Vivax series:

Plasmodium vivax under the microscope: the Aotus model

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The Aotus model for vivax malaria is extremely useful both as a source of living parasites in non-endemic areas, and as a model for vaccine and drug development research. Several species of New World primates can be infected with numerous different strains of Plasmodium vivax. This article reviews some aspects of the Aotus model, discusses the frequently observed hematological changes that can confound interpretation of hemogram data during the course of vivax infection, and provides a partial atlas of parasite forms and Aotus nancymai blood cells.

In all species of Plasmodium that infect humans, natural infection is initiated when an infected anopheline mosquito inoculates a host with sporozoites, which then rapidly invade hepatocytes. Asymptomatic hepatic schizogony is followed by the release of parasite merozoites, which invade host erythrocytes in successive waves of erythrocytic schizogony. The rupture of mature erythrocyte schizonts induces the classical clinical picture of malaria paroxysm, and the development of gametocytes from some merozoites provides the next step – infection of additional anopheline vectors. All human malaria parasite species can also be transmitted by blood transfusion.

Falciparum malaria receives the greatest share of international laboratory research attention because it causes the most fatalities worldwide and because the parasite has proven amenable to continuous laboratory culture. However, Plasmodium vivax also creates significant human morbidity, suffering and economic loss [1–3]. Increasing recognition of the global importance of targeting both parasite species for drug and vaccine developmental efforts, along with recognition of emerging drug resistance in Plasmodium falciparum [4,5], has stimulated significant human morbidity, suffering and economic loss [1–3]. Increasing recognition of the global importance of targeting both parasite species for drug and vaccine developmental efforts, along with recognition of emerging drug resistance in Plasmodium falciparum [4,5], has stimulated significant human morbidity, suffering and economic loss [1–3]. 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there are very active malaria research efforts in Colombia using this particular species [8,10]. Some other Aotus spp. are also reasonably good hosts, and Peruvian Aotus nancymai [23] are currently the most readily available species in other parts of the world. Although A. nancymai or Aotus vociferans (also occasionally available) are not the most optimal model because of their less reliable peak parasitemias and less predictable timing of detectable parasitemias, they are the most appropriate species available for many researchers. Both A. nancymai and A. lemurinus griseimembra are susceptible to blood-stage inocula of a wide variety of P. vivax strains [24] (Box 1).

A tremendous amount of work has been done adapting P. vivax strains to Aotus spp., characterizing the ensuing infections, demonstrating the infectivity of P. vivax gametocytes to various species of anophelines, and demonstrating subsequent sporozoite transmission back to Aotus, humans and other New World monkeys (reviewed in Refs [16,20,21] and see Ref. [27] for additional references). Blood-stage inocula establish P. vivax infection more easily and more consistently than sporozoite infections do [25]. Splenectomy of animals before or during infection by either route increases the reliability of infection and the peak and duration of parasitemia, and resultant gametocytes are more infective to mosquitoes [16,26]. Many human P. vivax isolates will adapt to Aotus monkeys [17], but not all isolates will adapt to any given Aotus species [19]. Work on the continued adaptation of new human isolates, particularly drug-resistant forms, continues [25,27,28,56]. For any situation, a combination of host species and parasite strain should be chosen carefully to optimize the objectives of the study, if availability issues make such a choice possible [29] (Box 1).

Using the model
Blood-stage infections, mainly of the widely used Chesson strain of P. vivax [26], are induced in splenectomized A. nancymai in support of ongoing culture and vaccine development efforts at Walter Reed Army Institute of Research (WRAIR; http://www.wrair.army.mil) [5]. Because these infections are induced by transfer of fresh or frozen blood-stage trophozoites from one animal to another, no observations on pre-erythrocytic stages or on true relapse from hypnozoites are possible. The Chesson strain was previously reported as fairly non-pathogenic in A. nancymai [30] but, as deaths have been reported [26,30], careful clinical monitoring is required. However, like other monkey species [31], signs of illness are rarely observed, although around one-third of monkeys exhibit a transient period of intermittent fever and partial anorexia associated with peak parasitemia. Hemogram values (the values collected during a routine complete blood count) are closely monitored to gauge the additive effect of bloodstream and malaria-induced anemia on hematocrit reduction; animals are immediately treated if their hematocrit falls below 25%, usually indicative of a 50% reduction. This has only occurred in two out of 24 total cases (one Chesson and one non-Chesson strain); generally, the parasitemia falls and the animals spontaneously recover without specific therapy. Subsequent recrudescences, although they usually do occur, are unpredictable in timing and frequency, and often produce a much lower peak parasitemia [21]. Therefore, the animals are treated to remove residual parasites after the primary parasitemia has waned, anywhere from four to 12 weeks after patenty. Hepatomegaly during the initial peak parasitemia has occurred in two out of 24 animals and was resolved promptly by chloroquine treatment.

Prepatent periods are highly variable, from four to 19 days (average 12 days) for diagnosis by microscopy. Limited examination of prepatent samples by polymerase chain reaction (PCR) and limited experience transfusing prepatent samples have indicated that parasites are present much earlier than four days in most cases. The length of the prepatent period has previously been associated with inoculum size and with the fresh or frozen state of the inoculum [25]. Because the viability of frozen parasites cannot be assessed in any other way, a prolonged prepatent period for frozen inocula could be a result of a reduced percentage of viable parasites in frozen preparations. Although it is true that longer prepatent periods are associated with smaller or frozen inocula, variations in
Anemia and response

One of the common clinical hallmarks of all species of human malaria is anemia. Acute anemia in humans has been theorized to be due to lysis and sequestration of infected erythrocytes, sequestration or lysis of non-infected erythrocytes, and parasite-induced or immune-mediator-induced direct or indirect (through renal damage) dyserythropoiesis [32–34]. Infection of either splenectomized or intact Aotus with P. falciparum, as in humans, causes rapid induction of anemia [35,36]. Careful comparison with cumulative parasitemia data in those studies had indicated that the drop in hematocrit is between five and 250 times larger than can be accounted for by destruction of infected erythrocytes alone. An attempt to demonstrate evidence for antibody- or complement-mediated lysis of non-infected cells to account for this discrepancy was not successful [36]. The observation that reticulocyte counts did not rise until after parasitemia had fallen supported the possibility of parasite-induced dyserythropoiesis.

Variable anemia can develop in P. vivax-infected A. nancymai. At one extreme, the anemia is very mild and can be almost accounted for by a combination of cumulative parasite lysis and the volume of sample harvested. At the other extreme, marked anemia can develop rapidly that represents a much greater cell loss than can be explained by the cumulative effect of parasitized cell lysis and blood sample withdrawal. In one case infected with the Chesson strain, an animal followed a more falciparum-like pattern: the hematocrit fell from 43% to 30% during the prepatent period, and then to 21% by the third day of patency. Chloroquine therapy was immediately instituted, but the monkey did not evince a reticulocyte response until that third day of patency. However, in general, the reticulocyte profile (determined by new methylene blue count) in the Aotus—Chesson model is different from that reported for P. falciparum infections in this and other Aotus spp. Baseline reticulocyte counts are often fairly high (1–5%), and they generally rise briskly upon infection with Chesson P. vivax. In two out of 21 cases infected with Chesson strain P. vivax, a marked increase in reticulocyte count was observed before the onset of detectable parasitemia. The majority of animals have rising reticulocyte counts that parallel or follow the parasitemia slightly. As with many other hematological

![Image](http://parasites.trends.com)
parameters, the timing, character and magnitude of the reticulocyte counts vary from one individual to another.

This brisk erythropoietic response is demonstrated not only by increasing reticulocytomia (to values as high as 15–25%), but also in some individuals by the appearance in the bloodstream of large numbers of nucleated erythrocytes (orthochromatich normoblasts) (Figure 2), and even earlier forms back to and including rubriblasts (basophilic normoblasts) [37] (Figure 3). Because these cells often register as lymphocytes in automated hemocytometers, they can give rise to a very misleading total white cell and lymphocyte count. Under these conditions, it is best to use an experienced microscopist who can determine the differential blood counts manually. In addition, a different stain (Wright’s) might be preferred to Giemsa for accurate evaluation of leukocytes. This indication of great responsive capacity of the erythropoietic system in this species is supported by the observation that it rarely takes more than 15–20 days for hematocrits in these animals to return to normal (50 ± 5%) either when parasite treatment has been instigated due to anemia or when parasitemia falls during natural recovery. There has been no indication, for example, by a reduction in mean corpuscular volume, that iron supplementation is required. Indeed, with the occasional occurrence of nutritional hemosiderosis in other captive populations of New World monkeys, iron supplementation would be undertaken reluctantly unless there were unambiguous indications of iron deficiency, and liver function was closely monitored.

Absolute leukocytosis, lymphocytosis, granulocytosis and monocytosis can all occur, and should be differentiated from falsely elevated counts caused by erythrocyte precursors. In conditions where very immature erythrocyte precursors are found in circulation, occasional precursors for other cell lineages can also be seen (Figure 3). Band neutrophils become common, and even younger granulocyte precursors (myeloblasts) can sometimes be seen. These infrequent earlier precursor cells are large, can have scattered granules in their cytoplasm, and large oval or indented nuclei that on first glance resemble monocytes or extraordinarily large granular lymphocytes. To help distinguish them, note that monocyte/macrophage chromatin appears focused, has a finely fibrillar nature and sometimes contains sharply defined tiny vacuoles, and the cytoplasm often contains large clear vacuoles or malaria pigment. The fuzzier, less clearly defined chromatin of band neutrophils closely resembles that of their immediate precursors. These and other normal Aotus leukocytes are included for comparison in Figure 3.

Immunity
There does not appear to be any reason to exclude animals previously infected with P. falciparum from subsequent

\[ \text{Figure 2. Abnormalities in Aotus nancymai erythrocytes during infection with Plasmodium vivax. Very mature nucleated red blood cells often appear in circulation. In the final stages before extrusion of the nucleus, nucleated rubriblasts (late-stage erythrocyte precursor cells) are easily identified by their small, very dense nucleus and their cytoplasm which is similar to that of polychromatophilic reticulocytes. As rubricyte maturity decreases (a–d), the cytoplasm can be seen to be less greyish-orange in colour and more smoky blue, and the nucleus is less condensed. As maturity decreases further, (e–g), cell and nuclear size become larger and there is more overlap with lymphocyte size and appearance. Rubricytes can be distinguished from lymphocytes by the combination of ruffled cell edges and homogeneous smoky blue cytoplasm. The cytoplasm of small lymphocytes (h) is more violet-blue and more granular in appearance, the clear perinuclear Golgi zone is often more prominent, and edge irregularities, if present, often appear as thin extensions rather than large ruffles. Erythrocyte fragments (schiztocytes), spherocytes, target cells and marked anisocytosis are often observed during infection, even in the absence of a spleen (i). Howell-Jolly bodies (j), which accompany asplenia and accelerated erythropoiesis, can be easily distinguished from parasites by the lack of associated pale blue cytoplasm. Single platelets superimposed on erythrocytes (k) can be mistaken for young parasites. They often lack two-color staining typical of platelets in clumps (l). They do not contain a distinct nucleus, unlike parasites (see Figure 1). (m–p) illustrate the new methylene blue-stained appearance of reticulocytes and rubricytes. Reticulocyte counts can become remarkably elevated in this species, as seen in (m) (26% reticulocytomia). Images are Giemsa stained at 1000x unless indicated otherwise. Scale bar = 10 μm.} \]
infection with *P. vivax* or vice versa [25, 38–39]. In fact, peak *P. falciparum* parasitemia can even increase in animals that were previously infected with *P. vivax* [38]. However, after the animal has resolved its initial *P. vivax* parasitemia, recurrences of the same strain in untreated individuals are often shorter in duration and usually attain only ~20% or less of the original peak parasitemia. Attempts to re-inflect monkeys with different *P. vivax* strains after an initial Cherson infection confirm other observations that the monkeys remain susceptible, but peak parasitemia is often greatly reduced [40, 41]. Limited data are available for re-use of *P. vivax*-infected *Aotus* with other simian malaria parasites [42].

**Perspective**

The use of *Aotus* monkeys will remain a valuable tool for the pre-clinical development of malaria drug and vaccine candidates [8, 16, 43] because they can be challenged with both *P. vivax* and *P. falciparum*. Although the selection of immunologic markers is more limited than in immunogenicity models in rhesus macaques and other Old World species, several useful lymphocyte phenotype markers and cytokine assays are available [44]. Partial human homologies for some *Aotus* major histocompatibility complex (MHC) [45–47], immunoglobulin [48], T-cell receptor [49, 50] and cytokine [51–53] genes have been published, which might help in the evaluation of immune responses to vaccine candidates [54]. However, it is important to remember that the *Aotus–P. vivax* interaction is only a model, and represents an abnormal host–parasite relationship. The use of this model in predicting ultimate vaccine efficacy in humans has yet to be evaluated [55].

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