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Circumsporozoite Genotyping of Global Isolates of *Plasmodium vivax* from Dried Blood Specimens

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The prevalence and global distribution of two circumsporozoite (CS) genotypes of *Plasmodium vivax* (VK210 and VK247) were determined by genetic analysis of isolates from 234 malaria-infected patients. Whole blood specimens were collected on filter paper from patients infected with malaria in Thailand, Mexico, Papua New Guinea, Peru, Afghanistan (Pakistan), India, and western Africa and from 50 asymptomatic smear-negative controls. Following extraction of DNA from the filter paper samples, the CS gene was amplified by the polymerase chain reaction and genotyped by using oligoprobes specific for the VK210 and VK247 repeat epitopes. The sensitivity of genotyping from a single blood dot was 95.2%. The VK247 CS genotype was identified in the blood of patients from all seven study areas and was the predominant form present in samples from Thailand (83%) and Papua New Guinea (90%). In contrast, VK247 DNA was present in only 9% of isolates from Mexico. Individuals infected with both genotypes simultaneously were identified in all study areas except Mexico and were particularly common in Thailand (58%) and Papua New Guinea (60%). These findings indicate that the VK247 genotype of *P. vivax* is widely distributed but that its prevalence varies geographically. In addition, we conclude that use of samples of whole blood on filter paper is a practical and sensitive method for determining the genotypes of large numbers of malaria isolates collected in field settings.

The main focus of recombinant vaccine development for *Plasmodium vivax* has been on the central immunodominant repeat region of the circumsporozoite (CS) protein (7) that until recently was believed to be invariant within that species (14). Progress on a vaccine has recently been complicated by the observation of polymorphism within the CS gene and the protein that it encodes (12). Fourteen percent of patients in a remote area of western Thailand were observed to be infected with a variant strain of *P. vivax* (VK247) that produces a CS protein with a repeat unit (ANGAGNQPG) distinct from that found in the predominant strain VK210 (GDRAA/DGQPA). Subsequent vaccine design and efficacy will depend, at least in part, on the prevalence and global distribution of infection with these forms of *P. vivax*. However, little is known regarding the frequency and distribution of natural infections with the VK210 and VK247 genotypes of *P. vivax*. Studies to determine the prevalence and global spread of infections caused by these different strains of *P. vivax* are complicated by the inability to maintain this parasite in culture and by the difficulty in acquiring from field sites sufficient *P. vivax* DNA for genetic analysis. Previous studies of antigenic diversity in *P. vivax* CS protein used cumbersome xenodiagnostic methods (12), maintenance of *P. vivax* strains in monkeys (1), or amplification of the CS gene from purified genomic DNA and then cloning and sequencing (11). None of these methods is appropriate for large epidemiologic surveys in the field. Seroepidemiologic studies by enzyme immunoassays and immunofluorescent antibody detection assays have been reported as an alternative approach for determination of the distribution of the polymorphic forms of the CS protein (4, 13). However, at present these methods are of limited sensitivity (2, 10) and questionable specificity for the VK247 form of *P. vivax* (4). In addition, they give little or no information about current or mixed infections.

We recently reported a rapid method for determination of genetic variation within *Plasmodium* sp. by using enzymatically amplified DNA from dried blood spot specimens collected in the field and used it to analyze small numbers of malaria-infected patients (8). This approach offers many advantages, including ease of collection and transportation, stability of samples, economy of sample volume, little biohazard risk, and centralization and batching of specimens for processing and analysis (3, 8). In addition, the antibodies eluted from companion dots can be used to examine humoral immune responses to the infecting strain of *P. vivax* (10).

The objectives of the present study were to (i) validate the practicality and sensitivity of using dried blood dot specimens to determine the CS genotype (VK210 or VK247) from epidemiologically relevant numbers of *P. vivax* samples collected primarily in the field and (ii) to determine the relative frequency of infections with the two CS genotypes of *P. vivax* in large numbers of consecutively studied patients from different geographic locations.

Study population. The study sites were chosen so that

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geographically diverse regions that are endemic for *P. vivax* were included. Patients presenting with *P. vivax* malaria between September 1990 and May 1991 to the (i) Armed Forces Research Institute of Medical Sciences Medical Research Unit on the Thai-Cambodian border; (ii) Centro de Investigacion de Paludismo in Tapachula, Mexico; (iii) Gaubin Hospital, Karkar Island, or Madang General Hospital, Papua New Guinea; (iv) Naval Medical Research Institute in Lima, Peru; (v) Afghanistan Refugee Camp in Peshawar, Pakistan; and (vi) Tropical Disease Unit, Toronto General Hospital, were eligible for entry into the study. In addition, patients from Thailand and Papua New Guinea with *Plasmodium falciparum* and *Plasmodium malariae* infections were included as controls. During this period, 234 malaria-infected individuals and 50 asymptomatic smear-negative controls were analyzed. The infections were acquired on four continents and two subcontinents in which *P. vivax* is endemic. Two hundred twenty-nine of the study subjects (98%) were residents of the study areas; the remaining 5 individuals (2%) acquired infection while traveling in the regions identified above (India, $n = 3$; Africa, $n = 2$). Study subjects from Thailand were members of the Royal Thai Army and were part of a larger *P. falciparum* vaccine efficacy study. Soldiers were eligible for inclusion in the present study if they developed *P. vivax* malaria. All episodes of *P. vivax* malaria that occurred in these individuals during the study period were included in the present analysis. Cases from regions other than Thailand represented consecutive patients studied. Twenty-four of the cases in the present study have been reported previously (10).

Specimen handling. Blood (25 μl) was collected by fingerprick or venipuncture from subjects with smear-positive *P. vivax*, *P. falciparum*, or *P. malariae* and from healthy controls. Blood was blotted in triplicate onto Whatman 3M chr paper (Whatman, Maidstone, United Kingdom). Samples were air dried, individually wrapped in plastic bags, and shipped by regular airmail to the Walter Reed Army Institute of Research. Each sample was processed for enzymatic amplification of the CS gene as described previously (8, 9). Plasmodial DNA was released from filter paper samples and was amplified by the polymerase chain reaction (PCR) (8). A limited number of specimens were diluted 1:10 (with distilled sterile H_2O), and PCR was repeated.

Slot blot and oligoprobe hybridization. Ten microliters of each PCR mixture was subjected to electrophoresis in a 0.8% agarose gel. The predicted and observed amplification product sizes varied between 657 and 711 bp, depending on the number of repeats present within the CS gene (12). Ten microliters of each PCR product was slot blotted in triplicate onto a nylon membrane according to the instructions of the manufacturer (Schleicher & Schell, Inc., Keene, N.H.). The membrane-bound DNA fragments were hybridized with ^{32}P-end-labeled oligoprobes, VK210 and VK247 and PV9 (5′-GCATGCATCCGCCAACGGTAGCTCTAACTTT-3′), as described previously (8). VK210 and VK247 are complementary to the predominant and variant forms, respectively, of the CS gene (12). PV9 is a constant region probe that is complementary to a conserved region of the CS gene (12). Membranes were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and once with 0.1× SSC–1% sodium dodecyl sulfate at 50°C. Filters were exposed to radiographic film with intensifying screens for 2 to 6 h at −80°C.

Statistical analysis. Numeric data were analyzed by using a two-tailed chi-square test with Yates' correction or Fisher's exact test, as appropriate.

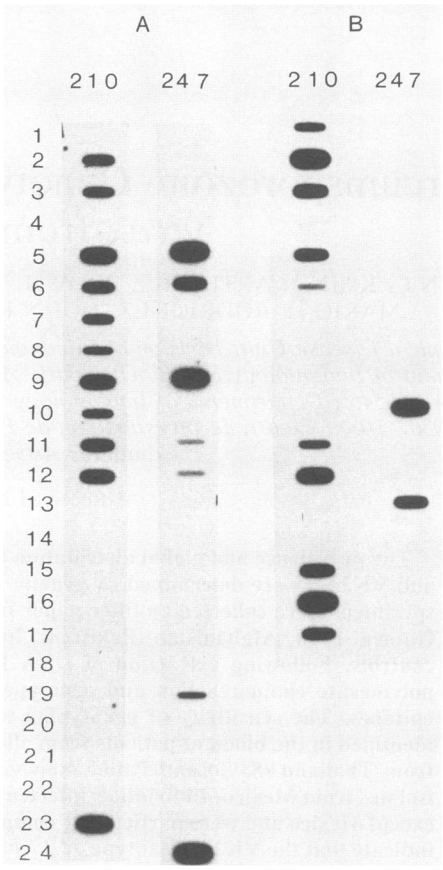

FIG. 1. Representative results of PCR-oligoprobe hybridization for the VK210 and VK247 CS genotypes from Thailand (A) and Mexico (B). The numbers 1 to 24 refer to individual patients or controls. (A) Patients 5, 6, 9, 11, 12, and 23 were from Thailand and were coinfected with both the VK210 and VK247 strains of *P. vivax*; patients 1 and 14 to 21 were from Thailand and were infected with *P. falciparum*; patient 19 had a mixed *P. vivax* and *P. falciparum* infection. Patients 4, 13, and 22 were negative controls. (B) Patients 10 and 13 had pure infections with VK247; none of the Mexican patients had mixed infections; patients 4, 7, 9, and 14 were negative controls; patient 8 was positive by smear but negative by PCR.

Filter paper samples from 234 patients with smear-positive malaria (*P. vivax* [$n = 188$], *P. falciparum* [$n = 43$], and *P. malariae* [$n = 3$]) and 50 controls without malaria were analyzed for the presence of *P. vivax* VK210 or VK247 CS DNA. Representative results are shown in Fig. 1. Malaria infections were acquired in Peru ($n = 7$), Thailand ($n = 106$ with *P. vivax*; $n = 32$ with *P. falciparum*), India ($n = 3$), Mexico ($n = 57$), Papua New Guinea ($n = 10$ with *P. vivax*; $n = 11$ with *P. falciparum*; $n = 3$ with *P. malariae*), Africa ($n = 2$), and Afghanistan (Pakistan) ($n = 3$). In addition, asymptomatic smear-negative controls from Thailand ($n = 20$), Papua New Guinea ($n = 5$), and Washington, D.C. ($n = 25$) were analyzed for the presence of *P. vivax* DNA.

Overall, of the 188 smear-positive cases of *P. vivax*, 179 (95.2%) were probe positive for VK210, VK247, or both when a single blood dot was analyzed (Table 1). The nine negative specimens were reanalyzed by using a 1/10 dilution of a new blood dot extract. Consequently, 2 additional samples became positive, permitting 96.3% (181 of 188) of the samples to be genotyped. VK247 DNA was identified in

TABLE 1. CS genotyping of *P. vivax* isolates

Location	No. of isolates	No. (%) of isolates of the following genotype:			
		VK210	VK247	Mixed	None
Thailand	106	13	26	62	5
Mexico	57	48	5		4
Papua New Guinea	10	1	3	6	
Peru	7	2		5	
India	3	1		2	
Afghanistan (Pakistan)	3	1	1	1	
Africa	2			2	
Total	188	66 (35)	35 (19)	78 (41)	9 (5)

60% (113 of 188) of *P. vivax* cases, and VK210 DNA was identified in 77% (144 of 188) of *P. vivax* cases. The VK247 variant was found in individuals from all regions analyzed, although the percentage of cases associated with the VK247 strain either alone or in combination with VK210 varied geographically. Eighty-three percent (88 of 106) of *P. vivax* infections from Thailand were associated with VK247 DNA, whereas 71% (75 of 106) were associated with VK210 DNA ($P = 0.05$). In Papua New Guinea, 9 of 10 infections were associated with VK247, whereas 7 of 10 were associated with VK210 (*P* was not significant). In both regions, mixed infections were common (58% in Thailand; 60% in Papua New Guinea). In contrast, the VK247 CS genotype was infrequently identified in Mexican cases of *P. vivax* (5 of 57 versus 88 of 106 for Thailand [$P < 0.0001$] and 9 of 10 for Papua New Guinea [$P < 0.0001$]), and mixed infections were not observed (0 of 57 versus 62 of 106 infections for Thailand [$P < 0.0001$] and 6 of 10 infections for Papua New Guinea [$P < 0.0001$]).

Forty-six cases of smear-positive *P. falciparum* or *P. malariae* infections from individuals in Thailand or Papua New Guinea were also analyzed for the presence of *P. vivax* DNA. One case of *P. falciparum* from Thailand was positive for both VK210 and VK247 DNA. This slide was reviewed, and after extensive examination, trophozoites of *P. vivax* were observed. None of 50 negative control blood samples was positive for either VK210 or VK247.

The results of this study confirm and extend our previous observations that the VK247 variant of *P. vivax* is globally distributed and frequently occurs as a mixed infection with the VK210 genotype (8). In addition, the findings from our analysis of 284 samples validate the sensitivity and practicality of genotyping large numbers of malaria isolates from field sites by using whole blood-impregnated filter paper discs. This approach has proven to be particularly useful for genetic analysis of *P. vivax*, which cannot be propagated in culture.

The VK247 CS variant was identified in individuals from all seven collection sites in this study. In the majority of cases in Thailand and Papua New Guinea, VK247 DNA was present either alone or as part of a mixed infection with the predominant VK210 form. In contrast to Thailand and Papua New Guinea, the VK247 genotype was infrequent and mixed infections were not observed in Mexican cases. Our observation of geographic differences in the proportion of cases associated with the VK247 genotype is supported by recent sequence data on *P. vivax* isolates from Brazil and Papua New Guinea (11). In that study, the VK247 repeat sequence predominated in isolates from Papua New Guinea; however, the VK210 sequence was more prevalent in Brazilian isolates. While the reasons for this global variation are unknown, they may reflect differences in transmission intensity in these regions, variability in vector competence for the different genotypes of *P. vivax*, or variations in the times that new genotypes entered or emerged in new regions (6).

Our findings are also in agreement with several serologic studies in which the distribution of the 247 strain in wild-type *P. vivax* infections was examined (4, 13). Unlike serologic studies, however, genotyping by PCR and oligoprobe hybridization provides direct information about the current infecting strain and the presence of mixed infections and is more sensitive than serologic assays performed in parallel on a companion blood dot (10). Since results of all previous studies and our own study agree that the VK247 genotype is widely distributed and frequently detected, we propose that the "predominant" and "variant" designations for VK210 and VK247, respectively, be dropped.

In this study, the sensitivity of a single blood dot for CS genotyping was 95.2%. On the basis of the thick smears, there were nine false-negative samples. Potential explanations for these negative findings include the following: a new CS variant that is not recognized by the VK210 or VK247 probes may have been present, the microscopic species diagnosis may have been wrong, the level of parasitemia may have been below the limit of detection by the filter paper technique, there may have been a technical error in collecting or processing samples, or an inhibitor to enzymatic amplification may have been present in the samples. We attempted to address some of these possibilities by repeating the extraction and amplification procedures on a new blood dot collected at the same time that the original one was. It seemed unlikely that the level of parasitemia alone (range 610 to 16,006/mm^3) was responsible for the false-negative results, because we routinely amplified parasitemias of this level. Two of the five Thai samples became positive after dilution and repeat boiling, suggesting, as have others (5), that an undefined inhibitor of *Taq* polymerase may be present in some patient samples. We also probed each sample with a CS constant-region probe in order to detect the presence of a new variant in the remaining cases. None was reactive with the constant-region probe.

On the basis of the findings of this and other studies (4, 11–13), the current VK210-based CS vaccine, even with improved immunogenicity, would likely be of limited efficacy throughout the world. However, recent data indicate that polymorphism within the CS protein appears to be limited (11). Therefore, if this proves to be true, a *P. vivax* CS repeat vaccine which includes the VK210, VK247, and any other variant CS epitopes may still be effective.

In summary, in this study we used filter paper samples containing whole blood from 234 malaria-infected patients from around the world to genotype field isolates of *P. vivax*. We confirmed and extended our earlier work which indicated that the VK247 CS genotype is global in distribution and is frequently associated with *P. vivax* infection. In addition, we validated the sensitivity and practicality of this technique for the analysis of genetic heterogeneity of large numbers of field isolates. The question of the biologic significance of the two different CS genotypes of *P. vivax* remains to be answered.

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