DIFFERENT PREVALENCES OF *Plasmodium vivax* PHENOTYPES VK210 AND VK247 ASSOCIATED WITH THE DISTRIBUTION OF *Anopheles albimanus* AND *Anopheles pseudopunctipennis* IN MEXICO

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Abstract. The geographic distribution of *Plasmodium vivax* circumsporozoite protein phenotypes from patient blood used to infect colonized *Anopheles albimanus* and *A. pseudopunctipennis* was investigated in southern Mexico. Parasite phenotype types were determined in blood samples by a polymerase chain reaction and oligoprobe hybridization or by immunofluorescent assay of sporozoites. The proportion of infected mosquitoes and the number of oocysts per mosquito confirmed previous *in vitro* observations indicating that *A. albimanus* is more susceptible to VK210 and that *A. pseudopunctipennis* is more susceptible to VK247. All patients living on the coast were infected with VK210 and most patients living above 170 meters above sea level had VK247. Both phenotypes infected patients from intermediate altitudes. These results concur with the distribution of the anophelines, indicating that *A. albimanus* is the main vector of the phenotype VK210, but that *A. pseudopunctipennis* transmits both phenotypes. These conditions have direct implications on parasite transmission rates and malaria epidemiology in Mexico.

Fifty percent of the Mexican territory is occupied by potentially malarious areas. In these areas, *Anopheles albimanus* is the primary vector in the coastal plains of the Gulf of Mexico and the Pacific Ocean, and *A. pseudopunctipennis* is the main vector in the foothills and low mountainous areas bordering the Pacific Ocean coast. Malaria control activities have traditionally better success in areas infested by *A. albimanus*, whereas permanent transmission foci persist in areas infested by *A. pseudopunctipennis*, in spite of more intensive control interventions.

*Plasmodium vivax* produces 98% of all malaria cases in the country. The infective stage of malaria parasites, the sporozoite, is covered by a surface coat predominantly made of a single protein (circumsporozoite protein [CS]). Two CS phenotypes of *P. vivax* have been identified according to the amino acid sequence of the repeat units located in their central region, (GDRAA/DGQPA) in phenotype VK210 and (ANGAGNQPG) in phenotype VK247. These phenotypes of worldwide distribution have also been identified in Mexico.

Using enzymatic amplification of *P. vivax* DNA from a limited number of blood samples of malaria patients from southern Mexico, we found that all infections in patients living in the coastal plain were caused by VK210; in contrast, 50% of the cases occurring in the foothills had VK247. Preliminary experimental infections comparing the susceptibility of colonized *A. albimanus* and *A. pseudopunctipennis* indicated that *A. albimanus* is more susceptible to the phenotype VK210, and that *A. pseudopunctipennis* is more susceptible to the phenotype VK247. We have now extended these observations to include more simultaneous infections of both mosquito species using blood from *P. vivax*-infected patients. Additionally, we have examined a single protein (circumsporozoite protein [CS]). Two CS protein types in stored sporozoites samples obtained from experiments conducted since 1996. We present herein results from both sets of experiments, which confirm that these vectors display different susceptibilities to the parasite phenotypes. Furthermore, an analysis of the geographic distribution of malaria cases indicates that the prevalence of the VK210 and VK247 parasite polymorphs is directly associated with the distribution of the corresponding vector.

MATERIALS AND METHODS

Patients. Gametocyte-carrying, *P. vivax*-infected subjects, diagnosed by Giemsa-stained blood smears at the Center for Malaria Research (CIP) in Tapachula, Chiapas, provided 5-ml blood samples. Venous blood samples withdrawn in heparin-treated syringes and maintained at 37°C were used to feed mosquitoes. The age, sex, history of previous malaria episodes, and place of residence were obtained from each patient. An informed consent form was signed previous to blood withdrawal by all patients or by the parents of minors. Treatment with chloroquine and primaquine was provided to all patients following blood withdrawal. The procedures were approved by the Ethics Committee of the Ministry of Health, Mexico.

Mosquito infections. *Anopheles albimanus* (white stripped phenotype) and a strain of *A. pseudopunctipennis* were used in these experiments. Female mosquitoes 2–6 days post-emergence were fed on *P. vivax*-infected blood through Parafilm® (American National Can, Neenah, WI) membranes. Since immune factors present in patient sera may affect the parasite infectivity to mosquitoes, the original serum was substituted with a pool of sera negative for antibodies to *Plasmodium* from subjects with no history of malaria. The blood from the same patient was used to feed both mosquito species simultaneously, but in experiments conducted in 1996 and 1997, when a colony of *A. pseudopunctipennis* was unavailable, only *A. albimanus* were fed. Blood-fed mosquitoes were maintained at 23–28°C and a relative humidity of 70–80% in an environment-controlled insectary and were offered 10% sucrose in a soaked cotton pad. All mosquitoes fed with 1 patient’s blood were considered as 1 cohort.
To estimate infection rates, the surviving mosquitoes from each species lot were dissected on day 7 postinfective blood meal. Midguts were stained with 1% mercuric chloride11 and oocyst numbers were recorded using a light microscope (magnification 400×). In the case of experiments using only An. albimanus, a sample of 10 surviving mosquitoes were analyzed by dissecting salivary glands on day 14 postinfection. All infected glands were pooled and used in parasite phenotype identification by immunofluorescent assay (IFA).

Identification of parasite phenotypes. Parasite phenotype identification was conducted either by identification of the gene codifying for each phenotype in patient blood samples or by immunofluorescence. Samples from the blood used to feed mosquitoes were blotted onto Whatman (Maidstone, United Kingdom) filter paper no. 2, air-dried, and stored in individual plastic bags at −20°C until shipped to The Toronto Hospital. Genomic DNA was extracted from dried blood dots using Qiagen columns (Qiagen, Chatsworth, CA) according to the manufacturers’ directions. A portion of the P. vivax CS gene was amplified by a polymerase chain reaction (PCR) from each sample using primers PV5A and PV6A as described.10,18 The PV5A and PV6A primer pair produce an ~700 basepair fragment as determined by gel electrophoresis and visualization under UV light after staining with ethidium bromide. Positive and negative blood and DNA samples were included in each amplification run. Cross-contamination was prevented as previously described.19 Samples (10 µl) of each amplification reaction were slot-blotted in triplicate, together with negative and gonucleotide probes to VK210 and VK247 as described.10,18

Air-dried salivary gland sporozoites were obtained by dissection on day 14 postinfective blood meal and stored at −70°C until used in immunofluorescence assays.20 Parasite phenotype identification was carried out using monoclonal antibodies that recognize either the P. vivax CS protein-210 repeat region or the CS protein-247 repeat region.21 These assays and the genotyping analysis were performed blinded to the results of entomologic and demographic investigations.

Statistical analysis. Data were analyzed using a statistical package (Stata Statistical Sofware, Release 5.0; Stata Corporation, College Station, TX). In experiments where both mosquito species were fed simultaneously, only those where at least 1 species was infected were included in the analysis. In experiments using exclusively An. albimanus, only those where samples of salivary gland sporozoites were obtained were analyzed. Maximum Likelihood Estimation methods were used to estimate the parameters of 2 generalized linear models.22 This modeling technique allows one to fit linear models to variables with distributions other than a normal distribution, including the binomial and Poisson distributions. To estimate the proportions of infected mosquitoes, their odds ratio (OR) and 95% confidence interval (CI), a binomial distribution was assumed to fit a logistic model. To test for differences in the estimated proportions, a significance test for the model coefficients and their linear combinations was made. Likewise, to estimate the mean oocyst number per mosquito and its ratios, a Poisson distribution was assumed to fit a Poisson model.23 To account for over-dispersion found in the data, we used a further generalization of this methodology (quasi-likelihood estimation)24 that estimates the variance of the model coefficients taking the data over-dispersion into account. All tests were made at the 95% confidence level. The proportion of infected mosquitoes (mean infection rate) was modeled using a logistic regression according to mosquito species and parasite phenotype. The number of oocysts per infected mosquito (mean oocyst infection intensity) was analyzed using a Poisson model. Variables included in the models were the CS protein polymorph, mosquito species and their interaction. All tests were carried out at a 95% significance level (α = 0.05). The geographic distribution of malaria cases was examined using MapInfo desktop mapping software (MapInfo Corporation, Troy, NY), and a binomial test was used to compare the proportions of patients infected with each parasite phenotype type in 3 altitude categories (coast, intermediate, and foothills).23

RESULTS

Identification of parasite phenotypes. Ninety-six P. vivax mosquito infections using infected patients were included in the study. In 37 experiments, the parasite phenotype was identified by both PCR genotyping and by immunofluorescence of salivary gland sporozoites. The concordance between the 2 methods was 100%. All simultaneous infections of the 2 mosquito colonies were included in this group. Only PCR genotyping was conducted in 7 experiments and only sporozoite immunofluorescence was conducted in 52. All of these corresponded to experiments using only An. albimanus. Both parasite phenotypes were detected by PCR in only 1 patient from the foothills. This patient was excluded from the study.

Simultaneous infections of both mosquito species. Thirty-one simultaneous P. vivax infections of An. albimanus and An. pseudopunctipennis mosquitoes were obtained using blood from an equal number of different patients. Circumsporozoite genotyping indicated that 9 infective blood meals corresponded to CS protein phenotype VK210 and 22 to VK247. The proportion of infected mosquitoes, as well as the mean number of oocysts per mosquito per experiment, are shown in Table 1. Because fewer An. pseudopunctipennis took a blood meal from the artificial membranes, more An. albimanus were dissected than in An. pseudopunctipennis. A total of 590 An. albimanus and 208 An. pseudopunctipennis were examined. Mosquito infections were associated with CS protein phenotypes; P. vivax VK 210 infected predominately An. albimanus, but a few An. pseudopunctipennis were infected. Conversely, P. vivax VK 247 infected predominantly An. pseudopunctipennis, but a few An. albimanus were infected.

The fitted proportions of infections with VK210 and VK247 in An. albimanus and An. pseudopunctipennis obtained in the logistic model were very close to the observed ones (Table 1). The proportion of infection with VK210 was significantly higher in An. albimanus than in An. pseudopunctipennis (OR = 5.56, 95% CI = 3.06–10.13, P < 0.0001). In the same way, the proportion of An. pseudo-
TABLE 1
Proportion of infected mosquitoes and mean number of *Plasmodium vivax* oocysts per mosquito in experiments where infected patient blood was simultaneously fed to *Anopheles albimanus* and *Anopheles pseudopunctipennis*, and in experiments using *An. albimanus* only

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>Parasite variant</th>
<th>n*</th>
<th>Proportion of infected mosquitoes</th>
<th>Mean ± SD</th>
<th>Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. albimanus</em></td>
<td>VK210</td>
<td>145</td>
<td>0.72</td>
<td>58.19 ± 45.61</td>
<td>43 (2–100)</td>
</tr>
<tr>
<td><em>A. pseudopunctipennis</em></td>
<td>VK210</td>
<td>78</td>
<td>0.32</td>
<td>8.00 ± 9.70</td>
<td>3 (1–31)</td>
</tr>
<tr>
<td><em>A. albimanus</em></td>
<td>VK247</td>
<td>445</td>
<td>0.08</td>
<td>7.18 ± 13.46</td>
<td>2 (1–66)</td>
</tr>
<tr>
<td><em>A. pseudopunctipennis</em></td>
<td>VK247</td>
<td>130</td>
<td>0.89</td>
<td>24.05 ± 25.18</td>
<td>16 (1–135)</td>
</tr>
</tbody>
</table>

Experiments using 2 mosquito species

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>Parasite variant</th>
<th>n*</th>
<th>Proportion of infected mosquitoes</th>
<th>Mean ± SD</th>
<th>Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. albimanus</em></td>
<td>VK210</td>
<td>50</td>
<td>0.71</td>
<td>41.64 ± 44.74</td>
<td>27 (1–318)</td>
</tr>
<tr>
<td><em>A. albimanus</em></td>
<td>VK247</td>
<td>416</td>
<td>0.24</td>
<td>11.36 ± 14.91</td>
<td>5 (1–95)</td>
</tr>
</tbody>
</table>

* n = number of mosquitoes examined.

*punctipennis* infected with VK247 was significantly higher than that of *An. albimanus* (OR = 17.57, 95% CI = 51.98–46–36.47, *P* < 0.0001). The expected mean oocyst counts per mosquito in the Poisson model were also very similar to the observed ones. This model demonstrated significant differences by species and CS protein polymorphs (*P* < 0.0001).

Infections of *An. albimanus* only. In 65 experiments, only *An. albimanus* were infected; of these, 45 corresponded to infections with the phenotype VK210 and 20 with VK247 based on the IFA results using specific monoclonal antibodies. The mean infection rate and the mean oocyst infection intensity with *P. vivax* VK 210 were higher than in infections with VK 247 (Table 1). Differences in infection rates for these experiments were also tested using a logistic regression analysis, which demonstrated significant differences by parasite phenotype (OR = 7.58, 95% CI = 5.64–10.18, *P* < 0.0001). Significant differences in oocyst infection intensities were documented using a Poisson model (*P* < 0.0001).

Geographic distribution of malaria cases. The total number of malaria cases diagnosed in the study area between 1996 and 1998 was 1,281; 611 cases were from the foothills, 284 from the coast, and 386 from the Tapachula area. The CIP diagnosed 651 cases (84 from the coast, 284 from the foothills and 283 from Tapachula). Assuming that the proportions of parasite phenotypes was uniform in the study area, the calculated sample size needed to estimate the proportion of each parasite phenotype (*P* = 0.05, power = 0.9), using the normal approximation of the binomial distribution with a finite population correction,\(^2\) was 89 cases (we examined 96 patients). The geographic distribution of these malaria cases is shown in Figure 1. The majority of infections (83.34%, 30 of 36 cases) at higher altitudes were produced by the polymorph VK247. The phenotype VK210 was responsible for 70% (28 of 40 cases) of infections in people living at intermediate altitudes (170 meters above sea level, Tapachula City) while VK247 produced 12 of these cases. All malaria cases in individuals living on the coastal plains (20 cases) corresponded to infections with the phenotype VK210 (Figure 2). Assuming that the prevalence of VK247 in the coastal plains was similar to that in the foothills (83.3%) a sample of 20 cases would have detected a prevalence of this phenotype between 70% and 90%, with a significance level of 90%.\(^2\) A binomial test showed that the proportions of the parasite phenotypes in the coast and the foothills were significantly different (all *P* < 0.0001).

All mosquito infections (from 30 patients) occurring in 1996 were produced by *P. vivax* VK210. In 1997, 5 of 24 infections (24%) were produced by VK247, but in 1998, 37 of 42 infections (88%) corresponded to this phenotype (Figure 2). Assuming that the prevalence of VK247 in the coastal plains was similar to that in the foothills (83.3%) a sample of 20 cases would have detected a prevalence of this phenotype between 70% and 90%, with a significance level of 90%.\(^2\) A binomial test showed that the proportions of the parasite phenotypes in the coast and the foothills were significantly different (all *P* < 0.0001).

Secondary malaria episodes. Of 54 patients infected with *P. vivax* VK210, 22 reported previous malaria episodes within 2 years, of which 19 occurred within the last year (9 of which occurred within the preceding 6 months). In 5 of these patients, the parasite phenotype was identified during the primary and secondary episodes, and corresponded in all cases
would expect to observe degenerated oocysts in mosquitoes that failed to become infected. Because these were not observed, it is difficult to directly associate the CS protein phenotypes with the resistance process. Other possible unidentified molecular differences in early sporogonic stages of the parasite phenotypes may exist that, in interaction with each mosquito species’ midgut structure and physiology, could determine mosquito species-specific parasite infectivity.

The fact that *P. vivax* polymorphs behaved exactly the opposite in the 2 anopheline species tested in this study may have resulted from selection of mosquito strains during colonization. The *An. albimanus* strain has been in the laboratory for more than 10 years and the white-striped strain was maintained for its high susceptibility to *P. vivax*. However, new infectivity tests with our 3 *An. albimanus* colony strains (white stripe, green, and brown) indicated that although the white-striped strain was more susceptible to both *P. vivax* phenotypes than the other 2 strains, all of them were more susceptible to the phenotype VK210 (Gonzalez-Ceron L and others, unpublished data). In the case of *An. pseudopunctipennis*, of recent colonization (1 year), preliminary experiments using a different colony raised from *An. pseudopunctipennis* collected in northern Mexico indicate that both strains are more susceptible to the phenotype VK247, thus confirming the preferential susceptibility of both species.

The recorded differential susceptibilities are consistent with the distribution of the parasite polymorphs, which follows the geographic distribution of the 2 anopheline vectors in the region. *Anopheles albimanus* larval habitats occur in water collections that form in the coastal plain during the rainy season. *Anopheles pseudopunctipennis* breeds on water collections in the foothills. Accordingly, in this study all patients residing in villages located on the coast were infected with *P. vivax* VK210, indicating an association of this phenotype with *An. albimanus*, the local vector. Most infections at altitudes more than 170 meters above sea level were produced by VK247, indicating an association of this phenotype with *An. pseudopunctipennis*, the local vector. Previous records of *An. pseudopunctipennis* collected in the foothill area suggest that although this species is more susceptible to VK247, it is able to transmit both parasite phenotypes. At intermediate altitudes where both vectors are present, infections were produced by both parasite phenotypes, but 70% were caused by VK210. These observations are consistent with the exclusive infection with the phenotype VK210 in humans living in the Chiapas Pacific Ocean Coast and the infection with both phenotypes in the foothills, which was previously observed. In a study including 1,711 experiments where *An. dirus* were infected with *P. vivax*-infected patient blood, Suwanabun and others documented different phenotype prevalences in 5 endemic villages in Thailand, and suggested that this could have resulted from the highly focal nature of malaria transmission. Focalization of transmission could contribute to the distribution pattern of *P. vivax* polymorphs in southern Chiapas, but a serologic study on military personnel indicates that the association of the parasite polymorphs with the mosquito vectors may also occur in larger areas in Mexico. Accordingly, the seroprevalence to VK210 was high in *An. albimanus* areas (Gulf of Mexico Coast and Yucatan Peninsula), while sero-
prevalence to VK247 was higher in An. pseudopunctipennis regions (Pacific Coast, north of Chiapas).

In the Thai study, a seasonal distribution with a higher prevalence of the phenotype VK 210 during the dry season was documented. Several possible explanations were offered: shorter persistence in humans, longer extrinsic cycle of VK247, or seasonal abundance of susceptible vectors. In our experiments, the temporal distribution of the parasite phenotypes in the malaria cases used to infect mosquitoes did not correlate with the seasonal abundance of both malaria vectors. This may be the result of the way patients were recruited in this study in that they came voluntarily to CIP for diagnosis and treatment.

The different distribution of parasite phenotypes and their association with a particular vector may provide new insights into our understanding of malaria epidemiology in Mexico, where malaria control has been more difficult in An. pseudopunctipennis areas. In Papua New Guinea, Burkot and others documented a fluctuation in the proportion of the local vector (An. punctulatus) infected with the 2 P. vivax polymorphs, indicating the possibility of a periodic selection due to immune pressure by the human population on each phenotype at the time. This temporal fluctuation was not seen over a 5-year period in Thailand.

In our patients, a clear shift from VK210 to VK247 infections was observed from 1996 to 1998. Since An. albimanus and An. pseudopunctipennis dwell in different ecologic systems and because An. albimanus seems to transmit preferentially (if not only) VK210 on the coast, the effect of human immunity to each parasite phenotype would not be expected to induce the fluctuations observed in Papua New Guinea. This suggests that malaria control may be easier in the coastal areas. However, infections with both parasite polymorphs were recorded in the foothills, indicating that the shift in the phenotype type infections we observed is occurring in these places. Fluctuations in the immune pressure to 1 phenotype might induce a preferred transmission of the other and thus might facilitate the continuous presence of malaria transmission in the region where both polymorphs exist.

A contributing factor to maintaining P. vivax transmission in endemic areas is the rate of relapsing episodes (secondary cases due to reactivation of dormant liver parasites). Of our cases, only 2 patients infected with P. vivax VK247 indicated they had previously had malaria (within 2 years), whereas 35% of those with VK210 recalled bouts of malaria during the previous 3–12 months. Although it is not possible to separate relapsing episodes from reinfections, the same parasite phenotype was identified in 5 of these cases. Additional VK210 secondary episodes could also result from more intense malaria transmission on the coast than in the foothills. However, since malaria transmission was decreasing on the coast as opposed to the foothills (Figure 2), this is unlikely.

These apparently different relapsing rates between parasite polymorphs require confirmation, and our observations should be extended to more patients in other malarious areas of the country. If confirmed, they would be the first clinical indication that the P. vivax polymorphs differ as much in their CS protein as in other characteristics determining their interaction with their vertebrate host. Kain and others reported that VK210 and VK247 responded differently to chloroquine treatment, and it is possible that their differences in relapsing rate could be a result of differences in their susceptibility to treatment with primaquine.

It is worth noting that a low relapsing rate in P. vivax VK247 would be a disadvantage to its survival under pressure from intervention. This would indicate that the difficulties in dealing with the disease in the foothills originate mainly from the ability of An. pseudopunctipennis to transmit both parasite phenotypes and the favored contact of this mosquito with humans, as well as its relatively high vectorial capacity.

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