The Santa Lucia Strain of *Plasmodium falciparum* in *Aotus* Monkeys

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Abstract. The Santa Lucia strain of *Plasmodium falciparum* was studied in 150 *Aotus lemurinus griseimembra*, 30 *A. azarae boliviensis*, 103 *A. nancymaeae*, and 121 *A. vociferans* monkeys. All four of these splenectomized hosts supported the production of gametocytes infective to *Anopheles freeborni* mosquitoes. Transmission through sporozoites from *A. freeborni*, *An. stephensi*, *An. maculatus*, and *A. albimanus* mosquitoes was successful to all four species of *Aotus* on a total of 100 occasions with a median pre-patent period of 21 days. For the production of infective mosquitoes for vaccine challenge studies, *A. l. griseimembra* and *A. vociferans* were the most predictable hosts.

INTRODUCTION

For more than four decades, we have studied 35 different strains of *Plasmodium falciparum* in > 2,000 *Aotus* monkeys in varied and numerous research projects. A major goal has been to establish sporozoite transmission models for each of the isolates for use in vaccine and chemotherapeutic challenge studies. This has proven difficult because the majority of the isolates once adapted to monkeys rarely produced gametocytes infective to the available laboratory-reared vectors. Studies were conducted in four different species of *Aotus*. As a result of these studies, the Santa Lucia strain from El Salvador was selected as the primary *P. falciparum* parasite to be used to assess transmission-blocking, anti-sporozoite, and liver stage vaccines using different species of *Aotus* monkeys.1–3 Reported here is a summary of the comparative studies made in four different species of *Aotus* monkeys.

In 1975, the Santa Lucia isolate of *P. falciparum* was obtained from a 3-year-old Salvadoran female who presented at the Santa Lucia Clinic in La Paz Department, El Salvador.4 Blood was passaged to an *Aotus lemurinus griseimembra* monkey. Once infection was established, blood was carried to the laboratory in Chamblee, GA, where studies were conducted in other species of New World monkeys and different species of *Anopheles* mosquitoes.5 The initial report indicated successful transmission in 20 of 27 attempts to splenectomized *A. lemurinus griseimembra* monkeys through the bites of *An. freeborni*, *An. maculatus*, and *An. albimanus* mosquitoes; subsequent studies indicated the comparative infectivity of the Santa Lucia strain to different species and phenotypes of anopheline mosquitoes.6–8 The Santa Lucia strain was used to study the effect of sequential infections with *P. falciparum* and *P. vivax* in *A. l. griseimembra* monkeys.9

After an embargo on the export of *A. l. griseimembra* from Colombia, other species of *Aotus* were examined as alternative hosts for studies with the Santa Lucia strain, including *Aotus azarae boliviensis* from Bolivia10 and *Aotus vociferans* from Peru.11 The strain, when combined with *Aotus nancymaeae* monkeys, provided a robust transmission model and was proposed for the testing of anti-sporozoite and liver stage vaccines.3

The reasons for the selection of the Santa Lucia strain was that, of all the strains of *P. falciparum* that have been available, 1) it was the strain most predictably infective to mosquitoes and 2) it was the strain most readily transmissible to monkeys. Intact animals either do not produce gametocytes in high numbers or rapidly lose their ability to produced gametocytes after serial passage. Thus, by working with splenectomized monkeys, gametocytemia is maintained in a particular strain of the parasite. Once the ability to produce gametocytes is lost, it has been impossible to restore it. Reported here is the development of this parasite in the four different species of *Aotus* monkeys that we have studied (*A. lemurinus griseimembra*, *A. azarae boliviensis*, *A. nancymaeae*, and *A. vociferans*) and the infectivity of this parasite using *An. freeborni* mosquitoes as the indicator vector. Previous comparative studies indicated that the strain is also infective to a broad range of laboratory-reared species of *Anopheles*.43

MATERIALS AND METHODS

*Aotus lemurinus griseimembra* and *A. azarae boliviensis* were wild caught and imported from Colombia and Bolivia during periods when export was allowed from these countries; after that, animals were laboratory bred. *Aotus nancymaeae* and *A. vociferans* monkeys were primarily wild-caught animals and imported from Peru; a limited number of these animals were laboratory bred. On arrival at the facility, all animals were quarantined for a 2-month conditioning period, weighed, and tested for tuberculosis. Parasitologic and serologic examination indicated that the animals were free of infection with malaria parasites before inoculation with the Santa Lucia strain, although many of the animals had been previously infected with other strains and species of *Plasmodium*. All monkeys were splenectomized before exposure. All surgeries were performed in an AAALAC (Association for the Assessment and Accreditation of Laboratory Animal Care, International)-approved surgical suite appropriate for aseptic surgery. Protocols were reviewed and approved by the Centers for Disease Control and Prevention Institutional Animal Care and Use Committee, in accordance with procedures described in US Public Health Policy, 1986.

Monkeys generally were housed doubly or, in some cases, singly to avoid injuries caused by fighting with cage mates. Space recommendations for laboratory animals were followed as set forth in the NIH Guide for the Care and use of

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Laboratory Animals. All animals were fed a diet that has been proven to provide adequate nutrition and calories in captive *Aotus* monkeys used in malaria-related research. Feed was free of contaminants and freshly prepared. Daily observations of the animals’ behavior, appetite, stool, and condition were recorded. All were treated as medical conditions arose by an attending veterinarian.

*Anopheles freeborni* (F-1 strain originally from California), *An. stephensi* (from Delhi, India), *An. maculatus* (from Malaysia), *An. gambiae* (from The Gambia), and *An. albimanus* (from El Salvador) were laboratory-reared and maintained at the CDC/DPD insectaries. During periods when gametocytes were present in blood, mosquitoes of different species were allowed to feed on tranquilized monkeys. Mosquitoes were contained in pint-sized ice-cream carton cages and allowed to feed directly through netting on the shaved belly of the animal until engorged to repletion. After feeding, mosquitoes were held in an incubator at 25°C until examined 1 week later for the presence of oocysts on their midguts. If oocysts were present, mosquitoes were kept until sporozoites were present in the salivary glands, which was usually after 14 or more days of extrinsic incubation.

For transmission, sporozoites were dissected from the salivary glands into 20% fetal bovine serum/saline and crushed under a coverslip, and the sporozoites were transferred to a vial. After being counted using a Neubauer Cell Counting Chamber, the sporozoites were injected intravenously into the femoral vein of the recipient monkey. Alternatively, mosquitoes were fed on animals only after being counted using a Neubauer Cell Counting Chamber, the sporozoites were injected intravenously into the femoral vein of the recipient monkey. Alternatively, mosquitoes were allowed to feed directly on a tranquilized animal. After feeding, mosquitoes were held in an incubator at 25°C until examined 1 week later for the presence of oocysts on their midguts. If oocysts were present, mosquitoes were kept until sporozoites were present in the salivary glands, which was usually after 14 or more days of extrinsic incubation.

Blood stage parasitemia was monitored and quantified by the daily examination of thick- and thin-blood films by the method of Earle and Perez. Parasite counts were recorded per microliter of blood. If the parasite count was very high, the count was estimated from the thin film using the conversion of 1.0% red blood cell (RBC) infection = 40,000 parasites/µL. Infections were terminated by treatment with chloroquine (10 mg daily × 3 days). Drugs were administered by oral intubation. Infections were controlled by administration of subcurative doses of quinine (50 mg) or chlorguanide (2 or 4 mg) that were also given orally to allow development of gametocytemia while controlling the asexual parasitemia.

RESULTS

The development of the Santa Lucia strain was examined in each of the four different species of *Aotus* monkeys. For this evaluation, only the *An. freeborni* mosquito feedings were considered because this was the species most frequently fed and the one that usually resulted in the highest percentage of infection. Thus, it was the most sensitive indicator for infection.

*Aotus lemurinus griseimembra*. A total of 150 splenectomized *A. lemurinus griseimembra* were infected, 94 through trophozoite and 56 through sporozoite injection. Of those that were trophozoite induced and had no previous malarial experience, the maximum parasite counts ranged from 11,160 to 3,000,000/µL, with a median of 400,000/µL. The 21 animals that had been previously infected with *P. vivax* had maximum parasite counts that ranged from 651 to 2,220,000/µL, with a median of 212,000/µL (approximately one half of that of the animals with no previous infection). The 18 monkeys that had been previously infected with *P. falciparum* or *P. falciparum* and *P. vivax* before being splenectomized and infected with the Santa Lucia strain had maximum parasite counts that ranged from 90 to 280,000/µL, with a median of 6,325/µL.

There were 56 sporozoite-induced infections with pre-patent periods that ranged from 10 to 45 days, with a median of 22 days (Figure 1). Transmission was obtained by *An. freeborni* (34 times), *An. stephensi* (8 times), *An. gambiae* (10 times), *An. albimanus* (3 times), and *An. maculatus* (1 time). Maximum parasite counts for the 31 animals with no previous infection ranged from 1,800 to 1,920,000/µL, with a median of 264,000/µL. For the 18 with previous infection with *P. vivax* only, the maximum parasite counts ranged from 46,500 to 1,820,000/µL, with a median of 412,920/µL. This pattern was the reverse of that seen with the trophozoite-induced infections.

It was apparent that maximum parasite counts in these splenectomized *A. l. griseimembra* varied greatly (Figure 2) as did the pre-patent periods (Figure 1). Nonetheless, high-density parasite counts and sporozoite transmissions were readily obtained. There appeared to be little prediction as to whether an animal would need treatment for survival or would be able to control the infection on its own.

Mosquito infection. During the first 10 linear passages in *A. l. griseimembra* monkeys, *An. freeborni* mosquitoes were fed on 11 blood-induced infections a total of 463 times; on 198 of the days (42.8%), mosquitoes were infected. *An. freeborni* were fed on 29 sporozoite-induced infections during the first 10 linear passages in this species, a total of 1,345 times, on 871 of the days (64.8%), mosquitoes were infected. After 10 linear passages, *An. freeborni* mosquitoes were fed on 19 blood-induced infections 397 times and 9 sporozoite-induced infections 60 times. On 141 of the days (30.9%), mosquitoes were infected. There was a suggestion in the data that mosquito infection decreased with increased linear passage in monkeys. Mosquito infection was frequently obtained for sustained periods as shown in Figure 3. Mosquitoes were fed on animals only during their initial infections with *P. falciparum*, although they were fed during recrudescent periods with that infection.

![Figure 1. Pre-patent periods for sporozoite-induced infections in 56 A. l. griseimembra (ALG), 7 A. a. bolivianus (AAB), 25 A. nancymae (AN), and 12 A. vociferans (AV) monkeys with the Santa Lucia strain of P. falciparum. Arrow indicates mean pre-patent period for all 100 successful transmissions. This figure appears in color at www.ajtmh.org.](image-url)
Aotus vociferans. A total of 121 splenectomized A. vociferans were infected, 109 through trophozoite and 12 through sporozoite injection. All occurred after 12 linear passages in Aotus monkeys and continued through 32 passages after isolation from the patient. Fifty-one of the animals that were trophozoite induced had no previous malarial experience and had maximum parasite counts that ranged from 9,720 to 1,760,000/µL, with a median count of 228,000/µL (Figure 2). As with other species of Aotus monkeys, many of the A. vociferans animals had been infected with heterologous strains before being splenectomized and challenged with the Santa Lucia strain. In monkeys thus challenged, the maximum parasite counts ranged from 810 to 540,000/µL, with a median count of 104,000/µL.

There were 12 sporozoite-induced infections, with prepatent periods that ranged from 16 to 39 days, with a median of 22 days (Figure 1). Transmissions were by sporozoites from An. stephensi (eight times) and An. freeborni (four times) mosquitoes, administered either by bites or intravenous injection. In 10 monkeys with no previous infections, the maximum parasite counts ranged from 540 to 392,000/µL, with a median of 264,000/µL.

Mosquito infection. Anopheles freeborni were fed on 78 trophozoite-induced infections during periods when gametocytes were present; 47 of the animals were infective on at least one occasion. Mosquitoes were fed on these 47 blood-induced infections a total of 1,568 times; on 407 of the days (26.0%), mosquitoes were infected. In 10 monkeys with no previous infections, the maximum parasite counts ranged from 540 to 392,000/µL, with a median of 264,000/µL.

Mosquito infection. Anopheles freeborni were fed on 78 trophozoite-induced infections during periods when gametocytes were present; 47 of the animals were infective on at least one occasion. Mosquitoes were fed on these 47 blood-induced infections a total of 1,568 times; on 407 of the days (26.0%), mosquitoes were infected. In 10 monkeys with no previous infections, the maximum parasite counts ranged from 540 to 392,000/µL, with a median of 264,000/µL.

Aotus nancymaae. A total of 103 splenectomized A. nancymaeae were infected, 78 through trophozoite and 25 through sporozoite injection. All occurred after 12 linear passages in Aotus monkeys and continued through 32 passages after isolation from the patient. Thirty-five of the animals that were trophozoite induced had no previous malarial experience and had maximum parasite counts ranging from 4,557 to 1,936,000/µL, with a median of 194,000/µL. Sixteen of the animals had been previously infected with P. vivax; maximum parasite counts ranged from 837 to 1,000,000/µL, and the median maximum parasite count was 258,000/µL. Twenty-four of the animals had previously been infected with heterologous strains of P. falciparum before being splenectomized and infected with the Santa Lucia strain. Little protection was evident in that the maximum parasite counts ranged from 3,162 to 1,040,000/µL, with a median maximum parasite count of 68,000/µL.

There were 25 sporozoite-induced infections with prepatent periods that ranged from 16 to 30 days, with a median...
of 20 days (Figure 1). Transmissions were by sporozoites from \textit{An. stephensi} (7 times) and \textit{An. freeborni} (18 times) mosquitoes, administered either by bites or intravenous injection. In 12 monkeys with no previous infections, the maximum parasite counts ranged from 3,420 to 2,200,000/µL, with a median of 238,200/µL. Again, six monkeys that had been infected with heterologous strains of \textit{P. falciparum} before splenectomy and challenge with the Santa Lucia strain had maximum parasite counts that ranged from 23,760 to 360,000/µL. An examination of the maximum parasite counts (Figure 2) indicated that, even with a previous history of infection with \textit{P. falciparum}, these monkeys supported the production of high-density parasite counts with the Santa Lucia strain after splenectomy. Apparently, any protective effect of prior infection was eliminated or drastically reduced by removal of the spleen.

\textbf{Mosquito infection.} Mosquitoes were not fed on \textit{A. nancy-mae} monkeys until the 13th monkey passage after human infection. Gametocytes continued to be produced, but at a reduced level by this strain. Most other isolates of \textit{P. falciparum}, after this many linear passages, have lost the capacity to produce infective gametocytes; however, probably as a result of the frequent sporozoite passage, mosquito infection was obtained but at a reduced and less predictable level.

\textit{Anopheles freeborni} mosquitoes were fed on 40 trophozoite-induced infections during periods when gametocytes were present; 20 of the animals were infective on at least one occasion. Mosquitoes were fed on these 20 blood-induced infections a total of 487 times; on 141 of the days (29.0%), mosquitoes were infected. When \textit{An. freeborni} were fed on 13 sporozoite-induced infections 186 times, seven animals were infected; on 28 of the days (15.1%), mosquitoes were infected. Several animals, such as AI-2710 (Figure 3), infected mosquitoes over a period of several weeks.

\textit{Aotus azarae boliviensis}. A total of 30 splenectomized \textit{A. azarae boliviensis} were infected, 23 through trophozoite and 7 through sporozoite injection. Seven of the animals that were trophozoite induced had no previous malarial experience and had maximum parasite counts ranging from 434 to 2,760,000/µL, with a median of 43,152/µL. Thirteen animals that had been previously infected with \textit{P. vivax} or \textit{P. vivax} and \textit{P. malariae} had maximum parasite counts that ranged from 31 to 980,000/µL. It was apparent that maximum counts for this parasite in \textit{A. azarae boliviensis} were lower and seldom required drug intervention for survival of the animal.

There were seven sporozoite-induced infections with prepatent periods that ranged from 15 to 56 days, with a median of 22 days (Figure 1). All transmissions were through sporozoites from \textit{An. stephensi} mosquitoes administered either by bites or intravenous injection. Maximum parasite counts in these seven animals ranged from 41,580 to 672,000/µL, with a median of 116,000/µL.

\textbf{Mosquito infection.} Because of the lower density parasite counts and lower levels of gametocytemia, mosquitoes were fed on only 11 of the animals and only 1 of these infected \textit{An. freeborni} mosquitoes consistently. Infection was obtained on 14 of the 15 days on which mosquitoes were fed (Figure 3). On the other 10 animals, mosquitoes were fed on 62 occasions when gametocytes were present without obtaining infection. In total, 54 of 133 lots (40.6%) of \textit{An. freeborni} fed were infected.

In summary, 404 splenectomized \textit{Aotus} monkeys were infected, 304 by trophozoites and 100 by sporozoite injection. Maximum responses for all the animals (Figure 2) indicated that high-density parasite counts were obtained for most animals in all four species of \textit{Aotus}. High-density parasite counts usually were followed by high levels of gametocytemia. Mosquito infection was obtained frequently, especially when they fed on \textit{A. lemurinus griseimembra}.

\section*{DISCUSSION}

The introduction of \textit{P. falciparum} into monkeys was a major breakthrough in efforts to bring this parasite into the laboratory for study. After its subsequent adaptation to \textit{in vitro} culture, many different isolates of the parasite became available to any laboratory with basic microbiological techniques and equipment. The parasite in non-human primates became preserved for more specific studies such as immunologic and vaccine trials, where the more complex responses of the host to infection were investigated, rather than for drug screening that could now be done with \textit{in vitro} culture. The Santa Lucia strain of \textit{P. falciparum} from El Salvador has been shown to be a very useful parasite for the testing of anti-sporozoite and liver stage vaccines because of the potential for mosquito infection and sporozoite transmission. An examination of this parasite indicates that it develops well in four different species of \textit{Aotus}; maximum parasite counts in \textit{A. lemurinus griseimembra}, \textit{A. vociferans}, and \textit{A. nancy-mae} that have had no previous infection were uniformly high (Figure 2). Mosquito infection was more predictable in the first two species than in \textit{A. nancy-mae}. \textit{A. a. boliviensis} was the least predictable host for infection with this parasite.

The Santa Lucia strain is the best example available of a \textit{P. falciparum} strain able to maintain the capacity to produce infective gametocytes through at least 30 passages since its isolation from humans. Superior mosquito infection and frequent sporozoite transmission to other splenectomized monkeys was obtained during the first 10 passages in \textit{A. l. griseimembra}. Infection of 25% of the lots of mosquitoes fed on \textit{A. vociferans} indicates that these monkeys from Peru are also capable of infecting mosquitoes on a predictable basis. As previously indicated, \textit{A. nancy-mae} is a predictable model for the testing of anti-sporozoite and liver-stage vaccines when challenged with sporozoites of the Santa Lucia strain.\textsuperscript{3} \textit{A. nancy-mae}, however, is somewhat less predictable regarding mosquito infection than \textit{A. l. griseimembra} and \textit{A. vociferans}.

Unfortunately, there has been a lack of coordination between many investigators working on vaccine development with \textit{in vitro} culture and those working with parasites in monkeys. Many of the parasites extensively studied \textit{in vitro} were not the same as those adapted to growth in monkeys. Thus, many of the candidate blood-stage vaccines were developed against strains of parasites that would only grow in culture and not be able to infect New World primates such as \textit{Aotus} monkeys. These vaccines based on these culture strains could not be used in immunization and challenge studies in monkeys against the homologous parasites. It had been assumed that these culture-adapted parasites, such as NF-54, 3D7, and 7G8, could be subsequently adapted to grow in monkeys. Such was not the case.

Experience has indicated that much less than a majority of isolates of \textit{P. falciparum} ever adapt to growth in New World monkeys. Even more evident was the fact that parasites maintained \textit{in vitro} soon lose any potential to develop in monkeys.
In general, a new isolate is slow to adapt to grow in Aotus monkeys but will almost never be established after long-term culture in vitro.

Previous studies also indicated that rapid serial trophozoite passage of a strain of P. falciparum in monkeys results in uniform maximum parasite counts in intact Aotus monkeys and a loss in the ability of that strain to produce infective gametocytes. This characteristic of uniformity was very useful in the development of models for the testing of blood-stage vaccines, where a predictable parasite count was desired after blood-stage challenge. This was used in the development of predictable models, such as the Vietnam Oak Knoll strain for the testing of blood-stage vaccines.13–16 However, serial passage of parasites through trophozoites in monkeys that soon results in the reduction or loss in the production of infective gametocytes seems to be irreversible; only by frequent sporozoite passage of gametocyte-producing strains can production of infective gametocytes be maintained. Thus, mosquito passage or preservation of frozen seed stocks, collected very early in the passage history, must be used to successfully maintain the gametocyte production of a particular strain of a parasite. Fortunately, the Santa Lucia strain has been frequently passed through sporozoites; in addition, numerous aliquots have been stored frozen at different stages in the passage genealogy of this parasite. This has resulted in a somewhat unpredictable response regarding asexual parasitemia, with a wide range in maximum parasite counts in splenectomized animals, regardless of the species of Aotus monkey used. Low-level parasite counts occasionally occur that result in a failure to produce large numbers of infective gametocytes; at other times, there is the production of very high asexual parasite counts that require drug treatment of the animal’s survival.

Both A. nancymaeae and A. vociferans are still imported in limited numbers from Peru. The failure to support breeding programs for A. l. griseimembra and the dependence on importation of A. vociferans animals will always place domestic programs in jeopardy for the testing of sporozoite and liver-stage vaccines. Malaria vaccine development and testing should have high interest and priority, and the establishment of breeding programs for these two species would seem to be an essential ingredient.

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