SEROLOGIC AND GENETIC CHARACTERIZATION OF PLASMODIUM VIVAX FROM WHOLE BLOOD-IMPREGNATED FILTER PAPER DISCS

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Abstract. The presence in the New World of a variant strain of Plasmodium vivax (VK247) containing a unique circumsporozoite (CS) repeat domain, was determined by the detection of antibodies to the variant CS protein and by genetic analysis of the CS gene from field isolates. Whole blood specimens were collected on filter paper from patients infected with P. vivax in Mexico and Peru. Plasmodium vivax DNA was eluted from filter paper samples and the CS gene was amplified by the polymerase chain reaction (PCR) and analyzed for the presence of VK247 or VK210 DNA by oligoprobe hybridization. Sera eluted from a companion filter paper sample were screened for antibodies reactive with the predominant and variant repeat peptides by enzyme-linked immunosorbent assays (ELISA) and with sporozoites by the immunofluorescent antibody (IFA) test. All 24 patients were positive by PCR and oligoprobe hybridization for either VK210 (16 of 24), VK247 (3 of 24), or both (5 of 24). Mixed infections were common (5 of 7) in Peru, but not observed in the Mexican isolates (0 of 17). All three VK247 infections from Mexico occurred in residents of the foothills above Tapachula (P = 0.02). Of patients with smear-positive P. vivax infection, 42% (10 of 24) had detectable antibodies eluted from dried blood dots that were reactive with the CS protein by IFA or ELISA. These findings establish the widespread distribution of the P. vivax variant CS protein in the New World and indicate that dried blood filter paper samples represent a valuable source of material for the serologic and molecular analysis of plasmodial infections.

The predominant surface or circumsporozoite (CS) protein of malaria sporozoites is a major target for the development of an effective vaccine. In Plasmodium vivax, the main focus of recombinant vaccine development has been on the central immunodominant repeat region (GDRRAA/DGQPP), which was believed to be invariant within this species. Recently, a strain of P. vivax (VK247) from western Thailand has been described with a variant repeat domain (ANGAGNQPG) that is not recognized by any of the available CS monoclonal antibodies. The prevalence of infection and worldwide distribution of this variant will have implications for the efficacy of current vaccines based on the predominant repeat domain. A rapid and simple method to determine the extent of infection of VK247 would be of great value. Seroproteanologic studies demonstrating antibodies against the predominant and variant CS repeats by both immunofluorescent antibody (IFA) and enzyme-linked immunosorbent assays (ELISA) have been reported as one approach to determine the distribution of this P. vivax variant. In order to assess the usefulness of these approaches for the characterization of P. vivax infections, we compared the serologic detection of VK247 and VK210 infection by IFA and ELISA with that of CS gene amplification and oligoprobe hybridization from whole blood–impregnated filter paper samples.

MATERIALS AND METHODS

Study population

Two geographically separate locations were chosen for analysis of CS variation in the New World. All study subjects were residents of these regions. One study site was in Tapachula, Mexico
located near the Guatemalan border, and the other was in Lima, Peru. Patients presenting with
P. vivax malaria to the Centro de Investigacion de Paludismo in Tapachula or referred to the
Naval Medical Research Institute Detachment in Lima between September 1990 and February
1991 were eligible for entry into the study. During this period, 17 individuals from the Mexican
site and seven from the Peruvian site were analyzed. These cases represented consecutive pa-

tients studied, and they were not selected from a larger study population. Subjects from the
Mexican site resided in either a coastal region or in the neighboring foothills. Cases from Peru
included infections acquired in both coastal and jungle regions of Peru.

Specimen handling

Blood (20 µl) was collected by finger prick or
by venipuncture from subjects with smear-posi-
tive P. vivax malaria and from healthy controls,
and spotted in triplicate onto Whatman (Hills-
boro, OR) 3M chromatography paper. Filter paper
samples were air dried, individually placed
in plastic bags, and shipped by regular air mail
at room temperature to the Walter Reed Army
Institute of Research (WRAIR). Samples were
processed within 1–3 months of arrival at
WRAIR. One filter paper sample from each case
was eluted at 4°C overnight in 400 µl of phos-
phate-buffered saline with 0.5% bovine casein and
0.1% Tween 20. These 1:20 dilutions were
stored at −20°C until assayed by ELISA and IFA.

A second filter paper sample from each case
was processed for enzymatic amplification of the
CS gene as previously described. Briefly, each
sample was excised and added to 180 µl of a 5%
weight/volume (w/v) Chelex-100 (Bio-Rad,
Richmond, CA) solution that was preheated to
100°C. Plasmodium vivax DNA was released from
the filter paper by vortexing (30 sec) and
boiling (10 min). The samples were centrifuged
(12,000 × g for 1.5 min) and the supernatant
was removed and centrifuged again. The super-
natant was then stored at 4°C, for up to four
months, until amplified by PCR. Both the se-
rologic and the amplification analysis was per-
formed in a blinded fashion.

Indirect immunofluorescence assay

Plasmodium vivax sporozoites of the predom-
inant (VK210) CS protein strain were produced
for IFA by membrane feeding Anopheles step-
henisi mosquitoes on blood from a splenectom-
ized chimpanzee (Pan troglodytes) infected with
the Salvador 1 isolate. Variant (VK247) CS pro-
tein strain sporozoites were produced by feeding
laboratory-reared An. dirus mosquitoes on in-
fected Thai men, as described by Rosenberg and
others. Salivary gland sporozoites were pro-
cessed and IFAs were conducted as described by
Wirtz and others, using fluorescein-labeled anti-
human IgG (heavy and light chain) secondary
antibody. The limited availability of variant spo-
rozotes permitted the testing of only selected
serum extracts. Strain-specific mouse monoe-

clonal antibodies (R. A. Wirtz and others, un-
published data) reacted only with the homologous
sporozoite, as did Thai positive control sera.

Enzyme-linked immunosorbent assay

The ELISAs for detection of anti-CS antibody
were conducted as described by Wirtz and oth-
ers. The capture antigens used were the P. vivax
recombinant CS protein vaccine NSIV20, which
contains the predominant CS protein repeat
(GDRAA/PQGO/A), and a synthetic peptide of
the variant repeat (ANGAGNQPC), that was
glutaraldehyde-conjugated to bovine casein.
Samples were analyzed in triplicate and all ex-
periments were repeated.

Amplification of the CS gene

The DNA released from the filter paper sam-
ples was subjected to amplification in a thermal
cycler (Perkin Elmer Cetus, Norwalk, CT). Five
microliters of supernatant from each processed
sample was added to an amplification mixture
(50 mM KCl, 10 mM Tris, pH 8.0, 0.01% (w/v)
gelatin, 3 mM MgCl2, 400 µM dNTPs, and 2.5
units of AmpliTaq (Perkin Elmer Cetus) contain-
ing the oligonucleotide primers (50 pmoles
each) PVS and PV6 (Table 1) complementary to
the conserved regions I and II of the P. vivax CS
gene. The reaction mixture was initially dena-
tured at 95°C for 5 min, followed by 45 cycles of
amplification (94°C for 30 sec, 37°C for 30 sec,
and 72°C for 2 min). Positive and negative blood
samples were included with each amplification
assay. Duplicate samples were analyzed and all
experiments were repeated. To prevent cross-
contamination, separate pieces of equipment and
rooms were used for the preparation of samples
and the handling of amplified products.
Slot-blot and oligoprobe hybridization

Ten microliters of each PCR reaction mixture were fractionated electrophoretically on a 0.8% agarose gel, and the amplified products were visualized under ultraviolet (UV) light after staining with ethidium bromide. Ten microliters of each PCR reaction mixture was slot-blotted in triplicate onto a nylon membrane according to the manufacturer's (Schleicher & Schuell, Keene, NH) instructions, and cross-linked with UV light using the auto-link mode on a Stratallinker (Stratagene, La Jolla, CA). The DNA fragments on the membrane were hybridized with a P-end-labeled internal oligoprobes VK210 and VK247 (Table 1), which are complimentary to the predominant and variant form, respectively, of the *P. vivax* CS gene. The membranes were washed with 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) at room temperature, and with 0.1× SSC containing 1% sodium dodecyl sulfate at 50°C. The membranes were then exposed to radiograph film with intensifying screens for 2–14 hr at −80°C.

**RESULTS**

**Genetic analysis of the CS gene from *P. vivax* isolates**

In all 24 cases of *P. vivax infecion* analyzed from Mexico and Peru, the CS gene was successfully amplified from filter paper samples. All patients studied were positive for both VK210 and VK247, or both (Table 2 and Figure 1). The parasitemias, as determined from stained smears, ranged from 1.600 to 13,280/mm³ in Mexico and from 79 to 37,920/mm³ in Peru (Table 2). Five of the seven cases (71%) from Peru were mixed infections, with both VK210 and VK247 identified by oligoprobng the amplified CS gene. In two cases, only VK210 DNA was identified. When compared with cases of VK210 *P. vivax* infection, there were no associations noted between the presence of VK247 DNA and the level of parasitemia, age or sex of the subjects, the area of acquisition of infection, unique symptoms, or the presence of unusual morphology on stained smears.

In contrast, Peruvian isolates, all cases from Mexico were pure infections. Three of 17 cases (18%) were positive with the VK247 probe and the remaining 14 (82%) were positive only with the VK210 CS probe. All three infections with VK247 occurred in residents of the foothills (3 of 6 versus 0 of 11; P < 0.029, by Fisher's exact two-tailed test). Infections with VK247 occurred in a younger age group than that observed with VK210 (mean 11 years versus 24.5 years). In cases from Peru, no unusual symptoms or distinctive morphology on stained smears was reported for VK247 infections from Mexico. None of the eight negative control blood samples was positive for either VK210 or VK247.

**Circumsporozoite protein antibody responses in *P. vivax*-infected patients**

An ELISA was used to screen sera eluted from filter paper samples for reactivity with the predominant (VK210) and variant (VK247) CS epitopes. Overall, 35% (6 of 17) of the Mexican sera and 57% (4 of 7) of the Peruvian sera reacted with either the VK210 or the VK247 repeat domains. In patients with VK210 DNA detected by PCR, 43% (9 of 21) of these patients showed detectable antibody responses to the predominant CS repeat domain. In contrast, only 13% (1 of 8) patients infected with the variant strain of *P. vivax* had antibodies reactive with the VK247 variant peptide.

In all cases examined, there was agreement between PCR/oligoprobe hybridization results and serologic data. Of the nine patients with antibodies reactive with VK210 polypeptide, all had VK210 DNA identified by the PCR/oligoprobe. Similarly, in the one individual with sera reactive with the variant peptide, VK247 DNA was amplified from the filter paper sample.

Most sera (7 of 10) that reacted with the variant or the predominant repeat proteins in the
Table 2

Summary of genetic analysis (polymerase chain reaction [PCR]/oligoprobing) and serologic response (enzyme-linked immunosorbent assay [ELISA] and immunofluorescent antibody [IFA] test) in Plasmodium vivax infections from the New World

<table>
<thead>
<tr>
<th>Patient age/sex</th>
<th>Location</th>
<th>Parasitemia (parasites/μL)</th>
<th>ELISA*</th>
<th>IFAT*</th>
<th>Probed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>VK130</td>
<td>VK247</td>
<td>VK120</td>
<td>VK247</td>
</tr>
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<table>
<thead>
<tr>
<th>Group 1: Mexico</th>
<th></th>
<th></th>
<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 (19/M)</td>
<td>Foothills</td>
<td>8,800</td>
<td>0.05</td>
<td>0.00</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>M2 (11/F)</td>
<td>Foothills</td>
<td>-104</td>
<td>0.09</td>
<td>0.01</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>M3 (50/F)</td>
<td>Coast</td>
<td>9,000</td>
<td>0.00</td>
<td>0.00</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>M4 (8/M)</td>
<td>Coast</td>
<td>ND?</td>
<td>0.26</td>
<td>0.00</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>M5 (10/M)</td>
<td>Coast</td>
<td>ND</td>
<td>0.00</td>
<td>0.01</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>M6 (33/F)</td>
<td>Foothills</td>
<td>13,280</td>
<td>0.03</td>
<td>0.00</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>M7 (45/M)</td>
<td>Coast</td>
<td>2,400</td>
<td>0.22</td>
<td>0.01</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>M8 (13/M)</td>
<td>Foothills</td>
<td>2,200</td>
<td>0.03</td>
<td>0.01</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M9 (22/M)</td>
<td>Coast</td>
<td>4,500</td>
<td>0.03</td>
<td>0.02</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M10 (17/F)</td>
<td>Coast</td>
<td>1,600</td>
<td>0.11</td>
<td>0.00</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>M11 (12/F)</td>
<td>Coast</td>
<td>5,760</td>
<td>0.10</td>
<td>0.00</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>M12 (19/F)</td>
<td>Coast</td>
<td>&gt;10§</td>
<td>0.06</td>
<td>0.00</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>M13 (9/M)</td>
<td>Foothills</td>
<td>9,200</td>
<td>0.00</td>
<td>0.67</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>M14 (27/F)</td>
<td>Coast</td>
<td>2,480</td>
<td>0.16</td>
<td>0.02</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>M15 (39/F)</td>
<td>Coast</td>
<td>&gt;10§</td>
<td>0.19</td>
<td>0.00</td>
<td>+2</td>
<td>NT</td>
</tr>
<tr>
<td>M16 (32/F)</td>
<td>Coast</td>
<td>2,600</td>
<td>0.02</td>
<td>0.00</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>M17 (10/M)</td>
<td>Coast</td>
<td>2,800</td>
<td>0.23</td>
<td>0.05</td>
<td>+1</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 2: Peru</th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 (26/M)</td>
<td>Jungle</td>
<td>11,769</td>
<td>0.04</td>
<td>0.02</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>P2 (19/M)</td>
<td>Jungle</td>
<td>37,920</td>
<td>0.04</td>
<td>0.02</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>P3 (22/M)</td>
<td>Jungle</td>
<td>6,518</td>
<td>0.71</td>
<td>0.04</td>
<td>+3</td>
<td>-</td>
</tr>
<tr>
<td>P4 (55/M)</td>
<td>Coast</td>
<td>14,400</td>
<td>0.78</td>
<td>0.00</td>
<td>+1</td>
<td>-</td>
</tr>
<tr>
<td>P5 (32/M)</td>
<td>Jungle</td>
<td>78</td>
<td>0.22</td>
<td>0.00</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>P6 (21/M)</td>
<td>Jungle</td>
<td>20,000</td>
<td>0.10</td>
<td>0.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P7 (21/M)</td>
<td>Jungle</td>
<td>16,000</td>
<td>0.18</td>
<td>0.03</td>
<td>+1</td>
<td>-</td>
</tr>
</tbody>
</table>

Positive controls (n = 210) | >2.0 | - | + |
Positive controls (n = 247) | >2.0 | - | + |

* Optical density values at 414 nm (OD414) for 1:20 dilutions of sera diluted from filter paper disks. Results are the mean of absorbance (OD) in antigen containing wells - OD in antigen without antigen. A positive ELISA result was defined as a net absorbance ≥0.15.

† Values for 1:20 dilutions of sera diluted from filter paper disks. Fluorescence levels were graded from negative (−) to +*, with negative values corresponding to background fluorescence and +* to intense uniform fluorescence over the entire sporozoite. NT = not tested.

* ND = not determined.

ELISA also gave a positive fluorescence with homologous P. vivax variant or predominant sporozoites, respectively (Table 2).

**DISCUSSION**

The determination of genetic heterogeneity within plasmodial isolates and the characterization of immune responses in infected individuals are important tools in understanding the complex relationship between host and parasite, and in facilitating the development of effective vaccines against malaria. The results of the present study indicate that dried blood filter paper specimens are a practical source of material for both serologic and molecular analysis of P. vivax infections.

Using enzymatic amplification of P. vivax DNA released from filter paper samples, we observed several interesting findings. First, we report here for the first time the detection of the VK247 variant in Mexico, supporting previous observations that the VK247 variant of P. vivax is present in the New World. Second, P. vivax infections in Peru were frequently mixed, with both the predominant and the variant forms present simultaneously. In five of seven cases studied, patients had both VK247 and VK210
DNA identified in a single filter paper sample. In contrast, mixed VK210/VK247 infections were not observed in Mexico. While the reasons for these geographic differences are not clear, it is interesting to speculate that a lower prevalence of infection in Mexico may reduce the opportunity for vectors to acquire and transmit mixed infections. The presence of VK247 in Mexico and Peru suggests that a vaccine based on the predominant repeat domain may select for the variant, particularly in patients with mixed infections. Finally, a significant association was noted between VK247 infection and residence in the foothills near Tapachula, Mexico. Three of six infections acquired in the foothills were caused by VK247. In contrast, none of 11 infected residents of the coastal region had VK247 DNA detected. Different vectors have been associated with P. vivax transmission in the coastal areas (Anopheles albimanus) versus the foothills (An. pseudopunctipennis).* Studies are underway to explore the intriguing possibility that the variant form of P. vivax may be preferentially transmitted by An. pseudopunctipennis in the foothills in this region of Mexico.

An alternative method to determine the global distribution of VK247 infections is to demonstrate humoral responses to the variant CS protein in sera from P. vivax-endemic areas. The availability and ease of automation of ELISAs makes them an ideal screening technique for this purpose. However, we found, as have others, that the ELISA is relatively insensitive in the detection of infection with the variant form of P. vivax. Only 42% of patients with smear-positive P. vivax infections had detectable antibodies to the CS protein, and only one of eight (13%) of VK247-infected individuals, as determined by CS gene amplification, had antibodies to the variant peptide. The sensitivity of the ELISA in this study might have been improved by the collection and analysis of paired serum samples. However, in a recent study by Brown and others, only 62% (16 of 26) of patients who were smear-positive for P. vivax had detectable antibodies against the CS protein, even when multiple sera samples were examined. In addition, the speci-
ficity of the ELISA using the variant peptide is uncertain. Cochrane and others recently reported that 7% (18 of 256) of sera from the Brazilian Amazon reacted with the variant CS repeat by ELISA. However, 72% (13 of 18) of the sera samples cross-reacted with \textit{Plasmodium} or \textit{P. malariae}. Preabsorbing the sera with \textit{P. brasilianum} repeat peptide completely abolished reactivity with the variant \textit{P. vivax} sporozoites. In this study, as in others, IFA results generally paralleled those of the ELISA, but its sensitivity was even lower. Clearly, problems with specificity and requirements for multiple patient samples for improved sensitivity limits the use of serologic detection alone for the characterization of CS variants in field studies.

In contrast to the ELISA and IFA, we found that PCR amplification of plasmodial DNA from filter paper was a sensitive method in characterizing current and mixed \textit{P. vivax} infections. All 24 smear-positive patients were positive by PCR/oligoprobe hybridization analysis. In addition, the use of filter paper samples made the collection and transportation of field samples practical, eliminating the need for refrigeration, sample shipping requirements, and lowering the biological hazard associated with shipping blood products.

Although serologic detection of \textit{P. vivax} from filter paper samples was not optimal, the ability to perform serologic and genetic analysis from the same easily collected and transported samples represents a unique opportunity to examine the immune responses of individuals to their invading parasite strain(s). For \textit{P. falciparum}, serologic analysis of patients infected with wild isolates is frequently performed against long-term cultured strains. While this allows some degree of standardization, laboratory isolates raised without immune pressure may bear little relationship to wild-type strains. In addition, many laboratory strains are cloned isolates, whereas we have recently shown that patients are frequently infected with mixed populations of parasites. Similarly, genetic analysis of cultured isolates may be misleading. Deletions of important genes such as the cytoadherence ligand for CD36 may be lost in cultured isolates of \textit{P. falciparum}. The inability to maintain \textit{P. vivax} in long-term culture makes its analysis even more difficult. The ability to use filter paper samples for both antibody and PCR analysis provides an opportunity to address these issues. It is now possible to characterize the parasite strain(s) of an individual in light of their pre-existing immune status. This could have particular importance in the evaluation of breakthroughs postimmunization with plasmodial vaccine candidate antigens. Dried blood filter paper samples are a practical method to determine whether vaccine failure is secondary to infection with new variants, or is due to suboptimal immune responses to the vaccine at the time of infection. This evaluation is currently possible only through tedious procedures for extracting plasmodial DNA from whole blood, which are not ideal for large-scale epidemiologic studies.

In summary, in this study we compared the usefulness of ELISA, IFA, and enzymatic amplification for the detection of VK247 and VK210 infection in the New World. Using whole blood filter paper samples from 24 infected patients, only CS gene amplification and oligoprobe hybridization were sufficiently sensitive to allow characterization of all isolates. Although the ELISA and IFA were less sensitive, the ability to determine humoral responses from the same samples used for genetic analysis is clearly advantageous and may help define the complex relationship between parasite and host immune responses.

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