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Adaptation of *Plasmodium Vivax* to Growth in Owl Monkeys (*Aotus nancymai*)

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The purpose of this study was reactivation and adaptation of a strain of *Plasmodium vivax* to *Aotus nancymai* monkeys. A need arose for malarial parasites for use in serologic and molecular studies and for teaching slides. This particular strain of parasite had been characterized previously as producing high-density parasitemia in splenectomized New World monkeys and therefore represented a good candidate for reactivation. *P. vivax* (Vietnam II), isolated in 1970, was reactivated after adaptation in *Aotus lemurinus griseimembra* monkeys nearly 33 years earlier and adapted to *A. nancymai* monkeys. Passage was achieved by intravenous inoculation of parasite blood stages into splenectomized *A. nancymai* monkeys. Parasitemia was determined by analyzing daily blood smears stained with Giemsa. Maximum parasite counts ranged from 10,630 to 94,000 parasites/μl; the mean maximum parasite count for the four animals was 39,565 parasites/μl. Parasite counts of >10,000/μl were maintained for 2 to 64 days. After only three passages of the parasite, attempts to reactive were successful. *A. nancymai* proved a suitable animal model for the recovery of this parasite. In conclusion, successful reactivation and adaptation of this parasite offers the capability to perform a series of diagnostic, immunologic, and molecular studies as well as to provide otherwise potentially unavailable teaching materials to healthcare professionals.

Adaptation of various human disease-causing organisms to animals has become a very important and often necessary approach to the study of human disease. This process has facilitated great advances in human health in the areas of disease pathogenesis, drug and vaccine development, assay development, and teaching materials. Whereas some studies examine natural host–parasite interactions, the complex nature of many organisms and their interactions with the mammalian host makes it necessary to adapt these organisms to live, susceptible animal species. This situation certainly has been the case for the study of malarial parasites. In the present study, a strain of *Plasmodium vivax* that was isolated in 1970 was reactivated and adapted to *Aotus nancymai* monkeys after nearly 33 years of frozen storage.

Malaria is one of the most severe public health problems worldwide. It is a leading cause of death and morbidity in many developing countries. Some disease manifestations include fever, chills, headache, muscle ache, vomiting, malaise, and other flu-like symptoms. Some patients also develop complications such as brain disease (cerebral malaria), severe anemia, and kidney failure (6). Young children and pregnant women are the most susceptible population. Malaria is caused by a protozoan parasite from the genus *Plasmodium*, naturally transmitted through the bite of the female *Anopheles* mosquito (10). Four species of *Plasmodium* are known to cause disease in humans: *P. vivax, P. ovale, P. malariae*, and *P. falciparum*, which is the most virulent species and the leading cause of mortality (11). However, it has become increasingly clear that severe malaria is often merely the consequence of inappropriate or delayed treatment (10). Studies made possible by the use of animal models contribute greatly to understanding the pathogenesis and various treatments for this disease. Further, teaching materials made possible through experiments such as the present study help healthcare providers receive appropriate training needed for the proper identification of the organism in smears (Fig. 1).

Efforts by the Centers for Disease Control (CDC) to eradicate malaria were ended in 1978 and redirected to develop methods for control (6). As recently as the summer of 2005, the President of the United States announced a new government initiative to dramatically reduce the number of children dying of malaria in sub-Saharan Africa, pledging to increase funding for malaria prevention and control by more than $1.2 billion over 5 years (7). Extensive research has been done and continues in the area of vaccine and drug development, much of which has involved the use of nonhuman primates, specifically New World primates like *Aotus*.

The *Aotus* monkey has long been identified as the animal model of choice for malaria work. Owl monkeys (*Aotus spp.*) are small (usually 700 to 1200 g), nocturnal primates that are arboreal and live as monogamous pairs within small family units of two to three offspring (1-4, 18, 20). The monogamous mating system is correlated with having males present to help raise offspring (27). The male is the main carrier of the infant and only gives the infant to the mother to suckle. The young stay with their parents until between 2.5 to 3.5 years old.

Also known as the night monkey or douroucouli, *Aotus* monkeys are used widely in a variety of animal model paradigms, including malaria vaccine and drug development and testing, viral pathogenesis, and ophthalmic studies (5). Owl monkeys live in a

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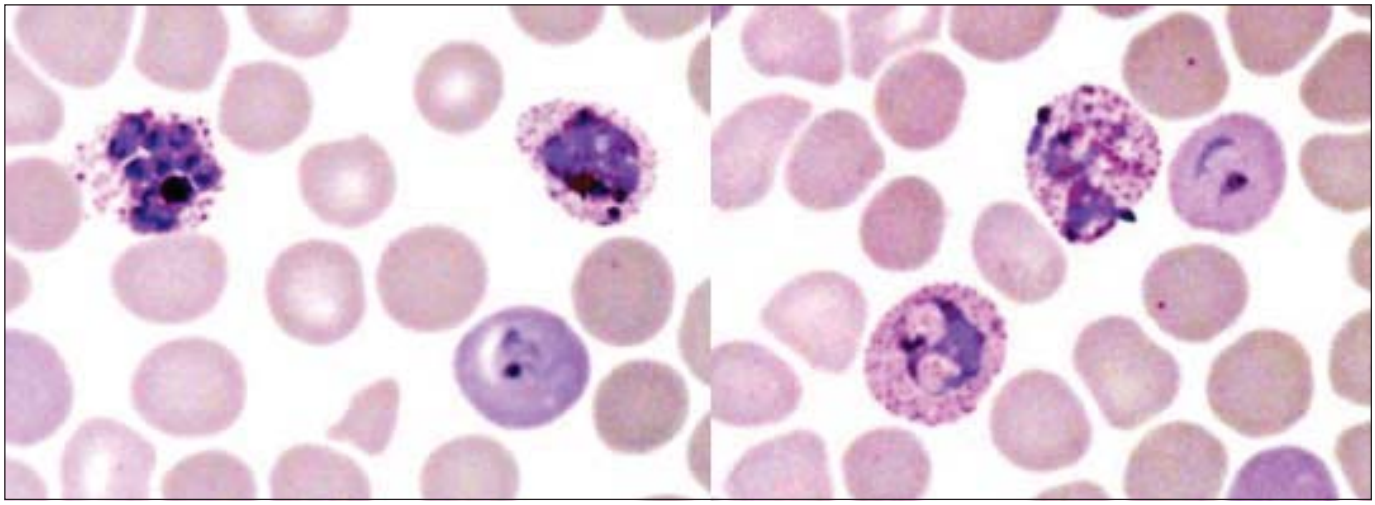

Figure 1. Photomicrograph of different blood stages of the Vietnam II strain of *Plasmodium vivax* from an *Aotus nancymai* monkey. Magnification, ×1000.

widely distributed range throughout Central and South America ranging from Panama to Argentina and from the mouth of the Amazon River west into Peru and Ecuador.

Taxonomic designations for members of the genus *Aotus* vary and will not be covered in detail in this paper. Briefly, the designation used most widely in the biomedical research community is that of Hershkovitz (15), who describes two phenotypic groups of *Aotus* composed of nine species and four subspecies distinguished by karyotype, pelage patterns, and neck color. The gray-necked species group contains *A. brombacki, A. lemurinus lemurinus, A. lemurinus griseimembra,* and *A. vociferans.* The red-necked species group contains *A. nancymai, A. nigriceps, A. trivirgatus, A. infulatus, A. micronax, A. azarae azarae,* and *A. azarae boliviensis.* Others characterizing the taxonomic designations include Wilson and Redder (26) and Ford (13).

The different species of *Aotus* vary widely in their susceptibility to experimental infection with malarial parasites (15-18). It has been reported that *A. lemurinus griseimembra* is highly susceptible to *P. falciparum*, whereas *A. nancymai* is less susceptible to some strains and resistant to others (22-24). Although the *Aotus* genus provides several species susceptible to both *P. falciparum* and *P. vivax*, *A. lemurinus griseimembra* represents the best current malaria parasite model because of its high susceptibility to infection by blood forms and sporozoites of both of these species of *Plasmodium* (14). However, this *Aotus* species is no longer being imported for use in biomedical research (25). Fortunately, once an isolate of a human malaria parasite is established in one species of *Aotus*, it can be readily passaged to other species (5).

Drug development, serologic, immunologic, and molecular studies are hampered by the decrease in *Aotus* availability for biomedical research. All species of *Aotus* are listed in the Convention of International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix II. These New World primates, according to CITES, are not currently threatened with extinction but may become so unless trade is strictly regulated. Between 1968 and 1972, the United States imported 20,869 owl monkeys, primarily from Colombia and Bolivia. These included *A. lemurinus*, *A. nancymai*, and *A. azarae* (5, 19). The number dramatically declined between 1976 and 1980 to 3300 monkeys (5, 19). The decline primarily was due to government bans imposed in the 1970s on exportation from source countries. These government importation bans, combined with the destruction of natural habitats and lack of national and international supervision during capture and transport of these neotropical primates, made these animals inaccessible (5, 19). Today it is still nearly impossible to import *A. lemurinus griseimembra*. Because of the limited availability of *Aotus* and their value to malarial work, judicious use of these New World primates as a biomedical resource is paramount. By augmenting the use of the *Aotus* as an animal model for the adaptation of various *Plasmodium* spp., researchers are able to better refine, replace, and reduce the number of these animals needed in the continuing efforts to prevent and control malaria.

When the need arose at the CDC for malarial parasites for use in serologic and molecular studies and for teaching slides, the Vietnam II strain, previously characterized for producing high-density parasitemia in splenectomized *Aotus,* was the ideal candidate for reactivation. However, it was isolated in 1970 and adapted to *A. lemurinus griseimembra,* which are no longer readily available. Recently, it has been reported that the Vietnam IV Palo Alto strain of *P. vivax* produces high-density parasite counts in *A. nancymai* monkeys (9). However, this strain has never been infectious to mosquitoes and therefore is suboptimal for many vaccine and biologic studies. In contrast, early observations with the Vietnam II strain indicated that it produced infective gametocytes in *A. lemurinus griseimembra* monkeys and therefore was useful for a wider variety of applications. In this study, we explored the possibility of reactivating this parasite after nearly 33 years of frozen storage and adapting it to a different species, *A. nancymai*. Promising results indicate that long archived strains of parasites previously adapted through one species of *Aotus* can be reactivated and adapted to another, more readily available species of *Aotus*.

Materials and Methods

A total of 50 wild-caught *Aotus* monkeys were received by the CDC on 22 April 2003. The shipment comprised 12 *A. vociferans* and 38 *A. nancymai* previously used at the National Institutes of Health (NIH) in *P. falciparum* studies. All animals were quarantined upon arrival at the CDC facility, which is fully accredited

by the Association for Assessment and Accreditation of Laboratory Animal Care International, Inc. While in quarantine, the animals were examined, weighed, and tested for tuberculosis. Parasitologic and serologic examination of animals indicated that they were free of infection with malarial parasites, *Trypanosoma cruzi*, and microfilaria before inoculation. Protocols were reviewed and approved by the CDC Institutional Animal Care and Use Committee. This research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals* (19).

One *A. vociferans* and three *A. nancymai* monkeys were used in this study. *A. vociferans* have been reported to be more susceptible to infection with *Plasmodium* spp. than *A. nancymai* (8). However, *A. nancymai* monkeys are more often available than *A. vociferans* (8). Each animal weighed at least 700 g and was approximately 3 to 5 years old. Animals were housed in pairs unless a suitable cagemate was not identified. Space recommendations as set forth in the *Guide* were followed; animals were housed in 6.0-ft^2 (ca. 0.56-m^2) stainless steel caging. All cages were equipped with plastic-tube perching and convenient nest logs. The rooms were temperature- and humidity-controlled at 75 to 80°F (ca. 23.1 to 26.7°C) and 30 to 70%, respectively. Ventilation was supplied at 10 to 15 air changes per h. A 12:12-h dark: light cycle also was maintained. All animals were fed a balanced commercial diet (New World Primate Monkey Chow 5040, Purina, St. Louis, Mo.), provided ad libitum and supplemented with a variety of fresh fruit, vegetables, and other treats daily. Water was provided through an automatic watering system furnished to each cage. Monkeys also were provided with various enrichment devices and other manipulata. Twice-daily observations of the animals' behavior, appetite, stool and urine output, and general health condition were recorded. As medical conditions arose, all animals were examined and treated, if needed, by the attending veterinarian.

Surgical procedures were performed under general anesthesia by a qualified laboratory animal veterinarian. Gas anesthesia with 2 to 3% isoflurane (IsoFlo, Abbott Laboratories, North Chicago, Ill.) was provided via a closed oxygen delivery system. All animals were fed lightly the night prior to surgery. Preanesthesia consisted of an injection of ketamine hydrochloride (10 mg/kg; Ketaset, Fort Dodge Animal Health, Fort Dodge, Iowa) into the caudal thigh muscle. The ventral abdomen from the xyphoid to the pubis was clipped with a #40 surgical blade and aseptically prepped by scrubbing for at least 5 min with povidone–iodine scrub (West-Vet Prepodyne Scrub; West Agro, Inc, Kansas City, Mo.) and 70% isopropanol (70% Alcohol Buffered, The Butler Company, Columbus, Ohio). A small strip of opthalmic ointment (Artificial Tears, The Butler Company) was placed in each eye. The surgical field was draped with an autoclaved paper drape fenestrated for an appropriate surgical approach.

A 1- to 2-cm midline incision above the umbilicus was made through the skin and continued through the linea alba. The spleen was exteriorized, and the vasculature was ligated with 3-0 chromic nonabsorbable suture (B|BRAUN, Aesculap, Center Valley, Pa.) to maintain hemostasis. Metzenbaum scissors were used to excise the spleen. The ligatures were checked to ensure hemostasis, and then the ligated blood vessels were allowed to return gently into the peritoneal cavity. The linea and subcutaneous tissue were closed with 4-0 synthetic absorbable suture (PDS II, ETHICON, a Johnson & Johnson Company, Somerville, N.J.) in a simple continuous pattern. The skin was closed with surgical glue (Nexaban, Abbott Animal Health, North Chicago, Ill.). Postoperative analgesia was provided with buprenorphine hydrochloride (0.01 mg/kg; Buprenex, Reckitt Benckiser Pharmaceuticals, Richmond, Va.) administered into the subcutaneous tissue on the dorsum between the shoulder blades. Animals then were placed in a prewarmed incubator for recovery and were monitored closely until full recovery. Upon recovery, animals were returned to their home cages and were monitored closely for 10 days postoperatively.

The parasite, *P. vivax*, was isolated from a soldier who returned to the United States in 1970 from service in Vietnam. Initial passages and adaptation were accomplished in 1970 in *A. lemurinus griseimembra* monkeys by intravenous inoculation into the femoral vein with parasitized erythrocytes from the patient. Reactivation was accomplished by inoculating the 1971 frozen stabilate of 0.5 ml parasitized blood containing approximately 20,000 parasites from an *A. lemurinus griseimembra* monkey (AO-0213) into a splenectomized *A. vociferans* monkey (T-0960) in 2004. The exact dose of inoculum is not known, but at the time of collection and storage in 1971, the blood from AO-0213 contained 40,000,000 parasites/ml. Subsequent inoculations were done with fresh parasitized erythrocytes by intravenous injection into the femoral veins of the *Aotus* monkeys. Monkey T-960 was used to infect T-1156, who received 450,000 parasites in 1.0 ml of blood. Monkey T-1156 was used to infect T-1181 and T-1171, who each received 225,000 parasites in 0.5 ml of blood.

To monitor parasitemia, daily thick and thin blood films were made after a hemolet was used to prick the lateral calf of the monkeys; parasite counts were determined by the method of Earle and Perez (12). After films were stained with Giemsa (prepared at the CDC), the parasite counts were recorded as the number of parasites/μl blood. All animals ultimately were given a curative dose of 50 mg quinine sulfate (Zenith Goldline, Miami, Fla.) and 20 mg lariam mefloquine (Roche, Basel, Switzerland) or 30 mg of aralen chloroquine (Sanofi-Synthelabo, New York, N.Y.) by mouth (8).

Results

Initial adaptation of Vietnam II strain of *P. vivax* to a monkey host occurred in *A. lemurinus griseimembra* monkeys in 1970 after five passages. The genealogy of the Vietnam II strain is shown in Fig. 2. Monkey AO-0191 was splenectomized before inoculation and developed a maximum parasite count of 39,600/μl. The parasite count exceeded 10,000/μl on seven different days, indicating that this particular isolate had the potential for producing higher-than-normal parasite counts in *Aotus* monkeys (8).

The parasite then was passaged to splenectomized monkey AO-0197. A maximal parasite count of 120,000/μl was obtained. Counts of > 10,000/μl were maintained for 9 days, and this rate corresponds to approximately 3% of erythrocytes infected. At this point, this animal was treated with 30 mg chloroquine by mouth to prevent potential adverse clinical signs often associated with severe malaria infection. Blood from AO-0197 then was passaged to an intact (non-splenectomized) monkey, AO-0225. The maximal parasite count of this animal was only 4092/μl, effectively demonstrating the role the spleen plays in the sequestration of erythrocytes. All of the splenectomized monkeys developed more extensive parasitemia, as evidenced by counts of > 10,000/μl (8).

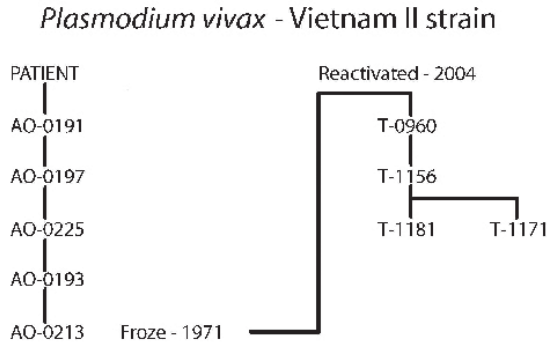

Figure 2. Genealogy of the Vietnam II strain of *Plasmodium vivax* in *Aotus* monkeys AO-191, AO-197, AO-225, AO-193 and AO-213 = *Aotus lemurinus griseimembra*; T-960 = *Aotus vociferans*; T-1156, T-1181 and T-1171 = *Aotus nancymai.*

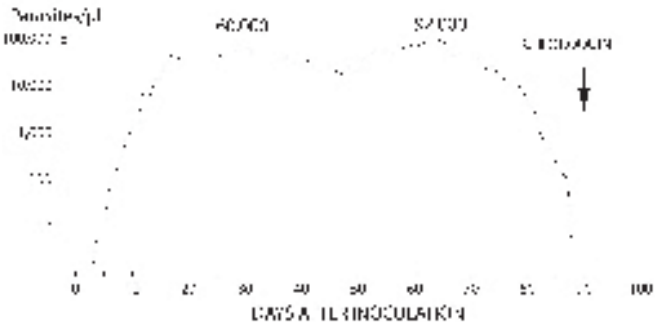

Figure 3. Course of asexual parasitemia after inoculation of *Aotus nancymai* monkey T-1171 with the Vietnam II strain of *Plasmodium vivax*. Maximal parasite counts were seen approximately 30 and 60 days after inoculation. Infection subsequently was cured by treatment with 30 mg chloroquine.

Next the parasite was passaged to the splenectomized monkey AO-0193. A maximal parasite count of only 12,770/µl occurred, but the parasite count was > 10,000/µl on seven consecutive days. Final passage to *A. lemurinus griseimembra* monkey AO-0213 resulted in a maximal parasite count of 68,880/µl. In addition, the parasite count was maintained at > 10,000/µl for 32 consecutive days. At this point (1971), the parasite was frozen in 10% glycerine in saline and stored over liquid nitrogen (8).

We used four monkeys to adapt the Vietnam II strain of *P. vivax* to *A. nancymai*. Maximal counts for the four animals ranged from 10,630 (T-1156) to 94,000 (T-1171) parasites/µl (mean, 39,565 parasites/µl). Parasite counts of > 10,000/µl were maintained for 2 to 64 days.

A sample from AO-0213 that was frozen in 1971 was thawed in 2004 and injected in the femoral vein of splenectomized *A. vociferans* monkey T-0960. A maximal parasite count of 18,630/µl was obtained from T-0960, and parasite counts of > 10,000/µl were maintained for 11 days. Blood from T-0960 was then passaged to splenectomized *A. nancymai* monkey T-1156. A maximum parasite count of 10,680/µl occurred and counts > 10,000/µl occurred on 2 days of the infection.

Finally, blood was passaged to 2 splenectomized *A. nancymai* monkeys (T-1181 and T-1171). Peak parasite counts for *A. nancymai* monkey T-1171 are shown in Fig. 3. The maximum parasite counts for monkeys T-1181 and T-1171 were 35,000 and 94,000/µl, respectively, and counts of > 10,000/µl were maintained for 22 and 64 days, respectively. Table 1 is a summary of the parasitemia during each passage during the adaptation of the Vietnam II strain of *P. vivax* from human to *Aotus*.

Discussion

In 2004, we thawed and reactivated the frozen sample of *P. vivax* from AO-0213. Given the volume of material required and immediate need, this strain of *P. vivax* was a practical selection because it was known to produce high-density parasitemia (9). Although the bacterium had been passaged to splenectomized *A. lemurinus griseimembra*, this species of monkey was no longer being imported and not available for use. Therefore, efforts were made to adapt this parasite to a different species of *Aotus* monkey. Parasite counts for *A. lemurinus griseimembra* monkeys were clearly higher than those seen for the species of *Aotus* we used, but prepatent periods in *A. nancymai* were notably shorter. This rapid and remarkable adaptation to *A. nancymai* monkeys makes them an extremely useful model system. Only three splenectomized *A. nancymai* monkeys were required to successfully reactivate and passage the Vietnam II strain of *P. vivax*.

Splenectomy is often essential to the study of malaria. Most monkeys used in malarial studies are splenectomized either before or during infection (5). The low parasitemia after passage from AO-0197 is likely the result of splenic sequestration of the parasitized erythrocytes. The spleen is a ductless, vertebrate gland that is associated closely with the circulatory system and is an important component of the reticuloendothelial system. The spleen functions primarily in the destruction of old or damaged red blood cells and in the removal of other debris from the bloodstream. It is also a holding reservoir for blood. In many species of animals, the spleen sequesters a large number of red blood cells. Therefore, removal of the spleen facilitates successful and rapid infection in the monkeys by allowing parasitized red blood cells to remain in circulation. They multiply, and marked parasitemia results.

Historically, *A. lemurinus griseimembra* monkeys are the most susceptible for primary adaptation of *Plasmodium* spp. (5, 8, 14). As discussed earlier, *A. lemurinus griseimembra* and *A. nancymai* belong to different groups. These two groups differ in serum proteins and levels of susceptibility to experimental infection with malarial parasites (15). Some *Aotus* species do not allow a good hepatic cycle of the parasite, and native *Plasmodium* spp. sporozoites reproducibly infect only *A. lemurinus griseimembra* (14). Serial subpassage through many animals is often necessary before a parasite grows well in more than one species of monkey (5, 8, 14).

Currently, only *A. nancymai* and *A. vociferans* are imported into the United States for malarial studies (22). Because accessibility to *A. lemurinus griseimembra* monkeys is very limited at best and availability of these animals is not likely to improve in the near future, it is increasingly important to make use of the more readily available *A. nancymai* and *A. vociferans*. The present study showed that *A. nancymai* as an equivalent model to the earlier model of *A. lemurinus griseimembra* for this strain of *P. vivax*. Prepatent periods were notably short, and only four animals were used. Increased passage of this parasite by blood decreases the number of gametocytes, thereby decreasing the number of mosquito infections. Ultimately, the goal is to passage the parasite by mosquito infection to maintain the gametocyte stage, otherwise the ability to infect mosquitoes would be lost. The shortened prepatent period allows maximal potential for exponential growth of the parasite and suggests very good adap-

Table 1. Summary of the adaptation of *Plasmodium vivax* (Vietnam II strain) to *Aotus nancymai*

Monkey	Species of *Aotus*	Passage no.	Prepatent period (days)	Maximal parasite count (/μl)	No. days parasite count was > 10,000/μl
AO-191	*A. lemurinus griseimembra*	1	17	39,600	7
AO-0197	*A. lemurinus griseimembra*	2	7	120,000	9[a]
AO-0225	*A. lemurinus griseimembra*	3	28	4092	0[b]
AO-0193	*A. lemurinus griseimembra*	4	13	12,770	7
AO-0213	*A. lemurinus griseimembra*	5	5	68,880	32
T-0960	*A. vociferans*	6	19	18,630	11
T-1156	*A. nancymai*	7	3	10,630	2
T-1181	*A. nancymai*	8	5	35,000	22
T-1171	*A. nancymai*	9	5	94,000	64

[a]This animal was treated with 30mg of chloroquine by mouth.
[b]This animal was not splenectomized, unlike all others presented.

tation in the host.

The use of *Aotus* as an animal model for studying malaria pathogenesis and drug and vaccine development has been well established (5, 8, 14, 21, 25). There is an immense need for these parasites for teaching, diagnostic testing, and molecular analysis applications. Without the ability to reactivate and adapt varying species and strains of *Plasmodium* to the more readily available *A. nancymai*, our capability to perform serologic and molecular studies and provide teaching materials would be hindered greatly. The importance of being able to adapt various strains of malarial parasites from one species to another is fundamental in the continuing efforts to control this devastating disease. In this study we showed successful reactivation and passage of a very old, yet valuable, sample.

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