

Artemisinin Resistance–Associated *K13* Polymorphisms of *Plasmodium falciparum* in Southern Rwanda, 2010–2015

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Abstract. Emerging artemisinin resistance is a threat to global malaria control. Mutations in the *Plasmodium falciparum* Kelch 13 (*K13*) propeller domain confer artemisinin resistance and constitute molecular markers for its detection and monitoring. We sequenced 222 *P. falciparum* isolates obtained from community children in the Huye District of southern Rwanda in 2010, 2014, and 2015 to investigate the presence of *K13* polymorphisms. No polymorphisms were observed in 2010 but they were present in 2.5% and 4.5% in 2014 and 2015, respectively. In 2015, two isolates showed candidate *K13* resistance mutations (P574L and A675V), which are common in southeast Asia and associated with delayed parasite clearance. *K13* polymorphisms in southern Rwanda are infrequent but include variants associated with artemisinin resistance. Establishing correlations with local treatment response and in vitro resistance assays are needed in addition to further monitoring *K13* polymorphisms in the study area.

Artemisinin-based combination therapy (ACT) is the mainstay of malaria treatment and control. However, emerging resistance of *Plasmodium falciparum* to artemisinin derivatives (ART) in southeast Asia may threaten the achievements of the last decade in reducing malaria morbidity and mortality. So far, ART resistance refers to delayed parasite clearance and in vitro findings, whereas actual clinical treatment failure still is rare.^{1–3} Delayed parasite clearance is increasingly observed in the Greater Mekong sub-region (GMS) in southeast Asia e.g., in Cambodia,^{3,4} but only occasionally in sub-Saharan Africa (SSA), for example, in three patients from Uganda.⁵ ART resistance poses a serious threat to public health in SSA, potentially leading to a recurrence of the excess mortality due to drug resistance seen before the implementation of the ACTs.⁶ Surveillance of ART resistance by clinical trials in SSA is therefore desirable, but its actual performance and coverage are limited by costs and logistics, above all.

Recently, Kelch 13 (*K13*) propeller variants have been identified as molecular markers of ART resistance facilitating large-scale screening and monitoring of resistance emergence and spread.⁷ The *P. falciparum* *K13* gene encodes a Kelch protein of 727 amino acids considered to be involved in the parasite's cytoprotective and antioxidant responses.^{8,9} More than 180 non-synonymous *K13* mutations have been identified so far. World Health Organization recently updated the role of *K13* polymorphisms in ART resistance³: validated *K13* mutations are associated with both, delayed parasite clearance as well as resistance as indicated by the in vitro ring-stage survival assay,¹⁰ whereas candidate mutations meet only one of these requirements. Validated polymorphisms include C580Y, the most common mutant in resistant parasites,¹¹ in addition to Y493H, R539T, I543T, and R561H. Candidate mutations involve P574L and A675V, among others. The list of mutations associated with ART resistance is still evolving, however.^{3,12}

In SSA, available evidence points to a multitude of rare non-synonymous *K13* polymorphisms and almost absence of the validated mutations seen in southeast Asia.^{3,12–14} In Rwanda, east Africa, malaria morbidity and mortality declined greatly between 2005 and 2011 following the enforcement of control activities including the adoption of artemether-lumefantrine (AL) as first-line treatment in 2005. In particular, community-level case management programs have contributed greatly to the large-scale deployment of ACTs.¹⁵ Correspondingly, the pattern of *pfmdr1* alleles in clinical isolates from Huye District, southern Rwanda, which we examined in 2010, was suggestive of intense AL pressure on the parasite population.¹⁶ In this study, we aimed at assessing the presence of *K13* polymorphisms in *P. falciparum* isolates collected between 2010 and 2015 in Huye District, southern Rwanda.

Plasmodium falciparum isolates were collected at three occasions between 2010 and 2015 from infected children residing in the Huye District of southern Rwanda. Huye District (population 330,000) is located on Rwanda's central plateau (average altitude, 1600–1800 m; mean temperature, 19°C; yearly rainfall, approximately 1,200 mm). Malaria transmission in the area is perennial but low; *P. falciparum* is the predominating species.¹⁷ In 2010, children under the age of 5 years were examined to determine the prevalence of common childhood diseases.¹⁷ In 2014 and 2015, blood samples were collected from school children alongside monitoring the effectiveness of routine deworming (manuscript in preparation). Informed consent for the participation in these studies was obtained from the children's parents or legal guardians, and the study protocols were approved by the Rwanda National Ethics Committee. Eighty-five microscopically positive samples were available for analysis from 2010, and we randomly selected the same number of *P. falciparum*-positive samples (by either microscopy or polymerase chain reaction [PCR]) from 2014 and 2015. Genomic DNA was extracted from full blood aliquots (2010, 2014) or filter paper blood spots (Whatman 3MM, Whatman, Buckinghamshire, UK; 2015) by QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). All 255 samples were confirmed to be *P. falciparum* positive by semi-nested PCR assays.¹⁸ The *K13* propeller domain was amplified by previously published PCR assays.⁷ PCR products were bidirectional sequenced (Source BioScience, Berlin,

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TABLE 1
Prevalence of *K13* mutations in *Plasmodium falciparum* isolates from southern Rwanda, 2010–2015

Year	Number of sequenced samples	Non-synonymous mutations, <i>n</i> (%)	Amino acid and nucleotide changes
2010	75	0	–
2014	81	2 (2.5)	V555A (GTA→GCA) A626S (GCA→TCA)
2015	66	3 (4.5)	P574L (CCT→CTT) D648H (GAT→CAT) A675V (GCT→GTT)

Germany), and multiple sequence alignment was performed to detect *K13* polymorphisms using BioEdit v.7.2.5 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and SnapGene v.3.1 (GSL Biotech, Chicago, IL) software. The *K13* sequence of *P. falciparum* 3D7 (PF3D7_1343700) retrieved from PlasmoDB was used as reference for the alignment. Of 255 samples, 222 (87%) *K13* amplicons were successfully sequenced.

The median age of the 222 children from whom the isolates were obtained was 8 years (range, 0.3–11), and 51.8% (115/222) were girls. The geometric mean parasite density was 4,217/μL (95% confidence interval, 3,175–5,601, 199 samples); 10.4% (23/222) of samples were submicroscopic, that is, positive by PCR only. Fever (axillary temperature ≥ 37.5°C) was present in 20.8% (42/221) of the children. Intake of antimalarials, generally AL, was stated for 6.7% (5/75; preceding 2 weeks) of children in 2010, and for 42.7% (32/75, preceding month) in 2014 (no data for 2015).

Five of the 222 *P. falciparum* isolates (2.3%) revealed single nucleotide polymorphisms in the *K13* propeller domain (Table 1), all were non-synonymous. Notably, there was a nonsignificant trend toward increasing prevalence of polymorphisms, that is, none in 2010, 2.5% in 2014, and 4.5% in 2015 (χ^2 trend = 3.3; $P = 0.07$). Among the three *K13* polymorphisms detected in isolates from 2015, two, that is, P574L and A675V, are candidate mutations associated with artemisinin resistance.³ The remaining three polymorphisms identified were novel (D648H, V555A, A626S). The presence of *K13* polymorphisms was not associated with age, sex, parasite density, fever, or pretreatment (data not shown).

We show that *K13* polymorphisms are present in southern highland Rwanda at a low frequency but include two candidate mutations previously observed in southeast Asia and associated with ART resistance. The relationship of *K13* polymorphisms with ART resistance is complex and interpretation is hampered by a multitude of naturally occurring *K13* variants and a lack of linked phenotypical data. So far, more than 20 *K13* polymorphisms associated with delayed parasite clearance have been reported with identical mutants arising independently at different locations.^{3,7,12,19}

In two previous large-scale studies on African isolates, validated *K13* mutations had not been observed. In one survey across 14 African sites including 1,184 *P. falciparum* isolates collected between 2002 and 2011, 23 different mutations (15 coding ones) were observed, of which 18 were restricted to single geographical sites. Two candidate mutations were detected, namely G449D in Mali and P553L in Kenya.¹³ Another study on 1,212 more recently collected African isolates (2013–2014) found 22 *K13* polymorphisms (seven non-synonymous) at allele frequencies of 1–3%.¹⁴ A recent assessment of *K13* variants by the MalariaGEN consortium

revealed 64 *K13* polymorphisms in 1,648 African samples. Of 26 non-synonymous polymorphisms, 14 were also present in isolates from southeast Asia, and seven of those are considered to confer ART resistance. The majority of African *K13* polymorphisms appeared to be of local origin, and, as compared with southeast Asian isolates, there were substantially more rare polymorphisms. The authors considered the heterogeneity of mostly rare non-synonymous polymorphisms in the African isolates as reflecting the only recent (and not universal) access to ACTs in that region and, thus, limited drug pressure and selection as compared with southeast Asia. In line with this notion, further analysis of the African isolates suggested neutral evolution of the rare *K13* polymorphisms, that is, a large reservoir of “natural” *K13*-propeller variants in SSA.¹²

In the present study from southern Rwanda, we detected the *K13* candidate mutations P574L and A675V. Although the A675V variant has been detected only once among more than 4,000 African *P. falciparum* isolates,^{12–14} the *K13* candidate mutation P574L is reported here for the first time from Africa. Both variants are common in southeast Asia,^{7,12} and both are associated with delayed parasite clearance.¹¹ Among the three novel polymorphisms, *K13* V555A was one of five polymorphisms detected among isolates from different areas of Rwanda in the recent KARMA (*K13* Artemisinin Resistance Multicenter Rapid Assessment) project. In those isolates collected in 2012–2013, no candidate mutations were observed.²⁰

The proportion of *K13* polymorphisms tended to increase over time. Although derived from a rather small single-center study, this observation may reflect the increased availability of ACTs in Rwanda during recent years.¹⁵ Moreover, the incidence of malaria in this country has increased since 2011,¹⁵ possibly increasing the likelihood of the random occurrence of *K13* variants. Already in 2010, we observed a *pfmdr1* allele constellation in the study area (40% *pfmdr1* N86 F184 D1246), which is indicative of intense AL drug pressure and reappearing parasitemia following treatment.¹⁶ Against such background of a parasite population with affected susceptibility to the non-artemisinin partner drug, the potential of spreading *K13* candidate mutations is worrisome. However, and as a limitation of our study, the actual role of *K13* polymorphisms in SSA is far from being understood. It has been suggested that the link with resistance may differ geographically,¹⁴ and that specific, non-*K13* genetic factors in the local parasite population may predispose to the emergence of resistance-causing mutations.¹¹ Information on the respective genetic makeup of the parasite population in the study area is therefore needed in addition to results of up-to-date ACT efficacy trials and in vitro ring-stage survival assays as well as the evaluation of association between these phenotypic resistance indicators and local *K13* variants.

Received June 15, 2016. Accepted for publication July 22, 2016.

Published online August 29, 2016.

Financial support: This study was supported by the German Federal Ministry of Education and Research (grant 01DG13006A, MOPACUR) and by the German Federal Ministry for Economic Cooperation and Development via the ESTHER program. Costanza Tacoli is financially supported by grant GRK2046 from the German Research Foundation (DFG), and Prabhanjan P. Gai by DFG grant GRK1673 and a stipend of the Sonnenfeld-Foundation, Berlin. The funding bodies had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

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