Molecular Analysis of Strains of *Plasmodium vivax* from Paired Primary and Relapse Infections

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Relapse infections are an important obstacle to the successful treatment and control of *Plasmodium vivax* malaria, but the molecular basis of relapse remains poorly understood. To provide insight into the molecular mechanisms of relapse, paired primary and relapse isolates of *P. vivax* were subjected to single-strand conformational polymorphism (SSCP) and sequence analysis of the circumsporozoite and merozoite surface protein 1 genes. All unrelated isolates were unique by both SSCP and sequence analysis, but 5 of 6 relapse isolates were identical to or were clones of their matched primary isolates. These results indicate that most relapses are caused by the same parasite populations that circulate during the primary infection and do not arise from a genetically distinct subpopulation.

Plasmodium vivax is the most widely distributed of the human malaria–causing parasites and is the most common cause of malaria imported into many areas in which malaria is not endemic [1, 2]. In contrast to *Plasmodium falciparum,* the management and control of *P. vivax* is complicated by its ability to cause relapse infections, a phenomenon that has intrigued parasitologists for more than a century but the mechanisms of which remain enigmatic [3]. Several experimental and clinical studies have investigated vivax malaria infections acquired in different geographic and climatic regions and have demonstrated striking differences in their patterns of relapse [4–6]. Strains of *P. vivax* originating from the tropics are characterized by an early primary infection followed by a short latent period (5–10 weeks) before the appearance of frequent relapse activity. In contrast, *P. vivax* strains acquired in temperate zones are characterized by a variable period before primary infection followed by a long latent period (5–10 months) before the onset of relapses (reviewed in [6, 7]). The relapse patterns associated with specific strains of *P. vivax* are reproducible in different hosts and appear to be largely independent of the infected host's immune response [3]. It has therefore been suggested that the timing of relapses is strain-dependent and determined by the genetic makeup of the individual sporozoites, leading to the concept of tachysporozoites (strains with short latent periods) and bradysporozoites (strains with long latent periods) [7, 8]. However, relapse remains poorly understood at a molecular level.

The objective of this study was to examine the genetic relationship between paired primary and relapse isolates of *P. vivax* in an effort to provide insight into the molecular mechanisms of relapse. Our analysis is based on the extensive genetic diversity displayed by the genes encoding the merozoite surface protein 1 (MSP1) and the circumsporozoite protein (CS), making them potentially useful markers to genotype *P. vivax* isolates [9–16]. However, as is the case with *P. falciparum,* the degree of genetic polymorphism exhibited within these genes is not discernible with hybridization or size polymorphism studies alone, and consequently these methods may lack sufficient resolution to distinguish between related and unrelated isolates of *P. vivax* [16–18]. For this reason, we applied single-strand conformational polymorphism (SSCP) analysis as a method to type *P. vivax* isolates. SSCP is a sensitive and simple technique capable of distinguishing single base pair differences between DNA fragments [19]. SSCP analysis involves denaturation of double-stranded DNA into single-stranded DNA (ssDNA) followed by electrophoresis on a nondenaturing polyacrylamide gel. Each ssDNA molecule will adopt a tertiary conformation, based on its nucleotide sequence, that results in differences in relative mobility. SSCP has been successfully used to genotype microorganisms, such as hepatitis B virus [20] and human immunodeficiency virus type 1 [21].

Materials and Methods

Study population, blood samples, DNA purification. The study population comprised *P. vivax*–infected travelers who relapsed following standard antimalarial therapy [22] during the study period from May 1993 to June 1995. Paired primary and relapse blood samples were collected from 5 consecutive patients who relapsed 6–36 weeks after treatment and from 1 patient with multiple relapses (table 1).

Whole blood samples were also serially collected over a 5-month period from a single monkey inoculated with the Sal-1 strain of *P. vivax* (provided by W. E. Collins, CDC, Atlanta) [10]. DNA was extracted from whole blood samples using Qiagen DNA

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This study was approved by the Ethical Review Committee of the Toronto Hospital and all participants gave informed consent.

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Table 1. Demographic characteristics of patients and paired primary and relapse *P. vivax* isolates.

Patient, isolate	Day/month/year	Age (years)	Area of acquisition	Parasitemia (infected RBC/μL)
TDU1, P	03/05/93	59	South America	N/A
TDU1, R	17/06/93			17,910
TDU2, R1	16/06/94	25	Southeast Asia	3822
TDU2, R2	09/10/94			6435
TDU3, P	04/04/94	23	West Africa	10,392
TDU3, R	25/05/94			1932
TDU4, P	21/06/94	59	New Guinea	1848
TDU4, R	17/08/94			908
TDU5, P	22/08/94	53	India	10,880
TDU5, R	10/05/95			4640
TDU6, P	10/04/95	45	South America	10,200
TDU6, R	27/06/95			1260

NOTE. P, primary infection; R, relapse infection; R1, 1st relapse; R2, 2nd relapse; NA, not available; RBC, red blood cells. All patients were male.

columns (Qiagen, Chatsworth, CA), and the purified DNA was resuspended in 50 μL of sterile water.

Polymerase chain reaction (PCR) amplification. A portion of the MSP1 gene was amplified by PCR from the 6 paired primary and relapse isolates and the sequential Sal-1 monkey-adapted isolates. Oligonucleotide primers were synthesized corresponding to the conserved sequences flanking the variable region ICB5–ICB6 of the MSP1 gene (PV200-1: 5′-TACTACTTGATGGTCCTC-3′; PV200-2: 5′-CCTTCTGGTACAGCTCAATG-3′) [11] and region I and II of the CS gene (PV5A: 5′-GAAAATAAGCTGAAACAACC-3′; PV6A: 5′-TCCACAGGTTACACTGCAT-3′) [23]. Amplification consisted of a preheat at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min for the MSP1 primer pair or 45°C for 1 min for the CS primer pair, and 72°C for 2 min.

Amplification products were analyzed by gel electrophoresis on a 4% agarose gel using 1-kb DNA markers (Life Technologies GIBCO BRL, Gaithersburg, MD). All gels were stained with ethidium bromide and visualized under UV light. The PV200 primer pair generates an amplified product ranging from 500 to 620 bp, and the PV5A/PV6A product ranges from 600 to 720 bp.

MSP1 and CS amplification products were gel-purified using a PCR purification kit (Qiagen) and reamplified with primers PV200M13F (5′-TGTAAAACGACGGCCAGTTACTACTTGATGGTCCTC-3′) and PV200M13R (5′-GGCAGGAAACAGCTATGACCCCTTCTGGTACAGCTCAAT-3′) for the MSP1 gene and PV5AM13F (5′-TGTAAAACGACGGCCAGTGAAAATAAGCTGAAACAACC-3′) and PV6AM13R (5′-GGCAGGAAACAGCTATGACCTCCACAGGTTACACTGCAT-3′) for the CS gene, in preparation for direct PCR product sequencing. Amplification products were gel-purified before sequencing. Steps to prevent PCR contamination were taken as described [24].

SSCP analysis. The paired primary and relapse MSP1 and CS PCR products were analyzed by SSCP. MSP1 PCR products were analyzed with and without *Rsa*I enzyme digestion (New England Biolabs, Mississauga, Canada). After digestion, chloroform extraction, and ethanol precipitation, each sample was resuspended in 9 μL of sterile water. PCR products were denatured before SSCP analysis by adding 1 μL of 0.5 m*M* NaOH with 10 m*M* EDTA to 100–200 ng of amplified DNA in 9 μL of sterile water, followed by heating at 42°C for 5 min. Load buffer (0.5% bromophenol blue, formamide, and glycerol) was added, and each sample was placed on ice before electrophoresis on a nondenaturing 10% TRIS-borate-EDTA polyacrylamide minigel (1 mm; Novex, San Diego). Lanes containing 1-kb DNA markers or reference strains that had been similarly treated were used to standardize SSCP patterns. Samples were run under constant voltage with 0.8× glycerol–tolerant running buffer (USB, Cleveland) and 5% glycerol for 2250 V · h (MSP1 undigested samples), or 1500 V · h (digested MSP1), or 2625 V · h (CS). SSCP banding patterns were detected by silver staining (Bio-Rad, Hercules, CA).

Statistical analysis. Digested MSP1 DNA fragment SSCP patterns were scanned, digitalized, and analyzed by use of the Bio-Image System (version 3; Millipore, Ann Arbor, MI) with whole band analysis and by visual examination. The SSCP pattern of each relapse isolate was compared with each of the primary isolates, and relapse isolates were considered to be clones of the primary isolate if the SSCP pattern of the relapse isolate was the same as or was contained within the SSCP pattern of the primary isolate. Some primary infections consisted of >1 strain of *P. vivax,* which resulted in a primary SSCP pattern with one or more SSCP bands than was observed in the relapse pattern. For pattern-matching purposes, our criteria for identity between primary and relapse isolates was that the mobility of each relapse SSCP band had to match that of a band within the primary SSCP pattern. The number of shared SSCP bands was scored using a modification of the coefficient of similarity [25] with the formula $F = 2N_{xy}/(N_x + N_y)$, where N_x is the number of SSCP bands in the relapse isolate x and N_y is the total number of SSCP bands in the primary isolate minus the number in excess of those present in the relapse isolate. With this formula, an F value of 1.0 indicates that the relapse banding pattern is identical to or is contained within the SSCP pattern of the primary isolate.

Direct sequencing of PCR products. MSP1 and CS PCR products were directly sequenced using a PCR product sequencing kit (Sequenase 2.0; USB). Direct PCR sequencing was chosen because results are less influenced by PCR base incorporation errors than by standard cloning and sequencing approaches and are therefore

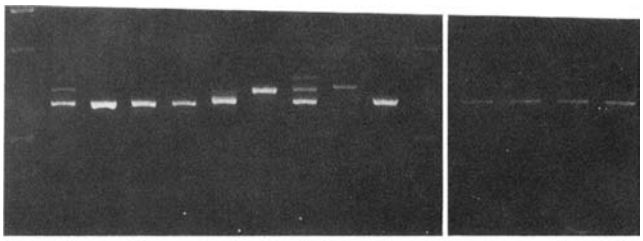

Figure 1. Size polymorphism of merozoite surface protein 1 gene fragments amplified by polymerase chain reaction from paired primary, relapse, and recrudescent *P. vivax* isolates. DNA was electrophoresed on 4% agarose gel with paired isolates run side by side. Lanes: 1 and 11, molecular weight markers (1-kb ladders); 2, TDU1 primary isolate; 3, TDU1 relapse isolate; 4, TDU2 1st relapse isolate; 5, TDU2 2nd relapse isolate; 6, TDU3 primary isolate; 7, TDU3 relapse isolate; 8, TDU4 primary isolate; 9, TDU4 recrudescent isolate; 10, TDU4 relapse isolate; 12, TDU5 primary isolate; 13, TDU5 relapse isolate; 14, TDU6 primary isolate; 15, TDU6 relapse isolate.

more reliable [16, 26]. For primary isolates with two or more different size PCR bands, each band was purified individually, and the band comigrating with the relapse band was sequenced. Comparative sequence analysis of relapse and primary isolates was done using MacDNAsis software (version 3.5; Hitachi, San Bruno, CA).

Results

Six *P. vivax*–infected patients who relapsed during the study period were identified. These patients acquired their infections in five geographically diverse areas in which vivax malaria is endemic (table 1). At presentation, all patients were treated with standard antimalarial regimens [22]. The possibility of reinfection was excluded because treatment and follow-up occurred entirely in Canada and no patient returned to an area in which malaria is endemic during or after therapy. Paired primary and relapse isolates were analyzed by PCR-SSCP and by direct PCR product sequencing of the MSP1 and CS genes.

A portion of the MSP1 gene was amplified from each isolate and analyzed by electrophoresis on a 4% agarose gel (figure 1). In the majority of samples, the PCR product of each relapse isolate comigrated with one of the PCR products from its corresponding primary isolate; however, in 1 pair (patient TDU3), the relapse PCR product did not match any product in the primary infection. Comparisons between isolate pairs indicated that it was not possible to discriminate between all unrelated infections using size polymorphism analysis alone.

To further discriminate between infections with similar size polymorphisms, SSCP was used to analyze the MSP1 gene from paired primary and relapse isolates of *P. vivax* in the presence and absence of glycerol (figure 2). Resolution and discrimination between isolates was improved using SSCP analysis compared with agarose gel electrophoresis alone. All relapse isolates, except the TDU3 pair, had a relapse SSCP pattern that either matched or was part of the pattern of their primary infection. The addition of glycerol to the running buffer generally improved the resolving capability of SSCP and therefore the discrimination of unrelated isolates. In the absence of glycerol, the SSCP patterns of some isolates were similar to unrelated isolates. However, in the presence of 5% glycerol, discrimination between unrelated isolates was possible: for example, patient samples TDU3 (figure 2B, lanes 5, 6) and TDU4 (figure 2B, lanes 7, 8) without glycerol versus TDU3 (figure 2A, lanes 4, 5) and TDU4 (figure 2A, lanes 8, 9) in the presence of 5% glycerol.

The optimal resolution of SSCP occurs with DNA fragments between 100 and 400 bp. Therefore, it was necessary to digest the amplified MSP1 products with *Rsa*I to obtain DNA fragments that could be maximally resolved by SSCP. Discrimination between all unrelated patient isolates was possible by restriction fragment length polymorphism (RFLP)–SSCP of the MSP1 gene (figure 3). Even when the analysis was repeated using higher resolution, all relapse isolates, with the exception of the TDU3 isolates, had an SSCP pattern that matched at least one population present in the original isolate. On the basis of the number and relative mobility of ssDNA fragments, the similarity values among SSCP patterns of *Rsa*I-digested MSP1 genes of each relapse isolate were calculated, and the relationship between the isolates was defined mathematically. Each relapse isolate was compared to all other primary isolates and assigned a coefficient of similarity value (F score) corresponding to the number of shared SSCP bands (table 2). All relapse isolates, except for TDU3, had an F score of 1.0 when compared with their matched primary isolate, indicating that the relapse SSCP pattern was identical to or a subpopulation of the primary SSCP pattern. In contrast, the paired TDU3 isolates and all unrelated isolates exhibited F scores <1.0, indicating nonidentity.

To assess the reliability of typing by SSCP and to confirm the relatedness of primary and relapse isolates, the MSP1 regions analyzed by SSCP were sequenced. The sequence of each isolate was compared with those of isolates from other subjects and to the 3 allelic types of the MSP1 gene previously reported [9–11]. In agreement with the SSCP results, molecular sequence analysis demonstrated that at the nucleotide level, all relapse isolates except the TDU3 pair were identical to their corresponding primary isolates and were different from all other unrelated isolates. Five general sequence types were observed (figure 4), and these corresponded to the MSP1 SSCP patterns as outlined in figure 2. Whole band computer analysis of SSCP patterns indicated that isolates with comparable patterns were of the same sequence type. For example, TDU5 primary and relapse isolates and TDU3 relapse isolate had similar SSCP patterns, and all were of sequence type 3B (figure 4). The region of the MSP1 gene amplified from the TDU6 isolates shared homology with the previously described Sal-1 strain [10], while all other isolates represented various intra-

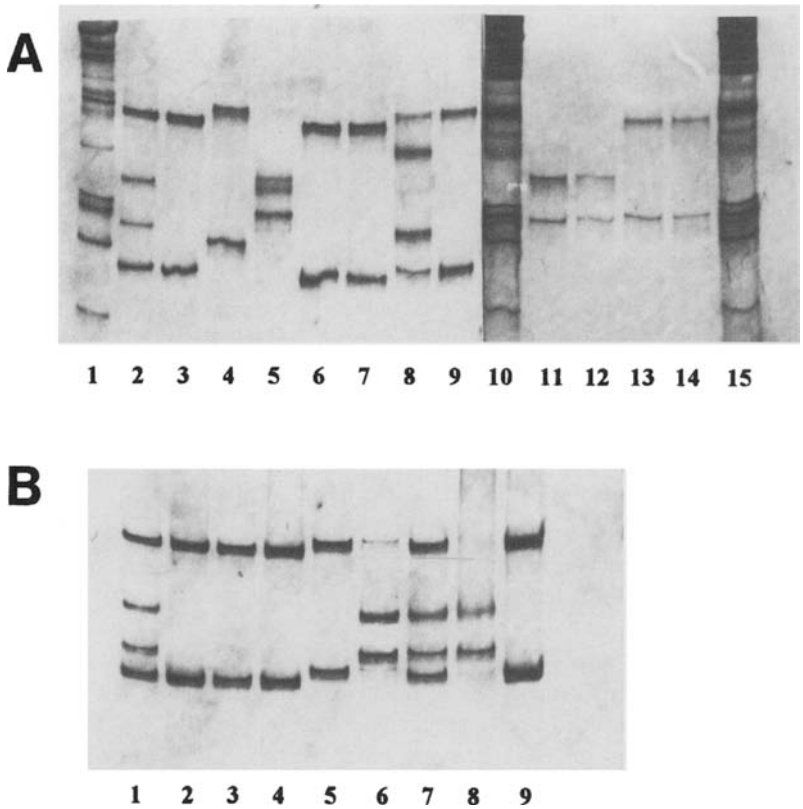

Figure 2. Genotyping of paired primary and relapse isolates of *P. vivax* by polymerase chain reaction–single-strand conformational polymorphism (SSCP) analysis of merozoite surface protein 1 (MSP1) gene. MSP1 gene fragments were amplified followed by SSCP analysis on nondenaturing 10% polyacrylamide gel with (**A**) or without (**B**) 5% glycerol. Primary and relapse isolates were run side by side. One patient (TDU4) had recrudescent and relapse infections. **A**, Lanes: 1, 10, and 15, molecular weight markers (1-kb ladders); 2, TDU1 primary isolate; 3, TDU1 relapse isolate; 4, TDU3 primary isolate; 5, TDU3 relapse isolate; 6, TDU2 1st relapse isolate; 7, TDU2 2nd relapse isolate; 8, TDU4 primary isolate; 9, TDU4 relapse isolate; 11, TDU5 primary isolate; 12, TDU5 relapse isolate; 13, TDU6 primary isolate; 14, TDU6 relapse isolate. **B**, Lanes: 1, TDU1 primary isolate; 2, TDU1 relapse isolate; 3, TDU2 1st relapse isolate; 4, TDU2 2nd relapse isolate; 5, TDU3 primary isolate; 7, TDU4 primary isolate; 8, TDU4 recrudescent isolate; 9, TDU4 relapse isolate.

genic recombinant forms of the prototype Sal-1 and Belem sequence types (figure 4). Of note, the paired TDU3 isolates were mirror image recombinants of each other.

A portion of the CS gene from each of the 6 paired isolates was also amplified and analyzed by SSCP and sequencing. All unrelated isolates were unique by SSCP and sequence analysis, and as in the MSP1 analysis, the SSCP patterns and sequence of all relapse isolates, except for patient TDU3, were identical to or a subpopulation of those of their matched primary isolate (figure 5). All sequenced CS genes contained type I repeats [13]. The TDU5 and TDU3 isolate pairs, acquired in India and West Africa, respectively, both contained a post–type I repeat insertion sequence that has been previously reported to be geographically restricted to Asia [26].

Interassay variability was assessed by repeating PCR and SSCP analyses at least five times. In each case, the resulting SSCP patterns were reproducible. The temporal stability of *P. vivax* SSCP patterns was assessed by performing SSCP analysis of the MSP1 gene on 5 isolates of the Sal-1 strain collected from an infected monkey over a 5-month period. The SSCP and RFLP-SSCP patterns of these isolates were identical over five time periods examined (figure 6), indicating that the polymorphisms detected by SSCP are stable in vivo.

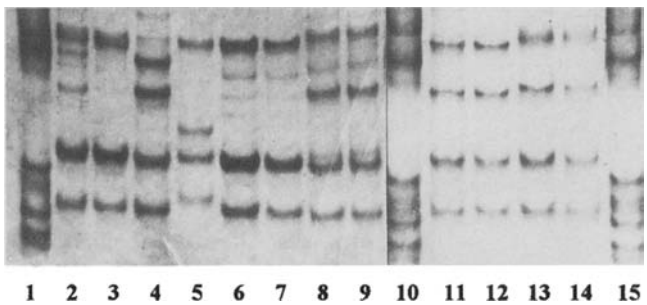

Figure 3. Genotyping of paired primary and relapse isolates of *P. vivax* by polymerase chain reaction–restriction fragment length polymorphism–single-strand conformational polymorphism (SSCP) analysis of merozoite surface protein 1 (MSP1) gene. Fragment of MSP1 gene was amplified, digested with *Rsa*I, and analyzed by SSCP on nondenaturing 10% polyacrylamide gel with 5% glycerol. Paired isolates were run side by side. Lanes: 1, 10, and 15, molecular weight markers (1-kb ladders); 2, TDU1 primary isolate; 3, TDU1 relapse isolate; 4, TDU3 primary isolate; 5, TDU3 relapse isolate; 6, TDU2 1st relapse isolate; 7, TDU2 2nd relapse isolate; 8, TDU4 primary isolate; 9, TDU4 relapse isolate; 11, TDU5 primary isolate; 12, TDU5 relapse isolate; 13, TDU6 primary isolate; 14, TDU6 relapse isolate.

Discussion

This study is, to our knowledge, the first to perform a molecular analysis of paired primary and relapse isolates of *P. vivax*. We demonstrate that 5 of 6 *P. vivax* isolates associated with relapse infections had MSP1 and CS gene sequences that were identical to those of clones circulating during the primary infec-

Table 2. Coefficient of similarity scores (*F* score) of restriction fragment length polymorphism–single-strand conformational polymorphism patterns of merozoite surface protein 1 gene of *P. vivax* relapse isolates compared with those of primary isolates.

Primary isolate	Relapse Isolate					
	TDU1	TDU2	TDU3	TDU4	TDU5	TDU6
TDU1	1.0	0	0.33	0.4	0.31	0.5
TDU2	0.15	1.0	0.22	0	0.15	0.29
TDU3	0.18	0.14	0	0.17	0.40	0.22
TDU4	0.31	0.17	0.18	1.0	0.17	0.18
TDU5	0.55	0.4	0.44	0.16	1.0	0.44
TDU6	0.4	0.22	0	0.18	0.44	1.0

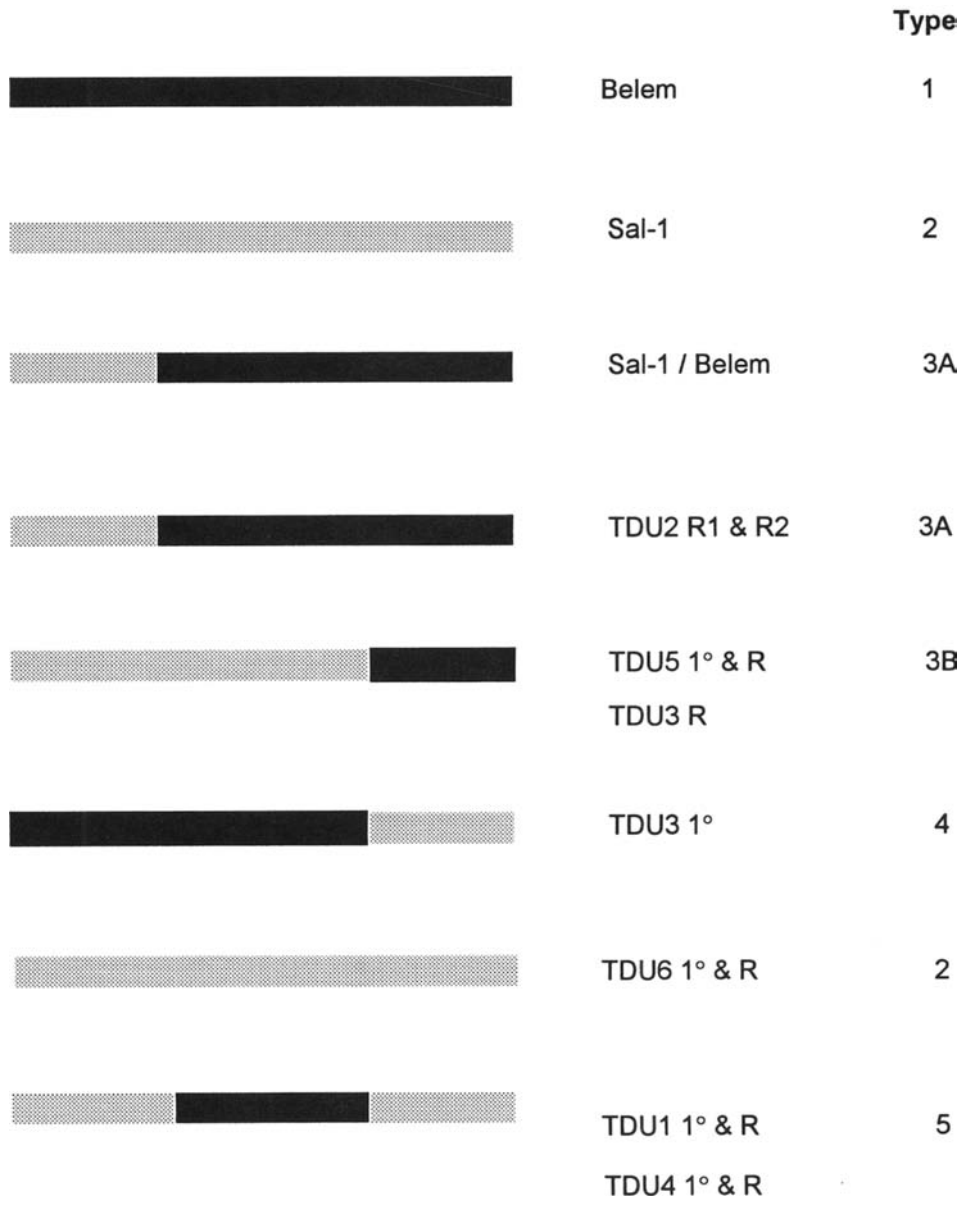

Figure 4. Schematic representation of nucleotide sequence of merozoite surface protein 1 sequence types. Shading identifies intragenic recombinant events between Sal-1 and Belem strains of *P. vivax*. Arbitrary numbers indicate sequence types (1 [9], 2 [10], 3a [11]). Sequences are from nt 2044–2629, according to sequence of del Portillo et al. [9]. 1° = primary isolate, R = relapse isolate, R1 = 1st relapse, R2 = 2nd relapse.

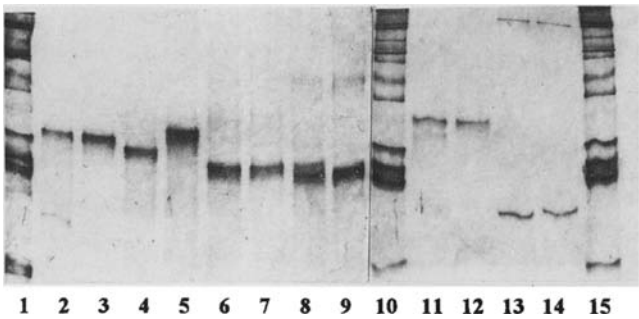

Figure 5. Polymerase chain reaction–single-strand conformational polymorphism (SSCP) analysis of circumsporozoite (CS) gene. Portion of CS gene was amplified and analyzed by SSCP on nondenaturing 10% polyacrylamide gel with 5% glycerol. Paired primary and relapse isolates of *P. vivax* were run side by side. Lanes: 1, 10, and 15, molecular weight markers (1-kb ladders); 2, TDU1 primary isolate; 3, TDU1 relapse isolate; 4, TDU3 primary isolate; 5, TDU3 relapse isolate; 6, TDU2 1st relapse isolate; 7, TDU2 2nd relapse isolate; 8, TDU4 primary isolate; 9, TDU4 relapse isolate; 11, TDU5 primary isolate; 12, TDU5 relapse isolate; 13, TDU6 primary isolate; 14, TDU6 relapse isolate.

tion. These observations were established by SSCP analysis and confirmed with PCR product sequencing. This study was done in a country without malaria transmission; therefore we can exclude reinfection as a confounding variable.

The MSP1 and CS gene fragments sequenced from paired primary and relapse isolates of *P. vivax* were identical at the nucleotide level except for the TDU3 pair. There are at least 8 known sequence variants for the MSP1 gene of *P. vivax*. In addition, we have sequenced this region of the MSP1 gene from another 21 *P. vivax* isolates (unpublished data), and to date, no 2 sequences are identical at the nucleotide level. Furthermore, size polymorphism, SSCP, and allele-specific hybridization studies of >100 *P. vivax* isolates indicate extensive polymorphism within this region of the MSP1 gene, even within isolates collected from geographically restricted areas during a single transmission season (unpublished data). The CS gene of *P. vivax* also displays considerable polymorphism, with at least 25 different sequence variants described, although there may be some regional bias in the distribution of these variants [16, 26]. Given the considerable polymorphism exhibited by these unlinked genes, the probability that a relapse would share the same nucleotide sequence as the primary isolate in both genes and not be related is very small. These results indicate that the majority of hypnozoites causing true relapses are identical to or closely related to parasite clones circulating during the primary infection and are not a genetically distinct subpopulation.

Patient TDU3 experienced a "relapse" with an isolate of *P. vivax* that was clearly different from his primary isolate. There are several potential explanations for this observation. Patient TDU3, during his extended travel, may have acquired and incubated a second unrelated infection caused by a parasite strain with a different incubation or prepatent period, thus appearing after the first infection as a "relapse." Another potential explanation may be that the parasite population responsible for his relapse was present in the primary infection at a parasitemia below the detection threshold of the primers used. For example, polyclonal infections with 1 parasite population in low number (<1:100 ratio) may be undetected by PCR because of competition by the more dominant populations [27], and this initially undetected subpopulation may then have caused the relapse. Finally, we cannot exclude the possibility that in some cases relapses may occur as a result of *P. vivax* strains that do not circulate during the primary infection. Analysis of additional paired isolates will be required to resolve which explanation is correct.

The ability to distinguish reinfections from relapses and recrudescences would be a major advance to treatment and vaccine studies in areas with active malaria transmission. Other potential methods to genotype *P. vivax* would include genomic RFLP analysis, PCR genotyping based on allele-specific hybridization, size polymorphism, or PCR-RFLP typing [18]. However, these methods are limited by their inability to detect small or matched additions, deletions, or base pair substitutions. Further, techniques such as genomic RFLP or PCR-RFLP typing cannot reliably detect polymorphisms present outside the recognition sequence for the corresponding restriction endonucleases chosen; therefore, these techniques may not be sufficiently powerful to distinguish between related isolates (primary, recrudescent, and relapse infections) and unrelated isolates causing reinfections. Sequence analysis, the reference standard for genotyping, is technically difficult, expensive, and impractical for large field studies. This study demonstrates that SSCP is a practical tool to type *P. vivax* and a suitable method to distinguish between related and unrelated vivax isolates.

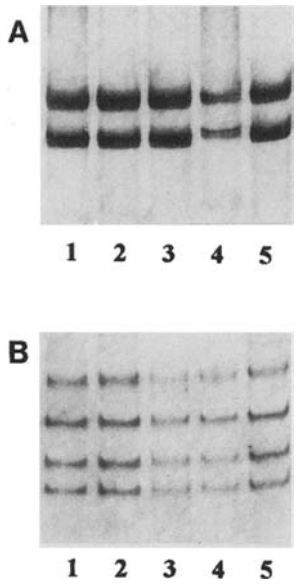

Figure 6. In vivo stability of polymerase chain reaction–single-strand conformational polymorphism (SSCP) patterns. Fragment of merozoite surface protein 1 gene was amplified from 5 samples collected over 5-month period from 1 monkey infected with Sal-1 strain of *P. vivax*. Each sample was either analyzed directly (**A**) or first digested with *Rsa*I (**B**) before SSCP analysis on 10% nondenaturing polyacrylamide gel with 5% glycerol. Lanes: 1, Sal-1 9/IV; 2, Sal-1 26/IV; 3, Sal-1 23/VI; 4, Sal-1 12/VIII; 5, Sal-1 2/IX.

SSCP offers the advantages of PCR-based approaches with a resolution approaching that of sequence analysis.

In summary, we have used both SSCP and sequence analysis to establish that most relapses of vivax malaria are caused by the same parasite populations that circulate during the primary infection and that relapse is not due to a genetically distinct subpopulation.

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