Adaptation of a Thai Multidrug-Resistant C2A Clone of *Plasmodium falciparum* to *Aotus* Monkeys and Its Preliminary in vivo Antimalarial Drug Efficacy-Resistance Profile

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**Abstract.** A multidrug-resistant (MDR) clone of *Plasmodium falciparum* (C2A) from Thailand was adapted through serial passage to *Aotus* monkeys. During adaptation, the parasite showed resistance to a single 20 or 40 mg/kg oral dose of mefloquine (MQ). Infection was only cured when MQ was administered orally at 40 mg/kg once in combination with intravenous artesunic acid at 20 mg/kg for 3 days. Similarly, the parasite clone was found to be resistant to quinine, failing at 20 mg/kg orally for 5 days in combination with an experimental dihydrofolate reductase (DHFR) inhibitor (WR297608) at 10, 20, or 40 mg/kg orally for 3 days, and with atovaquone/proguanil at 25 mg/kg for 3 days. This new model will allow *in vivo* testing of new antimalarial compounds or their combinations against a currently circulating MDR *P. falciparum* strain.

**INTRODUCTION**

Multidrug-resistant (MDR) *Plasmodium falciparum* parasites are increasing at an alarming rate in terms of both prevalence and severity. These strains have been detected along the Thai-Myanmar and Thai-Cambodian borders and their resistance to chloroquine, sulphadoxine-pyrimethamine, quinine, and mefloquine, and most recently to an artesunate-mefloquine combination, have been reported in humans.1–3

With the emergence of MDR falciparum malaria in different parts of the world,2,4 new drugs or drugs in new combinations are urgently needed. For this reason, the importance of a reliable animal model for *in vivo* testing of new antimalarial compounds or their combinations against these MDR isolates cannot be overemphasized.

The *Human P. falciparum/Aotus* monkey model is an excellent pre-clinical model that has been used extensively since it was first demonstrated in 1966 at Gorgas Memorial Laboratory in Panama, that *Aotus trivirgatus* monkeys could be infected with human plasmodia.4 For more than 40 years, the Panamanian *Aotus* (*Aotus lemurinus lemurinus*) has been used to adapt new strains of malaria1–5 and study its biology,6–12 pathogenesis of its infection,13–15 and to test the efficacy and pharmacokinetics of antimalarial compounds16–27 against these new strains. More recently, this model has also been used to test the efficacy and immunogenicity of antimalarial vaccines through the use of repeated challenge,28 plasmid DNA vaccines,29–32 temperature-sensitive mutants,33 synthetic peptides,34 recombinant proteins,35–37 and even to test the immunogenicity of hepatitis B DNA vaccines.38

Herein, we describe the adaptation of a new MDR C2A clone of *P. falciparum* originally from Thailand to *Aotus l. lemurinus* monkeys and present preliminary *in vivo* data on its drug sensitivity-resistance profile. This new model will allow testing the efficacy of new therapies against MDR malaria parasites.

**MATERIALS AND METHODS**

**Animals.** Data were obtained from 18 (male and female) *Aotus l. lemurinus* monkeys Karyotype VIII & IX,39 6 spleen

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Oral administration of drugs was by gastric intubation using a 14 French catheter. The total volume of fluid administered, drug solution or suspension, and rinse was 14 mL. The IV administration was carried out by way of the saphenous vein with a 25-g butterfly needle catheter. The following drugs were tested during the course of these experiments: artesunic acid (AS) (WR256293) at 8 and 20 mg/kg × 3 days IV; artelinic acid (AL) (WR255663) at 8 and 16 mg/kg × 3 days orally; MQ (WR142490) at 20 and 40 mg/kg once and × 3 days orally; quinine (QN) (WR297608) at 20 mg/kg × 5 days orally; WR297608 (PS) at 10, 20, and 40 mg/kg × 3 days orally; and atovaquone/proguanil (Malarone [MAL]) at 25 mg/kg × 3 days orally.

Response to treatment. Response to treatment was categorized as no effect (N), suppression without clearance (S), clearance and recrudescence (CL & R), or clearance and cure (CL & C). The day of clearance was defined as the first of 3 consecutive days in which the thick blood films were parasite negative. The day of recrudescence was the first of 3 consecutive days of positive thick blood films after a period of clearance. Suppression was defined as a transient decrease in parasite density post-treatment without clearance. Treatment failure of primary treatment (F1) or each successive re-treatment (F2–F4) was defined as parasitemia recrudescence, suppression of the parasitemia for more than 7 days with eventual clearance, suppression of parasitemia without clearance, an increase of parasite density > 25% on Day 3 after treatment, or if the animal was significantly anemic even without detectable parasitemia.

Statistics. The Mann-Whitney rank sum test using the SigmaStat software (Systat Software Inc., Richmond, CA) were applied to non-parametric data with a significant difference level defined at $P < 0.05$.

RESULTS

Passage history. Between December 14, 1998 and April 17, 2008 the C2A clone of *P. falciparum* was serially passaged nine times in splenectomized and intact *Aotus* monkeys, as shown in Figure 1. Although *Aotus* monkey MN89005 was eventually positive for the new C2A clone on Day 73 PI, both *Aotus* MN89012 and MN12907, which were inoculated with the C2B and WR75 clones of *P. falciparum*, respectively, remained negative for more than 100 days of follow-up and were therefore considered negative/failed passages. Serial passage of the C2A clone in spleen intact animals at passage levels III–VI and IX was carried out in one animal per passage (Figure 1) (Tables 1 and 2).

Parasitologic profile. As shown in Table 1, parasitemia became patent in original *Aotus* MN89008 on Day 73 PI. This animal had a peak asexual parasitemia of $10.5 \times 10^3 \times \mu L$ on Day 84 PI that cleared on Day 90 PI without treatment and had an asexual parasitemia < 10 parasites × µL that began on Day 77 PI and cleared on Day 106 PI (Figure 2, panel A). From passage level II up to level IV, when infected citrated whole blood from a donor monkey was passaged into MN92015 (Figure 2, panel B) MN92034 and MN93014, respectively, the
day of parasitemia level was established between Days 1–3 PI regardless of parasitemia level. It was only during those four passages that sexual parasites were detected. In contrast, when frozen stock whole blood was inoculated IP for the later passages (Figure 2, panel C), no sexual parasites were detected again. In the animals that received a frozen stabilate IP (MN12801, MN12753) and or IV (MN24049), patency occurred between 4 and 15 days PI and parasitemia reached a peak of 266.76 × 10^3 × µL in Aotus MN24049 on Day 12 PI, the highest parasitemia recorded in all of the inoculated animals during the study (Figure 3). Statistical significant differences were found in the mean peak parasitemia [54 (confidence interval [CI] 95%; 8, 100)] (P = 0.002) and day of clearance [49 (CI 95%; 29, 69)] (P = 0.003) between splenectomized and spleen intact animals (Table 2). Although no significant difference was found in the mean day of peak parasitemia between splenectomized [21 (CI 95%; 8, 34)] and spleen intact animals [11 (CI 95%; 2, 20)] (P = 0.257), the spleen intact group as shown in Table 2 all developed low grade parasitemias only [0.51 (CI 95%; 0.05, 1.07)], that peaked on average on Day 11 (CI 95%; 2, 20) but cleared spontaneously on average by Day 14 (CI 95%; 9, 19) (Figure 2, panel D). No sexual forms of the parasite were detected with the exception of MN12971 (passage level III) that was positive for only 1 day (4 PI). Only Aotus MN12961 (passage level VI) cleared but recrudesce on Day 23 PI. No further parasite specimens were collected from these animals because of their low grade parasitemias.

**Antimalarial drug treatments.** The antimalarial drug sensitivity-resistance profile of the MDR C2A clone of *P. falciparum* in splenectomized Aotus monkeys is shown in Table 3. Resistance to MQ was detected on primary treatment (F1) when administered at 40 mg/kg in Aotus MN92015 during passage level II and at 20 mg/kg in MN92034 in passage level III. The F1 resistance to MQ at 20 mg/kg was recorded during passage level V in Aotus MN12801 and during passage level VII at 40 mg/kg in MN12753. Resistance to re-treatment (F2) was detected during passage level VII in Aotus MN13112. Cures with MQ did occur on Day 39 when given alone once at 40 mg/kg to Aotus MN12907, which had previously failed QN at 20 mg/kg for 5 days and PS at 20 mg/kg × 3 days in F1 and F2, respectively, and also when given at 40 mg/kg once in combination with AS at 8 mg/kg × 3 days IV in Aotus MN12753, which had previously failed MQ at 40 mg/kg once in F1 and AS at 20 mg/kg × 3 days in F2 on Day 84 PI. The remainder of the compounds such as AL at 8 or 16 mg/kg, QN at 20 mg/kg × 5 days orally, PS at 10, 20, or 40 mg/kg × 3 days, or MAL at 25 mg/kg × 3 days orally all failed, as shown in Table 3.

**DISCUSSION**

Recent reports of failure of the artesunate-mefloquine combination therapy for uncomplicated *P. falciparum* malaria from southern Cambodia, where it has been deployed since 1995 and 2000, are of great concern. The establishment of a reliable animal model to test the efficacy of new antimalarial drugs alone or in combination against these MDR isolates is urgently needed.

In 1998, we initiated the attempted adaptation of three multidrug-resistant clones of *P. falciparum* from Thailand with three splenectomized Aotus monkeys that were inoculated with the C2A, C2B, and WR75 clones. After 73 days PI, only the C2A inoculated Aotus monkey became positive. Eight more passages in splenectomized animals were necessary to obtain a good sustainable parasitemia in a single animal as shown in Figure 3. In contrast, when the parasites were passed to spleen intact animals (passage levels III, IV–VI, and IX) only a low-density parasitemia was established (Table 2). This observa-

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**Table 1**

<table>
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<tr>
<th>Passage level</th>
<th>Aotus ID</th>
<th>Inoculation date</th>
<th>Inoculum size</th>
<th>Day of patency</th>
<th>Peak parasitemia × µL × 10^3</th>
<th>Day of peak</th>
<th>Day of primary treatment</th>
<th>Day of clearance</th>
<th>Day of recrudescence</th>
<th>Day of retreatment</th>
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<td>2/26/1999</td>
<td>&gt; 10</td>
<td>2</td>
<td>20.12</td>
<td>22</td>
<td>73</td>
<td>94</td>
<td></td>
<td></td>
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<tr>
<td>III</td>
<td>92034</td>
<td>5/7/1999</td>
<td>18120</td>
<td>1</td>
<td>85.12</td>
<td>11</td>
<td>11</td>
<td>55</td>
<td>95</td>
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<tr>
<td>IV</td>
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<td>&gt; 10</td>
<td>3</td>
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<td>22</td>
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<td>41</td>
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<td>Frozen</td>
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<td>Frozen</td>
<td>4</td>
<td>266.76</td>
<td>12</td>
<td>12</td>
<td>25</td>
<td>45</td>
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X(95% confidence interval) 54 (8, 100) 21 (8, 34) 24 (11, 38) 49 (29, 69)

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**Table 2**

<table>
<thead>
<tr>
<th>Passage level</th>
<th>Aotus ID</th>
<th>Inoculation date</th>
<th>Inoculum size × µL</th>
<th>Day of patency</th>
<th>Peak parasitemia × µL × 10^3</th>
<th>Day of peak</th>
<th>Day of primary treatment</th>
<th>Day of clearance</th>
<th>Day of recrudescence</th>
<th>Day of retreatment</th>
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<tr>
<td>IV</td>
<td>12955</td>
<td>8/12/1999</td>
<td>&gt; 10</td>
<td>3</td>
<td>1.71</td>
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<td>19</td>
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<td>V</td>
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<td>5</td>
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<td>VI</td>
<td>12961</td>
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<td>12</td>
<td>0.96</td>
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<td>15</td>
<td>23</td>
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<td>12977</td>
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<td>31620</td>
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X(95% confidence interval) 0.51 (0.05, 1.07) 11 (2, 20) 14 (9, 19)
tion confirms that splenectomy seems to be a pre-requisite for malaria parasites adaptation in *Aotus* monkeys, and that there exists different degrees of susceptibility among *Aotus* of different geographic and genetic backgrounds. The monkeys used in this study were all from the ICGES colony, known to be composed of karyotypes VIII and IX animals.

During this study it was observed that at low passage levels (levels I–IV), the parasites had a tendency to develop a medium- to high-density parasitemia in most of the animals regardless of the inoculum size (Table 1), and gametocytes were always present in blood smears examined (Figure 2, panel A and B). Once a change in protocol led to the infected blood being collected with alsevers-glycerol frozen and stored as described, and further passed by the IP route, gametocytes were not detected again (Figure 2, panel C). As the passage levels increased, the pathogenicity and mefloquine resistance (Table 3) of the parasites seemed to increase as well. During passage levels II–III into *Aotus* MN92015 and MN92034 mefloquine 40 mg/kg initially resulted in suppression and clearance but then progressed to a similar suppression and clearance but with eventual recrudescence as well at 20 mg/kg during passage level III.

Passage level VIII with inoculums of fresh citrated whole blood from donor monkey MN12753 that had been part of a malaria plasmid DNA vaccine protocol 11 years earlier, induced only a low-density parasitemia. This animal, however, still required treatment with MQ on Day 60 PI because of anemia. This is a pattern that is frequently observed in spleen intact animals suggestive of partial immunity (Figure 2 panel D). In this case, the spleen seemed not to have been a pre-requisite for anemia development, but other factors such as host immune status (perhaps from previous plasmid DNA immunization or challenge), increased parasite pathogenicity, or iatrogenic factors such as excessive bleedings during follow-up could have all contributed to its development.

In contrast, during the same passage level VIII to a malaria naive splenectomized *Aotus* (MN24009) this time inoculated IV with Glycerolyte cryopreserved infected blood from the same donor MN12753, we observed the highest recorded peak parasitemia (266.75 × 10^3 × µL) of the study. This suggests that the previous two inoculated animals (MN12753 and MN90025) had residual immunity or perhaps simply that the Glycerolyte blood cryopreservation method used to inoculate MN24009 was better than the alsevers-glycerol method described by Rossan and used previously.

From these observations it could be inferred that freezing with alsevers-glycerol preservative and the use of the IP inoculation route, as was done during passage level V–VII.

Figure 2. Parasitemia plots of splenectomized *Aotus l. lemurinus* monkeys (panels A, B, and C) and spleen intact (panel D) infected with a C2A clone of *Plasmodium falciparum* from Thailand.
ADAPTATION OF MULTIDRUG-RESISTANT *P. FALCIPARUM* TO *AOTUS* MONKEYS

Figure 3. Parasitemia plot of a splenectomized *Aotus* monkey infected with a multiple drug-resistant C2A clone of *Plasmodium falciparum* showing the efficacy of artesunic (AS) acid and atovaquone/proguanil (MAL) alone and AS in combination with mefloquine (MQ) against infection.

(Table 1), could have caused a parasite sub-population die-off, and thereby selected for parasites with these induced phenotypic and possibly genotypic changes. This same phenomenon may not have occurred when the parasites were cryopreserved in Glycerolyte and inoculated IV. The fact that the parasite clone studied here, rather than increasing its adaptation while passage levels increased, instead seemed to have gradually lost its capacity to induce medium- to high-density parasitemias in splenectomized animals before the change to the use of Glycerolyte supports this observation. Genotyping the parasite at different passage levels and correlating it with other host immune markers could help elucidate which genes were involved in this seemingly unusual adaptation behavior. Additionally, we observed that the parasite increased its MQ resistance from a moderate resistant phenotype during passage levels I–IV to a highly resistant one during passage levels VII–VIII. It is possible that this is caused by the widely varying blood levels and apparent resistance resulting from inadequate blood levels that MQ is known to produce, or perhaps that there was a selection of a single malaria isolate from the heterogeneous population of parasites exhibiting much more MQ resistance. We know from previous studies that a single oral dose of 20 mg/kg of MQ cures *P. falciparum* Vietnam oak knoll (FVO) infections in intact *Aotus* monkeys. It is also known that MQ resistance may be caused by increased copy numbers of the *pfmdr1* gene amplified during infection. It is therefore possible that the resistance observed in this experiment may have been induced by the host immune response forcing the parasite to use its tremendous adaptation plasticity to enter into different physiologic stages before becoming completely adapted to growth within the *Aotus*.

In conclusion, it appears that a MDR C2A clone of *P. falciparum* from Thailand has been successfully adapted to growth within spleenectomized *Aotus* monkeys. This parasite has demonstrated resistance to a single 20 or 40 mg/kg oral dose of MQ, but was cured when MQ was administered orally at 40 mg/kg once in combination with intravenous AS at 20 mg/kg for 3 days. Similarly, this parasite clone was found to be resistant to QN at 20 mg/kg orally for 5 days, an experimental DHFR inhibitor WR297608 at 10, 20, or 40 mg/kg orally for 3 days and to MAL at 25 mg/kg for 3 days. If confirmed and validated by future studies, this new model may provide an exciting new tool for testing the efficacy of new antimalarial compounds alone or in combination against parasites that are representative of the currently MDR strains circulating in the population.

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Disclosure: The experiments reported here were conducted according to the principles set forth in the “Guide for the Care and Use of Laboratory Animals,” Institute of Laboratory Animal Resources, National Research Council (Department of Health and Human Services, National Institutes of Health publication, 1996).

Disclaimer: The opinions and assertions contained herein are the private ones of the author and are not to be construed as official or reflecting the views of the U.S. Army.

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Table 3

Efficacy of artelinic acid (AL), WR283178 (PS), quinine (QN), artesunic acid (AS), mefloquine (MQ), and atovaquone/proguanil (MAL) alone or AS and MQ in combination in 11 splenectomized *Aotus* monkeys serially infected with a multiple drug resistant C2A clone of *Plasmodium falciparum*.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Passage level</th>
<th>Treatment efficacy (Post-Inoculation day of treatment: number or days)*</th>
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<td>8</td>
<td>S (8, 3)</td>
<td>S (12, 3)</td>
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<tr>
<td></td>
<td>16</td>
<td>S (22, 3)</td>
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</tr>
<tr>
<td>PS</td>
<td>10</td>
<td>S (12, 3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
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<td></td>
<td>40</td>
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<td>20</td>
<td>S (48, 3)</td>
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<td></td>
<td>(42, 5)</td>
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</tr>
<tr>
<td>AS</td>
<td>20</td>
<td>S, CL &amp; R (11, 1)</td>
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<td></td>
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<td>MAL</td>
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<td>CL &amp; C (69, 3)</td>
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*Table 3: Efficacy of artelinic acid (AL), WR283178 (PS), quinine (QN), artesunic acid (AS), mefloquine (MQ), and atovaquone/proguanil (MAL) alone or AS and MQ in combination in 11 splenectomized *Aotus* monkeys serially infected with a multiple drug resistant C2A clone of *Plasmodium falciparum*. Treatment efficacy over parasitemia: S = suppressed; CL = cleared; R = recrudesce; C = cured; N = no effect.†Animal was negative but anemic when treatment started.‡Treatment result: SC = self-cured; C = cured; F = failed; F1 = failed primary treatment; F2–F4 = failed re-treatments 2–4.
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2. Krudsood S, Patel SN, Tangpukdee N, Thanachartwet W, of Public Health, University of South Florida, Tampa, FL 33612. MD 20910; present address: Department of Global Health, College of Public Health, University of South Florida, Tampa, FL 33612.


