ASSOCIATION OF PLASMODIUM FALCIPARUM ISOLATES ENCODING THE P. FALCIPARUM CHLOROQUINE RESISTANCE TRANSPORTER GENE K76T POLYMORPHISM WITH ANEMIA AND SPLENOMEGALY, BUT NOT WITH MULTIPLE INFECTIONS

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Abstract. The aim of the study was to assess whether infections with Plasmodium falciparum isolates encoding the P. falciparum chloroquine resistance transporter (pfcrt) gene K76T polymorphism, a molecular marker for chloroquine resistance, are associated with multiple infections, age, or clinical signs of malaria in a semi-immune population in a holoendemic area of Burkina Faso. The parameters of interest were investigated in 210 P. falciparum-positive inhabitants. Logistic regression analysis showed that pfcrt K76T-carrying isolates are significantly more likely to cause anemia and splenomegaly. Furthermore, we found that infections with P. falciparum isolates encoding pfcrt K76T are dependent on age rather than multiple infections. Our findings suggest that pfcrt K76T might serve as a valuable marker for assessing the long-term clinical effect of chronic infections with chloroquine-resistant P. falciparum isolates in populations, without the need of drug efficacy trials.

INTRODUCTION

The spread of drug-resistant Plasmodium falciparum isolates has complicated the clinical management of malaria, which now requires regular surveillance of the efficacy of first-line antimalarial drugs in the field. Until recently, the efficacy of chloroquine (CQ) could only be monitored by observing clinical and parasitologic responses, which include time-consuming and labor-intensive procedures. These conventional methods bear a high risk of loss to follow-up, not to mention the possible delay in administering effective treatment. The recent finding that CQ resistance is genetically linked with polymorphisms within a gene termed the P. falciparum CQ resistance transporter (pfcrt) has facilitated rapid polymerase chain reaction (PCR)–based screening protocols to monitor CQ resistance in endemic areas. These PCR-based protocols take advantage of a restriction fragment length polymorphism (RFLP) generated by a conserved K76T mutation within pfcrt that discriminates between the wild-type allele and alleles associated with CQ resistance (CQR).

Other studies have shown that clinical resistance is more common in young children and that CQ treatment failure increases the risk of developing malaria specific signs. However, the implications of P. falciparum isolates carrying the pfcrt K76T allele on epidemiologic parameters, such as age, multiplicity of infection, and clinical aspects have not yet been considered. We hypothesized that infections with isolates encoding the pfcrt K76T polymorphism may be correlated with young age and clinical signs of malaria. To verify this hypothesis, we have investigated a possible association between the pfcrt K76T allele and clinical and epidemiologic aspects of malaria, including age, anemia, splenomegaly, and infection with multiple P. falciparum isolates, using a logistic regression model.

MATERIALS AND METHODS

Study area and study design. The study was conducted in Bourasso, a village in western Burkina Faso. The area has been under intense investigation for more than 10 years by researchers of the Center de Recherche en Santé à Nouna (CRSN) and the University of Heidelberg. Malaria is holoendemic in this area, and a clinical CQR rate of 12% in children has been reported. In October 2000, during the rainy season, a cross-sectional study on malaria was conducted over a three-day period and covered all inhabitants, excluding pregnant women, present in Bourasso village (n = 1,504) (Stich A and others, unpublished data). The following clinical parameters were assessed: fever (axillary body temperature ≥ 37.5°C using a digital thermometer), pallor of conjunctival or oral mucous membranes as a marker for anemia, and splenomegaly (applying the Hackett score). Peripheral blood samples were taken by finger prick, spotted onto Whatman (Brentford, United Kingdom) 3MM-chromatography paper, air-dried, and individually stored in plastic bags for further examination at the Parasitology Department of the Institute of Hygiene (Heidelberg, Germany). An additional drop of blood was obtained for a thick film, which was stained with standard Giemsa solution the same day and read in the laboratory of the CRSN. All parasitemic patients were treated with a full course of CQ, according to the national protocol.

Two hundred twenty-nine participants, selected at random, were investigated further as a subset of the overall study population. Ethical approval was obtained from the local ethical board in Nouna and from the ethical committee of the University of Heidelberg. Informed consent was obtained from all study participants.

Molecular epidemiologic analyses. DNA was extracted from filter papers by the Chelex-100 DNA extraction method. Plasmodium falciparum DNA was detected using a species-specific nested PCR as previously described. As controls, DNA from the P. falciparum laboratory isolates 3D7, Dd2, HB3, and FCR3 was investigated in parallel. A PCR product of 205 basepairs was considered indicative of P. falciparum. In some cases, this was confirmed by RFLP analysis and sequencing of the PCR products. Multiple P. falciparum infections were detected using a size polymorphism PCR of highly variable regions within the merozoite surface protein 1 (msp1) and msp2 genes of P. falciparum. To investigate the K76T point mutation within the pfcrt gene of P. falciparum, DNA was extracted from filter papers by the Chelex-100 DNA extraction method, which was then used for nested PCR and RFLP analysis.
falciparum, a reported marker for CQR,\textsuperscript{2,3} the PCR combined with the RFLP was carried out as previously described.\textsuperscript{3} Results were compared with control reactions using DNA from the CQR \textit{P. falciparum} laboratory clone Dd2 and the CQ-sensitive laboratory clone HB3. Some samples showed both the wild-type and a CQR \textit{pfcrt} allele.

**Statistical analysis.** All statistical calculations were performed using the STATA7 statistical program (Stata Corporation, College Station, TX). Stratified analysis was done for all relevant parameters using participants less than and greater than 10 years old as substrata. This cut-off was chosen because it is well known that malaria in holoendemic areas presents entirely different in small children as compared with older subjects.\textsuperscript{8,15}

### RESULTS

During the rainy season in 2000, we conducted an epidemiologic survey on malaria in a village in a holoendemic area in western Burkina Faso. A total of 1,504 villagers were examined for fever, signs of anemia, splenomegaly, and parasitized erythrocytes as determined by microscopic examination of Giemsa-stained blood smears. Blood samples from 229 participants selected at random were then investigated in more detail using PCR-based molecular epidemiologic techniques. Prevalences of representative base line variables in the subset and the total village population are shown in Table 1.

\textit{Plasmodium falciparum} DNA was detected by PCR in 210 of 229 blood samples, corresponding to a prevalence rate of \textit{P. falciparum} infection of 91.7%. Only \textit{P. falciparum}-positive (by PCR) participants were included in the statistical analysis. The prevalence rate of infection with \textit{P. falciparum} isolates encoding the \textit{pfcrt} K76T mutation was 43.3% (91 of 210; Table 2). A total of 20.5% (43 of 210) of the patients exclusively carried \textit{pfcrt} K76T encoding isolates, 56.7% (119 of 210) had only \textit{pfcrt} wild-type isolates with respect to amino acid 76, and 22.9% (48/210) had multiple infections with at least one isolate encoding the \textit{pfcrt} K76T mutation and one isolate encoding the \textit{pfcrt} wild-type allele (Table 2). Children < 10 years old appeared to be more likely to carry parasites with the \textit{pfcrt} K76T polymorphism ($\chi^2 = 4.54, P = 0.033$).

A total of 82.4% (173 of 210; Table 2) of the infected participants had infections with multiple \textit{P. falciparum} isolates, as determined by assessing size polymorphisms within the \textit{msp1} and \textit{msp2} genes. Multiple \textit{P. falciparum} infections were significantly more common in children < 10 years old ($\chi^2 = 12.30, P < 0.001$), consistent with previous reports.\textsuperscript{16,17} No relevant association of \textit{pfcrt} K76T status with multiplicity of infection was found for all 210 infected participants ($\chi^2 = 0.52, P = 0.472$). Age-stratified analysis could not be performed for children alone due to the low number of cases with single infection in this age stratum (n = 5). Age-stratified analysis for older subjects showed a non-significant association of \textit{pfcrt} K76T with single infection ($\chi^2 = 1.60, P = 0.206$).

A total of 4.8% (10 of 210) of the infected participants had fever (axillary body temperature $\geq 37.5^\circ$C), 17.1% (36 of 210) showed clinical signs of anemia, and 50.0% (105 of 210) had a palpable spleen (Table 2). To investigate possible correlations between clinical and molecular parameters, we performed a regression analysis with the clinical signs fever, anemia, and splenomegaly as dependent variables and age, multiple \textit{P. falciparum} infection, and infection with isolates carrying the \textit{pfcrt} K76T allele as independent variables. The following correlations were found. Anemia and splenomegaly were significantly associated with the presence of the \textit{pfcrt} K76T allele (anemia: odds ratio [OR] = 2.71, 95% confidence interval [CI] = 1.25–5.84, $P = 0.011$ and splenomegaly: OR = 2.46, 95% CI = 1.30–4.65, $P = 0.005$) and with age (anemia: OR = 0.95, 95% CI = 0.93–0.98, $P = 0.002$ and splenomegaly: OR = 0.95, 95% CI = 0.93–0.97, $P < 0.001$ (Table 3). Fever was not significantly associated with either age or the \textit{pfcrt} K76T allele (Table 3). The combination of anemia and splenomegaly was significantly associated with the \textit{pfcrt} K76T allele (OR = 2.71, 95% CI = 1.15–6.40, $P = 0.022$) and age (OR = 0.93, 95% CI = 0.89–0.97, $P = 0.001$) (Table 3). The combination of anemia, splenomegaly, and fever could not be analyzed due to the small number of cases (n = 3).

The same regression model was also calculated for nonfebrile patients. This did not result in any relevant changes of the associations found.

### DISCUSSION

Previous studies have established a causative link between clinical and parasitologic CQR and polymorphisms within the \textit{P. falciparum} \textit{pfcrt} gene.\textsuperscript{1,2} Within our study population, 43.3% of the participants infected with \textit{P. falciparum} carried isolates encoding the \textit{pfcrt} K76T mutation associated with CQR. This percentile exceeds the rate of clinical CQR of 12% recently reported from the same study area.\textsuperscript{7} The discrepancy between clinical CQR and the prevalence of isolates carrying the \textit{pfcrt} K76T allele has also been noted in several other studies.\textsuperscript{18,19} Current models to explain this discrepancy suggest that the \textit{pfcrt} K76T polymorphism is only one among other parasite factors that, in addition to host factors, ultimately define CQ treatment failure. Djimde and others described a model to estimate the rate of clinical CQR based on the prevalence of the \textit{pfcrt} K76T allele in a given population.\textsuperscript{5} According to this model, the prevalence of the \textit{pfcrt} K76T allele is 2–3-fold higher than the rate of therapeutic failure in endemic areas with low to moderate CQR. The data from our study area are consistent with this model.

The aim of our study was to assess whether the \textit{pfcrt} K76T marker, as a proxy measure for CQR, can be used to investigate the association between CQR malaria and increased risk of malaria manifestations. Interestingly, the \textit{pfcrt} K76T

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**Table 1**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Subset (n = 229)</th>
<th>Total study population (n = 1,504)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>49.3%</td>
<td>49.3%</td>
</tr>
<tr>
<td>Age &lt;10 years</td>
<td>36.2%</td>
<td>37.8%</td>
</tr>
<tr>
<td>\textit{Plasmodium falciparum} infections*</td>
<td>80.6%</td>
<td>76.3%</td>
</tr>
<tr>
<td>Fever</td>
<td>4.4%</td>
<td>5.5%</td>
</tr>
<tr>
<td>Anemia</td>
<td>16.6%</td>
<td>23.5%</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>48.0%</td>
<td>49.7%</td>
</tr>
</tbody>
</table>

* Based on microscopic examinations of Giemsa-stained blood smears.
Anemia and OR/H11505

We expected a higher risk of having drug-resistant malaria, 5,21 and since children have a lower immune status and the drug selection pressure.19,22,24 Adults who have acquired a premunition to malaria appear to be more capable of eliminating parasites in the presence of CQ than children do, irrespective of the susceptibility of the clone to CQ. Conversely, persistent levels of CQ increase the selection pressure on parasites and result in a higher rate of CQR P. falciparum infections.25

TABLE 2
Age distribution of parasitologic and clinical findings of malaria in the study population

<table>
<thead>
<tr>
<th>Age groups (years)</th>
<th>Number (%)</th>
<th>Plasmodium falciparum infections (%)</th>
<th>Patients positive for pfcrt K76T (%)</th>
<th>Simultaneous infection with pfcrt K76T encoding isolates and wild-type isolates (%)</th>
<th>Multiple infections (%)</th>
<th>Axillary temperature ≥ 37.5°C</th>
<th>Anemia</th>
<th>Splenomegaly</th>
<th>Anemia and splenomegaly</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 10</td>
<td>83 (36.2)</td>
<td>82/283 (98.8)</td>
<td>43/82 (52.4%)</td>
<td>23/82 (28.1)</td>
<td>77/82 (93.9)</td>
<td>5/82 (6.1)</td>
<td>26/82 (31.7)</td>
<td>66/82 (80.5)</td>
<td>22/82 (26.8)</td>
</tr>
<tr>
<td>≥ 10</td>
<td>146 (63.8)</td>
<td>128/229 (87.7)</td>
<td>48/128 (37.5)</td>
<td>25/128 (19.5)</td>
<td>96/128 (78.5)</td>
<td>5/128 (3.9)</td>
<td>10/128 (7.8)</td>
<td>39/128 (30.5)</td>
<td>7/128 (5.5)</td>
</tr>
<tr>
<td>Total</td>
<td>229 (100)</td>
<td>210/229 (91.7)</td>
<td>91/210 (43.3)</td>
<td>48/210 (22.9)</td>
<td>173/210 (82.4)</td>
<td>10/210 (4.8)</td>
<td>36/210 (17.1)</td>
<td>105/210 (50.0)</td>
<td>29/210 (13.8)</td>
</tr>
</tbody>
</table>

* pfcrt = P. falciparum chloroquine resistance transporter.
† Association with age at P = 0.033.
‡ Association with age at P < 0.001.

The observed correlation of the pfcrt K76T allele with young age has also been noted in a study from Nigeria.22 This correlation is substantially increased when associated with detectable CQ serum levels.22 Similarly, drug efficacy trials have shown that children have a higher rate of clinically CQR infection than older persons.2,4,23 Two variables are currently discussed to explain the age distribution of CQR P. falciparum isolates: immune status and the drug selection pressure.19,22,24 Adults who have acquired a premunition to malaria appear to be more capable of eliminating parasites in the presence of CQ than children do, irrespective of the susceptibility of the clone to CQ. Conversely, persistent levels of CQ increase the selection pressure on parasites and result in a higher rate of pfcrt K76T-carrying isolates. The age distribution of the pfcrt K76T allele in infected subjects from different areas could be a dependent variable of the distribution of immunity and CQ levels among the different age groups.

Up to now detection of CQR in vivo has only been possible in drug efficacy trials. This always implied some sort of additional intervention in the study population. Our data may suggest that pfcrt K76T as a proxy measure for CQR is a valuable marker in epidemiologic studies for assessing morbidity due to prolonged infection with CQR P. falciparum isolates, without the need to interfere with the natural course of asymptomatic infection. Further prospective studies are encouraged to validate this conclusion.

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