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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 were 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 (GDRAA/DGQPA),[1] which was believed to be invariant within this species.[2] 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.[3] 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.[1] A rapid and simple method to determine the extent of infection of VK247 would be of great value. Seroepidemiologic 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.[4, 5] We recently described an alternative approach based on genetic analysis of the CS gene that was amplified from whole blood–impregnated filter paper samples by the polymerase chain reaction (PCR).[6] 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 patients 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 fingerprick or by venipuncture from subjects with smear-positive *P. vivax* malaria and from healthy controls, and blotted in triplicate onto Whatman (Hillsboro, 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 phosphate-buffered saline with 0.5% boiled casein and 0.1% Tween 20.[5–7] 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.[6] 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 supernatant was then stored at 4°C, for up to four months, until amplified by PCR. Both the serologic and the amplification analysis was performed in a blinded fashion.

Indirect immunofluorescence assay

Plasmodium vivax sporozoites of the predominant (VK210) CS protein strain were produced for IFA by membrane feeding *Anopheles stephensi* mosquitoes on blood from a splenectomized chimpanzee (*Pan troglodytes*) infected with the Salvador 1 isolate. Variant (VK247) CS protein strain sporozoites were produced by feeding laboratory-reared *An. dirus* mosquitoes on infected Thai men, as described by Rosenberg and others.[3] Salivary gland sporozoites were processed and IFAs were conducted as described by Wirtz and others,[7] using fluorescein-labeled anti-human IgG (heavy and light chain) secondary antibody. The limited availability of variant sporozoites permitted the testing of only selected serum extracts. Strain-specific mouse monoclonal antibodies (R. A. Wirtz and others, unpublished data) reacted only with the homologous sporozoite, as did Thai positive control sera.[3]

Enzyme-linked immunosorbent assay

The ELISAs for detection of anti-CS antibody were conducted as described by Wirtz and others.[5] The capture antigens used were the *P. vivax* recombinant CS protein vaccine NS1V20, which contains the predominant CS protein repeat $(GDRAA/DGQPA)_{20}$, and a synthetic peptide of the variant repeat $(ANGAGNQPG)_3$ that was glutaraldehyde-conjugated to boiled casein. Samples were analyzed in triplicate and all experiments were repeated.

Amplification of the CS gene

The DNA released from the filter paper samples 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 $MgCl_2$, 400 μM dNTPs, and 2.5 units of Amplitaq [Perkin Elmer Cetus]) containing the oligonucleotide primers (50 pmoles each) PV5 and PV6 (Table 1) complimentary to the conserved regions I and II of the *P. vivax* CS gene.[3] The reaction mixture was initially denatured 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 electrophorectically 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 Stratalinker (Stratagene, La Jolla, CA). The DNA fragments on the membrane were hybridized with ^{32}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.[3] 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 radiographic film with intensifying screens for 2–14 hr at −80°C.

TABLE 1

Sequences of Plasmodium vivax *primers and oligoprobes*

Primer or probe	Sequence
Primers	
PV5	+5'-GTCGGAATTCAATAAGCT-GAAACAACC-3'
PV6	−5'-CAGCGGATCCACAGGTTA-CACTGCAT-3'
Oligonucleotide probes	
VK210	+5'-CCAGCAGGTGATAGAGCAG-3'
VK247	+5'-GGCAATCAACCAG-GAGCAAATGG-3'

RESULTS

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

In all 24 cases of *P. vivax* infection analyzed from Mexico and Peru, the CS gene was successfully amplified from filter paper samples. All patients studied were probe positive for VK210, VK247, or both (Table 2 and Figure 1). The parasitemias, as determined from stained smears, ranged from 1,600 to 13,280/mm^3 in Mexico and from 79 to 37,920/mm^3 in Peru (Table 2).

Five of the seven cases (71%) from Peru were mixed infections, with both VK210 and VK247 identified by oligoprobing the amplified CS gene. In two cases, only the 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 symptomatology, or the presence of unusual morphology on stained smears.

In contrast to 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). As 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) had 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 responses (enzyme-linked immunosorbent assay [ELISA] and immunofluorescent antibody [IFA] test) in Plasmodium vivax *infections from the New World*

Patient (age/sex)	Location	Parasitemia (parasites/mm³)	ELISA*		IFA†		Probe‡	
			VK210	VK247	VK210	VK247	VK210	VK247
Group 1: Mexico								
M1 (19/M)	Foothills	8,800	0.05	0.00	–	NT	+	–
M2 (11/F)	Foothills	>10§	0.09	0.01	–	–	–	+
M3 (50/F)	Coast	9,000	0.00	0.00	–	NT	+	–
M4 (8/M)	Coast	ND¶	0.26	0.00	+1	–	+	–
M5 (10/M)	Coast	ND	0.00	0.01	–	NT	+	–
M6 (33/F)	Foothills	13,280	0.03	0.00	–	NT	+	–
M7 (45/M)	Coast	2,400	0.22	0.01	+1	–	+	–
M8 (13/M)	Foothills	2,200	0.03	0.01	–	–	–	+
M9 (22/M)	Coast	4,500	0.03	0.02	–	NT	+	–
M10 (17/F)	Coast	1,600	0.11	0.00	–	NT	+	–
M11 (12/F)	Foothills	5,760	0.10	0.00	–	NT	+	–
M12 (19/F)	Coast	>10§	0.06	0.00	–	NT	+	–
M13 (9/M)	Foothills	9,200	0.00	0.67	–	+1	–	+
M14 (27/F)	Coast	2,480	0.16	0.02	–	NT	+	–
M15 (39/F)	Coast	>10§	0.19	0.00	+2	NT	+	–
M16 (32/F)	Coast	2,600	0.02	0.00	–	NT	+	–
M17 (10/M)	Coast	2,800	0.23	0.05	+1	–	+	–
Group 2: Peru								
P1 (26/M)	Jungle	11,769	0.04	0.02	–	NT	+	+
P2 (19/M)	Jungle	37,920	0.04	0.02	–	NT	–	+
P3 (22/M)	Jungle	6,316	0.71	0.04	+3	–	+	–
P4 (55/M)	Coast	14,400	0.78	0.00	+1	–	+	+
P5 (32/M)	Jungle	79	0.22	0.00	–	–	+	–
P6 (21/M)	Jungle	20,000	0.10	0.00	–	–	+	+
P7 (21/M)	Jungle	16,000	0.18	0.03	–	–	–	+
Positive controls (n = 210)			>2.0	–	+4	–	+	–
Positive controls (n = 247)			–	>2.0	–	+2	–	+

* Optical density values at 414 nm (OD_{414}) for 1:20 dilutions of sera eluted from filter paper discs. Results are the mean net absorbance (OD in antigen containing wells − OD in wells without antigen). A positive ELISA (underlined) was defined as a net absorbance ≥0.15.

† Values for 1:20 dilutions of sera eluted from filter paper discs. Fluorescence levels were graded from negative (–) to 4+, with negative values corresponding to background fluorescence and 4+ to intense uniform fluorescence over the entire sporozoite. NT = not tested.

‡ Hybridization results were graded after a 12-hr exposure.

§ >10 trophozoites or gametocytes per 1,000× oil immersion field.

¶ 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.[4–6] 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

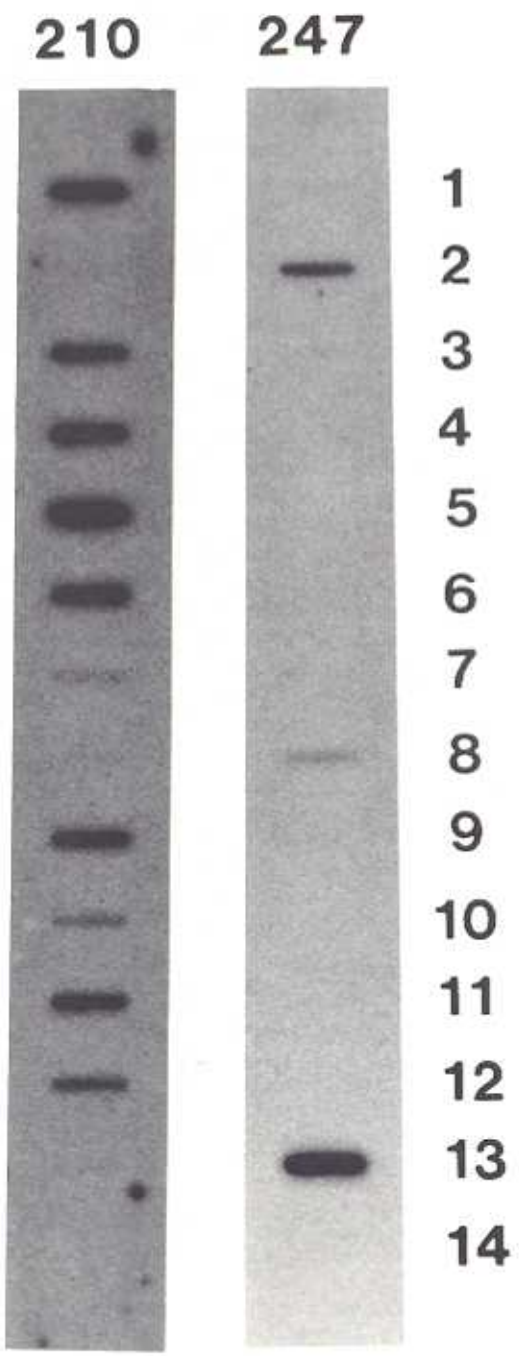

FIGURE 1. Representative results of a polymerase chain reaction/oligoprobe hybridization for the predominant (VK210) and variant (VK247) circumsporozoite repeat domains. The autoradiograph shows a slot-blot of *Plasmodium vivax* DNA that was eluted and amplified from dried blood filter paper samples. Patient numbers are indicated on the right side of the figure. Patients 1–14 are from the region around Tapachula, Mexico. Patients 2, 8, and 13, who are from the foothills, had only VK247 DNA identified. Other Mexican patients were infected with the VK210 strain of *P. vivax*.

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*).[8] 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,[7,9] 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,[9] 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[4] 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 *P. brasilianum* or *P. malariae*. Preabsorbing the sera with *P. brasilianum* repeat peptide completely abolished reactivity with the variant *P. vivax* sporozoites. In this study, as in others,[5, 7, 10] IFA results generally paralleled those of the ELISA, but its sensitivity was even lower. Clearly, problems with specificity and requirements for multiple patient sampling 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 *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, special shipping requirements, and lowering the biological hazard associated with shipping blood products.

Although serologic detection of *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 *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.[6, 11] 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 *P. falciparum*.[12, 13] The inability to maintain *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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