EVIDENCE FOR TRANSMISSION OF PLASMODIUM VIVAX AMONG A DUFFY ANTIGEN NEGATIVE POPULATION IN WESTERN KENYA

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Abstract. We present evidence that a parasite with characteristics of Plasmodium vivax is being transmitted among Duffy blood group–negative inhabitants of Kenya. Thirty-two of 4,901 Anopheles gambiae and An. funestus (0.65%) collected in Nyanza Province were ELISA positive for the P. vivax circumsporozoite protein VK 247. All positives were found late in the rainy season, when An. funestus predominated, and disproportionately many were found at a single village. A P. vivax specific sequence of the SSU rRNA gene was amplified from three of six ELISA positive positives. Erythrocytes from 31 children, including 9 microscopically diagnosed as infected with P. vivax, were negative by flow cytometry for the Fy3 or Fy6 epitopes, which indicate Duffy blood group expression. A DNA fragment specific for the C terminus of the gene for P. vivax merozoite surface protein 1 (MSP-1) was amplified from the blood of four of these children and subsequently sequenced from two.

INTRODUCTION

Plasmodium vivax, the most widespread of the four malaria parasite species infecting humans, is rare in most of tropical Africa.1 Inhabitants of West Africa are predominately homozygous for a mutation that prevents expression in erythrocytes of either major allele of the Duffy blood group gene, Fyα and Fyβ.2 The determinant, a glycoprotein that traverses the membrane seven times, includes a 35-amino acid epitope (Fy6) in the extracellular, N-terminal domain that mediates erythrocyte invasion by P. vivax merozoites.3–5 Little is known about the distribution of Duffy phenotypes in East Africa. Occasional reports of P. vivax in Kenya have commonly been ascribed, a priori, to confusion with the morphologically similar P. ovale or to the genetic contribution from Hametic or other populations who are largely Duffy positive.6,7 We report here, however, evidence that a parasite having molecular and antigenic characteristics specific for P. vivax is being transmitted among Fyα−b− indigenes of western Kenya. We were first alerted to this possibility when four wild Anopheles, among a group being used to validate a new dipstick assay,8 consistently gave positive results for P. vivax circumsporozoite protein, phenotype VK 247.9

MATERIALS AND METHODS

Mosquito collections. The source of the specimens used for Plasmodium DNA amplification and speciation was abdomen from 793 Anopheles that had been collected for an unrelated study on vector immune response during May 2000 from the Indian Ocean coastal village of Majenjeni and the western Kenyan village of Kamonye.10 The head-thorax of each was enzyme-linked immunosorbent assay (ELISA) tested for circumsporozoite (CS) protein, as described below; DNA for species identification was extracted from abdomens preserved in 70% ethanol.

The source of specimens for other ELISAs was ≥ 31,000 Anopheles collected from human landing, indoor resting, and light trap captures during 2000–2001 at three villages—Miwani, Kombewa, and Kamonye—in Nyanza Province, western Kenya. These collections were made to study various aspects of malaria transmission unrelated to P. vivax, but all specimens had been processed alike: each mosquito was identified by capture location, method, date, and time. The abdomen of each An. gambiae sensu latu was preserved for molecular speciation,11 and the remainder of its body was preserved for P. falciparum and P. vivax CS ELISA; whole bodies of other species were used for CS ELISA. Triturates not used for initial ELISA were stored at −70°C. For this study 5,000 specimens were selected beginning with the October 2000 collections and ending in January 2001, encompassing the annual “short rains” malaria transmission season. Specimens were selected in running order based on the proportion of the total catch (Miwani = 3,328, Kamonye = 1,262, Kombewa = 408), re-coded by one of the authors, and assayed blinded under the direction of a second investigator.

CS assays. CS ELISAs specific for P. falciparum,12 P. vivax phenotype VK210,13 and P. vivax phenotype VK24714 were used to quantitatively measure antigen directly; because of limited availability of specific monoclonal antibody, P. malariae ELISAs were not done. Each ELISA plate contained negative controls and an array of antigen standards required to generate a standard curve and quantitatively estimate the amount of CS antigen present in each sample.15 Because of low sample volume, the three ELISAs were run consecutively by transferring samples from one assay to the next after the 2-hour sample incubation step. During initial screening, 127 of 4,989 specimens produced results indicating P. vivax antigen (VK 247 only); these were retested under more stringent conditions, yielding 32 specimens containing ≥ 1.0 pg P. vivax antigen.

Sporozoite DNA. DNA prepared from abdomens of ELISA-positive Anopheles, as described above, were assayed by nested polymerase chain reaction (PCR), with an initial (nest 1) amplification using genus-specific primers followed by a second amplification that used molecular beacon probes16,17 and real-time PCR to detect Plasmodium species.
The first, genus-specific PCR reaction amplified a ~500-bp region toward the 5' end of the small sub-unit ribosomal RNA (SSU rRNA) gene. Nest 1 used the following primers—forward: 5'-GGCTGAGAAATAGCTACACAT-3'; reverse: 5'-GTTGTTCAATTTTGTTATCCAGTCT-3'. Reaction conditions for nest 1 were 95°C for 120 seconds, 35 cycles of 95°C for 10 seconds, 54°C for 20 seconds, and 72°C for 30 seconds; a Cepheid SmartCycler (Sunnyvale, CA) was used with fluorescent imaging at 54°C. The second, nested reaction again used genus-specific primers to amplify an internal segment with species-specific variability of 133–136 nucleotides (P. falciparum, 136; P. malariae and P. ovale, 135; P. vivax, 133). Nest 2 primers were as follows—forward: 5'-GCCGCTAAATACCCAATCT-3'; reverse: 5'-CCAGACTTGCCCTCAATTT-3'. Reaction conditions used for nest 2 were 95°C for 120 seconds, 35 cycles of 95°C for 10 seconds, 54°C for 20 seconds, and 72°C for 10 seconds.

The variable region, located at the approximate midpoint of the amplified segment (position 61–78 of the consensus sequence), showed a range of nucleotide substitutions and deletions, with between two and five nucleotide differences in pairwise comparison: Pf, 5'-CAATTTTTGTTTGTGAAA-3'; Pm, 5'-CAATTTTTGTTTGTGAAA-3'; Pv, 5'-CATTTCAATTTTTGTGAAA-3'; P. vivax, 5'-CAATTTTTGTTTGTGAAA-3'.

Using this region of variability, specific probes were designed for each species present: PF (FAM), 5'-CCTG(CCAATTTTTGTTTGTGAAA)-CCTTGG; PM (ROX), 5'-C(CAACGCTTTTTGTTTGTGAAA)-CCTTGG; PO (ROX), 5'-C(CAACGCTTTTTGTTTGTGAAA)-CCTTGG; PV (TET), 5'-CAATTTTTGTTTGTGAAA-3'. Sequences within parentheses represent hybridizing sequences. Probes were synthesized by MWG Biotech (High Point, NC) and Sigma Genosys (Woodlands, TX). Fluorophores were attached to the 5' ends and DABCYL [4-(4 dimethylaminophenylazo)benzoic acid] quenchers to the 3' ends. Sequences within parentheses represent hybridizing sequences. Probes were synthesized by MWG Biotech (High Point, NC) and Sigma Genosys (Woodlands, TX).

Nest 2 amplification products were run on a 1.5% agarose gel (Sigma-Aldrich, St. Louis, MO) for confirmation of genus-specific product. All reactions were repeated twice with both positive and negative controls. Positive controls were cloned plasmids, developed with Promega MiniPrep and pGEM-T Easy Vector cloning kits (Promega, Madison WI), using DNA isolated from specimens provided by the Walter Reed Army Institute of Research, Silver Spring, MD: P. falciparum, clone 3D7 maintained in culture (V. A. Stewart); P. vivax, Chессon (CDC) strain maintained in Aotus trivirgatus (V. A. Stewart); and P. malariae and P. ovale maintained as cryopreserved specimens, collected at Kisumu, Kenya (P. E. Duffy). All positive plasmids were confirmed by sequencing. Negative controls were both no-template and non-infected blood controls.

**Blood collection and examination.** Apparent P. vivax–positive cases were coincidental findings from children enrolled in a case-control study of P. falciparum and severe malaria. Cases were identified from the pediatric ward of the Nyanza Provincial General Hospital (NPGH), and controls were identified from the community and/or the outpatient clinic of the NPGH, Kisumu, western Kenya, during 1999–2000.18 Scientific and ethical approval for this study was obtained from the Kenya Medical Research Institute, Nairobi, Kenya, and the Human Subjects Research Review Board, Office of the Surgeon General, US Army, Washington, DC. NPGH serves the holoendemic malaria region of the Lake Victoria basin. Nearly all participants were from the Luo ethnic group. Informed consent was obtained from each parent or guardian at the time of enrollment. Cases of severe anemia were defined as children with asexual P. falciparum parasitemia by blood smear and a hemoglobin of 5 g/dL or lower in the absence of clinical evidence of any other infectious process or malignancy. Duplicate thick and thin blood films from each child were prepared and stained with Giemsa and examined by experienced microscopists at Kisumu using ×1000 magnification; the number of parasites of each species seen was enumerated for fields containing 500 white blood cells. Seven slides were sent to the Armed Forces Research Institute of Medical Science (AFRIMS), Bangkok, Thailand, to be independently re-examined by two microscopists with > 30 years each experience identifying P. vivax and P. ovale. For molecular analysis and blood typing, 2.5–5 mL of blood was collected by venipuncture and aliquoted into ethylenediaminetetraacetic acid (EDTA) and heparin tubes.

**Duffy antigen.** The presence of Fy6 and Fy3 epitopes were determined by flow cytometry. Monoclonal antibody (MAb) recognizing Fy6 epitope (NYBC-BG6/K6H9)19 was supplied by the Centers for Disease Control and Prevention, Atlanta, GA. MAB for Fy3 (CBC-512) was kindly provided by Dr. Makoto Uchikawa of the Japanese Red Cross Central Blood Center.20 EDTA-anticoagulated blood from field samples, healthy white volunteers, or commercial Panocell standards (Immucor, Norcross, GA) was diluted 1:100 in Alsever's buffer (Sigma-Aldrich), centrifuged, resuspended in the same volume of buffer, and stored at 4°C until used. Flow cytometry was usually carried out within 72 hours of sample arrival. Before staining, 100 μL of diluted erythrocytes was placed in wells of 96-well plates, centrifuged at 500g for 2 minutes, and resuspended in 200 μL of RPMI 1640 (Sigma-Aldrich). After a second wash, the cells were resuspended in no antibody (unstained), isotype control IgG1, or anti-Fy3 or anti-Fy6 antibodies. Optimal dilution of primary antibodies was determined in preliminary experiments. Primary antibodies were diluted in RPMI 1640/1% bovine serum albumin (BSA; wash buffer) at 1:50 (Fy3) or 1:200 (Fy6) and incubated at room temperature while rocking for 30 minutes. Isotype control (IgG1 κ, M-5284) (Becton Dickinson, Erembodegem, Belgium) was diluted 1:50 in the same buffer. After the primary incubation, the cells were washed twice in wash buffer and incubated at room temperature for 30 minutes with antimouse IgG FITC (BD 340931; Becton-Dickinson) diluted 1:50 in wash buffer. After a final wash step in wash buffer, the cells were resuspended in 200 μL of 1% paraformaldehyde. For acquisition, 40 μL of fixed cells was added to 1 mL of sheath fluid (Becton-Dickinson). Cells were acquired using a FACSscan flow cytometer. Erythrocytes were identified on the basis of their forward and side scatter characteristics using logarithmic amplification. Median fluorescence intensity (MFI) was measured using logarithmic amplification.

**Parasite DNA.** DNA was extracted from frozen pellets of infected erythrocytes using the general procedure described by Galinsky et al.,21 although with QIAGEN extraction (Valencia, CA). Primers, conditions, and procedures for amplification followed closely those published for each of the segments being sought: malaria surface protein 1 carboxy termi-
nus (MSP-1 C terminus)\textsuperscript{22}; MSP-1 polymorphic region\textsuperscript{23}; Duffy binding protein (DBP)\textsuperscript{24}, circumsporozoite protein\textsuperscript{9}, and species specific SSU rRNA gene fragments.\textsuperscript{25} Amplified DNA fragments from subjects SA376 and SA396 were subsequently cloned into the PCRscript plasmid vector, and three clones from each amplification were sequenced using an ABI 3100 sequencer by dideoxy chain-termination methodology.\textsuperscript{26} Ten positive controls for \emph{P. vivax} (both PVK 210 and PVK 247 phenotypes) were kindly provided by the Armed Forces Research Institute of Medical Science (AFRIMS), Bangkok, as frozen parasitized red blood cells (RBCs); \emph{P. falciparum}–positive control DNA was derived from an isolate from subject SA136 that was kept in continuous culture. For other species, DNA was extracted from whole blood of individuals with single infections: \emph{P. malariae} from subject SA325 and \emph{P. ovale} from subject SA296.

RESULTS

\textbf{CS ELISA.} Of the 5,000 specimens selected, 4,989 were successfully tested. Mosquitoes were either \emph{An. funestus} or \emph{An. gambiae} sensu stricto, except for 88 \emph{An. pharoensis} and \emph{An. coustani} caught in light traps, all of which were ELISA negative and were excluded from further analysis. Species identifications for \emph{Anopheles} collected at Kombewa had only been recorded for those initially tested as \emph{P. falciparum} positive and, therefore, the proportion of \emph{An. gambiae} to \emph{An. funestus} caught in that village is unknown. At the other two villages, the total numbers of \emph{An. funestus} (2,372) and \emph{An. gambiae} (2,123) collected were the same, but the proportion that was \emph{An. gambiae} was twice as high at Kamonye (79%) than at Miwani (35%). The seasonal distributions of the two species were typical for western Kenya: \emph{An. gambiae} peaked early in the rains, during October and November, whereas \emph{An. funestus} peaked in December, as the rains abated (Table 1).

Of those ELISA tested, 268 were positive for \emph{P. falciparum} (0.054) and 32 were positive for \emph{P. vivax} (0.006); all \emph{P. vivax} were phenotype VK 247. The \emph{P. vivax} positives ranged from 1.0 to 504.7 pg, or −15 to > 6,400 sporozoites; 78% of the positives were between 1.0 and 5.0 pg. \emph{P. falciparum} positives occurred throughout the transmission season in general proportionality to the abundance of the two vector species. With the exception of a single specimen, however, there were no \emph{P. vivax} positives before December (Table 1). The largest number of \emph{P. vivax} positives came from the Miwani collection, but the highest proportion of positives was at Kombewa (0.022), followed by Miwani (0.006) and Kamonye (0.004). Although \emph{An. funestus} harbored 83% (\(N = 19\)) of the infections in identified mosquitoes, this seems to be a consequence of the relatively large number of \emph{An. funestus} found at Miwani; at Kamonye, four of five \emph{P. vivax} positives were discovered in \emph{An. gambiae} in December.

\textbf{Sporogonic parasite DNA.} We were unable to consistently amplify DNA from the ELISA lysates. Consequently, we used DNA prepared from abdomens that had been preserved in ethanol from six \emph{Anopheles} collected in May 2000 from the coastal village Majenjeni and the western village of Kamonye,\textsuperscript{10} and whose heads and thoraces had been found at Miwani; at Kombewa had only been recorded for those initially tested as \emph{P. falciparum} positive and, therefore, the proportion of \emph{An. gambiae} to \emph{An. funestus} caught in that village is unknown. At the other two villages, the total numbers of \emph{An. funestus} (2,372) and \emph{An. gambiae} (2,123) collected were the same, but the proportion that was \emph{An. gambiae} was twice as high at Kamonye (79%) than at Miwani (35%). The seasonal distributions of the two species were typical for western Kenya: \emph{An. gambiae} peaked early in the rains, during October and November, whereas \emph{An. funestus} peaked in December, as the rains abated (Table 1).

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\textbf{Blood parasites.} From July 1999 through June 2000, nine participants in the severe anemia study (one child twice) were identified as putatively infected with \emph{P. vivax} on the basis of microscopic examination of blood films; an additional two cases were found among those who presented themselves to our clinic but were not participants in the study. There were, therefore, 12 cases microscopically diagnosed with \emph{P. vivax}; the first of these was noted independently of the first indication of \emph{P. vivax} from the sporozoite ELISA. All densities were low and, because of the case definition used in the severe anemia study, were also infected with \emph{P. falciparum}; seven were read as additionally mixed with \emph{P. ovale} and three with \emph{P. malariae}. Slides from the first seven found were sent to Thailand for independent, blinded examination. Giemsa

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\textbf{Table 1}

Circumsporozoite ELISA results for \emph{Anopheles} subsample collected October 1999 to January 2000 at three sites, Nyanza Province, Kenya

<table>
<thead>
<tr>
<th>Site and species\textsuperscript{*}</th>
<th>October</th>
<th>November</th>
<th>December</th>
<th>January</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miwani \emph{An. funestus}</td>
<td>281</td>
<td>689</td>
<td>935</td>
<td>203</td>
<td>2,108</td>
</tr>
<tr>
<td>\emph{Pf}</td>
<td>4</td>
<td>16</td>
<td>29</td>
<td>8</td>
<td>56</td>
</tr>
<tr>
<td>\emph{Pv}</td>
<td>0</td>
<td>1</td>
<td>17</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>\emph{An. gambiae}</td>
<td>459</td>
<td>346</td>
<td>161</td>
<td>165</td>
<td>1,131</td>
</tr>
<tr>
<td>\emph{Pf}</td>
<td>14</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>\emph{Pv}</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Kamonye \emph{An. funestus}</td>
<td>0</td>
<td>96</td>
<td>101</td>
<td>101</td>
<td>298</td>
</tr>
<tr>
<td>\emph{Pf}</td>
<td>0</td>
<td>12</td>
<td>12</td>
<td>13</td>
<td>37</td>
</tr>
<tr>
<td>\emph{Pv}</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>\emph{An. gambiae}</td>
<td>98</td>
<td>409</td>
<td>296</td>
<td>73</td>
<td>876</td>
</tr>
<tr>
<td>\emph{Pf}</td>
<td>4</td>
<td>66</td>
<td>22</td>
<td>21</td>
<td>113</td>
</tr>
<tr>
<td>\emph{Pv}</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Kombewa \emph{Anopheles spec.\textsuperscript{†}}</td>
<td>75</td>
<td>202</td>
<td>60</td>
<td>69</td>
<td>406</td>
</tr>
<tr>
<td>\emph{Pf}</td>
<td>7</td>
<td>17</td>
<td>8</td>
<td>9</td>
<td>41</td>
</tr>
<tr>
<td>\emph{Pv}</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>6</td>
<td>9</td>
</tr>
</tbody>
</table>

\textbf{Table 2}

Fluoroprobe identification of \emph{Plasmodium} DNA recovered from \emph{Anopheles} ELISA positive for \emph{P. vivax} circumsporozoite protein

<table>
<thead>
<tr>
<th>Sample</th>
<th>\emph{Anopheles}</th>
<th>Site</th>
<th>Generic PCR\textsuperscript{*}</th>
<th>\emph{Pv}</th>
<th>\emph{Pf}</th>
<th>\emph{Po}</th>
<th>\emph{Pm}</th>
</tr>
</thead>
<tbody>
<tr>
<td>169</td>
<td>\emph{gambiae}</td>
<td>Kamonye</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>230</td>
<td>\emph{gambiae}</td>
<td>Kamonye</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>240</td>
<td>\emph{gambiae}</td>
<td>Kamonye</td>
<td>-</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>\emph{gambiae}</td>
<td>Majenjeni</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>480</td>
<td>\emph{gambiae}</td>
<td>Majenjeni</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>\emph{arabiensis}</td>
<td>Majenjeni</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{*} Nest 1, \emph{Plasmodium} amplification.
\textsuperscript{†} Not all \emph{Anopheles} identifications were recorded for Kombewa collection.
staining, although adequate for routine microscopy, was suboptimal for definitively differentiating \textit{P. vivax} from \textit{P. ovale}. One Thai examiner confirmed \textit{P. vivax} in all, including in three of six initially scored as mixed \textit{P. vivax} and \textit{P. ovale}; the second, equally experienced Thai microscopist, identified only \textit{P. ovale} as present in the thin films.

The small amounts of blood available, scanty parasitemia, and ubiquity of mixed infections made DNA amplification inconsistent. A sequence diagnostic for \textit{P. vivax}, the MSP-1 C terminus, did amplify from four specimens, producing distinct bands at ~350 bp, as in the Thai controls. Two of these (SA376 and SA396) were independently amplified in Atlanta from genomic DNA and sequenced. Both were the same as the reference \textit{P. vivax}, SAL-1 (GenBank accession no. L36237), except for a single point mutation in each (Figure 1).

In SA376, guanine substituted for adenine at position 213, affecting a codon change of lysine to glutamic acid that had been previously recorded from Southeast Asia.\textsuperscript{22} In SA396, a previously unrecognized substitution of adenine for guanine occurred at position 263, mutating serine to asparagine. No DNA was amplified from the Kenyan specimens using primers for the N-terminal MSP-1 polymorphic region 2 or DBP. Attempts to amplify CS routinely produced faint bands at 700–750 bp, consistent with Thai controls, but all attempts to sequence these failed. Primers specific for \textit{P. vivax} SSU rRNA genes\textsuperscript{23} amplified lightly staining bands of 190 bp for seven specimens and 150 bp for two, rather than at 117 bp for the Thai controls, but these bands often also appeared in specimens in which only \textit{P. falciparum}, \textit{P. malariae}, or \textit{P. ovale} were detected microscopically.

\textbf{Duffy antigen.} Erythrocytes from 9 of 11 subjects microscopically positive for \textit{P. vivax} were tested for Duffy blood group by flow cytometry, as were 22 children positive only for the other three species (Table 3). The lack of staining with the isotype control showed the specificity of recognition by the FY3 and FY6 antibodies. None of the children were FY3 positive, including SA376, from whom the MSP-1 C terminus was sequenced, nor were any of the nine additionally tested for FY6 found positive. Absence of either the FY3 extracellular loop or of the FY6 extracellular N terminus indicates down-regulation of the Duffy glycoprotein mRNA in all the subjects and, therefore, Duffy blood group negativity.

\section*{DISCUSSION}

Since the pioneering work of Miller and others 30 years ago,\textsuperscript{29} the mechanism by which \textit{P. vivax} uses the Duffy antigen to invade erythrocytes has been much studied. Invasion proceeds in two general stages: first, the merozoite recognizes, attaches, and orients itself on the erythrocyte surface, after which a moving junction forms, through which the parasite enters the cell.\textsuperscript{29} It is this second step that requires Duffy determinants. A 35-amino acid segment of the FY6 domain of the glycoprotein\textsuperscript{3} interacts with the Duffy binding protein, produced in the merozoite’s micromeres, to initiate the junction.\textsuperscript{6} The source of FY negativity in the co-dominant alleles is

\begin{table}[h]
\centering
\caption{Presence of \textit{Plasmodium} species in thin films in 9 of 11 Thai subjects studied.}
\begin{tabular}{lcccc}
\hline
Subjects & \textit{P. vivax} & \textit{P. falciparum} & \textit{P. malariae} & \textit{P. ovale} \\
\hline
SA 224 & f & 2.3 & 2.4 & 2.4 \\
SA 253 & f & 2.6 & 2.6 & 2.6 \\
SA 332 & f, o & 2.6 & 2.6 & 2.7 \\
SA 352 & f, o, m & 2.5 & 2.3 & 2.5 \\
SA 366 & f, o & 2.4 & 2.5 & 2.5 \\
SA 367 & f & 2.7 & 2.7 & 2.7 \\
SA 368 & f & 2.8 & 2.9 & 3.0 \\
SA 369 & f & 2.5 & 2.5 & 2.4 \\
SA 370 & f & 2.5 & 2.5 & 2.6 \\
SA 371 & f & 3.9 & 4.4 & 4.1 \\
SA 372 & f & 3.4 & 3.4 & 3.5 \\
SA 373 & f & 2.9 & 2.9 & 3.0 \\
SA 374 & f & 2.9 & 3.0 & 2.9 \\
SA 375 & f & 2.9 & 3.0 & 2.9 \\
SA 377 & f & 2.4 & 3.0 & 3.0 \\
SA 378 & f & 2.6 & 2.8 & 2.5 \\
SA 379 & f & 2.5 & 2.6 & 2.6 \\
SA 382 & f & 3.1 & 3.1 & 3.1 \\
SA 383 & f, m & 2.9 & 2.9 & 3.0 \\
SA 384 & f, g & 3.1 & 3.1 & 3.1 \\
SA 385 & f & 3.0 & 3.1 & 3.2 \\
SA 386 & f & 3.6 & 3.7 & 3.6 \\
\hline
\end{tabular}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Alignment of translated sequences of MSP-1 C-terminal gene sequences amplified from Kenya DNA samples 376 and 396 with known \textit{P. vivax} MSP-1 sequences from strains Belem, NYU-Thai and Salvador 1.}
\end{figure}
the same single nucleotide substitution, which prevents binding of the erythroid transcription factor, GATA-1. Homozygosity for this trait is nearly fixed among those populations of tropical Africa who have been tested, providing a satisfying explanation for the apparent absence of P. vivax from West Africa and, by extension, among most African Americans. It has never, however, been clear to what extent P. vivax is indeed missing from equatorial Africa. P. ovale and P. vivax so closely resemble each other microscopically that, even when staining is perfect, expert microscopists find them difficult to differentiate. Recently, a small but persistent number of travelers to West and Central Africa have been molecularly diagnosed with P. vivax. There have been sporadic reports of P. vivax from Kenya since the early 20th century; for example, Garnham, who was unlikely to have confused P. vivax with P. ovale, reported a P. vivax epidemic during 1943 in the Chelupunga Forest, abutting Nyanza Province on the east, and other cases were reported at high altitude. Generally such findings are explained a priori, in the absence of Duffy testing, as resulting from P. vivax transmitted locally among groups not homozygous for the negative alleles.

The paradox of the evidence we present is that P. vivax seems to be transmitted among people who are Duffy negative. Assuming that our procedures were properly executed, possible explanations for these data are that the parasite is not P. vivax and does not require Duffy for invasion, that the parasite is P. vivax that has evolved to use receptors other than Duffy, or that the study population has a unique blood group that serves in P. vivax junction formation. We will examine each of these possibilities in light of the limited data we have presented.

Although no one piece of our evidence is proof, the combination of the parts makes the identification of the parasite as P. vivax compelling. The circumsporozoite MAB 2E10 is highly specific for VK 247, excluding even P. ovale, whose CS has yet to be sequenced. It is possible that an as yet to be described feral Plasmodium or variant of P. malariae or P. ovale VK247, produced false positives, but this does not explain the amplification of P. vivax-specific SSU rDNA from sporozoites or of MSP-1 C terminus from blood. The case for P. vivax based on gene identification would be more convincing if other specific genes had been sequenced. Our procedures worked consistently to amplify from the Thai controls the expected bands for each targeted gene. The low parasite densities and near ubiquity of mixed infections may have prevented greater success with the Kenyan specimens, but may also indicate genes that have drifted substantially from the norm.

Plasmodium knowlesi, the primate model for P. vivax, can exploit receptors other than Duffy on monkey RBCs, as can the murine model, P. yoelii, on mouse RBCs. The sequence of the DBP domain of P. vivax that recognizes Fy6 has been shown, in specimens collected in Papua New Guinea, to be highly polymorphic, suggesting the merozoite may have the capacity for rapid adaptation. There is evidence that P. vivax can invade Aotus and Saimiri RBCs even when the Fy6 receptor has been blocked. Notably, all the CS ELISA results positive for P. vivax were of only the phenotype VK247, which in Asia and Latin America is scarcer than is VK210; in Ethiopia, only VK210 was found in 14 ELISA-positive Anopheles arabiensis (R. A. Wirtz, personal communica-

tion). Although sometimes referred to as “variants,” VK210 and VK247 are the only CS antigenic phenotypes yet discovered and, in fact, each exhibits limited, mostly silent base variation. There is evidence from Mexico that the two phenotypes are differentially infective to local vectors, suggesting biologic differences unrelated to CS protein function. It is possible that VK247 marks a P. vivax population capable of using receptors other than Duffy.

The children we studied were Luo, a Nilotic ethnic group that began to emigrate from southern Sudan > 500 years ago. Little blood typing has been published for the Luo, and nothing about Duffy before now. It is possible that the Luo uniquely express on erythrocytes molecules other than Duffy that can serve in junction formation. It is also possible that a relatively rare, alternate receptor is widely distributed; P. knowlesi invades Duffy negative RBCs treated with trypsin, suggesting the availability of alternates that are normally masked. The number of Duffy-negative humans who have been tested either experimentally or empirically for susceptibility to P. vivax invasion has been small.

Neither the large number of positives that were An. funestus nor the relatively high positivity rate at one village are necessarily significant. Malaria transmission has often been found to be focal, and small differences in drainage, farming practices, and vegetation can have profound effects on the mix and abundance of Anopheles. The appearance of nearly all the P. vivax positives late in the transmission season is, however, striking; in seasonal, temperate zone transmission, P. vivax traditionally peaks before P. falciparum. The late pattern suggests a very small number of gametocyte carriers early in the season and possibly immune suppression by P. falciparum.

The meaning of our findings will only become clear with directed research. In particular, the identification of even a single volunteer donor with a density of the putative P. vivax sufficiently high to allow comprehensive testing of both blood and parasite will be necessary before additional epidemiologic studies are done.
REFERENCES


