AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

EPIDEMIOLOGY AND SURVEILLANCE

Changing Pattern of *Plasmodium falciparum pfmdr1* Gene Polymorphisms in Southern Rwanda

Welmoed van Loon,[a] Clara Bergmann,[a] Felix Habarugira,[b] Costanza Tacoli,[a]* Darius Savelsberg,[a] Rafael Oliveira,[a] Djibril Mbarushimana,[b] Jules Ndoli,[b] Augustin Sendegeya,[b] Claude Bayingana,[c] Frank P. Mockenhaupt[a]

[a]Charité–Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Institute of Tropical Medicine and International Health, Berlin, Germany

[b]University Teaching Hospital of Butare, Huye, Rwanda

[c]University of Rwanda, Kigali, Rwanda

ABSTRACT *Plasmodium falciparum multidrug resistance-1* gene (*pfmdr1*) polymorphisms associate with altered antimalarial susceptibility. Between 2010 and 2018/2019, we observed that the prevalence of the wild-type allele N86 and the wild-type combination NYD increased 10-fold (4% versus 40%) and more than 2-fold (18% versus 44%), respectively. Haplotypes other than NYD or NFD declined by up to >90%. Our molecular data suggest the *pfmdr1* pattern shifted toward one associated with artemether-lumefantrine resistance.

KEYWORDS multidrug resistance, *Plasmodium falciparum*, malaria, Rwanda

Treatment of *Plasmodium falciparum* malaria relies on artemisinin-based combination therapies (ACTs), comprising a fast-acting artemisinin derivative and a slowly eliminated partner drug. *P. falciparum kelch-13* (*pfkelch13*) single-nucleotide polymorphisms (SNPs) associate with decreased artemisinin susceptibility. When conferring reduced *in vitro* sensitivity and delayed parasite clearance *in vivo*, they are termed validated mutations, common in Southeast Asia (1). Recently, these were detected in East Africa (Rwanda) and associated with delayed parasite clearance (2–4). Although ACT failure remains rare in Sub-Saharan Africa (1), the emergence of non-artemisinin partner drug resistance is feared. Susceptibility to these antimalarials, including lumefantrine (LF) and amodiaquine (AQ), is influenced by the *Plasmodium falciparum multidrug resistance-1* gene (*pfmdr1*) SNPs N86Y, Y184F, and D1246Y (5–9). Individual allele combinations, or haplotypes (e.g., N86-Y184-D1246, NYD, wild-type haplotype), exhibit specific susceptibility phenotypes (9). Notably, *pfmdr1* 86Y associates with increased sensitivity to LF, mefloquine, and dihydro-artemisinin and decreased chloroquine and AQ sensitivity (5). Resistant strains spread under drug pressure but may decline without (10, 11). Rwanda has used AL as a first-line antimalarial since 2006 (12). In 2010, we reported a predominant *pfmdr1* pattern (NFD) suggestive of intense AL pressure in mostly asymptomatic preschool children in Huye, Rwanda (13). Almost a decade later, we reassessed *pfmdr1* alleles in symptomatic and largely adult patients in Huye and compared them to the 2010 findings.

In March–June 2018 and September–December 2019, we recruited 295 uncomplicated malaria patients at Sovu Health Centre and Kabutare District Hospital, Huye district, Rwanda. All reported fever in the preceding 48 h or were febrile (164/276; ≥37.5°C, axillary). The study was approved by the Rwanda National Ethics Committee, and participants or caregivers provided informed written consent. Patients were clinically examined, malaria was microscopically confirmed, and venous blood was collected into EDTA. Following DNA extraction (QIAamp DNA blood minikit; Qiagen, Germany), *Plasmodium* species was confirmed by PCR (14) in 2018 and by real-time PCR (TIB MolBiol, Germany) in 2019. Two *pfmdr1* regions (codons 61 to 236 and 1023 to 1288) were PCR amplified (15), sequenced (Eurofins Genomics,

Citation van Loon W, Bergmann C, Habarugira F, Tacoli C, Savelsberg D, Oliveira R, Mbarushimana D, Ndoli J, Sendegeya A, Bayingana C, Mockenhaupt FP. 2021. Changing pattern of *Plasmodium falciparum pfmdr1* gene polymorphisms in southern Rwanda. Antimicrob Agents Chemother 65:e00901-21. https://doi.org/10.1128/AAC.00901-21.

Address correspondence to Welmoed van Loon, welmoed.van-loon@charite.de.

* Present address: Costanza Tacoli, Malaria Molecular Epidemiology Unit, Institut Pasteur du Cambodge, Phnom Penh, Cambodia.

Received 30 April 2021
Returned for modification 19 May 2021
Accepted 28 June 2021
Accepted manuscript posted online 6 July 2021
Published 17 August 2021

TABLE 1 Observed prevalence of *pfmdr1* polymorphisms and allele combinations in Huye, Rwanda, in 2010 and in 2018/2019

pfmdr1 allele or allele combination[a]	2010 (13), *n* = 104 [% (*n*)]	2018 and 2019,[b] *n* = 212 [% (*n*)]
86Y	39.4 (41)	3.8 (8)*
184F	51.9 (54)	53.8 (114)
1246Y	12.5 (13)	2.4 (5)*
N86-Y184-D1246	18.3 (19)	44.3 (94)*
N86-**184F**-D1246	38.5 (40)	50.0 (106)
86Y-Y184-D1246	23.1 (24)	1.9 (4)*
86Y-**184F**-D1246	7.7 (8)	1.4 (3)*
86Y-Y184-**1246Y**	5.8 (6)	0.5 (1)*
N86-**184F**-**1246Y**	2.3 (3)	1.9 (4)*
86Y-**184F**-**1246Y**	2.3 (3)	0
N86-Y184-**1246Y**	1.0 (1)	0

[a]Mutations are presented in boldface.
[b]*, significantly different from the respective proportion in 2010.

Germany), and aligned to reference PF3D7_0523000 (PlasmoDB; https://plasmodb.org/plasmo/app/record/gene/PF3D7_0523000) using CodonCode Aligner 4.2.5. *Plasmodium* infections can contain genetically distinct parasites and have a multiplicity of infection (MOI) of >1. To include all isolates, also those with evidence of MOI of >1 (i.e., both wild-type and mutant alleles present), we grouped alleles into combinations. In a secondary analysis, we estimated haplotype frequencies using a Bayesian model (16), which integrates unknown MOIs. The same priors were used as described previously (17). SNP and allele combination prevalence as well as haplotype frequencies in 2010 and 2018/2019 were compared using Fisher's exact test. A *P* value of <0.05 was considered significant. We used R 3.6.3 for statistical analyses.

By PCR, 90.3% (234/259) of the malaria patients in 2018/2019 had *P. falciparum* infection. Of these, 50.6% (118/233) were female, median age was 17.5 years (range, 1 to 73), and mean temperature was 37.2°C (standard deviation [SD], ±1.3°C). Good-quality sequencing reads for both *pfmdr1* regions were obtained from 90.6% (212/234) of isolates. Evidence of an MOI of >1 was present in 17.9% (38/212) of samples. The observed mutation prevalence was the following: 86Y, 3.8%; 184F, 53.8%; and 1246Y, 2.4% (Table 1). As for observed allele combinations, NFD (50%) dominated over wild-type NYD (44%). Considering haplotype frequency estimates, the reverse was seen (i.e., 39% versus 56%) (Table 2). In any case, >90% of isolates showed NFD or NYD in 2018/2019. Other nonsynonymous polymorphisms were T199S (*n* = 4), V207I (*n* = 2), T222I (*n* = 8), and Q1198K (*n* = 1), but not S1034C or N1042D.

Compared to 2010 data from the same region, the 2018/2019 *pfmdr1* allele pattern has changed: the prevalence of the 86Y mutation declined 10-fold and that of 1246Y 5-fold, whereas 184F remained basically unchanged (Table 1). Consequently, both the observed prevalence and the estimated frequency of wild-type haplotype NYD more than doubled between 2010 and 2018/2019. Allele combinations or haplotypes other

TABLE 2 Estimated haplotype frequency in Huye, Rwanda, in 2010 and in 2018/2019[a]

pfmdr1 haplotype	2010 (13) [% (95% credibility interval)]	2018 and 2019 [% (95% credibility interval)]
N86-Y184-D1246	24.0 (17.0, 32.3)	56.3 (49.6, 62.9)
N86-**184F**-D1246	38.0 (29.6, 47.0)	39.2 (32.8, 45.9)
86Y-Y184-D1246	24.3 (17.4, 32.3)	1.2 (0.4, 2.6)
86Y-**184F**-D1246	3.4 (1.2, 7.2)	0.9 (0.3, 2.2)
86Y-Y184-**1246Y**	4.6 (1.9, 8.6)	0.4 (0.0, 1.3)
N86-**184F**-**1246Y**	2.0 (0.5, 5.1)	1.1 (0.3, 2.4)
86Y-**184F**-**1246Y**	0.7 (0.0, 2.9)	
N86-Y184-**1246Y**	1.5 (0.2, 4.5)	0.5 (0.1, 1.6)

[a]Haplotype frequencies are estimated by a Bayesian model accounting for multiplicity of infection (17). Mutations are presented in boldface.

than NYD or NFD present in 2010 declined by up to >90% or disappeared, resulting in reduced genetic diversity (Tables 1 and 2).

The trends in our study accord with observations across Africa, i.e., a shift toward *pfmdr1* N86 and D1246, where AL is the major antimalarial (11). The N86 wild-type allele confers decreased LF susceptibility and increased AL failure (6, 8). So far, AL treatment failure is rare in Rwanda (1), possibly due to partial immunity and clinical artemisinin effectiveness. However, susceptibility to dihydro-artemisinin is linked to *pfmdr1* 86Y (5, 8), which almost vanished from the local parasite population. Moreover, a validated marker of artemisinin resistance, *pfkelch13* R561H, occurs in 4.5% of *P. falciparum* isolates in the same population (2). This molecular constellation, the emergence of an artemisinin resistance allele together with >95% of *pfmdr1* N86, indicates a shift toward AL-resistant genotypes in this region.

Since almost 20% of samples had evidence of an MOI of >1, we modeled haplotype frequencies, which differed from observed allele combination prevalence. This illustrates that considering one mutated allele in samples with an MOI of >1 as mutated genotypes should not be mistaken as haplotype frequency. Using MOI in analyzing temporal and/or regional allele patterns is recommended to increase comparability (16).

As limitations, we assessed *pfmdr1* alleles at two time points only, in a confined region, and lack susceptibility data. Moreover, we did not type *pfmdr1* copy number or the *P. falciparum chloroquine resistance transporter* gene, which also interfere with artemether and LF sensitivity (6, 7). We compared randomly selected, mostly asymptomatic children (13) to symptomatic, largely adult patients. Manifestation associates with *pfmdr1* SNPs (18), but not to the extent observed in our study. A strength is the comparison of molecular markers in the same district almost a decade apart.

Fifteen years after the implementation of AL as a first-line antimalarial, our study suggests the pattern in *pfmdr1* SNPs shifts toward AL resistance-associated genotypes in the Huye region. The recently demonstrated independent emergence of artemisinin-resistant *P. falciparum* strains at two sites in Rwanda underlines the importance of focal surveillance (3, 4). These developments could be the first sign of an imminent health threat to the African continent, and, in the absence of novel antimalarials, triple ACTs might be considered (19).

Data availability. Data will be made available in the WWARN repository, as a .csv file, containing Pfmdr1 genotypes and basic patient characteristics. The doi will be available when the repository is confirmed. Code for the implementation of the haplotype frequency estimation model is available at https://github.com/welmoedvl/ARTHUR_pfmdr1_haplotypefreqest.

ACKNOWLEDGMENTS

We are grateful to the staff of Sovu Health Centre and Kabutare District Hospital for their collaboration and help.

This study was financially supported by grant GRK2046 from the German Research Foundation (DFG) to W.V.L. and C.T. C. Bergmann was supported by DFG grant GRK2290. The funding bodies had no role in designing the study, collecting, analyzing, or interpreting data, or writing the manuscript.

We have no conflicts of interest to declare.

C. Bayingana and F.P.M. designed the study. D.M., J.N., and A.S. supervised logistics. W.V.L., C. Bergmann, F.H., D.S., J.N., and A.S. were responsible for patient recruitment. W.V.L., C. Bergmann, C.T., and D.M. did the laboratory work. W.V.L. and R.O. analyzed the data. W.V.L. and F.P.M. wrote the manuscript. All authors contributed to and approved the manuscript.

REFERENCES

1. World Health Organization. 2019. Status report on artemisinin resistance and ACT efficacy 2019. World Health Organization, Geneva, Switzerland.
2. Bergmann C, van Loon W, Habarugira F, Tacoli C, Jäger JC, Savelsberg D, Nshimiyimana F, Rwamugema E, Mbarushimana D, Ndoli J, Sendegeya A, Bayingana C, Mockenhaupt FP. 2021. Increase in Kelch 13 polymorphisms in Plasmodium falciparum, Southern Rwanda. Emerg Infect Dis 27:294–296. https://doi.org/10.3201/eid2701.203527.
3. Uwimana A, Legrand E, Stokes BH, Ndikumana JLM, Warsame M, Umulisa N, Ngamije D, Munyaneza T, Mazarati JB, Munguti K, Campagne P, Criscuolo A, Ariey F, Murindahabi M, Ringwald P, Fidock DA, Mbituyumuremyi A, Menard

D. 2020. Emergence and clonal expansion of in vitro artemisinin-resistant Plasmodium falciparum kelch13 R561H mutant parasites in Rwanda. Nat Med 26:1602–1608. https://doi.org/10.1038/s41591-020-1005-2.
4. Uwimana A, Umulisa N, Venkatesan M, Svigel SS, Zhou Z, Munyaneza T, Habimana RM, Rucogoza A, Moriarty LF, Sandford R, Piercefield E, Goldman I, Ezema B, Talundzic E, Pacheco MA, Escalante AA, Ngamije D, Mangala JN, Kabera M, Munguti K, Murindahabi M, Brieger W, Musanabaganwa C, Mutesa L, Udhayakumar V, Mbituyumuremyi A, Halsey ES, Lucchi NW. 2021. Association of Plasmodium falciparum kelch13 R561H genotypes with delayed parasite clearance in Rwanda: an open-label, single-arm, multicentre, therapeutic efficacy study. Lancet Infect Dis https://doi.org/10.1016/S1473-3099(21)00142-0.
5. Veiga MI, Dhingra SK, Henrich PP, Straimer J, Gnädig N, Uhlemann AC, Martin RE, Lehane AM, Fidock DA. 2016. Globally prevalent PfMDR1 mutations modulate Plasmodium falciparum susceptibility to artemisinin-based combination therapies. Nat Commun 7:11553. https://doi.org/10.1038/ncomms11553.
6. Mwai L, Kiara SM, Abdirahman A, Pole L, Rippert A, Diriye A, Bull P, Marsh K, Borrmann S, Nzila A. 2009. In vitro activities of piperaquine, lumefantrine, and dihydroartemisinin in Kenyan plasmodium falciparum isolates and polymorphisms in pfcrt and pfmdr1. Antimicrob Agents Chemother 53:5069–5073. https://doi.org/10.1128/AAC.00638-09.
7. Tumwebaze P, Conrad MD, Walakira A, LeClair N, Byaruhanga O, Nakazibwe C, Kozak B, Bloome J, Okiring J, Kakuru A, Bigira V, Kapisi J, Legac J, Gut J, Cooper RA, Kamya MR, Havlir DV, Dorsey G, Greenhouse B, Nsobya SL, Rosenthal PJ. 2015. Impact of antimalarial treatment and chemoprevention on the drug sensitivity of malaria parasites isolated from Ugandan children. Antimicrob Agents Chemother 59:3018–3030. https://doi.org/10.1128/AAC.05141-14.
8. Venkatesan M, Gadalla NB, Stepniewska K, Dahal P, Nsanzabana C, Moriera C, Price RN, Mårtensson A, Rosenthal PJ, Dorsey G, Sutherland CJ, Guérin P, Davis TME, Ménard D, Adam I, Ademowo G, Arze C, Baliraine FN, Berens-Riha N, Björkman A, Borrmann S, Checchi F, Desai M, Dhorda M, Djimdé AA, El-Sayed BB, Eshetu T, Eyase F, Falade C, Faucher JF, Fröberg G, Grivoyannis A, Hamour S, Houzé S, Johnson J, Kamugisha E, Kariuki S, Kiechel JR, Kironde F, Kofoed PE, LeBras J, Malmberg M, Mwai L, Ngasala B, Nosten F, Nsobya SL, Nzila A, Oguike M, Otienoburu SD, Ogutu B, Ouédraogo JB, ASAQ Molecular Marker Study Group, et al. 2014. Polymorphisms in Plasmodium falciparum chloroquine resistance transporter and multidrug resistance 1 genes: parasite risk factors that affect treatment outcomes for P falciparum malaria after artemether-lumefantrine and artesunate-amodiaquine. Am J Trop Med Hyg 91:833–843. https://doi.org/10.4269/ajtmh.14-0031.
9. Malmberg M, Ferreira PE, Tarning J, Ursing J, Ngasala B, Björkman A, Mårtensson A, Gil JP. 2013. Plasmodium falciparum drug resistance phenotype as assessed by patient antimalarial drug levels and its association with pfmdr1 polymorphisms. J Infect Dis 207:842–847. https://doi.org/10.1093/infdis/jis747.
10. Laufer MK, Thesing PC, Eddington ND, Masonga R, Dzinjalamala FK, Takala SL, Taylor TE, Plowe CV. 2006. Return of chloroquine antimalarial efficacy in Malawi. N Engl J Med 355:1959–1966. https://doi.org/10.1056/NEJMoa062032.
11. Okell LC, Reiter LM, Ebbe LS, Baraka V, Bisanzio D, Watson OJ, Bennett A, Verity R, Gething P, Roper C, Alifrangis M. 2018. Emerging implications of policies on malaria treatment: genetic changes in the Pfmdr-1 gene affecting susceptibility to artemether–lumefantrine and artesunate–amodiaquine in Africa. BMJ Glob Health 3:e000999. https://doi.org/10.1136/bmjgh-2018-000999.
12. Uwimana A, Penkunas MJ, Nisingizwe MP, Uyizeye D, Hakizimana D, Musanabaganwa C, Musabyimana JP, Ngwije A, Turate I, Mbituyumuremyi A, Murindahabi M, Condo J. 2018. Expanding home-based management of malaria to all age groups in Rwanda: analysis of acceptability and facility-level time-series data. Trans R Soc Trop Med Hyg 112:513–521. https://doi.org/10.1093/trstmh/try093.
13. Zeile I, Gahutu JB, Shyirambere C, Steininger C, Musemakweri A, Sebahungu F, Karema C, Harms G, Eggelte TA, Mockenhaupt FP. 2012. Molecular markers of Plasmodium falciparum drug resistance in southern highland Rwanda. Acta Trop 121:50–54. https://doi.org/10.1016/j.actatropica.2011.09.009.
14. Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, do Rosario VE, Thaithong S, Brown KN. 1993. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol 61:315–320. https://doi.org/10.1016/0166-6851(93)90077-B.
15. Li J, Chen J, Xie D, Monte-Nguba SM, Eyi JUM, Matesa RA, Obono MMO, Ehapo CS, Yang L, Lu D, Yang H, Yang HT, Lin M. 2014. High prevalence of pfmdr1 n86y and y184f mutations in Plasmodium falciparum isolates from Bioko Island, Equatorial Guinea. Pathog Glob Health 108:339–440. https://doi.org/10.1179/2047773214Y.0000000158.
16. Taylor AR, Flegg JA, Nsobya SL, Yeka A, Kamya MR, Rosenthal PJ, Dorsey G, Sibley CH, Guerin PJ, Holmes CC. 2014. Estimation of malaria haplotype and genotype frequencies: a statistical approach to overcome the challenge associated with multiclonal infections. Malar J 13:102. https://doi.org/10.1186/1475-2875-13-102.
17. Taylor AR, Flegg JA, Holmes CC, Guérin PJ, Sibley CH, Conrad MD, Dorsey G, Rosenthal PJ. 2017. Artemether-lumefantrine and dihydroartemisinin-piperaquine exert inverse selective pressure on plasmodium falciparum drug sensitivity-associated haplotypes in Uganda. Open Forum Infect Dis 4:ofw229. https://doi.org/10.1093/ofid/ofw229.
18. Tukwasibwe S, Mugenyi L, Mbogo GW, Nankoberanyi S, Maiteki-Sebuguzi C, Joloba ML, Nsobya SL, Staedke SG, Rosenthal PJ. 2014. Differential prevalence of transporter polymorphisms in symptomatic and asymptomatic falciparum malaria infections in Uganda. J Infect Dis 210:154–157. https://doi.org/10.1093/infdis/jiu044.
19. Hanboonkunupakarn B, White NJ. 2016. The threat of antimalarial drug resistance. Trop Dis Travel Med Vaccines 2:10. https://doi.org/10.1186/s40794-016-0027-8.