MAJOR ARTICLE

Relapses of *Plasmodium vivax* Infection Result from Clonal Hypnozoites Activated at Predetermined Intervals

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(See the article by Imwong et al., on pages 927–33, and the editorial commentary by Collins, on pages 919–20.)

Plasmodium vivax **infections are characterized by varying numbers of relapses occurring at different intervals as a result of activation of liver-stage hypnozoites. Parasite or host factors that determine the number and timing of relapses are unclear. In the present article, we report the analysis of relapse patterns and molecular characterization of parasites collected from Australian soldiers experiencing relapses of vivax malaria after exposure in East Timor. Although high molecular diversity was observed, a single allelic type was identified in association with 99% of relapses. Importantly, in 71% of patients experiencing >1 relapse, the allelic types were clonal and different in the 2 different relapses. These results, combined with those from a computer simulation model, suggest that a single hypnozoite clone was activated, causing a relapse, and that multiple relapses most likely arose from coordinated activation of hypnozoites originating from different parasite strains. These findings suggest remarkable regulation of relapse intervals in vivax malaria.**

Plasmodium vivax is the dominant species of malaria parasite in most areas outside of Africa. Although the case fatality rate associated with *P. vivax* malaria is less than that associated with *P. falciparum* malaria, *P. vivax* causes considerable morbidity [1]. *P. vivax* infections are characterized by the occurrence of relapses at different intervals as a result of the activation of liver-stage hypnozoites. *P. vivax* strains from different geographic zones tend to display different relapse patterns. In general, strains from subtropical or tropical zones are associated with early primary infections followed by frequent relapses at short intervals, whereas strains from temperate zones are associated with primary infections that tend to be delayed, with fewer relapses [2]. Mixed relapse patterns are observed in some areas [3, 4].

To date, the determinant of *P. vivax* relapse patterns and other factors influencing relapses remain unclear. Limited molecular studies indicate that parasites associated with primary infection and relapse are not genetically different [5, 6]. A high sporozoite inoculum shortens the incubation period dramatically [7, 8]. In *Plasmodium cynomolgi*, the *Plasmodium* species most closely related to *P. vivax*, the number and integrity of inoculated sporozoites determine the number of relapses [9, 10]. These findings suggest that parasite factors can determine the latency and frequency of relapses. However, the frequent occurrence of *P. vivax* infection after treatment of *P. falciparum* malaria, when reexposure to infected mosquitoes is unlikely, suggests that a more general trigger, such as illness or drug treatment, may be responsible for activating hypnozoites in the liver [11].

In 1999 and 2000, Australian Defence Force (ADF) personnel participated in an international peacekeeping

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operation in East Timor. Despite receiving prophylaxis (doxycycline, 100 mg daily) during deployment and terminal prophylaxis (doxycycline, 100 mg daily, and primaquine, 7.5 mg 3 times daily or 15 mg twice daily for 14 days) at the end of deployment, a number of personnel developed vivax malaria [12]. Personnel who experienced their first clinical episode after returning to Australia were, in all probability, experiencing a hypnozoite-induced relapse, because prophylactic medication received in East Timor and terminal prophylaxis received after the return to Australia would have completely inhibited any blood-stage parasites produced as a result of the primary infection [13]. Analysis of the interval to relapse and parasite diversity in blood samples obtained from these personnel during these relapses provided a unique opportunity to investigate the relapse patterns and molecular characteristics of the parasites causing the relapses.

MATERIALS AND METHODS

Sample Collection

ADF personnel. Cases of malaria among ADF personnel, whether occurring overseas or in Australia, were notified and recorded on the central malaria register at the Australian Army Malaria Institute in Brisbane. Blood samples were obtained from personnel at the onset of illness, when possible, and were stored in 6 mol/L guanidine hydrochloride and/or were frozen as packed cells. In some circumstances, only Giemsa-stained blood smears were available for analysis. The use of these blood samples plus blood smears for this study was approved by the Australian Defence Health Research Ethics Committee (number 288/02).

East Timorese population. A separate epidemiological survey was conducted (from February through April 2001) to estimate the prevalence of parasites in the local population of Bobonaro, the second most populous district on the northern coast of East Timor [14]. Fully informed, written consent was obtained for each blood sample. Seventeen random *P. vivax* samples from the survey were used in this study to assess the level of genetic diversity and mixed-strain infections by genotyping.

Genomic DNA Extraction

P. vivax genomic DNA was extracted from guanidine hydrochloride–preserved blood, as described elsewhere [15], and from frozen-packed cells or Giemsa-stained blood smears, by use of a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions.

Genotyping Performed Using Nested Polymerase Chain Reaction (PCR)

Polymorphic regions of 3 *P. vivax* genes were used as markers for genotyping: a fragment between the interspecies-conserved blocks 5 and 6 of *pvmsp1* [16], a polymorphic region in *pvama1* [17, 18], and the repeat region of *pvcs* [19]. *pvmsp1* and *pvama1* were examined for all the parasite samples, whereas *pvcs* was used to further differentiate samples indistinguishable by genotyping *pvmsp1* and *pvama1*.

Amplification of *pvmsp1* was performed using primers PvMB5 (5′-AGACAGGCGAGAAACCTG-3′) and PvMB6 (5′-TATATATTGTAAACCATTTCC-3′) in the first round of PCR, and primers published elsewhere [16] were used in the second round of PCR. For the amplification of *pvama1,* the primers PvAF11 and PvAR11 [17] were used in the first round, and the primers PvAF5 [17] and PvAR5 (5′-ATCCCAATCATTACGCAC-3′) were used in the second round. Amplification of *pvcs* was achieved using primers published elsewhere [19]. PCR amplifications were performed in 50-μL reaction mixtures containing 1.5 μg/mL each primer, 200 μmol/L deoxynucleoside triphosphates, buffer (50 mmol/L KCl and 10 mmol/L Tris-HCl [pH 8.3]), $MgCl_2$ (2.5 mmol/L for first-round PCR and 2.0 mmol/L for second-round PCR), and 1.25 U of AmpliTaq Gold DNA Polymerase (PE Applied Sciences). PCR cycling conditions were as follows: for first-round PCR, 10 min at 94°C, followed by 40 cycles of 50 s at 94°C, 50 s at 50°C , and 1 min at 72°C; for second-round PCR, 10 min at 94°C, followed by 30 cycles of 50 s at 94°C, 50 s at 55°C, and 1 min at 72°C. The fragments were amplified, purified, and sequenced. Sequence polymorphisms were used to determine the allelic types for individual markers.

Determination of Allelic Types and Mixed Allelic Types

For each marker, DNA sequences obtained from the samples were analyzed using the programs PileUp and PrettyBox in the GCG set of programs provided by the Australian National Genomic Information Service. Sequence polymorphisms, including single-nucleotide polymorphisms and insertions/deletions in each individual marker, were used to determine the allelic types for individual markers. A 2-loci or 3-loci allelic type was assigned to each sample by combining the allelic types of individual markers. A sample was classified as having a mixture of allelic types when multiple bands were present for any of the markers (excised and sequence confirmed) or when a mixture of signals was detected at polymorphic sites in DNA sequences.

Statistical Analysis

The intervals between departure from East Timor and the onset of clinical malaria for soldiers developing malaria in East Timor and for soldiers who were not ill in East Timor were compared using the Mann-Whitney *U* test. For patients receiving chloroquine-primaquine treatment versus other treatment regimens, Fisher's exact test was used to compare the proportion of paired samples having the same genotype. Frequency dis-

tributions displaying data in monthly groupings were obtained with the assumption that there were 30 days in a month. All statistical analysis was conducted using the SPSS for Windows software package (version 13.0; SPSS).

Simulation Model

A mathematical model was developed to test the hypothesis that individual hypnozoites are genetically programmed to develop after a predetermined interval (e.g., a biological clock). The model assumes that each individual was infected with a defined number of hypnozoites according to his or her length of duty in East Timor. The results of an entomological survey conducted by ADF personnel from December 2000 through March 2001 suggested that the entomological inoculation rate in East Timor was ~1 infectious bite per person every 27–43 nights (R. Cooper, S. P. Frances, M. Edstein, N. Beebe, unpublished data). Peak transmission occurred during the rainy season (from January through May), although *P. vivax* prevalence was fairly stable all year round [14]. The seasonality of the mosquito vectors reflected malaria illness observed among defense personnel in East Timor, with all vivax malaria episodes occurring between December and April. Hence, the number of simulated sporozoite infections generating hypnozoites was determined under the assumption that transmission commenced at the start of December and finished when the individual left East Timor. It was assumed that only 1 hypnozoite from each infectious bite inoculating sporozoites survived primaquine treatment. The date of each inoculation was randomly assigned, with the assumption that distribution was uniform over the exposure period. At the time of the simulated infection, each hypnozoite was assigned a release date according to a 4-parameter Weibull distribution describing the number of days from inoculation to hypnozoite release (SigmaPlot software, version 8 [SPSS]):

$$\text{If } x < x0 - b\left(\frac{c-1}{c}\right)^{1/c},$$

$$P(x) = 0.$$

Otherwise,

$$P(x) = a\left[\frac{c-1}{c}\right]^{\frac{1-c}{c}}\left[\frac{x-x0}{b}+\left(\frac{c-1}{c}\right)\right]^{1/c} \times \exp\left\{-\left[\frac{x-x0}{b}+\left(\frac{c-1}{c}\right)^{1/c}\right]^{c}+\frac{c-1}{c}\right\}.$$

At the designated time, each hypnozoite was activated, and the consequences of its development were noted: (1) if the hypnozoite was released either while the individual was still in East Timor or within 15 days of the return of the individual to Australia, no clinical attack was simulated, because of the suppressive effects of the chemoprophylactic and terminal prophylaxis regimens, respectively; (2) if the hypnozoite was released within 40 days of receipt of previous treatment for a clinical attack, subsequent merozoites were killed by the residual drug and no clinical attack developed; or (3) if the hypnozoite was released at a time not satisfying conditions 1 or 2 above, a clinical attack was simulated. The number of "strains" present at the onset of a clinical attack was also documented by assuming that parasites developing from hypnozoites released within 3 days of each other would both be detected in PCR genotyping and would be classified as a mixed infection.

After simulating the infections for each individual, the dates of the simulated clinical malaria attacks were converted to intervals from the time of departure from East Timor to the time of the clinical attack, and they then were grouped into 1-month intervals (under the assumption that there were 30 days per month) to form a theoretical frequency distribution. This theoretical distribution (obtained from averaging the results of 50 simulation sets) was compared with the empirical distribution of the data (for all documented clinical episodes). The parameters of the Weibull distribution (b, c, and $x0$) used to allocate intervals between infection and hypnozoite release were systematically altered to achieve the best fit of the standardized theoretical distribution to the empirical distribution, by use of the sum of the absolute difference between points in the cumulative distributions as the criterion. The 2-sample Kolmogorov-Smirnov test was used to compare the theoretical and empirical distributions. The value of parameter a had no effect on the standard Weibull distribution and was not considered during the fitting process. Hypnozoite release times were converted from months (as output from the Weibull distribution) to days by multiplying by 30.

RESULTS

Patients, relapses, and treatment. Seventy-one patients, from whom ⩾1 blood sample was obtained and was successfully genotyped, were included in the present study (figure 1). Six patients developed symptomatic vivax malaria in East Timor, and all 6 experienced relapse after they returned to Australia. Three of these individuals experienced a second relapse. The remaining 65 patients experienced their first vivax malaria attack at various times after returning to Australia (figure 2). Of these patients, 20 had ⩾2 clinical episodes (13 patients had 2 episodes, and 5 and 2 patients had 3 and 4 clinical episodes, respectively). The majority of episodes of malaria (75%) were treated with chloroquine (1500 mg over 3 days) and primaquine (315 mg over 2 weeks), whereas the remaining episodes were treated with a variety of other regimens of known efficacy [12, 20].

Relapse intervals. The 6 patients who had acute vivax malaria in East Timor experienced their first relapse 110–777 days (median, 181 days) after the initial episode (figure 2). For 59

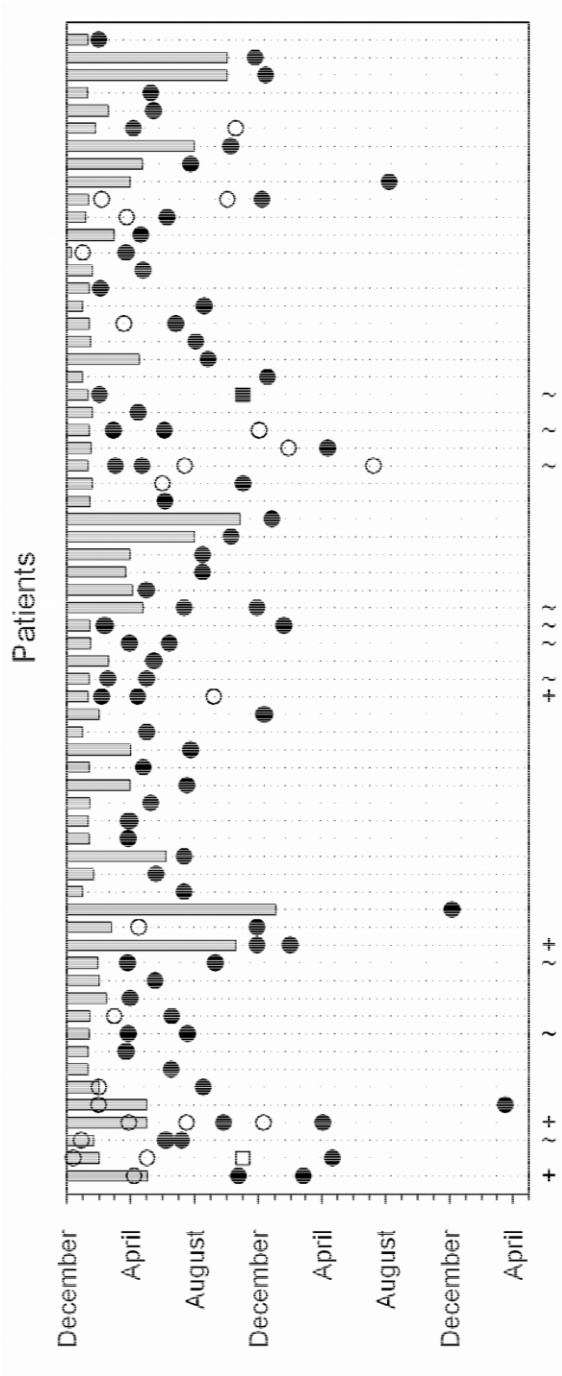

Figure 1. Summary of exposure, illness, and samples of Australian Defence Force personnel included in the present study. Horizontal bars denote when the individual was in East Timor, with circles denoting the clinical episode of vivax malaria. Squares denote clinical attacks with missing dates of onset. Although the actual date of onset of these attacks is unknown, their order relative to other attacks is known to be correct. Black symbols (■ and ●) and white symbols (□ and ○) show individuals with clinical attacks from whom parasite samples were collected and not collected, respectively. The symbols on the right side of the graph (+ and ~) denote identical and different genotypes in the paired samples analyzed, respectively. For each individual, the start date on the horizontal axis is the first of December before their return to Australia.

of the 65 patients who did not experience vivax malaria until returning to Australia, the date of departure from East Timor and the date of onset of the first clinical episode of malaria were known. The first clinical episode noted for this subgroup occurred 20–376 days (median, 76 days) after the return to Australia (figure 2). The interval between arrival in Australia and relapse was significantly longer for patients who were ill with malaria in East Timor than for those who were not ill (median interval, 157 days vs. 76 days; $P = .037$). The longer interval to relapse for those experiencing vivax malaria in East Timor may be related to residual levels of chloroquine suppressing relapsing parasites for 5–6 weeks after receipt of chloroquine therapy and the hypnozoiticidal activity of the additional course of primaquine. Second relapses were experienced by 23 (32%) of the 71 patients, with fewer relapses occurring after treatment with chloroquine plus primaquine (12 relapses among 54 patients) compared with other drug regimens (11 relapses among 17 patients). The range of the interval between the first and second relapses was 29–343 days (median, 99 days) (figure 2).

It was noted that a proportion of patients experienced their first relapse >300 days after returning to Australia: 1 of 6 patients who were ill in East Timor and 4 of 59 patients who were not ill in East Timor. There was no significant difference in the proportion of patients with such long relapse intervals between the 2 groups ($P = .394$).

Clonality and genetic diversity of* P. vivax *samples. Single alleles were found in 85 of the 86 *P. vivax* samples (collected from 71 ADF personnel) genotyped at the *pvmsp1* and *pvama1* (and, in some cases, *pvcs*) loci. This indicates that 99% of samples were obtained from patients with clonal infections.

Analysis of the DNA sequence data identified 45 different *pvmsp1* allelic types in 86 samples. The most prevalent allelic type was observed in 13 samples; however, most (31 of 45) of the *pvmsp1* allelic types were unique. Diversity was slightly less for *pvama1*, with 26 allelic types identified; 11 allelic types were unique, whereas 2 prevalent types were detected in 22 and 14 samples, respectively. When the *pvmsp1* and *pvama1* allelic types were considered together, 61 unique and 9 common genotypes were identified. The *pvcs* allelic types of the common genotypes were examined to further differentiate parasites. The combination of *pvmsp1*, *pvama1*, and *pvcs* allelic types revealed a total of 78 different genotypes in the 86 samples. Of the 78 different genotypes observed, 73 were unique and were seen once, 3 were seen twice, and 2 were found in 3 samples each.

Genotyping of *P. vivax* samples obtained from 17 East Timorese residents showed that 6 (35%) had >1 clone of *P. vivax*. Each of the 11 single-clone samples had a different allelic type, with 3 genotypes matching those seen in the ADF personnel. These data indicate that the population reservoir of *P. vivax* in

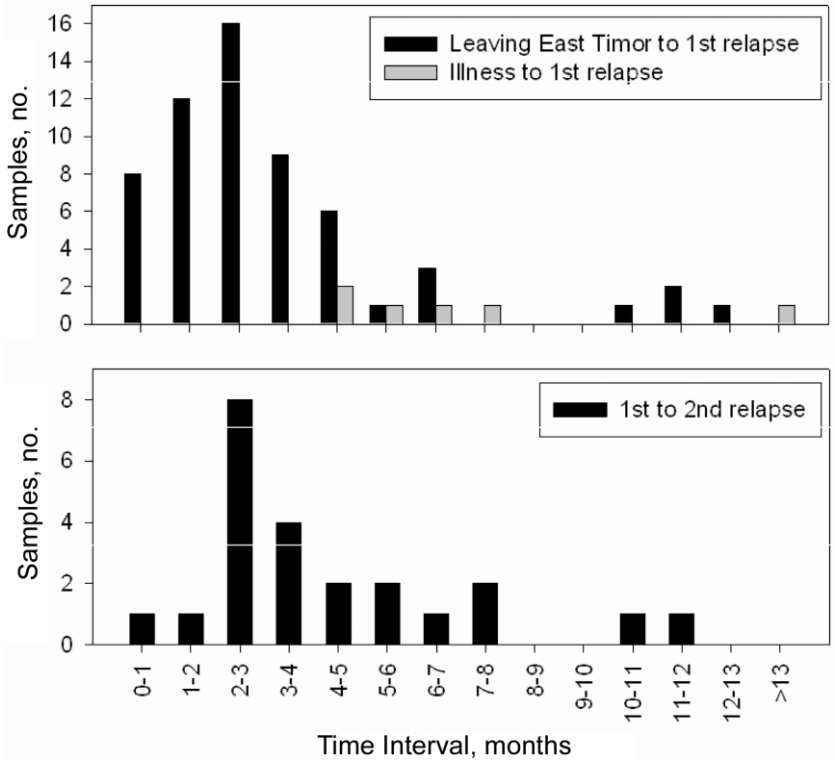

Figure 2. Frequency distribution of the relapse intervals. *Top,* the interval between the time of onset of clinical malaria in East Timor and the time of the first relapse (*gray bars; n* = 6), as well as the interval between the time of departure from East Timor and the time of onset of the first clinical episode for individuals not experiencing malaria in East Timor (*black bars; n* = 59). *Bottom,* the interval between the times when the first and second clinical episodes occurred in Australia (*n* = 23).

East Timor may be large and that there was no preferred genotype in the relapse samples.

Comparison of paired relapse samples. Paired samples obtained from different relapses in the same individual were available for 15 patients. Multiple genotypes within 1 of the samples precluded comparison of the paired samples for 1 patient. Parasites with single but different allelic types were detected in 10 (71%) of 14 paired samples, whereas identical 3-loci genotypes were identified in samples from the remaining 4 individuals (29%) (figure 1). There was no association between the treatment regimen received and homogeneity in the paired samples; 7 of 9 patients treated with chloroquine and primaquine had different allelic types, compared with 3 of the 5 patients treated with alternative drug combinations ($P = .58$).

Modelling relapses of* P. vivax *infection. A mathematical model was developed mimicking the pattern of primary infection and relapse observed in this study population. Using a Weibull distribution ($b = 5.1$, $c = 1.55$, and $x0 = 2.9$) to model the interval from infection to hypnozoite release produced a distribution of relapse intervals that was not significantly different from that observed (figure 3) ($P = .96$). On the basis of the estimated entomological infection rate in East Timor and the length of time that individuals were in the country, infection with a total of 161 hypnozoites in the 65 patients with known dates of departure from East Timor was simulated. The simulation resulted in a median of 99 clinical episodes (range, 90–113 episodes), compared with the 93 episodes observed. Of the ~38% of hypnozoites that did not generate clinical infections, an average of 23% were activated either while the soldier was still in East Timor or within 14 days of return. These infections were presumably suppressed by the antimalarials taken. The remaining 15% of hypnozoites were released in the 40 days after treatment of the primary malaria infection and were assumed to be suppressed by the residual drug. The random release of hypnozoites according to the fitted Weibull distribution resulted in a median of 1.6% of simulated clinical episodes (range, 0–9.4%) containing >1 parasite genotype, which compares well to the observed mixed-infection rate of 1.2% in the samples (i.e., 1 of 86 samples).

DISCUSSION

Relapses are a feature of vivax malaria that provide parasites with renewed opportunities for sexual reproduction and transmission. They cause recurring clinical episodes in people living in or visiting areas of endemicity and account for much mor-

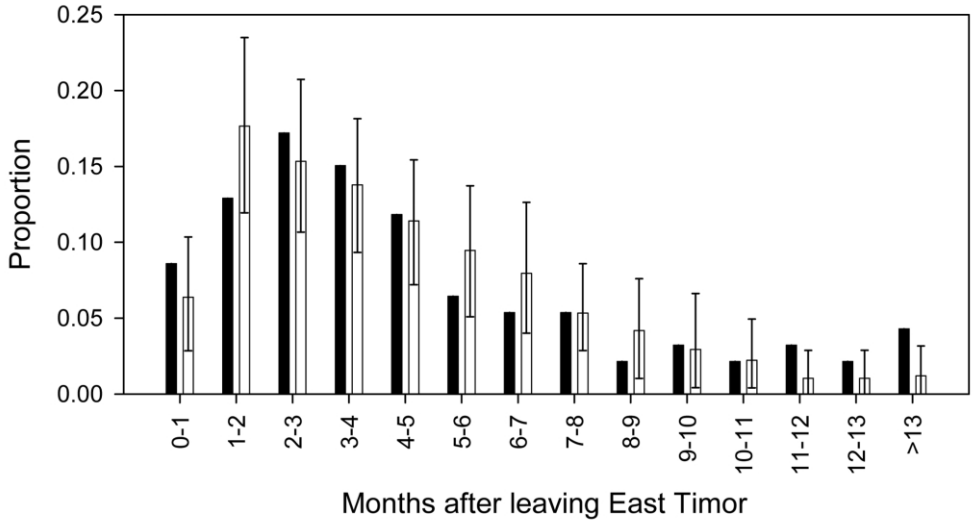

Figure 3. Comparison of intervals between the time of departure from East Timor and the time of relapse of vivax malaria, according to simulation output and observed data. Simulated *(white bars)* and observed *(black bars)* data are grouped according to 1-month intervals. Error bars for the simulated data denote the 5th and 95th percentiles of the 50 simulations. Values on the *X*-axis denote the no. of months after leaving East Timor, such that 0–1 month denotes 0–30 days, 1–2 months denote 31–60 days, etc. The values on the *Y*-axis denote the proportion of simulated and observed data.

bidity and lost productivity. Although clinical attacks of vivax malaria can be suppressed effectively while prophylactic medication is being taken, relapses occurring after departure from an area of endemicity present a significant health problem for civilians and military personnel [21, 22]. In addition, relapsing malaria can potentially initiate local transmission in such areas as northern Australia, where malaria has been eliminated but competent mosquito vectors remain [23]. Therefore, investigations into the factors influencing relapses have practical, as well as profound, biological implications.

Relapses of vivax malaria are common in the Western Pacific region, despite the administration of primaquine for treatment or terminal prophylaxis. Most of the 71 individuals in our study experienced their first attack of vivax malaria >30 days after their return to Australia. These cases were almost certainly relapses. However, 8 individuals experienced their first episode 20–30 days after their return to Australia. It is possible that some of these episodes could have been due to persistent blood stages, if these individuals were not in compliance with terminal prophylaxis. However, a more likely explanation is that parasites were released from the liver at the end of terminal prophylaxis and were no longer suppressed by doxycycline because of the relatively short pharmacological half-life of doxycycline. Relapses have been observed 2–4 weeks after receipt of effective tetracycline therapy [24] (K.R., personal communication). The lack of development of *P. falciparum* infections after the return to Australia, despite a much increased exposure of ADF personnel to *P. falciparum* [12], is further evidence of reasonably good compliance with terminal prophylaxis and the effective elimination of blood stages [24].

In the present study, all 71 patients experienced relapse after receiving terminal prophylaxis with primaquine, and at least 27% experienced a second relapse after treatment with chloroquine and primaquine, confirming the prevalence of primaquine tolerance in the region. Interestingly, 5 individuals developed their first relapse, and 22 their second relapse, at intervals of >10 months. Such long intervals have not been previously reported, with the region generally considered to be associated with short relapse intervals (range, 21–117 days) [25].

It is noted that, for patients not having malaria in East Timor, the median interval between departure from East Timor and the development of clinical malaria was significantly shorter than that for patients who were ill with malaria in East Timor. The most obvious difference between the 2 groups was that the latter group received a course of chloroquine plus primaquine as treatment for vivax infection while in East Timor. It is possible that (1) residual levels of chloroquine after treatment may have suppressed or killed relapsing parasites developing within 40 days of the therapy or that (2) the additional course of primaquine may have interfered with the development of hypnozoites, thus prolonging the intervals. Further research is required to more fully investigate the effect of treatment on the timing of subsequent relapses.

A large number of different alleles were observed in both the ADF samples (78 of 86 samples) and the samples obtained from the local population (11 of 11 samples). Most of the alleles

were seen only once, indicating that the size of the *P. vivax* population may be very large and that this combination of genetic markers has excellent power for distinguishing parasite allelic types circulating in the area. On the basis of the diversity of vivax strains, a person receiving >2 infectious bites would likely be infected with 2 different types of parasites.

Genotyping indicated that samples from ADF personnel with *P. vivax* relapses were almost exclusively clonal (99% of samples), compared with only 65% of East Timorese samples (presumably, both primary infection and relapse samples). This finding suggests that relapses result from activation of a single allelic type of hypnozoite. Although it is possible that some patients were infected with only 1 parasite, the availability of paired samples from multiple relapses in the same patient indicated that 71% of patients with ⩾2 genetically different hypnozoites present in their livers still experienced clonal relapses. The activation of a single hypnozoite genotype, when multiple genotypes were present in the liver, suggests coordination of hypnozoite activation and not triggering by a general (nonspecific) environmental or host factor. A nonspecific trigger would have been expected to activate both clones of hypnozoites, resulting in a mixed allelic infection in the relapse samples. This led us to hypothesize that the hypnozoites were activated according to a genetically determined biological clock (i.e., hypnozoites initiated blood-stage infections at definite, genetically predetermined intervals). This hypothesis about a biological clock was tested using a mathematical simulation model. The simulation output replicated the observed temporal pattern of malaria episodes and the proportion of mixed infections, which suggests that the hypothesis is feasible.

Our data suggest that a single clone of *P. vivax* can cause a primary infection and a few relapses at varying intervals. Infections with multiple *P. vivax* strains, whether through multiple infectious bites or through a single infectious bite inoculating multiple strains, give rise to multiple relapses at various predetermined intervals. Because prophylaxis and treatment are often unable to prevent vivax relapses, malaria control measures that decrease human-mosquito contact should be emphasized. Bed nets and other personal protection measures provide the simplest way to reduce the number of infectious bites and the number of subsequent relapses in civilian and military groups. Reducing the number of relapses would lower the rate of *P. vivax* transmission in areas of endemicity, minimize illness and lost productivity among civilian and military groups returning to malaria-free areas, and limit the reintroduction of malaria into such areas.

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