Various pfcr\textit{t} and pfmdr1 Genotypes of \textit{Plasmodium falciparum}
Cocirculate with \textit{P. malariae, P. ovale} spp., and \textit{P. vivax} in Northern Angola

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Artemisinin-based combination therapy for malaria has become widely available across Africa. Populations of \textit{Plasmodium falciparum} that were previously dominated by chloroquine (CQ)-resistant genotypes are now under different drug selection pressures. \textit{P. malariae, P. ovale curtisi}, and \textit{P. ovale wallikeri} are sympatric with \textit{P. falciparum} across the continent and are frequently present as coinfections. The prevalence of human \textit{Plasmodium} species was determined by PCR using DNA from blood spots collected during a cross-sectional survey in northern Angola. \textit{P. falciparum} was genotyped at resistance-associated loci in pfcr\textit{t} and pfmdr1 by real-time PCR or by direct sequencing of amplicons. Of the 3,316 samples collected, 541 (16.3\%) contained \textit{Plasmodium} species infections; 477 (88.2\%) of these were \textit{P. falciparum} alone, 6.5\% were \textit{P. falciparum} and \textit{P. malariae} together, and 1.1\% were \textit{P. vivax} alone. The majority of the remainder (3.7\%) harbored \textit{P. ovale curtisi} or \textit{P. ovale wallikeri} alone or in combination with other species. Of 430 \textit{P. falciparum} isolates genotyped for pfcr\textit{t}, 61.6\% carried the wild-type allele CVMNK at codons 72 to 76, either alone or in combination with the resistant allele CVIET. No other pfcr\textit{t} allele was found. Wild-type alleles dominated at codons 86, 184, 1034, 1042, and 1246 of the pfmdr1 locus among the sequenced isolates. In contrast to previous studies, \textit{P. falciparum} in the study area comprises an approximately equal mix of genotypes associated with CQ sensitivity and with CQ resistance, suggesting either lower drug pressure due to poor access to treatment in rural areas or a rapid impact of the policy change away from the use of standard monotherapies.

Malaria is a major public health problem in Angola, with 3.7 million cases per year reported by the National Malaria Control Program (NMCP), two-thirds of which occur in children under 5 (7, 49). Although incidence rates of malaria have been decreasing due to control measures, according to the NMCP, malaria remains the primary cause of morbidity and mortality among children under 5 and pregnant women, accounting for 35\% of the overall infant mortality, 60\% of hospital admissions in this age group, and 25\% of maternal deaths (7, 8, 16). The continuous and indiscriminate use of chloroquine (CQ) as monotherapy in the 20th century created strong selection pressure and led to an increased prevalence of CQ-resistant (CQR) parasites worldwide (11). The recent introduction of artemisinin-based combination therapy (ACT) has changed this drug pressure, and the recovery of CQ-sensitive (CQS) \textit{P. falciparum} populations has been reported in Malawi, China, and Kenya (24, 26, 33, 45). However, widespread ACT use may also increase the prevalence of ACT-tolerant phenotypes (46).

Mutations in the \textit{P. falciparum} genomic region encompassing amino acids 72 to 76 of the CQ resistance transporter gene (pfcr\textit{t}) are associated with resistance to CQ and amodiaquine (AQ) (1, 6, 21, 29, 44). The different pfcr\textit{t} mutant haplotypes have a consistent geographical distribution: the CQR haplotype CVIET is predominant in southeast Asia and Africa, whereas the AQ-resistant (AQR) haplotype SagtVMNT is predominant in Asia and South America, and StctVMNT, from South America, is very rare in Africa (16). The wild-type haplotype CVMNK is widespread (6, 16, 29, 38). A recent study reported the presence of the StctVMNT haplotype of pfcr\textit{t} in Angola, but this has not yet been verified by others (15, 37). Polymorphisms in the parasite protein Pgh-1, encoded by pfmdr1, are thought to modulate resistance to drugs such as quinine, mefloquine, halofantrine, artemisinin, lumefantrine, CQ, and AQ (1, 21, 32, 38). Mutations in this gene have some
association with geographic areas. Considering codons 86, 103, 1034, 1042, and 1246, the pfmdr1 CQR haplotype YYSND is common in Asia and Africa, whereas NFDyd has occurred more frequently in CQ-resistant parasites in the Americas (16, 24, 29, 38).

In this cross-sectional study conducted in periurban and rural northwest Angola, we evaluated drug resistance-associated haplotypes of pfcr and pfmdr1 among 525 P. falciparum-infected individuals, many of whom harbored additional coinfecting Plasmodium species, and discuss the implications of our findings for the treatment of malaria in the study area.

MATERIALS AND METHODS

Study area. This study was conducted within the CISA (Health Research Center in Angola) Project’s Health and Demographic Surveillance System (HDSS). The HDSS study area encompasses three communes (Caxito, Mabubas, and UCua) of the Dande municipality, Bengo Province, in northwestern Angola. This is a largely rural area 60 km north of the capital Luanda, spanning more than 4,700 km². The initial census, carried out in September 2009 and February 2010, registered 60,075 inhabitants in 15,643 households distributed in 69 hamlets (9). Bengo province is considered an area in which malaria is mesoendemic with stable transmission intensity (7). The main peak of malaria occurs in the rainy season, between November and May (8).

Sample collection. The blood samples used for this study were collected between May and August of 2010 as part of a malaria baseline prevalence survey in women and their preschool- and school-aged children in the HDSS study area (40). Households with at least one preschool child (0 to 5 years old) or one school-aged child (6 to 15 years old) and a mother/caregiver (ideally from 16 to 49 years of age) were randomly selected, as described by Sousa-Figueiredo et al. (40). During the survey, finger-prick blood samples were collected and spotted onto Whatman 3MMChr filter paper and slides. Blood spots on filter paper were left to air dry and were stored at 4°C until DNA extraction. A total of 3,316 samples were analyzed, 2,632 from children (1,217 females and 1,146 males) and 952 from their mothers or caregivers.

Microscopy. Thick and thin blood smears were prepared on the same slide, stained with 10% Giemsa for 15 min, and screened for malaria parasites by two independent microscope technicians (double blinded). Any discordance in whether or not parasites were present was resolved by a third reader.

DNA extraction. Genomic DNA was extracted from the dried filter papers using a QIAamp DNA minikit (Qiagen, United Kingdom) by following the manufacturer’s instructions. Positive-control blood spots prepared from known P. falciparum-positive samples and negative-control blood spots prepared from uninfected blood were also included and extracted in the same way as the field samples. DNA already extracted from samples of the other species was added to each PCR as further positive controls.

Nested PCR for RNA (small-subunit [SSU]-rRNA) amplification and species identification. For the determination of Plasmodium species, we performed an optimized nested PCR (39). The first PCR was performed with primers specific for the Plasmodium genus: RPLU6 and RPLU5New (the latter primer, CytTGTGTGCTTAAAACCTTC, was modified from the published primer, RPLU6, by the United Kingdom Malaria Reference Laboratory, London School of Hygiene and Tropical Medicine [LSHTM] [unpublished data]) (39). The second nested PCR was performed in 5 separate reactions, 4 containing Plasmodium species-specific primers as described by Snounou et al. and an additional reaction with primers for P. ovale wallikeri, rOVAv, described by Calderaro et al. (3, 39). DNA amplification was performed in a thermocycler (Gene Amp PCR System 9700; Applied Biosystems). PCR products were separated on a 2% agarose gel (Invitrogen) and stained with GelRed (Biotarget). Real-time and nested PCR for amplification of pfcr codons 72 to 76. Polymorphisms at codons 72 to 76 of the pfcr gene were successfully determined in 430 out of 525 P. falciparum PCR-positive samples by real-time quantitative PCR (qPCR) using dually labeled probes complementary to CVMNK (CQ sensitive; 3D7 type), CVIET (CQR; International Standard [IS] type), and SagtVMNT (AQR; 7G8 type) haplotypes (42). To increase sensitivity, a first-round PCR was carried out on all samples using primers P1 and P2 (21). The PCR product was then used in the qPCR procedure as described by Sutherland et al. and run on a Corbett Rotorgene 3000 thermocycler (Corbett Lifescience, Qiagen, Germany) (42).

Unpublished primer sequences and cycling conditions are listed in Table 1. For the analysis, a threshold was set manually by reference to the appropriate positive and negative controls. DNA from 3D7 (CVMNK), IS (CVIET from NIBSC, United Kingdom), and 7G8 (SagtVMNT) clones.
were used as positive controls. As the qPCR was nested in this format, only qualitative data were obtained.

To check for the possible presence of additional genotypes, such as StctVMNT, that may not be recognized by our three qPCR probes, all 525 samples positive for *P. falciparum*, including those with apparent failure to amplify in the qPCR, were also processed using the nested PCR protocols of Djimde et al. and run on 2% agarose gels (12). A selection of 42 samples was sequenced (see below).

Nested and seminested PCR for *pfmdr1* amplification. To identify *pfmdr1* haplotypes, we performed nested PCR on a subset of 184 samples for fragment 1 (codons 86 and 184) and 177 for fragments 3 (codons 1034 and 1042) and 4 (codon 1246) of the 525 *P. falciparum*-positive samples corresponding to the first two plates of extracted DNA (Fig. 1). Amplicons for sequencing were generated by nested and seminested PCR as described in reference 21 and in Table 1.

Sequencing protocol for *pfcrt* and *pfmdr1*. Polymorphisms in the *pfmdr1* gene were determined by direct sequencing of the amplicons resulting from the nested PCR described above using a BigDye Terminator v3.1 cycle sequencing kit in an ABI 3730 sequencer (Applied Biosystems). Data were analyzed using Geneious 5.4.4 Pro.

To solve discrepancies between nested and real-time PCR for *pfcrt*, in particular where a band was present in the nested PCR but no qPCR signal was obtained, 42 of the 51 discordant samples were sequenced on both strands to confirm single-nucleotide polymorphisms (SNPs) at codons 72 to 76 (Fig. 1).

Statistical analysis. Database entry methods utilized in the baseline prevalence survey have been previously described in detail by Sousa-Figueiredo et al. (40). *Plasmodium* mixed infections and resistance gene haplotype prevalences were determined through tabulations in STATA v11.0 software.

Ethical approval. Ethical approval for the study was obtained from the Angolan Ministry of Health Ethics Committee. Written informed consent was obtained before inclusion in the study, and antimalarial treatment was provided when appropriate.

**RESULTS**

*Plasmodium* species identification. Of the 3,316 blood smears analyzed after Giemsa staining, 525 samples were found to be infected with *P. falciparum* (15.8%) (40). No other *Plasmodium* species, either in single or mixed infections, were detected by the microscopists.

The same 3,316 samples were screened by SSU-rRNA-based nested PCR, with 541 samples (16.3%) detected as having a *Plasmodium* infection (Table 2). Of those infected, 493 individuals (91.1%) carried a single species only. The majority of the *Plasmodium* infections were due to *P. falciparum* single infections (88.2%), whereas *P. vivax* single infections were seen in 6 (1.1%) of the infected samples. *P. ovale curtisi*, *P. ovale wallikeri*, and *P. malariae* single infections were observed in less than 2% of the infected samples: 4 (0.7%), 3 (0.6%), and 3 (0.6%), respectively. The 48 mixed-species infections included 41 individuals (7.6%) infected with 2 species, 5 (0.9%) with triple infections, and 2 (0.4%) with quadruple infections (Table 2). The most commonly found double species infection was the combination of *P. falciparum* and *P. malariae*, which was observed in 35 (6.5%) individuals.

Characterization of *pfcrt* alleles. The 525 samples with *P. falciparum* (single or mixed) infections were further investigated by qPCR to determine each *pfcrt* haplotype. Alleles at *pfcrt* codons 72 to 76 were successfully identified in 430 out of 525 samples. A proportion of 63.5% of the samples harbored the CVIET (CQ-resistant) haplotype, whereas 61.6% harbored the CVMNK (wild-type) haplotype, including the ones present in mixed infections (25.1%) (Fig. 2). No other haplotype occurred among our isolates, including those which failed to generate a signal in the qPCR, as
sequencing at codons 72 to 76 in 42 samples failed to identify SVMNT or any other alleles.

Characterization of pfmdr1 alleles. Out of 184 samples sequenced for fragment 1 (codons 86 and 184), 138 produced readable sequences. Regarding codon 86, the wild-type N86 allele was the most prevalent (114/138; 82.6%), while 44/138 (31.9%) of the samples carried the tyrosine mutation (Y86) associated with CQ and AQ resistance (Fig. 2). At codon 184, the wild-type tyrosine

<table>
<thead>
<tr>
<th>Type of infection (n = 3,316)</th>
<th>Presence of:</th>
<th>P. falciparum</th>
<th>P. malariae</th>
<th>P. vivax</th>
<th>P. ovale curtisi</th>
<th>P. ovale wallikeri</th>
<th>No. positive (% of total)</th>
<th>% of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. falciparum</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>477 (14.4)</td>
<td>88.2</td>
</tr>
<tr>
<td>P. malariae</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>3 (0.1)</td>
<td>0.6</td>
</tr>
<tr>
<td>P. vivax</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>6 (0.2)</td>
<td>1.1</td>
</tr>
<tr>
<td>P. ovale curtisi</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<td>4 (0.1)</td>
<td>0.7</td>
</tr>
<tr>
<td>P. ovale wallikeri</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>3 (0.1)</td>
<td>0.6</td>
</tr>
<tr>
<td>P. falciparum + P. malariae</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>35 (1.1)</td>
<td>6.5</td>
</tr>
<tr>
<td>P. falciparum + P. ovale curtisi</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>2 (0.1)</td>
<td>0.4</td>
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<tr>
<td>P. falciparum + P. ovale wallikeri</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>4 (0.1)</td>
<td>0.7</td>
</tr>
<tr>
<td>P. falciparum + P. ovale curtisi + P. ovale wallikeri</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>1 (0.0)</td>
<td>0.2</td>
</tr>
<tr>
<td>P. falciparum + P. malariae + P. ovale curtisi</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>2 (0.1)</td>
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<td>+</td>
<td>−</td>
<td>2 (0.1)</td>
<td>0.4</td>
</tr>
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</table>

No. positive (% of total) 525 (15.8) 44 (1.3) 6 (0.2) 11 (0.3) 12 (0.4) 541 (16.3) 100
% of positive samples 97.0 8.1 1.1 2.0 2.2 100

FIG 2 Prevalence of haplotypes of pfcr72-76 and pfmdr1 codons 84, 184, 1034, 1042 and 1246. The prevalence of each haplotype was calculated with n/nt, where n is the number of positive samples and nt is the number of successfully sequenced samples. The pfmdr1 (codons 86, 184, 1034, 1042, and 1246) mixed haplotypes NY/FSND (7 samples), N/YYSND (8 samples), and N/YY/FSND (8 samples) were removed from the analyses.
The heterogeneous access to drugs across the country, other drugs may have been used until 2007 to 2008, when AL became broadly available (fightingmalaria.gov/technical/acts/angola_acts.html). The fact that, in this cross-sectional community study, the prevalence of the CQS haplotype CVMNK was high (61.6%), contrasted with studies conducted in 2004 and 2008 in health facilities in the Angolan provinces of Uíge and Luanda, where a very high prevalence of the pfcr7 76T haplotype was found (Fig. 2) (13, 30).

Alternatively, this result suggests that, as CQ was withdrawn, a loss of the survival advantage of CQR parasites occurred in the absence of drug pressure, leading to a resurgence in the relative abundance of wild-type CVMNK (20, 26). A gradual decrease in the prevalence of CQR haplotypes has also been observed in China and Kenya (33, 45). Although the antimalarial activity of CQ would likely still be poor in our study area (as the prevalence of pfcr codons 72 to 76 [CVIET] is close to 64%), reintroduction of CQ as a partner drug in ACTs or in intermittent presumptive treatment for adults (IPTa) may become possible in Angola should the CVMNK haplotype become predominant, as occurred in Malawi (22, 24, 26). Nevertheless, we should be aware that the decline in rates of CQR mutations depends on geographic, epidemiologic, and parasite genetic factors (26, 38). Additionally, efficacy trials would have to be undertaken to support in vivo the molecular findings described here.

The pfcr SVMNT haplotype is associated with AQ resistance; it is postulated to carry a lower fitness cost than the CVIET haplotype and to persist among the parasite population even after the removal of drug pressure, potentially jeopardizing the effectiveness of artesunate-AQ, which is widely used in west Africa (37, 38). Surprisingly, this haplotype, which is usually found in South America and parts of Asia, was detected in adult samples from Luanda in 2007 (15). Despite the proximity of Luanda to our study site, we have not found this haplotype among our samples, suggesting that AQ would still be effective in this area. Further studies are needed to determine which provinces harbor the SṯcVMNT haplotype and whether it compromises the efficacy of artesunate-AQ combination therapy.

Our pfmdr1 results (82.6% of the wild-type N86 allele) differ from those found in Luanda in 2007/2008, where Y86 was the most prevalent, and from Uíge in 2004, where similar proportions of Y86 and N86 were observed (Fig. 3) (13, 15, 30). There were no mutations at codons 1034, 1042, and 1246, i.e., 100% of the haplotype and whether it compromises the efficacy of artesunate-AQ combination therapy.

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leading to qualitative differences in drug pressure compared to rural districts.

Given these results, we postulate that a reversion process is also occurring regarding \textit{pfmdr1} (codons 86 and 184), consistent with \textit{in vitro} and \textit{in vivo} observations reporting a modest decrease in the frequency of N86Y mutations in the absence of CQ pressure (20) and the known selective advantage of the \textit{pfmdr1} haplotype NSFND under AL treatment (1, 14, 44). Given the limitations of clinical diagnosis and the limited laboratory confirmation of suspected cases (such that many febrile diseases are wrongly diagnosed as malaria), it is possible that AL is being overprescribed, exacerbating this selection upon \textit{pfmdr1}. The presence of the NSFND haplotype of \textit{pfmdr1} among our samples thus raises concern and suggests that efficient monitoring of ACT efficacy in Angola is needed. On the other hand, it has been postulated that limited reintroduction of CQ, along with the use of AQ in combination therapy, could help to prevent resistance to ACTs, as both CQ and AQ have well-described antagonistic interactions with artemisinins \textit{in vitro} and \textit{in vivo} (5, 21, 41, 46).

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47. Reference deleted.