroquine and primaquine either for 5 days (71.5 days) or 14 days (73 days).

DISCUSSION

P. vivax infections that recur after drug treatment may have 1 of 3 origins: recrudescence originating from asexual blood-stage parasites that survived drug treatment, reinfection resulting from a new mosquito inoculation, or relapse arising from the dormant liver stages (hypnozoites). In the present study, we conducted what, to our knowledge, is the first extensive within-patient comparative genetic analysis of primary infection and first relapse *P. vivax* populations. Transmission intensities in the 3 Asian countries were low, making early reinfection unlikely, and efficacious treatment regimens were used, making recrudescence unlikely [23, 30]. In the Thailand study, reinfection was impossible, so recurrences were certainly relapses, whereas, at the other 2 study sites, reinfection could not be excluded. Contrary to general expectation, the *P. vivax* infections causing relapses were usually caused by parasites that were genetically distinct from those that caused the acute infection. This unexpected observation provides novel insights into the biology of relapses and, if confirmed as a general phenomenon, confounds the use of parasite genotyping analyses to support in vivo drug efficacy trials.

The majority of episodes of recurrent vivax malaria in the present study presumably arose from hypnozoites that became reactivated after treatment of the initial acute infection. There are 2 generic explanations for this: either (1) there was simultaneous inoculation of different genotypes by a single mosquito or by different mosquitoes within a short period (i.e., 1 or 2 days); or (2) multiple inoculations occurred over a longer period, and the acute infection activated dormant hypnozoites originating from an earlier inoculation. If the first explanation is correct, then either the primary infection with the relapse genotype is at a density below the PCR-genotyping detection level or there is no primary infection and these later emerging parasites have a longer incubation period. The hypnozoites of different parasite strains may have different fixed periods of dormancy, as in the *P. vivax* strains from temperate and tropical zones. However, how activation of the hypnozoites of heterologous genotype occurs is unknown. Previously acquired genotype-specific immunity might preferentially suppress one genotype but would be inconsistent with the appearance of the suppressed genotype some 3 weeks later. A more likely explanation is that genotype-specific immunity develops only against the predominant blood-stage parasites in the mixed-genotype primary infection and that it preferentially suppresses this genotype when it later emerges at relapse. This would imply a threshold parasitemia for the induction of protective antibody responses. More information on the *P. vivax* genotypes carried by mosquitoes would help to resolve the question of inoculation times. More-sensitive genotype-specific detection methods would resolve the question of whether initial infections contain more genotypes. Irrespective of the timing of inoculation, these data indicate that the hypnozoites activated after treatment usually arise from a subset of sporozoites different from that which caused the initial infection. This implies an unexpected and remarkable regulation of within-host phenotypic behavior among different strains.

In conclusion, genotyping analysis of vivax malaria indicates a high rate of mixed-genotype infections even in settings where transmission is low, and it suggests a hitherto unsuspected phenomenon of heterologous activation of hypnozoite populations. These observations, as well as those presented in a companion report [31], provide important insights into the biology of the dormant stages of *P. vivax*.

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