AOTUS LEMURINUS GRISEIMEMBRA MONKEYS: A SUITABLE MODEL FOR PLASMODIUM VIVAX SPOROZOE INFECTION

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Abstract. This study describes a successful Plasmodium vivax sporozoite infection in Aotus lemurinus griseimembra. Twenty-eight naive or previously infected monkeys, either splenectomized or spleen intact, were inoculated intravenously or subcutaneously with Plasmodium vivax sporozoites of the Salvador I strain or with two wild isolates (VCC-4 and VCC-5; Vivax-Cali-Colombia). The monkeys were successfully infected regardless of the parasite strain, spleen presence, or inoculation route and showed prepatent periods that ranged from 16 to 89 days. Only one monkey inoculated intravenously failed to develop parasitemia. Since immune protection against malaria pre-erythrocytic forms is mediated by both helper and cytolytic T cells that may home in the spleen and P. vivax cultures are not yet available; the use of spleen-intact A. lemurinus griseimembra, susceptible to both adapted and non-adapted strains of P. vivax sporozoites, is a valuable model for evaluation of pre-erythrocytic vaccine candidates.

INTRODUCTION

Malaria is the most important parasitic disease of humans and, along with tuberculosis and infection with human immunodeficiency virus, is one of the three leading causes of morbidity and mortality in tropical areas of the world. Most clinical cases of malaria are caused by Plasmodium falciparum, the most prevalent parasite in Africa, and by Plasmodium vivax, which is responsible for most cases in Asia, Oceania, and Latin America. Approximately 80 million malaria cases are caused yearly by P. vivax. These two parasite species are the subject of intense study in the search for a malaria vaccine to complement the current control strategies. One limitation in the process of developing malaria vaccines has been the lack of reliable animal models for preclinical studies. Additionally, because P. vivax cannot be continuously cultured in vitro, animal models may represent a valuable source of parasites.

Two genera of New World monkeys, Aotus and Saimiri, have been used as models for malarial studies because they are small, convenient to breed and handle, relatively inexpensive to maintain, and susceptible to both P. vivax and P. falciparum parasite blood infections. As a result, these monkey species have been used successfully for preclinical assessment of malaria vaccine candidates targeting parasite blood stages, as well as for the assessment of new antimalarial lead compounds. However, infection by sporozoites has generally been more difficult to achieve. Previous studies have shown that several types of some Aotus species can be infected with sporozoites from P. vivax strains previously adapted to grow in monkey blood, although they have more prolonged prepatent periods than the Saimiri model. We addressed the question of whether Aotus lemurinus griseimembra, a primate species being successfully used to assess malaria vaccine candidates directed at controlling asexual blood stages, could be infected with P. vivax sporozoites in a reproducible manner, and thereby lead to a convenient model for vaccine testing. For this purpose we used A. lemurinus griseimembra monkeys and inoculated them with P. vivax sporozoites produced in colonized Anopheles albimanus mosquitoes artificially fed with parasites obtained from either malaria-infected patients or monkeys. Development of this model is of great value for the assessment of P. vivax pre-erythrocytic vaccines.

MATERIALS AND METHODS

Primates. Thirty-eight monkeys were used in the study. Thirty-two were A. lemurinus griseimembra adults (males and nonpregnant females) from the northern forest of Colombia that were either malaria naive or with a history of malaria and weighed more than 800 grams. Six were Saimiri sciureus adults (males or nonpregnant females) that were malaria naive and weighed more than 600 grams. They were born in captivity or in the Amazon rain forests and were kept in captivity at the Primate Center in Cali, Colombia. All animals had been in captivity more than 120 days and had been subjected to the corresponding quarantine periods (60 days), during which extensive laboratory testing and serologic analysis were conducted to test for intestinal and blood parasites or antimalarial antibodies, respectively. The experimental protocol was reviewed and approved by the Animal Ethical Committee of Universidad del Valle, and both handling and experimentation were conducted following National Institutes of Health guidelines for the Care and Use of Laboratory Animals.

Parasite. Plasmodium vivax–parasitized red blood cells (pRBCs) were maintained as frozen stables in liquid nitrogen or freshly obtained from malaria-infected patients. The P. vivax Salvador I strain (Sal I) was provided by Dr. William Collins (Centers for Diseases Control, Atlanta, GA) and was kept frozen until use. Parasites were thawed and injected intravenously into a donor monkey (M-126). Sporozoites were then produced from this monkey by mosquitoes that fed on gametocyte-carrying blood by artificial membrane feeding. Serial passages were performed.

Fresh P. vivax isolates were obtained from infected patients from a Colombian malaria-endemic area and were named VCC-4 and VCC-5 (Vivax-Cali-Colombia). After obtaining written informed consent, we took blood samples from P. vivax–infected patients attending an outpatient clinic in Buenaventura, Colombia. Samples were transported at 37°C to the Instituto de Inmunología at Universidad del Valle in

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Cali and were used to feed mosquitoes to obtain sporozoites that were used to infect experimental monkeys.

**Experimental design.** The descriptive study was divided into two experiments, the first one exploratory and the second comparative. The main objectives of experiment 1 were to complete the malaria cycle through the vertebrate and invertebrate hosts by serial passages performed using both pRBCs and sporozoites and to determine the infection pattern in every case. To accomplish this objective, 14 *Aotus* monkeys, 3 infected twice, and 6 *Saimiri sciureus* monkeys were arbitrarily selected from a larger group, splenectomized, and experimentally inoculated with either pRBCs or sporozoites. Blood was drawn from infected monkeys, and the level of trophozoites was determined. In some cases, parasitemias level in the inocula could not be determined (Table 1). *Plasmodium vivax* Sal I and VCC-4 isolates were serially passed in *P. falciparum*.*

Experiment 2 was carried out in 18 naïve monkeys that were arbitrarily assigned to three different groups of six monkeys each and inoculated with the same dose of VCC-5 sporozoites (20,000). The dose was selected based on results from experiment 1. Group I had intact spleens and was inoculated intravenously; group II was splenectomized and inoculated intravenously; group III had intact spleens and was inoculated subcutaneously. Prepatent periods were compared across groups.

**Sporozoite production.** We infected *An. albimanus* reared in our mosquito colony by an artificial membrane feeding assay (MFA).¹⁶ Fourteen days after infection, the salivary glands were dissected under a stereomicroscope, and sporozoites were collected in phosphate-buffered saline (PBS). We estimated the total number of sporozoites by counting them in a Neubauer cell-counting chamber. Aliquots of a variable quantity of sporozoites were diluted in 500 µL of PBS and were used to inoculate monkeys as previously described.¹⁶

**Splenectomy.** Splenectomies were performed either before or after parasite inoculation. Animals were anesthetized by intramuscular injection of midazolam (0.1 mg/kg) and ketamine chloride hydrate (15 mg/kg) and surgery was conducted by a qualified veterinary surgeon under aseptic conditions.

**Treatment.** After challenge, infections were treated when the parasitemia was 1% or when the hematocrit reach 20%. Curative treatment consisted of a combination sulfadoxine-pyrimethamine (25 mg/kg) in one dose given orally.

**Follow-up of malaria infection.** Parasitemia was followed using three different methods: thick and thin blood smears, polymerase chain reaction (PCR), and mosquito xenodiagnosis. The prepatent period was selected as the first day of infection diagnosed by any of the three methods.

Thick and thin blood films were made three times a week after 10 days post-inoculation. Slides were stained with Giemsa and analyzed for the presence of malaria parasites by two independent readers. Parasitemia levels (pRBCs/µL) were calculated by multiplying the number of parasites present in microscopic fields corresponding to 300 leukocytes by a mean of 14,100 leukocytes/mL of monkey blood.¹⁷ The presence of parasite DNA was diagnosed by PCR every seven days beginning 10 days after inoculation. Using the salting-out technique, we extracted genomic DNA from 500 µL of whole EDTA-stabilized blood. The DNA was used immediately or stored at 4°C. A nested PCR was conducted using primers complementary to the gene coding for the small ribosomal subunit RNA.¹⁸ The resulting PCR product was analyzed for the presence of a 121-basepair band after electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

Mosquito xenodiagnosis consisted of checking the infection of *Aotus* by the detection of parasites in mosquitoes fed with blood from *Aotus* previously inoculated with either trophozoites or sporozoites of *Sal I* or VCC-4 *P. vivax* strains. Xenodiagnosis was performed by artificial membrane feeding at least once during the follow-up of each monkey. Samples of mosquitoes from each batch were examined for oocysts on days 7–9 in the midguts and for sporozoites in the salivary glands on days 13–15. Their presence was a positive result.

We also checked the hematocrit level in all animals before inoculation and once a week during follow-up.

**Statistical analysis.** Length of the prepatent period, maximum level of parasitemia, peak day of parasitemia, and thick blood smear results are provided for monkeys included in experiment 1 by strain. For experiment 2, length of prepatent period, PCR, and thick smear results are summarized by ex-

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**Table 1**

<table>
<thead>
<tr>
<th>Monkey code¹</th>
<th>Previous malaria</th>
<th>Spplenectomy</th>
<th>Inoculum x 10⁷</th>
<th>Prepatent period (day)</th>
<th>Maximum parasitemia</th>
<th>Thick smear</th>
<th>Xenodiagnosis</th>
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<td></td>
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<td></td>
<td>Spz</td>
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<td>6</td>
<td>4,556</td>
<td>17</td>
<td>ND</td>
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<td>9</td>
<td>+</td>
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<td>†</td>
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<td>27</td>
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<tr>
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<td>†</td>
<td>2</td>
<td>544</td>
<td>2</td>
<td>5/41</td>
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<tr>
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<td>†</td>
<td>55</td>
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<td>M-215</td>
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<td>8</td>
<td>40</td>
<td>204</td>
<td>40</td>
<td>3/49</td>
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<td>M-126‡</td>
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<td>Yes</td>
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<td>32</td>
<td>1,360</td>
<td>32</td>
<td>13/23</td>
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<td>2,924</td>
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<td>V-69</td>
<td><em>P. falciparum</em></td>
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<td>89</td>
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<td></td>
<td>43</td>
<td>89</td>
<td>1,139</td>
<td>53</td>
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</tbody>
</table>

* PCR = polymerase chain reaction; PRBCs = parasitized red blood cells; ND = not done; Spz = sporozoites; P. f. = *P. falciparum*; P. v. = *P. vivax*.

† Monkey code with a V indicates an animal born in captivity at the Primate Center in Cali, Colombia.

‡ First infection.

§ Second infection.
perimental group. The results for all *A. lemurinus griseimembra* monkeys inoculated with sporozoites across the two experiments are summarized by spleen presence, strain, and infection route.

**RESULTS**

In the first experiment, all monkeys except *Aotus* M-213 developed patent asexual parasitemia as determined by the methods described in this report, and all were infective to the mosquitoes (sexual parasitemia) (Tables 1 and 2). We infected mosquitoes with gametocytes by artificial MFA, which resulted in isolation of more sporozoites. These were subsequently used to infect new monkeys (Figure 1). Our results allowed us to choose a minimum infective inoculum of 20,000 sporozoites that we used to carry out the second experiment.

**Cyclic passage of the *P. vivax Salvador I* strain.** A geneology of the serial passages for the *P. vivax* Sal I strain is shown in Figure 1A. Inoculation of $16 \times 10^5$ trophozoites derived from the frozen stabile induced patent parasitemia in one *Aotus* (M-126) within a period of one week; from this primary infection, three monkeys were infected and two lines were established. For the first line, *An. albimanus* mosquitoes were infected by artificial MFA and dissected two weeks later. Sporozoites from their salivary glands were inoculated into *Aotus* monkey M-215 and in *Saimiri* monkey S-05. No additional studies were done with this line. For the second line, *Aotus* monkey V-79 was infected with trophozoites, and blood parasites were successively passed from this monkey to both splenectomized and spleen-intact animals (M-129, M-135, and V-74). Sporozoite batches were produced from the same animal (V-79) and used to infect *Aotus* V-69 and *Saimiri* S-06. These monkeys were inoculated with $2 \times 10^4$ sporozoites each and from them multiple sporozoite batches were produced to induce the parasite cycle in other monkeys.

**Adaptation of the wild *P. vivax VCC-4* strain to monkeys.** To adapt new *P. vivax* parasites to grow in monkeys, we infected *An. albimanus* mosquitoes by feeding them with the infected patient blood delivered through the artificial feeder. Mosquitoes were dissected 14 days after infective feed, and the sporozoites produced were used to infect *Aotus* M-213 and *Saimiri* S-04 monkeys. From the latter, two lines of transmission were generated for serial sporozoite passages in six *Aotus* monkeys (M-131 was infected twice). Additionally, a third line of transmission was initiated by inoculation of parasitized blood into *Saimiri* monkey S-19. Sporozoites were again produced from blood of this animal and used to infect *Saimiri* monkey S-31 (Figure 1B).

**Parasitemia follow-up.** Monkeys infected by blood passages of the Salvador I strain showed a prepatent period that ranged from 2 to 55 days with a mean of 18 days, whereas for sporozoite infections, this period was 9–89 days with a mean of 43 days (Table 1). The maximum mean parasitemia for Sal I blood infections was $1,700$ parasites/μL (range = $544–4,556$ parasites/μL), whereas for sporozoite infections, the maximum mean parasitemia was $1,139$ parasites/μL (range = $68–2,924$ parasites/μL). We observed these parasitemia peaks between days 2 and 27 (mean = 13 days) for trophozoite infections and between 32 and 120 days (mean = 53 days) for

![Figure 1](image-url)  
**Figure 1.** Passage sequence of *Plasmodium vivax* strains in *Aotus* and *Saimiri* monkeys. **A**, Serial passages of the Sal I strain were made by trophozoite injection or sporozoite inoculation through either intact or splenectomized monkeys. **B**, Passage sequence of the VCC-4 strain in *Aotus* monkeys. Sporozoites produced from blood of an infected patient were inoculated into *Aotus* monkey M-213 and *Saimiri* monkey S-04. Serial passages were then made by sporozoite inoculation or trophozoite injection through either intact or splenectomized monkeys. M and V indicate *Aotus lemurinus griseimembra* monkeys and S indicates *Saimiri sciureus* monkeys. *Monkeys infected with parasitized red blood cells.
sporozoite infections. All monkeys developed a patent parasitemia (Table 1) and induced mosquito infection, allowing us to obtain sporozoites for new passages. For Saimiri monkeys S-05, S-06, and S-12, the prepatent period was 27, 52, and 37 days, respectively, and parasitemia peaks were observed on days 34, 52, and 100, respectively.

We found a high reproducibility of the VCC-4 wild parasite infection through the serial passages in Aotus monkeys (Table 2). The prepatent period was between 16 and 80 days with a mean of 28 days. However, Aotus monkey M-213 did not show patent parasitemia after the first injection. The peak parasitemia ranged from 272 to 4,216 parasites/µL with a mean of 1,914 parasites/µL. VCC-4 sporozoite inoculation into Saimiri monkey S-04 induced an infection that was first detected on day 16; parasitemia peaked on day 74. Saimiri monkey S-19 was infected with blood stages obtained from monkey S-04 with a prepatent period of 25 days, and its parasitemia peak was detected on day 74. Saimiri monkey S-31 showed a similar prepatent period (27 days) and peak parasitemia (83 days) after sporozoite inoculation.

*Plasmodium vivax* VCC-5 sporozoite challenge. Sixteen of the 18 monkeys in the *P. vivax* VCC-5 challenge became parasitemic as determined either by thick blood smear or PCR with prepatent periods ranging from 47 to 63 days (Table 3). The six animals that had been splenectomized and inoculated intravenously showed similar prepatent periods (47–63 days, mean = 51.8 days). Two of the six monkeys, Aotus monkeys 300*624 and 512*272, could be diagnosed by thick blood smear and had a maximum parasitemia of 68/µL at day 47; the other four monkeys were only diagnosed by PCR. The group of six spleen-intact monkeys inoculated intravenously had prepatent periods that ranged from 49 to 63 days with a mean of 53.2 days. Aotus monkey 612*562 died 16 days after inoculation, and monkey 083*892 had a parasitemia by thick blood smear of 68/µL on day 54. The other four monkeys developed low parasitemia that had to be diagnosed by PCR. The group of six spleen-intact monkeys inoculated subcutaneously had a prepatent period that ranged from 49 to 63 days with a mean of 54.2 days. Aotus monkey 342*336 died of renal failure after 80 days of follow-up and was negative by thick blood smear and PCR. Aotus monkey 606*523 had a maximum parasitemia of 1,088 on day 54; the rest of the monkeys were diagnosed by PCR. The results for the *A. lemurinus griseimembra* monkeys inoculated with sporozoites in both experiments are summarized in Table 4. Some of the monkeys showed resolution of infection and others recrudescence as determined by PCR.

### DISCUSSION

The development of *P. vivax* malaria vaccines has been significantly delayed by the lack of continuous *in vitro* cultures and by the scarcity of animal models in which to grow the parasite *in vivo*. The latter also limits our ability to assess the efficacy of vaccines in preclinical studies. Although *P. vivax* asexual blood stages can be easily frozen and stored and efficiently reactivated by passage through monkeys, cryopreserving sporozoites while maintaining their infectivity is not yet possible on a routine basis.

During the last few years, significant progress has been made toward developing *P. vivax* vaccines.19–22 However, continued progress urgently requires models with which to test their protective efficacy. *Aotus lemurinus griseimembra* monkeys supports growth of *P. vivax* asexual blood forms and therefore allow the assessment of vaccines targeting this parasite stages. Additionally, *P. vivax* blood infections in monkeys allow development of mature gametocytes that lead to successful mosquito infections. Therefore, *Aotus* monkeys are also useful to test transmission blocking vaccines. However, the successful development of liver forms is limited for both *P. falciparum* and for *P. vivax*. Here we describe the results obtained in establishing a *P. vivax* sporozoite challenge system in *Aotus* monkeys. Since immunologic protection against malaria pre-erythrocytic forms is mediated by both helper and cytolytic T cells that may home in the spleen,23,24 the use of spleen-intact primates for vaccine efficacy testing would be preferable.

In this study, we tested whether spleen-intact and splenectomized *A. lemurinus griseimembra* monkeys responded equally to sporozoite challenge. Additionally, we sought to determine whether a previously adapted *P. vivax* strain, compared with strains that were not adapted, would display any growth advantage in these animals.

Previous sporozoite infections done with adapted parasite strains in splenectomized *Aotus* and *Saimiri* monkeys have shown higher levels of parasitemia and shorter prepatent periods in *Saimiri* than in several species of *Aotus*; only 43.6% of the exposed *Aotus* could be infected with prepatent periods ranging from 14 to 55 days, which has led to the conclusion that *Saimiri* monkeys are more susceptible than *Aotus* monkeys for *P. vivax* sporozoite infection.12,15,25–27

Studies carried out in different species of *Aotus* have shown higher parasitemias (1,210–56,000/µL) than in our study (68–4,216/µL).4,5,8,15,28 However, we have shown that *A. lemurinus griseimembra* spleen-intact or splenectomized monkeys

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**Table 3**

Prepatent period in 18 naive *Aotus lemurinus griseimembra* monkeys inoculated with 20,000 sporozoites of *Plasmodium vivax*.

<table>
<thead>
<tr>
<th>Monkey code</th>
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<th>Infection route</th>
<th>Prepatent period</th>
<th>Parasitemia</th>
</tr>
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<td>018*087</td>
<td>No SC</td>
<td>IV</td>
<td>49</td>
<td>+</td>
</tr>
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<td>083*892</td>
<td>No SC</td>
<td>IV</td>
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<td>No SC</td>
<td>IV</td>
<td>272</td>
<td>+</td>
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<td>63</td>
<td>+</td>
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<td>083*892</td>
<td>No SC</td>
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<td>+</td>
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<tr>
<td>Mean</td>
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*PCR = polymerase chain reaction; IV = intravenous; SC = subcutaneous. Two monkeys died of renal failure not related to malaria.
can be reproducibly infected with sporozoites from both the monkey-adapted *P. vivax* Salvador I strain (two of two intact, two of two splenectomized) and from wild strain VCC-4 derived from malaria patients (four of four intact, four of four splenectomized). Transmission via intravenous and subcutaneous sporozoite inoculation with the nonadapted strain VCC-5 to *Aotus* was highly reproducible in both spleen-intact monkeys (five of five intravenous inoculations and five of six subcutaneous inoculations) and in splenectomized monkeys (six of six intravenous inoculations) and showed no difference between groups (Table 4).

Because *P. vivax* has not yet been adapted to continuously grow in culture, the sources of infected blood to feed mosquitoes are either experimentally infected monkeys or malaria patients. However, the use of primates for this purpose renders the system very expensive, particularly if the *Aotus* monkeys were only susceptible to *P. vivax* sporozoites previously adapted to monkeys. In this study, we observed that all the monkeys were similarly infected by adapted or nonadapted *P. vivax* sporozoites.

As expected, sporozoite infection always led to longer prepatent periods as well as to lower parasitemia than did intravenous inoculation of trophozoites. There are three potential explanation for lower parasitemia: 1) failure of a large number of the inoculated sporozoites to invade the liver in the first instance,2,20-22 2) co-expression of antigens between the liver and the blood stages may be able to induce strong immune responses that further limit the appearance and development of a solid blood infection, or 3) the variation in the prepatent period may be related to sporozoite dose.15,30-32 The occurrence of low-density transient parasitemia has been described as a common phenomenon in *P. vivax* sporozoite infection in nonhuman primates.29,33,34 This low density, plus the typical spontaneous resolution of parasitemia, necessitates the use of a sensitive diagnostic method such as PCR35,36 to study the immune dynamic responses to both the pre-erythrocytic and the blood stage phases of *P. vivax* infection.

In conclusion, the *A. lemurinus griseimembra* monkey is susceptible to sporozoite infection, regardless of spleen presence or strain adaptation, and represents an alternative model with which to test the immunogenicity and protective efficacy of pre-erythrocytic vaccine candidates.

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### REFERENCES


### Table 4

Summary of prepatent period for *Aotus lemurinus griseimembra* after inoculation of sporozoites, by infection route, spleen presence, and parasite strain

<table>
<thead>
<tr>
<th>Infection route</th>
<th>Splenectomy</th>
<th>Sal 1</th>
<th>VCC-4</th>
<th>VCC-5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>Mean (range)</td>
<td>No.</td>
</tr>
<tr>
<td>IV Yes</td>
<td>2</td>
<td>36 (32-40)</td>
<td></td>
<td>4†</td>
</tr>
<tr>
<td>IV No</td>
<td>2</td>
<td>49 (9-89)</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>SC Yes</td>
<td>2</td>
<td>36 (32-40)</td>
<td></td>
<td>4†</td>
</tr>
<tr>
<td>SC No</td>
<td>2</td>
<td>49 (9-89)</td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>

* IV = intravenous; SC = subcutaneous.
† Prepatent period missing for one monkey, although xenodiagnosis result was positive.
‡ Excludes one animal that died before infection was detected.
AOTUS MONKEY MODEL: P. VIVAX SPOROZOITE INFECTION


