MAJOR ARTICLE

Evaluation of *Plasmodium vivax* Genotyping Markers for Molecular Monitoring in Clinical Trials

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Background. Many antimalarial interventions are accompanied by molecular monitoring of parasite infections, and a number of molecular typing techniques based on different polymorphic marker genes are used. Here, we describe a genotyping technique that provides a fast and precise approach to study *Plasmodium vivax* infection dynamics during circumstances in which individual clones must be followed over time. The method was tested with samples from an in vivo drug efficacy study.

Methods. The sizes of polymerase chain reaction fragments were evaluated by capillary electrophoresis to determine the extent of size polymorphism for 9 potential genetic markers (5 genes of merozoite surface proteins *[msp]* and 4 microsatellites) in 93–108 *P. vivax*–positive blood samples from 3 villages in Papua New Guinea.

Results. The microsatellites MS16 and Pv3.27 showed the greatest diversity in the study area, with 66 and 31 different alleles, respectively, followed by 2 fragments of *msp1* and 2 other microsatellites. *msp3α*, *msp4*, and *msp5* revealed limited polymorphism.

Conclusions. Even for the most diverse markers, the highest allelic frequencies reached 6% (MS16) or 13% (Pv3.27). To reduce the theoretical probability of superinfection with parasites that have the same haplotype as that detected at baseline, we propose to combine at least 2 markers for genotyping individual *P. vivax* infections.

Plasmodium vivax is the second leading cause of human malaria and, together with *Plasmodium falciparum*, accounts for a huge majority of malaria cases worldwide. Although *P. falciparum* is dominant in large parts of Africa, *P. vivax* causes >50% of all malaria cases outside of Africa. In total, >2.5 billion inhabitants of the Middle East, Asia, Eastern Africa, Central and South America, and Oceania are exposed to *P. vivax*, resulting in an estimated 71–391 million cases of malaria per year [1–3]. In these areas, *P. vivax* is causing significant economic and social damage [2], and there is increasing evidence that severe illness and death due to *P. vivax* may be more common than previously appreciated [4–6]. Nevertheless, although considerable efforts were made during recent decades to understand and control *P. falciparum*, only limited resources have been invested in *P. vivax* vaccine and drug research and development [7].

It has been argued that malaria control programs focusing predominantly on *P. falciparum* may foster *P. vivax* endemicity [2, 8]. This effect might be caused by different transmission strategies of the 2 parasites and the ability of *P. vivax* to relapse from the hypnozoite stage, a long-lasting liver stage that is activated through unknown mechanisms [9]. Alternative approaches are thus needed to effectively control *P. vivax*. Although the development of a number of *P. vivax* vaccine candidates is progressing [7], new combination drug regimens are rarely rigorously tested for their efficacy against *P. vivax*. This is partly because standard methods to differentiate between true treatment failures and reinfections are lacking. Because relapses are often genetically different from infections present at the baseline of a drug trial [9], distinguishing between recrudescence, new infection, and relapse on the basis of genotyping results is further complicated.

Similarly, despite recent efforts to fill gaps in knowledge of the biology and epidemiology of *P. vivax*, little is known about the basic parameters describing the course

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of natural infections, such as the duration or multiplicity of infection (MOI) and the possible interaction with other *Plasmodium* species. Intensified research of *P. vivax* will increase the demand for genotyping tools, investigation of *P. vivax* infection dynamics, and molecular monitoring during clinical trials.

Previously, *P. vivax* genotyping was usually performed by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis or by sequencing polymorphic genes. Common markers were genes encoding merozoite surface proteins *(msp),* the gene encoding circumsporozoite protein *(csp),* the gene encoding gametocyte antigen *(gam-1),* the gene encoding Duffy binding protein *(dbp),* plus a number of microsatellites. PCR-RFLP analysis revealed that the most polymorphic markers were *msp3α*, with 49 different alleles detected in 94 isolates [10], and *msp1,* with 36 and 37 alleles detected in 100 and 151 isolates, respectively [11, 12]. Less polymorphic were *csp,* with 23 alleles detected in 100 isolates [11], and *gam-1,* with 5 alleles [13, 14]. Use of PCR-RFLP to study microsatellites revealed up to 15 different alleles [15, 16]. Sequencing revealed *msp1* as highly polymorphic, with 31 alleles among 40 isolates analyzed [17]. High diversity was also detected in *msp3β* [18], *msp5* [18, 19], and *dpb* [20–22].

Although PCR-RFLP is a cheap and reliable genotyping method, it requires time-consuming analysis of restriction fragments. Side-by-side runs on high-resolution gels are needed, especially when minor differences in fragment size occur. Multiclonal infections generate complex fragment patterns that are difficult to analyze. Sequencing, on the other hand, usually has a much higher resolution but is not suitable for multiple-clone infections unless a preceding cloning step is included.

As an alternative to these 2 widely used methods, size polymorphism detection by capillary electrophoresis combined with analysis software such as GeneMapper or GeneMarker has proven to be a valuable tool for high-resolution genotyping of *P. falciparum* [23]. Recently, this method was used to determine the size of *P. vivax* microsatellites [9, 24–27]. Up to 43 alleles were found at a single locus in samples from various regions worldwide, indicating that this method is highly suitable for *P. vivax* genotyping. The present study focuses on genetic diversity at the community level, because this reflects the situation likely encountered in drug surveillance or studies of infection dynamics, and it evaluates the extent of size polymorphism for 9 potential genotyping markers by means of capillary electrophoresis in 93–108 blood samples from Papua New Guinea.

SUBJECTS, MATERIALS, AND METHODS

Study sites, subjects, and therapeutic classification. In vivo drug-efficacy studies that accorded with the revised World Health Organization (WHO) protocol were conducted between October 2004 and April 2005 in 3 villages in Papua New Guinea to assess rates of amodiaquine failure and chloroquine plus sulfadoxine-pyrimethamine failure in persons with uncomplicated *P. falciparum* and *P. vivax* malaria [28]. *P. vivax* from 108 community samples was genotyped (because of a shortage in material, 15 samples could not be genotyped for microsatellites MS16 and Pv3.27). Of 108 cases of *P. vivax* infection in the in vivo studies, 13 involved children who received a diagnosis of *P. vivax* infection during the 28-day in vivo efficacy trial. For these 13 patients, available samples from days 7, 14, and 28 plus any day of treatment failure were genotyped.

For patients in the in vivo drug efficacy study, treatment failure was defined as clinical deterioration in the presence of *P. vivax* parasitemia; as parasitemia onset 3–28 days after treatment initiation and an axillary temperature of $\geqslant$37.5°C; or as parasitemia onset 7–28 days after treatment initiation, irrespective of clinical conditions [28]. For patients without clinical signs and recurrent detection of asexual parasites $\leqslant$28 days after treatment, the clinical and parasitological response to treatment was considered adequate. Detailed information on treatment, patients, and outcome classification was published previously [28].

Protocol approval and ethical clearance for the study were obtained from the Medical Research Advisory Committee (MRAC 05.19) of the Ministry of Health in Papua New Guinea, and consent was obtained from parents or legal guardians before recruitment of each patient. Blood samples, obtained via finger prick, were collected into EDTA microtainer tubes, and DNA was extracted using a QIAamp DNA blood kit (Qiagen) according to the manufacturer's instructions.

Genetic markers. Five of 9 markers tested are coding regions of merozoite surface genes. The *msp1* gene contains a series of conserved and variable stretches, which are described elsewhere [17]; 2 regions, *msp1*F1 and *msp1*F3, proved to be the most polymorphic in a previous study [11] and in our sequence alignments of published sequences. *msp3α*, *msp4,* and *msp5* each contain a region in which the number of repeats varies among parasites. The microsatellite markers Pv3.27, MS16, Pv1.501, and Pv3.502 were selected because of the previously reported extent of their polymorphisms [9, 24, 27].

PCR analysis. For amplification of all 9 genetic markers, a nested or seminested approach was used. To size PCR products by capillary electrophoresis, the forward primer of the nested PCR was labeled with a fluorescent dye (NED, VIC, or 6-FAM [Applied Biosystems]). Taq polymerase sometimes adds an adenine at the 3' end of the PCR product. Because size variation must be avoided, the reverse nested primer contained a proprietary 7-bp tail (Applied Biosystems) at its 5' end that promoted the addition of an adenine at the 3' end of the labeled fragment. The primer sequences are given in table 1. As negative controls, pooled DNA from humans without infection and a *P. falciparum* DNA sample were used.

PCR analyses were done in a total volume of 20 μL that contained 0.25 μmol/L of each primer (obtained from Operon for primary PCR and from Applied Biosystems for nested PCR), 200

Table 1. Primer sequences for 9 genetic markers in *Plasmodium vivax*.

Genetic marker, primer	Primer sequence
Microsatellite MS16	
Forward primary	TTCCTGATGACAATTTCGACGG
Reverse primary	TCTCTTCCCATTTGAGCATCGC
Forward nested[a]	CTTGTTGTGGTTGTTGATGGTG
Reverse nested	AGTACGTCAACCATGTGGGTAG
Microsatellite 3.27	
Forward primary	TTTTTCAACTTGCTGCCCCCTG
Forward nested[b]	GGACATTCCAAATGTATGTGCAGTCG
Reverse	CGTCATCGTCATTGCTCTGGAG
Microsatellite 1.501	
Forward primary[c]	TCCTGTAACTCCTGCTCTGT
Forward nested[c]	AATTGTAGTTCAGCCCATTG
Reverse[c]	CTTACTTCTACGTGCCCACT
*msp1*F3	
Forward primary	GGAGAACATAAGCTACCTGTCC
Reverse primary	GTTGTTACTTGGTCTTCCTCCC
Forward nested[d]	CAAGCCTACCAAGAATTGATCCCCAA
Reverse nested[d]	ATTACTTTGTCGTAGTCCTCGGCGTAGTCC
*msp1*F1	
Forward primary	TATGATTTGTTGAGGGCGAAGC
Reverse primary	TGCTTTCCATCATCTGGATTTTGC
Forward nested[e]	CGATATTGGAAAATTGGAGACCTTCAT
Reverse nested[e]	CTTTTGCGCCTCCTCCAGCT
Microsatellite 3.502	
Forward primary[c]	CCATGGACAACGGGTTAG
Forward nested[c]	GTGGACCGATGGACCTAT
Reverse[c]	TCCTACTCAGGGGGAATACT
msp3α	
Forward primary	GAAAGAACGACTCCCTCCC
Reverse primary	CTTTTGCCTTCGCCACTTCG
Forward nested	GCRACCAGTGTGATACCATTAACC
Reverse nested	TCCTCCTTTGCCACTACTGC
msp4	
Forward primary	AGCCACTTCAACATGTGGAACC
Reverse primary	GATGACTCCACACATTCGGTGC
Forward nested	ATGGAGATTACAACGAGCAGGG
Reverse nested	ATCTCCACATCCCCCATTGTTG
msp5	
Forward primary	GATGTGGACATGTTTGAGAGGG
Reverse primary	GATGCATATTTGGTCGTCTGCG
Forward nested	GCATACAAATGAGGCCCTTCCC
Reverse nested	CCGTTACTCCTTTCTCCACTCG

[a] Primer modified from Karunaweera et al. [25].
[b] Primer modified from Imwong et al. [27].
[c] Primers originally described by Imwong et al. [9].
[d] Primers originally described by Imwong et al. [11].
[e] Primers modified from Imwong et al. [11].

μmol/L of dNTPs, 2 μL of buffer B (Solis BioDyne), 2 mmol/L of $MgCl_2$, 1.5 U of Taq DNA polymerase (FirePol [Solis BioDyne]), and 0.5 μL of genomic DNA, corresponding to 0.5 μL of blood. One microliter of undiluted or diluted (at 1:100 or 1:1000) PCR product was used as template for the nested or seminested PCR (table 2).

PCR analyses were performed in an MJ Research PTC 100 or a Biometra T Professional Basic thermocycler under the following conditions: initial denaturation for 5 min at 95°C; 30 cycles (primary PCRs) or 25 cycles (nested PCRs) of denaturation for 1 min at 95°C, annealing for 1 min at 55°C–62°C (table 2), and elongation for 1 min at 72°C; and final elongation for 5 min at 72°C. PCR products were stored at 4°C in the dark until further analyses.

Capillary electrophoresis and data analyses. PCR products were first analyzed on a 2% agarose gel. For capillary electrophoresis, most nested PCR products were diluted to 1:20 in H_2O; a few samples were not diluted or were diluted to 1:40. A total of 2.5 μL of diluted PCR product was mixed with 10 μL of diluted size standard Rox-500 (Applied Biosystems; dilution 1:40 in H_2O).

The mix was dried overnight at room temperature in the dark; capillary electrophoresis was done at the MRC genomics core facility (London, United Kingdom), where 10 μL of highly deionized formamide (Applied Biosystems) was added to the sample. The sample was then incubated at room temperature for 45 min and run on a 3730xls DNA analyzer (Applied Biosystems).

Data were analyzed using GeneMarker, version 1.6 (SoftGenetics), to facilitate determination of fragment sizes and peak intensity and to set cutoff values for distinguishing peaks for existing fragments from background noise. All samples were checked visually, and fragment sizes were remeasured at a different dilution for peaks that were too high or too low. Alleles were grouped manually according to their size into 3-bp bins for coding regions and according to their repeat length for microsatellites (3 bp for MS16, 4 bp for Pv3.27, 7 bp for Pv1.501, and 8 bp for Pv3.502).

Measures of diversity. In addition to the total number of alleles distinguished, the distribution of allelic frequencies is an important characteristic for determining the quality of genotyping markers. Low and evenly distributed frequencies and a large number of alleles reduce the risk that a second infection involves the same allele by chance. For each individual marker, the probability P of being infected by 2 parasites carrying the same allele is equal to the expected homozygosity and was calculated using the formula $P = \Sigma p_i^2$, where p_i is the frequency of allele i [29].

To facilitate comparability with other published markers, the expected virtual heterozygosity H_E was also calculated, using the equation $[n/(n-1)] \times (1 - \Sigma p_i^2)$, where n is the number of samples analyzed. This variable can be defined as the probability that a randomly chosen pair of alleles differ from each other.

The likelihood that 2 infections carry the same genotype by chance is reduced by combining >1 unlinked genetic marker. On the assumption that different infections are independent, the probability of reinfection due to parasites with the same geno-

Table 2. PCR conditions for 9 genetic markers in *Plasmodium vivax*.

Genetic marker	Repeat length, bp	Annealing temperature, °C		Primary-product dilution for nested PCR	Expected nested-product size, bp[a]	Observed allele size, range, bp	Fluorescent dye
		Primary PCR	Nested PCR				
Microsatellite MS16	3	57	56	. . .	406	145–497	VIC
Microsatellite 3.27	4	58	58	. . .	301	184–325	6-FAM
Microsatellite 1.501	7	58	58	1:100	120	90–282	NED
*msp1*F3	3	59	60	. . .	260	226–372	VIC
*msp1*F1	3	55	55	. . .	400	344–533	NED
Microsatellite 3.502	8	58	58	. . .	168	131–231	6-FAM
msp3α	3	60	60	. . .	463	312–481	VIC
msp4	3	58	58	1:100	245	167–265	6-FAM
msp5	3	60	62	1:1000	332	246–432	6-FAM

[a] In reference strain Sal-1, including a 7-bp tail and a terminal adenine.

type can be calculated by multiplying the probabilities of different molecular markers.

Calculation of mean MOI. The MOI in 93–108 microscopy-positive samples was calculated for all 9 markers independently by dividing the total number of *P. vivax* clones detected by the number of samples PCR positive for this parasite.

Classification of genotyping results. To discriminate recrudescence from new infection, genotypes at the time parasites reappeared (defined as day *X*) were compared with genotypes in the sample obtained before treatment (defined as day 0). Follow-up samples were genotyped using *msp1*F1, *msp1*F3, the microsatellites 1.501 and 3.502, *msp3α*, *msp4*, and *msp5* as markers. According to the definition of new infection adopted by the WHO and MMV [30], a new infection is a subsequent occurring parasitemia in which all alleles in parasites from the posttreatment sample are different from those in the admission sample, for 1 or more loci tested. Any genotype detected in samples collected on both days indicated recrudescence. In rare cases, parasites from a patient with relapsing infection that carried the same genotype as parasites recovered on day 0 might be classified as recrudescence.

RESULTS

Diversity of 9 marker genes. To identify the most suitable *P. vivax* genotyping marker, we determined the prevalence of polymorphisms for 9 potential markers in 93–108 *P. vivax*–positive blood samples from Papua New Guinea. Figure 1 shows the numbers of alleles discriminated and the respective allelic frequencies for all 9 markers.

For some markers, 1–8 of the 93–108 samples tested remained PCR negative even after repeating the PCR under the same conditions. This may be a result of sequence variation in the primer binding sites or the detection limit of this particular PCR. A summary of PCR results for different markers is given in table 3.

Allelic frequencies. Diversity was highest for the microsatellites MS16 ($P = 0.023$; $H_E = 0.988$) and Pv3.27 ($P = 0.07$; $H_E = 0.94$), followed by *msp1*F3 ($P = 0.107$; $H_E = 0.902$) and *msp1*F1 ($P = 0.134$; $H_E = 0.875$) (table 3). If MS16 and Pv3.27—the most polymorphic microsatellites—were combined, the probability that 2 samples would have the same genotype by chance was reduced to 0.0016 for the combination. Table 4 lists the values of P for each marker and for combinations of 2–9 markers.

MOI. The mean MOI was highest (2.37) for microsatellite MS16 and lowest (1.46) for *msp5* (table 3 and table 5, which appears only in the electronic edition of the *Journal*). On the basis of 9 markers, 20 single-clone infections (MOI, 1) were found among 108 samples, and the highest MOI detected was 8.

msp1 ***F1 and F3 haplotypes in single infections.*** PCR fragments were obtained for the *msp1*F1 and *msp1*F3 regions in 18 of 20 single-clone infections. Because the F1 and F3 PCR products detected in these samples originated from a single *msp1* gene, the number of different *msp1* haplotypes could be determined. In 18 samples, 8 *msp1*F1 alleles and 10 *msp1*F3 alleles were detected, leading to 17 distinct *msp1* haplotypes. All except 1 appeared only once, indicating that *msp1* diversity is by far greater than that indicated by 1 marker alone. The long distance between the F1 and the F3 region (~2800 bp in reference strain Salvador 1) did not allow spanning a single PCR over both regions.

Distinguishing between treatment failures and new infections or relapses. *P. vivax* parasites in follow-up blood samples from 13 children were genotyped. In 11 children (84.6%), parasites were detected on day 28; clinical episodes occurred in 1 patient on day 6 and in another on day 20. Infection in 4 of 13 patients was classified as treatment failure (i.e., recrudescence). Among these were the 2 children in whom treatment failure was detected on days 6 and 20. Nine children had a new infection or relapse.

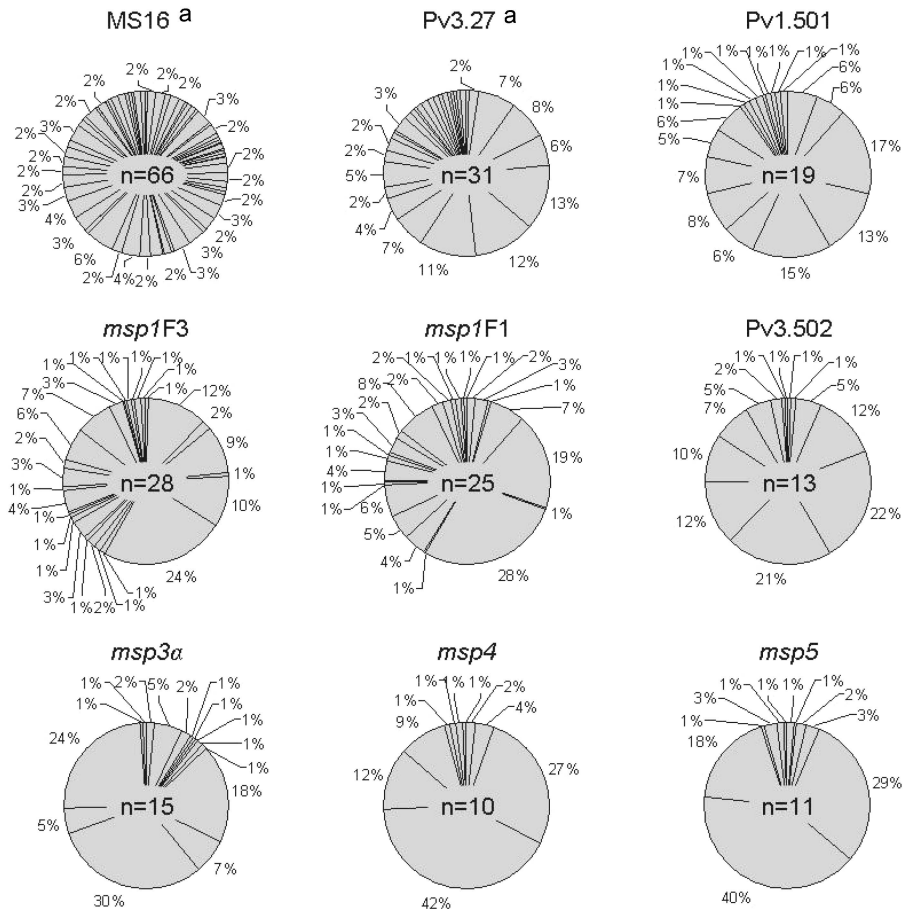

Figure 1. Allelic frequencies of 9 potential genetic markers among 93–108 *Plasmodium vivax*–positive samples. n, number of alleles. [a]Frequency of alleles without lettering is <1.5%.

DISCUSSION

Although *P. vivax* is the main causative agent of malaria outside of Africa, remarkably little is known about the infection dynamics of this pathogen under natural circumstances. A genotyping system that can be used in large-scale studies is urgently needed to strengthen the research on and control of *P. vivax*. Here, we show results of an evaluation of different molecular markers and demonstrate the usefulness of a precise and fast genotyping technique based on capillary electrophoresis for studies of *P. vivax* populations. This technique detected extensive diversity for microsatellites MS16 (16 alleles) and Pv3.27 (31 alleles); significant diversity was also found in 2 other microsatellites (Pv1.501 and Pv3.502), as well as in 2 regions of *msp1*. *msp1* has been genotyped previously by use of PCR followed by separation on agarose gels, leading to only 5 *msp1*F1 and 4 *msp1*F3 size variants in

Table 3. Diversity, PCR positivity, and multiplicity of infection (MOI) for 9 potential marker genes in *Plasmodium vivax*.

	MS16	Pv3.27	Pv1.501	*msp1*F3	*msp1*F1	Pv3.502	*msp3α*	*msp4*	*msp5*
Alleles, no.	67	31	19	28	25	13	15	10	11
PCR positivity[a]	90/93	90/93	107/108	108/108	100/108	108/108	106/108	108/108	108/108
Clones, no.	213	166	204	232	189	178	183	163	158
MOI	2.367	1.844	1.907	2.148	1.890	1.648	1.726	1.509	1.463
$P = \Sigma p_i^2$	0.023	0.070	0.099	0.106	0.134	0.145	0.202	0.273	0.288
H_E	0.988	0.940	0.909	0.902	0.875	0.863	0.806	0.734	0.718

NOTE. MOI was calculated for each marker by dividing the number of clones detected in all positive samples by the number of PCR-positive samples.

[a] Data are no. of samples with positive PCR results/no. of samples tested.

Table 4. Probability of the coincidence of the same genotype in independent *Plasmodium vivax* clones.

Molecular marker[a]	$P = \Sigma p_i^2$ for each individual marker	Combined probability[b]	πP_i
MS16	0.023	$P_1 = P_{MS16}$	0.02259252
Pv3.27	0.070	$P_2 = P_1 \times P_{Pv3.27}$	0.00158892
Pv1.501	0.099	$P_3 = P_2 \times P_{Pv1.501}$	0.00015794
*msp1*F3	0.107	$P_4 = P_3 \times P_{msp1F3}$	1.682×10^{-5}
*msp1*F1	0.134	$P_5 = P_4 \times P_{msp1F1}$	2.2539×10^{-6}
Pv3.502	0.145	$P_6 = P_5 \times P_{Pv3.502}$	3.2592×10^{-7}
msp3α	0.202	$P_7 = P_6 \times P_{msp3\alpha}$	6.5673×10^{-8}
msp4	0.273	$P_8 = P_7 \times P_{msp4}$	1.7902×10^{-8}
msp5	0.288	$P_9 = P_8 \times P_{msp5}$	5.1613×10^{-9}

[a] Ordered according to increasing values of P.

[b] The combined product πP_i, calculated as $P_1 \times P_2 \times \ldots P_i$, is the overall probability that the genotype of 2 independent clones coincides both at the locus corresponding to that line in the table and at all loci higher up in the table.

100 samples and indicating a restricted resolution from this technique. When RFLP analysis was applied to the F2 fragment of *msp1,* 2 individual digests led to 36 different alleles [11]. PCR-RFLP is labor-intensive, as it involves 2 digests per sample, and difficulties arise in fragment sizing on gels, particularly in the case of multiple-clone infections. The capillary electrophoresis approach overcomes these limitations and is thus highly suitable for large sample sets and for longitudinal tracking of individual clones in cases such as drug trials.

Recently, different studies have used PCR followed by capillary electrophoresis to evaluate a number of microsatellites, including those used in this study. The extent of size polymorphism in microsatellite MS16 was higher in our study than previously reported (66 alleles in 93 isolates, compared with 43 alleles in 164 isolates [24]). The other microsatellites (i.e., 31 alleles at locus 3.27, 19 alleles at locus 1.501, and 13 alleles at locus 3.502) showed polymorphisms comparable to those reported from Myanmar, Thailand, Laos, and India, where 80–92 samples were genotyped per country and 20–28 alleles at locus 3.27, 10–15 alleles at locus 1.501, and 7–14 alleles at locus 3.502 were found [9, 27]. In contrast, these loci had shown limited polymorphism in Colombia [27]. We determined an H_E value of 0.94 for the marker Pv3.27 among parasites recovered from a limited geographic region in Papua New Guinea, which is remarkably similar to the value of 0.95 we calculated on the basis of data, published elsewhere [27], for 90 *P. vivax*–infected blood samples from India.

Comparison of the genetic diversity of potential markers is complicated because H_E values for a single marker can greatly differ between study sites [27]. It is difficult to determine how much of the variation in discrimination power depends on the genotyping technique and choice of marker and how much is due to underlying differences in genetic diversity of the study populations. The usefulness of the markers we tested for genotyping in Papua New Guinea needs to be evaluated for each study site.

Some of our markers, such as *msp1,* are negatively affected by a high frequency of the most common allele (29% for the F1 fragment and 24% for the F3 fragment). Such a high allelic frequency increases the risk that a relapse or new infection carries by chance a genotype identical to the one detected at day 0. This unequal distribution of frequencies might be explained by natural selection. Allelic frequencies of microsatellites are generally more evenly distributed because of the absence of selective pressure.

The marker MS16 provided the highest MOI, with a mean of 2.4 *P. vivax* infections/carrier in our study, whereas in the same population, the mean MOI for *P. falciparum* was 1.7 estimated with the marker gene *Pfmsp2* (J. Marfurt, unpublished data). The higher MOI of *P. vivax* could reflect either greater complexity due to relapses and different transmission intensities or merely differences in the resolution of the 2 molecular markers used. Longitudinal studies are required to further elucidate rates of acquisition and elimination of *P. vivax* clones, compared with those of *P. falciparum*.

Previously, *P. vivax* genotyping studies had been performed in children from the same study area. The different results obtained illustrate effects due to resolution of the typing technique used. A mean MOI of 1.4 was detected by *msp3α* PCR-RFLP [31], and a mean MOI of 3.5 was found when cloned *msp3α* and Duffy binding protein *(dpbII)* alleles were sequenced [32].

Our genotyping method was used for samples from a drug-efficacy study. Among 13 patients who were *P. vivax* positive after day 0, 4 treatment failures (i.e., persistent infections with same genotype) and 9 new infections were found. Relapses in *P. vivax* complicate the interpretation of genotyping in drug trials as a novel genotype during the follow up period can either be true new infection or a relapse. However, because a relapse involving a different genotype would be considered a new infection and not a treatment failure, the discrimination "relapse/new infection" is of little relevance to PCR adjustment of trials of drugs against blood-stage parasites.

In principle, relapses can also lead to overestimation of recrudescences if the relapse is of the same genotype as the infection at baseline. Although *P. vivax* strains in areas of endemicity, such as New Guinea, are thought to have a short relapse interval [9, 33], it is still unclear how frequent an infection present at baseline and successfully treated relapses during the 28-day follow-up period. However, other studies indicate that parasites involved in relapse often carry a genotype different from those present at baseline [9]. Therefore, it is unlikely that many relapses after a baseline infection will falsely be considered as recrudescences.

Table 5. Multiplicity of infection (MOI) obtained by different genotyping markers for *Plasmodium vivax*.

The table is available in its entirety in the online edition of *The Journal of Infectious Diseases*.

Although no genotyping method can ultimately distinguish between relapses and new infections or recrudescences, genotyping improved the precision of trial findings considerably and thus represents a clear improvement, compared with outcomes based on microscopy findings only.

In conclusion, a high-throughput system based on PCR followed by capillary electrophoresis is well suited for genotyping *P. vivax*. For the PNG Institute of Medical Research in Papua New Guinea, we recommend use of the marker MS16, eventually in combination with microsatellite Pv3.27 or Pv1.501. As for fragment sizing by capillary electrophoresis, a maximum of 3 differently labeled PCR products (plus a size standard) can be combined [23], and no extra costs will occur when analyzing up to 3 markers. The probability that 2 independent infections carry the same alleles was estimated to be 0.15% when MS16 and Pv3.27 were analyzed in combination. If an even higher resolution is needed, the marker Pv.1.501 or *msp1*F3 can be included, further reducing the probability by a factor of almost 10 each. It remains to be shown whether these markers are suitable for genotyping in other geographic areas and, if so, which combinations are best. This easy to use and fast genotyping system can provide the basis for further investigations of the infection dynamics of *P. vivax*.

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Table 5. Multiplicity of infection (MOI) obtained by different genotyping markers for *Plasmodium vivax*.

MS16	3.27	1.501	*msp1*F3	*msp1*F1	3.502	*msp3α*	*msp4*	*msp5*	Maximal MOI of all markers
PCR finding									
ND	ND	1	1	1	1	1	1	1	1
ND	ND	1	1	1	1	1	2	1	1
1	1	1	1	1	1	1	1	1	1
ND	ND	1	1	–	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1
1	–	1	1	–	1	–	1	1	1
1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1
ND	ND	1	1	1	1	–	1	1	1
1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1
1	–	1	1	1	1	1	1	1	1
ND	ND	1	1	1	1	1	1	1	1
ND	ND	1	1	1	1	1	1	1	1
ND	ND	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1	1	2
2	1	1	2	1	1	1	1	1	2
1	1	2	2	2	2	1	2	2	2
2	2	1	2	2	1	2	1	1	2
ND	ND	2	1	–	1	1	2	1	2
2	1	2	1	1	1	1	2	2	2
1	1	2	2	2	1	2	1	1	2
ND	ND	1	1	1	1	2	1	1	2
1	1	1	2	2	1	2	1	2	2
2	2	2	2	2	2	2	1	1	2
2	1	1	1	1	1	1	1	1	2
1	1	2	2	1	2	2	1	1	2
ND	ND	2	2	2	1	1	2	1	2
2	1	1	2	–	1	1	1	1	2
2	1	1	1	1	1	1	1	1	2
2	2	1	2	1	2	1	2	1	2
1	1	1	2	1	1	2	1	1	2
2	1	1	2	1	1	1	1	1	2
1	1	1	1	2	1	1	1	1	2
1	2	1	1	2	2	2	2	1	2
1	2	1	1	1	1	1	1	1	2
2	2	2	2	2	2	2	1	2	2
2	1	1	1	1	1	1	2	1	2
3	2	2	2	1	2	2	1	1	3
ND	ND	2	2	3	2	1	1	2	3
2	1	1	3	1	1	1	1	1	3
3	1	1	1	1	1	1	2	1	3
3	1	2	2	2	1	1	1	1	3
3	2	2	3	1	3	2	2	2	3
3	2	5	2	2	2	2	3	2	3
3	2	2	2	1	1	2	2	2	3
1	1	1	2	2	1	3	1	1	3
3	1	1	1	1	1	1	2	1	3

(continued)

Table 5. ***(Continued.)***

MS16	3.27	1.501	*msp1*F3	*msp1*F1	3.502	*msp3α*	*msp4*	*msp5*	Maximal MOI of all markers
2	3	1	3	3	2	2	1	1	3
1	1	–	3	1	1	1	1	1	3
3	1	1	1	–	1	1	1	1	3
1	2	2	3	3	1	1	3	2	3
3	3	2	3	3	2	2	2	2	3
2	2	2	3	3	3	3	2	3	3
3	–	1	1	1	1	1	1	1	3
1	1	1	2	3	1	2	1	2	3
ND	ND	1	2	3	1	1	1	1	3
2	2	2	3	2	2	2	3	2	3
1	1	1	1	1	1	3	1	1	3
2	1	1	3	1	1	2	1	1	3
2	1	3	1	1	1	2	2	1	3
2	1	1	3	2	1	1	1	1	3
1	1	1	3	1	1	1	1	1	3
3	1	1	2	1	2	1	1	2	3
1	1	1	1	1	1	1	3	1	3
–	2	1	3	2	2	3	2	2	3
2	2	3	2	2	2	1	2	3	3
2	1	2	1	1	1	4	1	1	4
4	3	3	2	3	3	3	4	2	4
4	3	3	2	3	2	2	2	2	4
1	2	1	3	–	4	2	2	1	4
1	1	1	4	1	1	1	1	1	4
1	1	1	1	2	1	4	1	1	4
1	4	3	3	3	2	2	2	4	4
4	2	3	4	2	2	3	1	1	4
3	2	2	2	2	1	4	1	1	4
3	4	2	2	3	3	1	3	2	4
2	2	2	4	2	3	2	2	1	4
2	4	2	2	3	3	3	3	2	4
2	2	2	4	1	1	2	1	1	4
3	2	1	4	1	2	1	1	1	4
4	2	2	2	2	2	2	1	2	4
1	1	1	1	4	1	2	2	1	4
3	1	5	3	4	3	2	2	3	5
ND	ND	5	4	5	3	5	4	4	5
3	1	5	1	1	1	1	1	1	5
5	4	5	3	4	3	2	3	2	5
5	1	1	1	–	2	1	1	1	5
ND	ND	5	1	–	1	1	1	1	5
5	2	2	5	3	3	3	2	3	5
3	3	5	5	2	2	3	2	2	5
ND	ND	1	1	5	1	2	1	1	5
4	4	3	5	1	4	2	2	2	5
5	5	4	1	3	2	2	1	1	5
5	1	2	5	3	4	1	2	3	5
–	5	5	6	4	4	3	1	4	6
5	1	6	4	1	3	3	1	1	6
6	3	5	4	3	4	3	3	3	6
3	5	2	3	6	2	2	2	1	6
–	4	7	5	3	3	3	4	3	7
7	2	4	4	4	4	2	1	4	7
8	4	1	5	3	1	3	3	2	8
7	8	4	6	4	5	4	1	2	8

(continued)

Table 5. ***(Continued.)***

MS16	3.27	1.501	*msp1*F3	*msp1*F1	3.502	*msp3α*	*msp4*	*msp5*	Maximal MOI of all markers
Total clones, no.									
213	166	204	232	189	178	183	163	158	331
PCR-positive samples, no.									
90	90	107	108	100	108	106	108	108	108
MOI									
2.367	1.844	1.907	2.148	1.890	1.648	1.726	1.509	1.463	3.065

NOTE. ND, not done; −, PCR negative.