FACTORS INFLUENCING RESISTANCE TO REINFECTION
WITH PLASMODIUM FALCIPARUM

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Abstract. A treatment-reinfection study design was used to investigate the relationships between host immunologic and/or genetic factors and resistance to reinfection with Plasmodium falciparum. Sixty-one children in Gabon were enrolled in a cross-sectional study to measure the prevalence of each human plasmodial species. All were given amodiaquine for radical cure of parasites, and 40 were subsequently followed-up for 30 weeks. Successive blood smears were examined to measure the delay of reappearance in blood of asexual stages of P. falciparum parasites. Presence of infection during the cross-sectional survey was associated with male sex, non-deficient glucose-6-phosphate dehydrogenase activity, plasma interleukin-10 level, and anti-LSA-Rep antibody concentration. Resistance to reinfection was related to the presence of anti-LSA-J antibodies, and the absence of anti-LSA-Rep antibodies. Moreover, P. malariae-infected subjects were usually co-infected with P. falciparum, and were also more rapidly reinfected with P. falciparum after treatment, compared with those without P. malariae infection.

Most studies aimed to investigate the protection against Plasmodium falciparum malaria have used malaria attacks as criteria.1,2 However, these criteria require long and precise follow-up of the populations because malaria attacks occur rarely, even in hyperendemic areas where children who present more than 2 malaria attacks per year are rare.3 Moreover, such criteria mainly investigate protection against blood stages of the parasite and do not allow the assessment of protection against the pre-erythrocytic stages. The existence of such protection is shown by the fact that only a small proportion part of infective bites leads to the appearance of parasites in the peripheral blood.4 Similarly, the prevalence rates of blood parasite infections following antimalarial treatment are extremely lower than presumed in view of the number of infectious bites from mosquitoes.5 In this context, effector mechanisms may be directed against the sporozoites, the liver stages, or both. Protection against the sporozoites may result from a specific humoral response because it has been demonstrated for the antibody directed to the repetitive epitope (NANP)3 from the circumsporozoite (CS) protein.6 Antibody targeted against liver-stage antigens may also contribute to the reduction of the emergence of P. falciparum from the liver. The plasma levels of Th1-type and Th2-type cytokines are also thought to play a major role in individual levels of protection. To evaluate the level of protection against the sporozoite and liver stages of the parasite, we first performed a cross-sectional study to assess the parasitologic status of children living in Gabon, an area hyperendemic for malaria. All children then received a curative antimalarial treatment and were enrolled in a longitudinal follow-up to measure their delay of reinfection by natural challenge. Relationships between the immunologic factors and the infection rate at the beginning of the study, as well as the delay of reinfection by P. falciparum, were investigated. Lastly, we evaluated the impact of several red blood cell polymorphisms, such as the sickle cell trait and the glucose-6-phosphate dehydrogenase (G6PD) deficiency, on both asymptomatic malaria and resistance to the reinfection. These erythrocytic abnormalities are present at high frequencies in this African area and have been related to the resistance against clinical malaria.7

STUDY AREA. The study was conducted in Dienga, a village located in the Ogooué-Lolo province in southeastern Gabon. The equatorial climate is characterized by short and long dry seasons in January–February and June–August, respectively, separated by 2 rainy seasons. Malaria is highly endemic with seasonal variations in vector density and transmission (Elissa N and others, unpublished data). Plasmodium falciparum is the main malaria species encountered in the area. Several studies have been previously conducted in this village where a permanent base of the Centre International de Recherches Médicales de Franceville has been located for several years. The village is quite isolated and self-medication is highly reduced, having been shown to be in the range of 5–10% by detection of 4-aminoquinoline metabolites in the urine by the Saker-Solomon test.3 The study was initiated at the end of the long rains (May 1998) and lasted until the next rainy season (December 1998).

SUBJECTS. All children from 10 families were enrolled in the study after the study design was explained to the families and informed consent was obtained from the parents. The study design involved a cross-sectional study of malaria prevalence, followed by radical antimalarial treatment, and longitudinal follow-up to measure the delay of reinfection. Ethical clearance for the study was given by the Ethics Committee of the Centre International de Recherches Médicales de Franceville.

Cross-sectional survey of malaria prevalence and antimalarial cure. At enrollment, a thick blood smear was obtained from each of the 61 children. Blood samples were collected in Vacutainer® (Becton Dickinson, Meylan, France) tubes containing EDTA for the oldest children, and into capillary blood collection tubes (Microtainer® with EDTA; Becton Dickinson) for the youngest ones. Amodiaquine (Flavoquin®; Roussel, Paris, France) was then given orally, irrespective of the result of the thick smear, at a regimen of 10 mg/kg/day. Youngest children were treated for 2 days (total dose = 20 mg/kg), while older children were treated for 3 days (total dose = 30 mg/kg). Each drug dose was given by the research team. Efficacy of treatment was assessed by weekly thick blood smears. Treatment failure
was defined as the presence of malaria parasites in blood smears collected 1 or 2 weeks following initiation of treatment. The corresponding children were excluded from the second part of the study.

Assessment of the delay of reinfection. Following treatment, thick and thin smears were prepared from blood collected by fingerprick and stained with Giemsa. Follow-up started 1 week after initiation of treatment, and was conducted weekly up to the 11th week (W11), then every 2 weeks up to the 30th week (W30) to measure the delay of reinfection. Asexual blood-stage \( P. falciparum \) parasites were counted against 1,000 leukocytes. The parasite density was calculated on the basis of the mean number of leukocytes per microliter of blood (6,500 leukocytes/µl) observed in the subjects of the cohort. The threshold of sensitivity was therefore approximately 7 parasites per microliter of blood. All slides showing the first reappearance of parasites after treatment (as well as the last negative slides) were viewed by a second examiner.

Hematologic assays. At initiation of the study, blood group was determined by serology and sickle cell trait was detected by hemoglobin electrophoresis. The G6PD enzymatic activity was evaluated by the spot-test method. Briefly, glucose-6-phosphate was oxidized in the presence of G6PD and NADP+ producing 6-phosphogluconate, hydrogen, and NADPH. The production of NADPH was assessed under long-wave UV light using its physical property of fluorescence. This method detects red blood cells with less than 20% of the normal enzymatic activity. Consequently, most heterozygote females are not detected.

Immunologic investigations. Plasma was isolated by sedimentation and stored frozen at \(-60^\circ\text{C}\) until used for measurement of cytokine and antibody levels. The plasma concentrations of interferon-\(\gamma\) (IFN-\(\gamma\)) and interleukin-10 (IL-10) were measured by a standard capture and detection sandwich ELISA using the manufacturer’s instructions (IFN-\(\gamma\): Mabtech, Stockholm, Sweden and IL-10: Pharmingen, San Diego, CA). Recombinant human cytokines for the determination of standard curves were provided by Pharmingen. The threshold of sensitivity was 2 pg/ml.

Antibody directed against 2 synthetic peptides from the \( P. falciparum \) pre-erythrocytic stage antigen LSA-1 ((LAK-EKLQEQQSDLEQER), LAKELQ, referred to as LSA-Rep and ERRAKEKLQEQQRDLEQKRADTKK, referred to as LSA-J)\(^9\) and 2 synthetic peptides, (NANP)\(^5\) and (NAAG)\(^5\), corresponding to 5 repeats of the repetitive epitope of the CS proteins of \( P. falciparum \) and \( P. malariae \), respectively, were measured by ELISA as previously described.\(^6,10,11\) An anti-human IgG (Fc specific) conjugated to alkaline phosphatase (Sigma, St. Louis, MO) was used to measure the level of antibody bound to the peptides.

Statistical analysis. Genetic and immunologic factors were compared in groups of subjects with or without \( P. falciparum \) infections at the beginning of the study, as well as according to the delay of reinfection. Quantitative variables did not follow normal distributions, even after log-transformation, and were therefore compared by the Mann-Whitney U-test. Age was considered both as a quantitative variable and as a qualitative variable considering younger (\(<7\) years) and older (\(\geq7\) years) children. Differences between quantitative variables were assessed by the chi-square test. \(P\) values <0.05 were considered significant.

RESULTS

Sixty-one children from 10 families, including 34 boys and 27 girls with a mean ± SD age of 10.08 ± 4.99 years, were enrolled in the study. Blood was obtained from 59 children, but the amount of blood drawn was sometimes insufficient to allow all hematologic and immunologic assays.

Parasitologic, genetic, and immunologic parameters before treatment. Among the 61 children, \( P. falciparum \) was observed in blood smears from 41, while \( P. malariae \) and \( P. ovale \) were present in 15 and 1, respectively. Children infected with \( P. malariae \) had a the tendency to present more often with a concomitant \( P. falciparum \) infection (13 of 15, \( P = 0.065 \)), and to exhibit higher \( P. falciparum \) parasite densities than \( P. malariae\)-uninfected children (geometric mean \( P. falciparum \) parasite density = 212, 95% confidence interval [CI] = 184–240 for \( P. malariae\)-infected subjects and 149 [95% CI = 110–188] for \( P. malariae\)-uninfected subjects: \( P = 0.068 \)). At enrollment, none of the children presented with any symptom of a clinical malaria attack, as defined by the absence of fever.

Males were infected significantly more often than females (\(P = 0.023\); Table 1). Most (54%) of the children had blood group A, 37% had blood group O, 5% had blood group B, and 4% had blood group AB. No difference in parasite prevalence was observed in the children with different blood groups. The sickle cell trait was observed in 33% of the subjects. Among the 55 subjects tested for their G6PD activity, 11 had a deficient activity and 4 of these 11 subjects (36%) had \( P. falciparum \) parasites in the blood, while 35 (80%) of 44 non-deficient G6PD subjects were infected (\( P = 0.005 \)). When G6PD-deficient subjects (7 females and 4 males) were excluded from the analysis, the gender difference in parasite prevalence was highly reduced (\( P = 0.065 \)).

The level of plasma IL-10 showed a tendency to be higher in \( P. falciparum\)-infected subjects than in noninfected subjects (\( P = 0.057 \)). The level of plasma antibody to LSA-Rep, but not to the other peptides, was higher in \( P. falciparum\)-infected subjects than in non-infected subjects (\( P = 0.03 \)) (Table 1). Levels of anti-NAAG antibody were not related to infection with \( P. malariae \), but were correlated with levels of anti-NANP antibody (\( P = 0.02 \)). Furthermore, the level of plasma anti-NAAG antibody was higher in older than in younger children (median = [25th percentile–75th percentile] = 61.47 [28.54–86.49] and 23.06 [11.11–61.00], respectively; \( P = 0.05 \)).

Efficacy of amodiaquine treatment. Among the 41 children presenting with a \( P. falciparum \) infection at the beginning of the study, 7 were given a total dose of 20 mg/kg of amodiaquine, and 34 received 30 mg/kg of the drug. Treatment failed in 5 of the 7 infected children who received 20 mg/kg of amodiaquine and in 3 of the 34 \( P. falciparum\)-infected children who received 30 mg/kg of amodiaquine (\( P = 0.0002 \), by chi-square test) (Table 2). Three children were lost to follow-up at the first or second week. All children who were negative at enrollment were similarly treated, and all were negative at both W1 and W2.

Delay of reinfection. Children with treatment failure were
excluded, and the study was continued with the 50 children with a negative blood smear at the second week following treatment. Among these, 40 were correctly followed until either the reappearance of *P. falciparum* in the blood or the end of the follow-up at W30. Follow-up was considered correct for children with no more than 2 successive missing blood smears during the period preceding the reappearance of *P. falciparum* in blood or before the end of the follow-up. *Plasmodium falciparum* parasites reappeared in the blood of 33 children during the follow-up, while 7 remained uninfected until W30. For these individuals, the delay of reinfection was arbitrarily assigned to a value of 32 weeks, and thus may have been underestimated. Overall, the mean delay of reinfection in peripheral blood was 16.5 weeks. The frequency of reinfection was the highest at the beginning of the follow-up, corresponding to June and July, then low until October, and increased again in November and December, following the variation of transmission as may be expected from seasonal variation in rain (Figure 1).

During the follow-up, 16 of the 40 children presented with *P. malariae* infections. Among these 16 children, *P. falciparum* reinfection occurred earlier than in the other children (*P* = 0.001; Table 3). The delay of reinfection with *P. falciparum* was similar in children infected or uninfected with *P. falciparum* before treatment (Table 3).

The delay of reinfection with *P. falciparum* was then compared with antibody levels to LSA-Rep, LSA-J, NANP, and NAAG. Children with a high anti-LSA-J antibody level tended to be reinfected later than others (*P* = 0.051). No antibody levels were related to *P. malariae* infection during the follow-up.

**DISCUSSION**

In the initial cross-section, *P. falciparum* infection was more frequent in children with normal G6PD activity, and in those with a high plasma level of anti-LSA-Rep antibodies. During the follow-up, reinfection with *P. falciparum* occurred sooner in individuals that also showed a blood infection with *P. malariae* than in those without this infection. The delay of reinfection tended to be increased in individuals with a high level of plasma anti-LSA-J antibodies. Most therapeutic failures were observed in the group who received 20 mg/kg of amodiaquine, while the efficacy of a regimen of 30 mg/kg was higher (*P* = 0.0002 among infected sub-

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**TABLE 1**
Characteristics of children infected (n = 41) or not infected (n = 20) with *Plasmodium falciparum* at enrollment*

<table>
<thead>
<tr>
<th></th>
<th>Negative</th>
<th>Positive</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>General factors†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, years (mean ± SD)</td>
<td>9.30 ± 6.03</td>
<td>10.46 ± 4.44</td>
<td>0.46</td>
</tr>
<tr>
<td>Age groups‡</td>
<td>Young/old</td>
<td>9/11</td>
<td>12/29</td>
</tr>
<tr>
<td>Sex</td>
<td>Female/male</td>
<td>13/7</td>
<td>14/27</td>
</tr>
<tr>
<td>Blood group</td>
<td>O/non-O</td>
<td>7/13</td>
<td>15/24</td>
</tr>
<tr>
<td>Hb phenotype</td>
<td>AA/AS</td>
<td>9/6</td>
<td>25/11</td>
</tr>
<tr>
<td>G6PD phenotype</td>
<td>Normal/deficient</td>
<td>9/7</td>
<td>35/4</td>
</tr>
<tr>
<td>Immunologic factors§</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ab against LSA-Rep (arbitrary units)</td>
<td>0.00 (0.00–23.81)</td>
<td>30.43 (1.83–71.96)</td>
<td>0.03</td>
</tr>
<tr>
<td>Ab against LSA-J (arbitrary units)</td>
<td>12.04 (0.00–23.41)</td>
<td>32.65 (4.83–54.90)</td>
<td>0.13</td>
</tr>
<tr>
<td>Ab against NANP (arbitrary units)</td>
<td>19.34 (0.00–56.49)</td>
<td>12.26 (0.00–46.26)</td>
<td>0.75</td>
</tr>
<tr>
<td>Ab against NAAG (arbitrary units)</td>
<td>43.96 (16.28–71.82)</td>
<td>58.81 (24.34–87.46)</td>
<td>0.39</td>
</tr>
<tr>
<td>Plasma IFN-γ (pg/ml)</td>
<td>4.40 (1.00–80.35)</td>
<td>2.70 (1.00–39.30)</td>
<td>0.79</td>
</tr>
<tr>
<td>Plasma IL-10 (pg/ml)</td>
<td>16.89 (10.71–36.27)</td>
<td>38.17 (19.56–61.40)</td>
<td>0.057</td>
</tr>
</tbody>
</table>

* Differences between groups were assessed by the non-parametric Mann-Whitney *U*-test for immunologic factors and age, and by the chi-square test for other factors. Hb = hemoglobin; G6PD = glucose-6-phosphate dehydrogenase; Ab = antibody; IFN-γ = interferon-γ; IL-10 = interleukin-10.
† *n* = 51–61.
‡ Age groups are defined as young: < 7 years old; old: ≥ 7 years old.
§ *n* = 40–43. Values are medians (25th percentile–75th percentile).

**TABLE 2**
Antimalarial efficacy of amodiaquine treatment in children from Dienga, Gabon*

<table>
<thead>
<tr>
<th>Dose</th>
<th>20 mg/kg</th>
<th>30 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years (mean ± SD)</td>
<td>3.93 ± 3.71</td>
<td>12.09 ± 3.50</td>
</tr>
<tr>
<td><em>Plasmodium falciparum</em>–positive slides before treatment</td>
<td>7/15</td>
<td>34/46</td>
</tr>
<tr>
<td>GMPD (parasites/µl of blood)</td>
<td>470 (318–622)</td>
<td>135 (124–146)</td>
</tr>
<tr>
<td>Lost to follow-up</td>
<td>1/15</td>
<td>2/46</td>
</tr>
<tr>
<td>Positive at first or second week</td>
<td>5/14</td>
<td>3/46</td>
</tr>
</tbody>
</table>

* GMPD = geometric mean parasite density (95% confidence interval) of the positive slides.
that reciprocally, given that seasonal variations of the 2 species by *P. malariae* in the Garki area of Nigeria. Conversely, Black and others idly. Such a relationship has already been observed in the serological study.

Parasitologic factors

Antibody

*P. falciparum* infection at day 0, absence/presence

- In the present study, an association between *P. malariae* carriage and *P. falciparum* infection rate (at initiation of the study) or reinfection delay (during the follow-up) was observed. At enrollment, children infected with *P. malariae* presented more often with a *P. falciparum* infection and usually exhibited higher parasite densities than the other children, although the differences were not significant (both *P* < 0.07). During the follow-up, subjects who were infected by *P. malariae* were reinfected by *P. falciparum* more rapidly. Such a relationship has already been observed in the Garki area of Nigeria. Conversely, Black and others showed that symptomatic children not only presented with fewer mixed species infections, but also had less previous *P. malariae* infections than symptom-free children, as demonstrated by a serological study. Consequently, the influence of *P. malariae* parasites on *P. falciparum* infection may depend on the study population, as well as on epidemiologic factors. Clearly, *P. malariae* carriage appears to characterize subjects sensitive to *P. falciparum* infection. A simple explanation of this would be that both parasite species are transmitted by the same vector, and that any differences in exposure will apply to both of them. However, in Garki, this preferential association was found related to differences in acquired immunity, and not to differences in current exposure, suggesting that the levels of immunity to *P. falciparum* and to *P. malariae* were inter-related. It was not assumed that *P. malariae* infection favors *P. falciparum* infection or reciprocally, given that seasonal variations of the 2 species suggested a partial suppression of *P. malariae* by *P. falciparum*. The immunologic response towards sporozoite and liver stages plays a major role in the protection against infection, as demonstrated by the fact that the infection rate is much smaller than expected based on the number of infective bites received. In our study, the plasma level of anti-NANP antibody was not associated with the presence of *P. falciparum* parasites or the delay of reinfection. Such results agreed with those previously reported in other endemic areas. Conversely, in Burkina Faso, the anti-NANP antibody level was directly related to the rate of sporozoite inoculation but not to protection against infection. As observed with anti-NANP antibody, anti-NAAG antibody was not related to *P. falciparum* infection rate or reinfection delay, or to infection with *P. malariae* at enrollment or during the follow-up. This is not surprising since the presence of *P. malariae* in blood may follow a sporozoite inoculation, but parasites may also appear in the blood a long time after the exposure to sporozoites. Antibody levels against NANP and NAAG were correlated, suggesting that the intensity of exposure to both species is related. The higher level of anti-NAAG antibody in older children suggests that this immune response is dependent on the time of exposure to the infected bites. The anti-LSA-Rep antibody level was positively correlated with the presence of *P. falciparum* infection before treatment. Moreover, subjects with anti-LSA-Rep antibody tended to be more rapidly re-infected. Thus, anti-LSA-Rep antibody may be related to the level of exposure to infective bites, but does not appear to protect against infection. Nevertheless, anti-LSA-Rep antibody was positively correlated with the parasite clearance time in those with mild malaria. In our study, the anti-LSA-J antibody level was not related to the parasitologic status before treatment, but seems to be associated with resistance to reinfection. This data agrees with a recent study showing that the immune response to

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**Table 3**

<table>
<thead>
<tr>
<th>Delay of reinfection (weeks)</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.67</td>
</tr>
<tr>
<td>Age groups (young/old)</td>
<td>0.56</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>0.76</td>
</tr>
<tr>
<td>Blood group (O/non-O)</td>
<td>0.72</td>
</tr>
<tr>
<td>Hb phenotype (AA/AS)</td>
<td>0.32</td>
</tr>
<tr>
<td>G6PD phenotype (normal/deficient)</td>
<td>0.99</td>
</tr>
</tbody>
</table>

* Differences between groups were assessed by regression analysis for age, and by the non-parametric Mann-Whitney U-test for the other factors. For definitions of abbreviations, see Table 1.

† Age groups are defined as young: ≤ 7 years old; old: > 7 years old.

‡ n = 34–40.

§ n = 40.

# n = 25–28.
the LSA-J region was related to resistance to reinfection.\textsuperscript{20} The in vitro production of INF-\(\gamma\) was implicated in this immune response, thus involving pathways other than the humoral response we explored. Nevertheless, IFN-\(\gamma\) is thought to be the most potent cytokine acting directly upon the \textit{P. falciparum} liver stage.\textsuperscript{21} In the present study, plasma levels of INF-\(\gamma\) were not related to the presence of parasites before treatment, contrasting with previous data collected near Dienga.\textsuperscript{22} In fact, the level of plasma INF-\(\gamma\) is reported to be correlated with the level of parasitemia, and to be elevated in cases with clinical malaria attacks.\textsuperscript{22,23} The high level of plasma IL-10 tended to be related to the presence of parasites before treatment, in agreement with the higher level of anti-LSA-Rep antibody in infected subjects, since IL-10 plays a major role in the regulation of the humoral immune response. Studies including those with mild malaria attacks demonstrated the role of the antigen-specific IL-10-mediated antibody response in the control of parasitemia, and in the prediction of resistance to reinfection.\textsuperscript{19,24} The role of cytokines during asymptomatic malaria infection is still not well understood because as most studies focused on those with acute malaria.\textsuperscript{25-27}

In our cohort, blood group distribution (37% of the O group) and the sickle cell trait prevalence rate (33%) differed from those previously reported (54% and 23%, respectively) among school children in the same village.\textsuperscript{1} This discrepancy is explained by the method of selection of the subjects (subjects who were members of 10 families who were not representative of the entire population). The O blood group and the sickle cell trait were not associated with parasite density or the delay of reinfection, although they have been commonly associated with resistance to severe malaria.\textsuperscript{1} The G6PD deficiency has also been shown to protect against severe malaria and high parasite density.\textsuperscript{26,29} In our study, subjects with a G6PD deficiency were less frequently infected by \textit{P. falciparum} before treatment, but no effect was observed against the delay of reinfection, confirming that a G6PD deficiency protects against blood stages of the parasite, but not against pre-erythrocytic stages. Furthermore, none of the G6PD-deficient subjects were infected by \textit{P. malariae} at the initiation of the study (\(P = 0.03\)), suggesting that this deficiency may also protect against \textit{P. malariae}. Before treatment, boys were infected more often than girls. This result is probably related to, at least partially, the link between gender and G6PD deficiency. In favor of this hypothesis, the relationship between gender and \textit{P. falciparum} infection is no longer significant after excluding G6PD-deficient subjects from the analysis. In fact, heterozygous females for the G6PD deficiency are more resistant against malaria than homozygous females and hemizygous males.\textsuperscript{28} The phenotypic investigation of the G6PD deficiency we conducted did not fully identify heterozygotes women and a complete investigation of the G6PD genotype would be informative.

Our main observation was that \textit{P. malariae} and \textit{P. falciparum} infections are highly related, suggesting that the human host resistance to these 2 species shares some common mechanism. As discussed earlier, the association between the 2 species may be related to the ability to respond to malarial antigens, independently of the species of \textit{Plasmodium}. Individuals from the same family share genetic characteristics, some of them being related to the susceptibility to \textit{P. falciparum} malaria, as demonstrated by segregation analysis.\textsuperscript{30,31}

In our study, children from 10 families and a number of siblings showed a high frequency of slide positivity for \textit{P. malariae}, while other siblings showed a low frequency of slide positivity. Future studies will be conducted to find familial links to susceptibility or resistance to malaria.

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