GENETIC DIVERSITY OF PLASMODIUM VIVAX PVcsp AND PvMSP1 IN GUYANA, SOUTH AMERICA

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Abstract. Approximately 55% of malaria infections in the Guyana Amazon region are attributed to Plasmodium falciparum while the other 45% are attributed to non-falciparum, mostly Plasmodium vivax. However, little is known about the P. vivax strain types circulating in the region. Using PCR for Plasmodium detection and two genetic markers specific to P. vivax to detect the polymorphic circumsporozoite protein (CSP) and the conserved 19-kDa region of the merozoite surface protein-1 (MSP-1), we investigated the overall Plasmodium strain distribution and population diversity within P. vivax in isolates collected from the blood of infected individuals in the interior Amazon region of Guyana, South America. Out of a total of 250 samples positive for Plasmodium, P. vivax was detected in 30% (76/250) and P. falciparum was detected in 76% (189/250). Mixed infections containing both P. falciparum and P. vivax constituted 6% (15/250) of the total positive samples. Further analysis of P. vivax strains showed that 92% (56/61) of the P. vivax samples hybridized with a probe specific to type VK210, 39% (24/61) hybridized with a probe specific for type VK247, and 25% (15/61) hybridized with a probe specific for the P. vivax-like CS genotype. DNA sequencing of the 19-kDa C-terminal domain in block 13 of MSP-1 amplified from 61 samples from patients infected with P. vivax demonstrated that this region is highly conserved, and all samples were identical at the nucleotide level to the Belem Salvador-1 type. No synonymous or non synonymous mutations were observed in this region of the gene, indicating that current vaccine-development efforts based on the MSP-1α fragment would be applicable in Guyana.

INTRODUCTION

Plasmodium falciparum is most typically associated with morbidity and mortality due to malaria infections. However, severe morbidity and mortalities can also be attributed to malaria caused by Plasmodium vivax.1 Although typically considered more benign than falciparum malaria, infections caused by P. vivax induce anemia, hypoglycemia, effects on pregnancy, low birth weight, and even respiratory distress and cerebral malaria.1-3 Moreover, the negative economic impact and public health burden that P. vivax infections have on endemic countries is considerable. Escalating resistance to antimalarial drug therapies for P. vivax infections is compounding the problem as the standard drug regimens are becoming less effective at eliminating infections.4,5 To better control the impact of P. vivax infections, a better characterization and understanding of P. vivax strains circulating in a given region is necessary.

Importantly, the prevalence of P. vivax malaria has been on the rise globally and now accounts for > 50% of all malaria cases outside of Africa. Approximately 70–80 million cases are recorded annually, signifying that P. vivax malaria represents a significant public health burden in many parts of the world.6 However, despite the high prevalence of P. vivax worldwide, little is known about the genetic diversity and population structure of P. vivax.7 Parasite diversity in P. falciparum, relative to the distribution of Pfcsps and Pfmsp1 genotypes, has been under investigation for a number of years.8-11 More recently, a few studies investigating the genetic diversity of P. vivax have been reported.12-18 Understanding the dynamics of the strains circulating in a region is not only of academic interest but is also important for determining the usefulness of a particular vaccine or drug formulation in a malaria-endemic region.

The circumsporozoite protein (CSP) is an outer coat protein covering the surface membrane of the malaria sporozoites. The protein consists of an immunodominant central repeat domain that is flanked by two non-repeated amino and carboxyl sequences containing highly conserved protein stretches. The high degree of immunogenicity induced by the tandem repeats in the amino acid sequence of the CSP has been the main focus of recombinant vaccine development.19 However, genetic polymorphisms discovered in the central repeat domain of the P. vivax CSP20-22 has complicated vaccine development, and it has been suggested that strain variability exists in responses to chloroquine.22

The classic P. vivax VK210 strain has a CSP with an amino acid repeat GDRAA/DBGQA.23 A variant form, VK247, was later identified in Thailand with an amino acid repeat ANGAGNPQ in the tandemly repeated amino acid region.20 Later, a third variant form, P. vivax-like was described with an amino acid repeat APGANQEGGA.24 This repeat sequence is identical to that observed in the monkey malarial parasite P. simiovale.25 Using sequence-specific DNA hybridization techniques, the VK210 and VK247 variants of P. vivax have been found to be distributed worldwide.26 The P. vivax-like variant was also found to have a global distribution in blood samples from Papua New Guinea, Indonesia, Brazil, and Madagascar were tested and found positive for P. vivax-like DNA.21,27 However, another study did not confirm a worldwide distribution of this P. vivax-like genotype.28 More recently, several serological surveys in P. vivax-endemic areas of Brazil have described the prevalence of antibodies recognizing P. vivax-like synthetic polypeptides.29-31 Interestingly, the P. vivax-like genotype identified in Brazil was found to only occur in conjunction with the presence of strain VK210 and/or strain VK247.32

The merozoite surface protein-1 (MSP-1) of P. vivax is a large polypeptide of ~200 kDa expressed on the surface of the merozoite and is one of the leading vaccine candidate anti-
The gene has been divided into several variable blocks flanked by 10 conserved blocks, and these variable blocks have a dimorphic pattern. MSP-1 of *Plasmodium* species is synthesized as a high molecular weight precursor and then processed into several smaller fragments. When the merozoite invades the red blood cell, only a 19-kDa C-terminal fragment (MSP-1\(_{19}\)), which contains two epidermal growth-like factors, remains present on the surface. Antibodies against MSP-1\(_{19}\) have been shown to inhibit merozoite invasion and immunization of monkeys with MSP-1\(_{19}\) provides protection against challenging infections. Despite being considered conserved, polymorphism at several locations has been reported in the *P. falciparum* MSP-1 19-kDa domain among different isolates across different geographical areas. In *P. vivax*, this domain of the gene (block 13) has been reported to be highly conserved. However, very few studies have investigated the degree of vaccine development because different amino acid variations caused by single-point mutations can affect the immunogenic properties.

Because there has been an observed increase in the prevalence of *P. vivax* infections in the Amazon Basin of South America, we wanted to identify the strain types of *P. vivax* circulating in Guyana and characterize genetic information that could assist in future management of *P. vivax* malaria cases in the region. This study characterizes the distribution of *Pvmsp* genotypes of *P. vivax* circulating in Guyana and determines the genetic homogeneity/heterogeneity of the *PvMSP-1* 19-kDa domain.

**MATERIALS AND METHODS**

**Study population.** Blood samples were collected in June and November 2000 from individuals presenting to Guyanese health care clinics with symptoms of malaria. Patients described themselves as symptomatic for malaria (fever/chills), and blood samples were collected consecutively without bias toward confirmed-positive patients. IRB approval was obtained from both the University of Florida and the Guyana Ministry of Health, and informed consent was obtained from all individuals participating in the study before blood samples were obtained.

**Study site.** The study was completed in multiple sites in the Amazon region of Guyana. Samples were collected in Ministry of Health clinics, field stations, and gold mining camps throughout Region 8, including the town of Mahdia, known as the “crossroads” of the Amazon region in Guyana because it is fairly large for an interior Amazon village and contains a clinic with beds for overnight evaluation, a dirt landing strip for small planes, mining supply stores, generator power used to create electricity during the day, and recreation (bar/lodging/restaurant). Miners travel to this small town from many areas of the interior Amazon region and even from the border with Brazil. At least nine other field locations within Region 8 were used in this study. Blood samples were also collected in the large coastal capital city of Georgetown (Region 4) at the Ministry of Health Tropical Disease Clinic. It is important to note, however, that most of the malaria-infected individuals diagnosed in Georgetown had traveled to the city’s clinic from the interior Amazon region and had not become infected in the capital city.

**Blood sample collection.** Blood specimens were collected by venipuncture or finger stick with blood films prepared by Ministry of Health microscopists at the time of collection. Local health care workers read Giemsa-stained thick blood smears, and treatment was administered to those positive for malaria according to Guyana Ministry of Health directives. Blood collected using the finger-stick method was spotted in duplicate onto IsoCode STIX filter paper (Schleicher & Schuell, Keene, NH). After being dried in air, each filter paper card was individually placed into a plastic bag with a desiccant pouch. Whole-blood specimens were kept refrigerated for PCR and sequencing analysis.

**DNA extraction.** Fifty microliters of whole blood was transferred to a 1.5-mL centrifuge tube containing 2 μL of saponin and vortexed at room temperature. After 5 minutes, 650 μL of lysis solution (6 M guanidine thiocyanate, 0.1 M sodium acetate, 0.5% Sarkosyl, pH 7) and 5 μL of proteinase K (20 mg/mL) were added and the sample was vortexed for 15–30 seconds and placed in a water bath for 1 hour at 37°C. After incubation, 750 μL of phenol/chloroform/isooamyl alcohol was added and the sample was mixed well. The mixture was centrifuged at 10,000g at 4°C for 5 minutes. The upper phase was collected and transferred to a fresh tube containing 750 μL of chloroform/isooamyl alcohol, mixed by inversion, and centrifuged at 10,000g at 4°C for 5 minutes. The upper phase was collected and transferred to a tube containing 750 μL of ice-cold isopropanol, mixed by inversion, and placed at −20°C overnight. The sample was then centrifuged at 12,000g at 4°C for 20 min to pellet the nucleic acid. The pellet was washed with 75% ice-cold ethanol and centrifuged at 12,000g at 4°C for 20 minutes. The ethanol was carefully aspirated, and the pellet was dried in a vacuum centrifuge. The nucleic acid was resuspended in 200 μL of HPLC-grade water and frozen at −20°C before PCR amplification.

DNA preparation from the IsoCode STIX included adding 100 μL of HPLC-grade water to a blood-containing tip of the stick placed in a 1.5-mL centrifuge tube. The filter matrix was vortexed for 30 seconds and heated to 95°C for 30 minutes. The tube was vortexed again for 30 seconds and stored at −20°C before PCR amplification.

**Oligonucleotide primers and labeled probes.** Oligonucleotides were synthesized by Sigma Genosys (The Woodlands, TX). The *Pvmsp*-specific primers were AL60 and AL61, and the three internal oligonucleotide probes specific for strains VK210, VK247, and *P. vivax*-like were AL116, AL114, and AL240, respectively (Table 1). The internal oligonucleotide probes were labeled with digoxigenin-11-ddUTP using the digoxigenin oligonucleotide 3'-end labeling kit (Roche Biochemicals) according to the manufacturer’s instructions.

For PCR amplification of *Pvmsp*\(_{19}\) primers MSA48 and MSA53 were used (Table 1). For PCR detection of *P. falciparum*, primers Pf1 and Pf2 specific to the *Pfox1* gene were used (Table 1).

**PCR amplification.** Fifty-microliters of reaction buffer was combined with 5 μL of the DNA template for amplification. The amplification reaction contained 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl), 2.5 mM MgCl\(_2\), 200 mM each of deoxynucleoside triphosphate, 0.5 μM of each primer and 1.25 units of AmpliTaq DNA polymerase (Applied Biosystems). Amplification was initiated by a hot-start technique (95°C for 5 minutes) prior to introducing the Taq DNA polymerase to the reaction. The amplification involved 40 cycles.
of 1 min of denaturation at 94°C, 1 minute of annealing at 48°C (for *P. falciparum*) or 58°C (for *P. vivax*), and 1 minute of primer extension at 72°C. A final extension of 72°C for 7 minutes was included after the final cycle. Each experiment included a *P. vivax* DNA positive control and a negative DNA control from an uninfected individual.

**Detection of PCR products.** Eighteen microliters of the PCR product was electrophoresed in a 2% agarose gel containing 0.1 μg ethidium bromide per mL, and the bands were visualized by UV transillumination. For Southern blot hybridization, amplified DNA was transferred from the agarose gel to a positively charged nylon membrane using a Posiblotter (Stratagene, La Jolla, CA). The amplified DNA was alternatively transferred to a positively charged nylon membrane with a slot-blot manifold (Schleicher & Schuell). The PCR product was cross-linked with UV light and prehybridized for 1 hour at 50°C in hybridization buffer containing 5× SSC, 0.1% N-lauroylsarcosine, 0.02% sodium dodecyl sulfate (SDS), and 2% casein. Hybridization was carried out overnight in the same hybridization solution with 100 pmol of the labeled probe in 15 mL of buffer. The hybridization temperatures were 51°C for AL116, 49°C for AL114, and 47°C for AL240. After hybridization, the nylon membrane was washed twice for 5 minutes with 2× SSC-0.1% SDS at room temperature followed by two washes for 15 minutes with 0.1× SSC-0.1% SDS at 5°C below the hybridization temperature. The bound digoxigenin-11-dUTP-labeled probe was detected with an anti-digoxigenin antibody conjugated with alkaline phosphatase. The chemiluminescent substrate CDP-Star (Roche Biochemicals) was added, and the membrane was autoradiographed on film for 5-15 minutes.

**DNA sequencing.** The PCR amplicons were purified using Millipore Montage-PCR filter units and sequenced directly with both primers MSA48 and MSA53 to obtain a clean read of the entire amplicon. BigDye Terminator V3 Cycle Sequencing Kit (ABI) reagents were used for cycle sequencing. The primer sequences were removed from the acquired sequences prior to comparison with the type strains.

#### Table 1
Primer and probe sequences

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Sequence 5′-3′</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. vivax</em> amplification</td>
<td>AL60</td>
<td>GTCGGAATTCATGAAGAACCTATTGCTTCT</td>
</tr>
<tr>
<td>Hyb probe VK210</td>
<td>AL61</td>
<td>CACCGGATCCTTAATTGAAATATGGCTAGG</td>
</tr>
<tr>
<td><em>P. vivax</em> VK247</td>
<td>AL116</td>
<td>GGTGATAGGCAAGATGGGA</td>
</tr>
<tr>
<td><em>P. falciparum</em> VK210</td>
<td>AL114</td>
<td>ATCAACKAGGGAGCAAATG</td>
</tr>
<tr>
<td><em>P. falciparum</em> VK247</td>
<td>AL240</td>
<td>AGTGGGAGCAGCACG</td>
</tr>
<tr>
<td><em>P. vivax</em>-like</td>
<td>MSA48</td>
<td>ATGAGCTTCCGAGCACACACA</td>
</tr>
<tr>
<td>Pvcsp fragment amplification</td>
<td>MSA53</td>
<td>TAAAGCTCCACTGCACAGGA</td>
</tr>
<tr>
<td><em>P. falciparum</em> cox1 gene</td>
<td>PI1</td>
<td>GGAATTTATGGCATAAACAC</td>
</tr>
<tr>
<td></td>
<td>PI2</td>
<td>AATGAAGAGCTGTGTATC</td>
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#### Table 2
Plasmodium infections identified by microscopy and PCR in Guyanese people presenting to a clinic

<table>
<thead>
<tr>
<th>Species</th>
<th>Microscopy (%)</th>
<th>PCR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. falciparum</em></td>
<td>176 (66.4)</td>
<td>174 (69.6)</td>
</tr>
<tr>
<td><em>P. vivax</em></td>
<td>89 (33.6)</td>
<td>61 (24.4)</td>
</tr>
<tr>
<td><em>P. falciparum</em>/*P. vivax</td>
<td>0 (0)</td>
<td>15 (6.0)</td>
</tr>
<tr>
<td>Total</td>
<td>265 (100)</td>
<td>250 (100)</td>
</tr>
</tbody>
</table>

#### RESULTS

Out of 387 patient blood samples tested by PCR, a total of 250 samples were positive for malaria. Sixty-one (61) samples were positive for *P. vivax*, 174 samples were positive for *P. falciparum*, 15 samples were positive for both *P. falciparum* and *P. vivax*, and 137 were negative (Table 2). Local microscopists detected 265 samples positive for malaria, 176 samples positive for *P. falciparum*, 89 samples positive for *P. vivax*, and no samples positive for mixed *P. falciparum*/*P. vivax* infections (Table 2). Generally, the concordance between sample analysis methods was high except for the failure of the microscopists to detect mixed infections. Microscopists detected more *Plasmodium* spp. in PCR-negative samples than was detected in microscopy-negative PCR-positive samples. In > 80% of these discrepant samples, the PCR was performed from the dried blood spots; and in > 50% of these samples, the parasitemia was < 20 parasites/μL.

The probe for genotype VK210 hybridized to 92% (56/61) of the *P. vivax*-positive samples, and the genotype VK247 probe hybridized to 39% (24/61) of the samples (Table 3). The probe for the *P. vivax*-like genotype hybridized with 25% (15/61) of the samples. Genotype VK210 was the only genotype detected in 55.7% (34/61) of the *P. vivax*-positive samples, only genotype VK247 was detected in 4.9% (3/61), and *P. vivax*-like was not detected by itself in a sample (Table 3). Mixed genotype infections were detected in 39.3% (24/61) of the positive samples with all three genotypes detected in 16.4% (10/61) of the *P. vivax*-positive samples (Table 3).

Sequencing of block 13 of the *Pvmsp1* gene indicated that the gene locus was completely conserved among the 61 *P. vivax*-positive samples. All samples were identical at the nucleotide level to the Belem and Salvador-1 types.
DISCUSSION

Guyana, South America, is a country with high levels of endemic malaria and many other important infectious diseases, including leishmaniasis, filariasis, intestinal parasites, HIV, and dengue fever. Most of the malaria cases are acquired in the interior region of the country, which is part of the South American Amazon basin. Although falciparum malaria causes at least half of the estimated 50,000 cases of malaria annually in this country, P. vivax malaria is increasingly recognized as a significant problem. During this study, and in previous malaria studies completed in Guyana, patients often reported they would have six or more malaria infections per year, suggesting either an increase in drug-resistant strains, poor compliance with drug-treatment regimens, or a high level of reinfection.

In Guyana during the year 2000, the annual parasitic index (API) was 230.9, the annual index of P. falciparum (AFI) was 118.5, and the annual index of P. vivax (AVI) was 112.4; no annual index of P. malariae (AMI) was recorded. In 2004, the AFI was 209.2, the AVI was 88.7, the AVI was 116.9, and the AMI was 3.6. This indicates an increase in P. vivax infections relative to P. falciparum and the possible emergence of P. malariae in the region.

Because of increased drug resistance in P. vivax malaria in Guyana and personal observations of increased morbidity and mortality caused by P. vivax infections, we wanted to identify the strain types of P. vivax circulating in Guyana and characterize genetic information that could assist in future management of P. vivax malaria cases in the region. Establishment of a baseline distribution of P. vivax genotypes in the region could assist in further studies investigating the increase resistance to antimalarials and/or eventual field trials of experimental vaccines. It may also shed light on the contribution of the various genotypes to the increased morbidity observed with P. vivax infections in this region of the Amazon.

In addition, although information on the specific gene is not new, the results from the country of Guyana is a new finding because very little work on malaria strain types has been reported from Guyana. This is a real disservice to this country as it has had ongoing malaria problems for many years. It was not unusual for us to see miners who were on their sixth case of malaria in June. Whether due to reinfection or to inadequate elimination of the parasite in past episodes, however, the fact remains that they were sick from a disease that adversely affects their health, their ability to earn an income, and their overall quality of life. The population of Guyana is less than one million people; in 1999, this country reported over 50,000 cases of malaria. This is a very high occurrence in such a relatively small population base and suggests that malaria is a major contributor to morbidity in this country and has a significant economic impact on the population. Additionally, the regions where we worked were quite isolated, and little has been reported from these areas as it is difficult to reach and accommodations are extremely spartan (generator power is normally only available during the daytime at the clinic).

Results of P. vivax strain typing indicated that three different CSP genotypes of P. vivax (VK210, VK247, and P. vivax-like) are circulating in Guyana. Type VK210 was the most prevalent, occurring in 92% of samples, followed by type VK247, which occurred in 39% of samples, and type P. vivax-like was detected in 25% of samples. A high prevalence (39%) of samples contained mixed genotypes, indicating that multiple P. vivax strains often infect an individual (Table 3). A recent study in Iran found that only 12% of the samples were mixed-type (VK210/VK247) infections; however, all mixed infections were in samples taken from the tropical southeastern region of the country, where a higher frequency of Pvspl allelic variants and co-circulating P. falciparum infections is observed. In Thailand, 25.6% of samples had multiple infections with different P. vivax genotypes. Our data demonstrate a somewhat similar pattern of distribution of VK210 and VK247 observed in Iran (the tropical southeastern region) and Thailand but also demonstrate the presence of the P. vivax-like genotype. The identification of a highly unique genetic variant of Pvspl antibodies worldwide to determine where and when allelic variants arise.

Sequence of the Pmspl 19-kDa fragment demonstrated that this region of the gene was highly conserved among the 61 samples containing P. vivax. All samples were identical at the nucleotide level to the Belem and Salvador-1 types. No synonymous or nonsynonymous mutations were observed in this region of the gene, indicating that current vaccine-development efforts based on the Pvspl-1 fragment would be applicable in Guyana. Results of this present study parallel reports of P. vivax strain types in neighboring Brazil where circulation of all three strain types (VK210, VK247 and P. vivax-like) have been documented. Researchers in Brazil have also noted increasing levels of infection caused by P. vivax, chloroquine and primaquine drug resistance, and evidence of severe malaria in a subset of those infected with P. vivax. Thus, results from both Brazil and Guyana suggest that increasing health/treatment problems associated with P. vivax infections may be prevalent yet unrecorded throughout the South American Amazon region. Of great interest is the specific cause of the severe morbidity and mortality that have been observed in the subset of patients infected with P. vivax. Could these be due to infection with a specific strain type or strain type combination? This is an unanswered question that is of great public health importance to the Amazon Basin inhabitants.

We found a high correlation between the local microscopists’ diagnosis and PCR, although not in mixed Plasmodium infections. Mixed infections, however, are notoriously misdiagnosed. Microscopy appeared to have a higher sensitivity than PCR when the samples were collected and stored on filter paper rather than whole blood preserved in a tube. The reason for this is unknown. Perhaps the dried samples with low parasitemia would require a larger DNA inoculum into the PCR reaction or additional processing to release the nucleic acid from the strip than the manufacturer’s suggested protocol.

Another interesting point is a report from Mexico noting that Anopheles mosquitoes showed differential susceptibility to P. vivax strains. The researchers showed that An. Albimanus and An. pseudopunctipennis are preferentially infected with VK210 and VK247, respectively. In Guyana, An. darlingi is the most commonly recognized mosquito carrier of malaria. Recently, however, it was reported that another species of Anopheles, An. aquasalis, was found to be infected with P.
vivax. It would thus be of interest to investigate whether certain species of mosquito in Guyana preferentially become infected with different Plasmodium vivax strain types and the relationship to virulence of infection.

Although much more work needs to be completed on malaria strains in Guyana, results of this current research provide the first report on the strain types of Plasmodium vivax circulating in Guyana. Problems with drug resistance and increasing morbidity and mortality continue to increase, and we observed especially severe clinical presentations in some of the individuals infected with Plasmodium vivax malaria. Further characterization of Plasmodium vivax strains in Guyana needs to be completed to help determine the relationship between infections with different strain types and/or combinations of strain types and clinical presentation and outcomes.

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