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Molecular surveillance of *Plasmodium falciparum* drug resistance markers reveals partial recovery of Chloroquine susceptibility but sustained Sulfadoxine-Pyrimethamine resistance at two sites of different malaria transmission intensities in Rwanda

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Abstract

Faced with intense level of chloroquine (CQ) resistance in *Plasmodium falciparum* malaria, Rwanda replaced CQ with amodiaquine (AQ) + sulfadoxine-pyrimethamine (SP) in 2001, and subsequently with artemether–lumefantrine (AL) in 2006, as first-line treatment for uncomplicated

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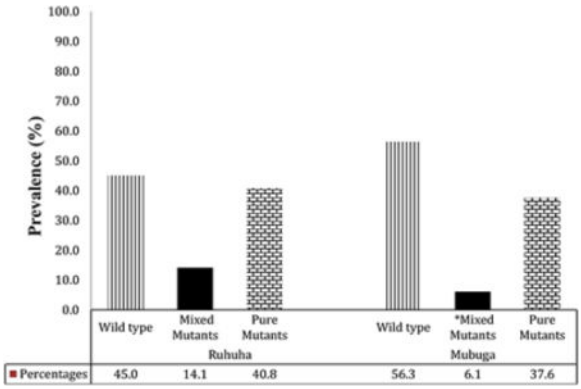
Authors' contributions: FK conceived the idea, designed the study, participated in performance of the experiments, analysed the data and drafted the manuscript. SLN participated in performing the experiments and revised the manuscript substantially. ST performed the experiments and revised the manuscript. NK helped in data analysis and writing and editing of the manuscript. EH, PFM, MPG and MvV contributed to the writing of the manuscript. KN supported study protocol development, and provided substantial input into the writing of the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors have declared that they have no competing interests.

malaria. Following years of discontinuation of CQ use, re-emergence of CQ-susceptible parasites has been reported in countries including Malawi, Kenya and Tanzania. In contrast, high levels of SP resistant mutant parasites continue to be reported even in countries of presumed reduced SP drug selection pressure. The prevalence and distributions of genetic polymorphisms linked with CQ and SP resistance at two sites of different malaria transmission intensities are described here to better understand drug-related genomic adaptations over time and exposure to varying drug pressures in Rwanda. Using filter paper blood isolates collected from *P. falciparum* infected patients, DNA was extracted and a nested PCR performed to identify resistance-mediating polymorphisms in the *pfcrt, pfmdr1, pfdhps* and *pfdhfr* genes. Amplicons from a total of 399 genotyped samples were analysed by ligase detection reaction fluorescent microsphere assay. CQ susceptible *pfcrt* 76K and *pfmdr*1 86N wild-type parasites were found in about 50% and 81% of isolates, respectively. Concurrently, SP susceptible *pfdhps* double (437G-540E), *pfdhfr* triple (108N-51I-59R), the quintuple *pfdhps* 437G-540E / *pfdhfr* 51I-59R-108N and sextuple haplotypes were found in about 84%, 85%, 74% and 18% of isolates, respectively. High-level SP resistance associated *pfdhfr* 164L and *pfdhps* 581G mutants were noted to decline. Mutations *pfcrt* 76T, *pfdhfr* 59R and *pfdhfr* 164L were found differentially distributed between the two study sites with the *pfdhfr* 164L mutants found restricted in at Ruhuha site, eastern Rwanda. Overall, sustained intense levels of SP resistance mutations and a recovery of CQ susceptible parasites were found in this study following 7 years and 14 years of the drug withdrawal from use, respectively. Most likely, the high prevalence of resistant parasites selected by the continued use of DHFR/DHPS inhibitors like trimethoprim-sulfamethoxazole (TS) for the treatment of and prophylaxis against bacterial infections among HIV infected individuals as well as the continued use of IPTp-SP within the East and Central African regions for malaria prevention among pregnant women may partly account for the observed sustained SP resistant parasite prevalent. With regard to CQ, the slow recovery of CQ susceptible parasites may have been caused partly by the continued use of CQ and/or CQ mimicking antimalarial drugs like AQ in spite of policies to withdraw it from Rwanda and the neighbouring countries of Uganda and Tanzania. Continued surveillance of *P. falciparum* CQ and SP associated polymorphisms is recommended for guiding future rational drug policy-making and mitigation of future risk of anti-malaria drug resistance development.

Graphical abstract



Keywords

Plasmodium falciparum ; chloroquine; sulfadoxine-pyrimethamine; resistance; polymorphisms

Background

Globally, malaria accounts for about 214 million cases and over 438,000 deaths annually (World Health Organization, 2015). A major hindrance to malaria control is the development of resistance in malaria parasites to available antimalarial therapies. Currently, artemisinin-based combination therapies (ACTs) (that consist of a combination of a fast-acting artemisinin component (artesunate, artemether, or dihydroartemisinin) and a longer-acting partner drug (lumefantrine, amodiaquine, piperaquine, or mefloquine) are widely used for treatment of *P. falciparum* malaria Although not a problem in Sub-Saharan Africa, ACTs clinical failure has been confirmed in Southeast Asia raising concerns of a possible lack of malaria treatment in the near future at a time when anti-malarial therapy options are limited (Dondorp et al., 2009; Ashley et al., 2015).

Prior to introduction of ACTs, widespread resistance to two more affordable and safe anti-malarial drugs; chloroquine (CQ) - a highly effective first line anti-malarial monotherapy used for about 50 years, and anti-folate drug sulfadoxine - pyrimethamine (SP), led to their withdrawal from primary use in many malaria-endemic settings (Young & Moore. 1961; Harinasuta et al., 1965; Enosse et al., 2008; Hastings et al., 2002; Kublin et al., 2003; Pearce et al., 2009, White. 1999). Although completely withdrawn from use in Rwanda in 2008, SP continues to be used for intermittent preventive treatment of malaria during pregnancy (IPTp-SP), infancy (IPTi) and for seasonal malaria chemoprevention (SMC) strategies in countries surrounding Rwanda. This practice poses a spillover risk of continued impact on SP resistance levels in Rwanda. Resistance to SP has been associated with polymorphisms in the *P. falciparum* dihydrofolate reductase (*pfdhfr*) and dihydropteroate synthase (*pfdhps*) genes while polymorphisms in the *P. falciparum* CQ resistance transporter (*pfcrt)* gene is the major mediator of resistance to CQ and amodiaquine (AQ) (Su et al., 1997; Fidock et al., 2000; Nzila-Mounda et al., 1998; Triglia et al., 1997). In addition, the *P. falciparum* multidrug resistance (*pfmdr*1) gene polymorphisms are associated with increased sensitivity to lumefantrine, mefloquine, and dihydroartemisinin, and to decreased sensitivity to CQ and AQ (Rosenthal, 2013; Koenderink et al., 2010).

Faced with emerging resistance in *P. falciparum* in Rwanda, CQ was replaced with AQ + SP in 2001 and the later subsequently replaced with artemether–lumefantrine (AL) in 2006, as first line antimalarial therapies for uncomplicated malaria (Zeile et al., 2012). For SP however, its use in intermittent preventive treatment of malaria in pregnancy (IPTp) continued until 2008 when it was withdrawn due to increasing anti-folate resistance (Karema et al., 2012). Elsewhere, after periods of complete CQ withdrawal, re-emergence of CQ-sensitive parasite strains, albeit at varying rates over time and across different geographic settings, has been reported suggesting that CQ-sensitive parasites may have a fitness advantage over resistant parasites in the absence of CQ drug selection pressure (Ndiaye et al., 2012; Mwai et al., 2009; Laufer et al., 2006). This is further evidenced by

the notably lower prevalence of mutant *pfcrt* 76T and *pfmdr*1 86Y alleles in low malaria transmission settings where drug pressure is presumably less (Ord et.al, 2007). Similar to the CQ experience, use of SP has been associated with ever increasing levels of resistance in *P. falciparum* in malaria endemic countries including Rwanda (Matondo et al., 2014; Karema et al., 2010). However, four years after cessation of SP use, high-level SP resistance was still observed in Rwanda (Karema et al., 2010).

Data on anti-malarial drug resistance is needed for rational drug policy-making, effective malaria management and designing strategies to mitigate risk and burden of drug resistance. For Rwanda, there is paucity of these data. This study measured the prevalence and distributions of *P. falciparum* molecular markers of resistance to CQ and SP, 14 and 7 years after a policy change involving withdrawal of these two drugs from use, respectively, at two sites of different transmission intensities.

Materials and methods

Study area and design

Rwanda is broadly divided into four malaria ecologic zones based on altitude, climate, level of transmission, and disease vector prevalence (President's Malaria Initiative, 2015). Topographically, malaria transmission is considered meso-endemic in the plain regions of eastern and southern provinces while being epidemic-prone in the high plateau and hill settings of northern and western provinces, respectively. Ruhuha sector, Bugesera district, eastern province is located within the high malaria transmission zone whilst Mubuga sector, Kalongi district, western province is located in the low transmission zone (President's Malaria Initiative, 2015) (Figure 1). *P. falciparum* infected isolates were collected from malaria confirmed cases seen at two rural health facilities located in the two sectors in a cross-sectional survey carried out between January and February 2015.

Study participant enrolment and assessments

Study inclusion was limited to health-facility area residents who were microscopically confirmed with *P. falciparum* infections and who were aged ≥ 6 months. Upon provision of a written informed consent, finger-prick blood samples were then collected and used for preparation of thick and thin blood film for microscopy and for blotting on to filter papers.

Ligase Detection Reaction-Fluorescent Microsphere (LDR-FM) Assay

DNA was extracted from filter paper bloodspots using Chelex® (Bio-Rad, Germany) as described elsewhere (Kain et al., 1991). Genomic DNA was used to analyse single nucleotide polymorphisms (SNPs) in *pfcrt, pfmdr1* and *pfdhfr* and *pfdhps* genes by nested PCR as previously described (LeClair et al., 2013). The ligase detection reaction-fluorescent microsphere assay was used to analyse all SNPs of interest (Nankoberanyi et al., 2014). SNPs were categorized as wild type (WT), mutant and mixed alleles against the comparator control reference strain DNA.

Statistical analysis

All statistical analyses were done using STATA version 13.1 (STATA Corp Inc., TX, USA). Differences in characteristics distribution of the study population for the different sites were tested by analysis of variance (ANOVA). Prevalence of SNPs was calculated for WT or mixed infections or pure mutants. In the final analysis, all pure mutant and mixed infections were summed up to generate the number of mutant genotypes per codon. Genotype proportions between the two study sites were compared using Pearson's chi square test. A p value of < 0.05 was considered statistically significant.

Ethical clearance

All adults and caregivers of children < 18 years were informed of the study purpose and procedures; recruitment was done only after obtaining informed written consent. The study was reviewed and approved by the National Health Research Committee (NHRC) and the Rwanda National Ethics Committee (No. 020/RNEC/2015), Kigali, Rwanda.

Results

Patient characteristics and variable distributions

Four hundred and two (402) patients aged 6 months to 73 years were enrolled. Of these, 399 patients whose isolates provided at least one genotype result were included in the current study. Table 1 describes study participant baseline data.

Genotyping efficacy at each codon

Per codon, typing was achieved in 97.2% of samples for *pfcrt* 76 (Figure 2); 95.7% for codon 86, 86.7% for codon 184, 94.7% for codon 1034, 97.5% for codon 1042 and 97.0% for codon 1246 at *pfmdr*1(Table 2); 94.2% for codon 51, 95.5% for codon 59, 95.0% for codon108 and 94.5% for codon 164 in *pfdhfr* ; 91.2% for codon 437, 91.0% for codon 540, 91.0% for codon 581, and 362 for codon 613 in *pfdhps* (Table 3). Among the typed polymorphisms, susceptible WT infections were observed for alleles *pfmdr1* 1042N and 1034S and *pfdhps* 613A, while mixed type infections were identified for *pfcrt* 76 (14%), *Pfmdr*1 86 (17%), 184 (32%) and 1246 (16%), *pfdhfr* 51 (1%) and 59 (17%) and for *pfdhps* 437 (8%), 540 (0.5%) and 581 (9%), respectively. Saturation (100%) levels for mutant strain were only identified in *pfdhfr* codon 108.

Age related association with in prevalence of mutations.

For each allele (*pfcrt* 76, *pfmdr1* 86, *Pfdhfr* (51, 59, 108) and *Pfdhps* (437, 540, 581), no statistically significant difference in mean number of mutant strains between age groups 0–5 years versus 6–15 years versus >15 years and age groups 0–5 years versus > 5 years was found (data not shown).

pfcrt gene

Overall, for the 388 total isolates typed, 50.8% carried the WT *pfcrt* 76K allele while 10.1% were pure mutants *pfcrt* 76T and 39.2% mixed infections. Stratified by site, WT, mixed and mutant *pfcrt* 76T genotype prevalence were 45.1%, 14.1%, 40.8%, respectively at

Ruhuha and 56.3%, 6.1%, and 37.6%, respectively at Mubuga (Figure 2). *pfcrt* 76T mutant (pure and mixed mutants combined) distribution varied significantly ($p = 0.026$) with higher proportions seen at Ruhuha (55%) compared to Mubuga (44%).

pfmdr1 gene

pfdmr1 WT 86N alleles were found in 80.9% isolates while 15.4% and 3.7% of isolates carried mixed and mutant type infection, respectively. WT alleles 184Y and 1246D were found in 40% and 80% isolates, respectively (Table 2). The distribution of all mutant alleles at typed *pfdmr1* codons were comparable for isolates from the two sites (Table 4).

pfdhps gene

High-level prevalence for *pfdhps* mutant (pure and mixed mutants combined) alleles 437G and 540E of 92.9% and 94.5%, respectively, was seen. At 581G and 613S codons, mutant prevalence was 24.2% and 0%, respectively. The distributions of 437G, 540E and 581G mutant alleles were comparable across the two study sites.

pfdhfr gene

pfdhfr mutant (pure and mixed mutants combined) allele prevalence at codons 51I, 59R, 108N and 164L were 99.7%, 90.3%, 100% and 5%, respectively, whilst the prevalence of the *pfdhfr* triple (108N-51I-59R) haplotype was 85% (Table 3). The distribution for each of *pfdhfr* 164L and 59R mutants varied significantly ($p < 0.0001$) by study sites. Notably, all 19 164L mutants were seen at the Ruhuha site of higher malaria endemicity. For the 59R mutants, a higher prevalence was observed at the lower malaria endemic Mubuga site (96.3%) compared to the Ruhuha site (84.2%).

Combination haplotypes

Of the 374 samples typed only 46 (12.2%) carried both *pfcrt* 76K and *pfdmr1* 86N WT alleles. Notably, the proportion of double *pfcrt* 76T and p*fdmr1* 86Y mutants was 2-fold higher at Ruhuha compared to Mubuga ($p = 0.018$). The prevalence of the *pfdhps* double (437G-540E), *pfdhfr* triple (108N-51I-59R), and *pfdhps*/*pfdhfr* quintuple haplotypes were 83.7%, 85.0% and 73.7%, respectively (Table 3). The proportions of the *pfdhps* double, *pfdhfr* triple (108N-51I-59R) and the *pfdhps* / *pfdhfr* quintuple polymorphisms were comparable across the two study sites (Table 4). In contrast, a borderline significant ($p = 0.06$) higher proportion of triple *pfdhps* 437G, 540E and 581G haplotype was seen at Ruhuha (25.3%) compared to the Mubuga (17.6%) site (Table 4). In total, about 18.3% (73 isolates) carried the sextuple (51I, 59R, 108N, 540E, 437G, 581G) mutant, with its occurrence being restricted to the Ruhuha site ($p = 0.005$).

Discussion

In Rwanda, CQ was replaced with AQ+SP in 2001 and the later combination was then used for only five years. No study or report on anti-malarial drug resistance neither before CQ withdrawal (2001) nor after AQ+SP withdrawal (2006) exists and thus the impact of AQ on CQ resistance has never been estimated.

In a study conducted in 2010 among under five-year old children in high malaria endemic southern Rwanda, a 74% *pfcrt* 76T mutant prevalence was reported (Zeile et al., 2012). In this study, conducted 5 years later, *pfcrt* 76T prevalence was 49%. Presuming a similar *pfcrt* 76T prevalence in the comparably high malaria transmission eastern and southern regions of Rwanda, ~ 25% recovery of WT *pfcrt* 76K strains was observed. Recovery of CQ-susceptibility after years of CQ withdrawal has shown a mixed pattern, both within and between countries. Whilst high CQ recovery rates (>85%) have been reported in Tanzania (Mohammed et al., 2013; Malmberg et al., 2013) and Malawi (Kublin et al., 2003), slower recovery rates have been reported elsewhere including Kenya (Mwai et al., 2009) and Uganda (Nsobya et al., 2010; Kamugisha et al., 2012). In our study, CQ recovery occurred in spite of the large-scale use of AL (which has been shown to select for the CQ-susceptible *pfcrt* 76K allele) similar other findings (Malmberg et al., 2013; Sisowath et al., 2009). Possible reasons for the observed slow CQ recovery of susceptibility include the continued use of CQ in spite of CQ withdraw policies as reported in Uganda, Rwanda and Tanzania (Karema et al., 2010; Frosch et al., 2011; Eriksen et al., 2005) and the continued use of CQ related antimalarial drugs like AQ. AQ use has been associated with limited recovery of CQ susceptibility after years of CQ withdrawal previously and has been shown to strongly select for the resistance conferring *pfcrt* 76T allele (Frank et al., 2011; Djimde et al., 2008). Other determinants of CQ recovery rates include time since actual CQ drug withdrawal from use, time since policy to withdraw CQ from use, baseline CQ resistance levels and malaria transmission intensities.

Polymorphisms in the *P. falciparum pfmdr*1 gene show mixed sensitivity responses for different anti-malarial drugs (Rosenthal, 2013). Our study showed a >80% prevalence for *pfmdr*1 WT 86N and 1246D alleles and a 75% prevalence for the *pfmdr*1 86N/1246D/184Y CQ susceptible triple haplotype. However, only 40% of isolates carried the WT 184Y alleles. Compared to a previous study in southern Rwanda where WT allelic prevalence of 61%, 88% and 48% for *pfmdr*1 86N, 1246D and 184Y, respectively, with ~ 60% prevalence for the *pfmdr*1 86N/184Y/1246D wild-type haplotype were reported, our study showed a slow recovery of WT 86N allele and the triple *pfmdr*1 (86N/184Y/1246D) haplotype but not the 1246Y and 184F mutants whose levels remained relatively stable (Gahutu et al., 2011). A comparably high 66% prevalence for the *pfmdr*1 86Y/184F/1246Y mutant haplotype was reported in Kenya (Okombo et al., 2014). This mixed selective pressure for CQ among alleles at this locus, with recovery reported for alleles 86N and 1246D but not 184Y, has been reported to be partly associated with scale-up in use of AL (Okombo et al., 2014). Our study findings are similar to those from Zanzibar, Burkina Faso, Tanzania where findings of increased prevalence of *pfmdr1* 86Y (Sisowath et al., 2005; Dokomajilar et al., 2006; Humphreys et al., 2007) and *pfmdr*1 184F have been noted to come under selection in settings of AL resistance (Vinayak et al., 2010). Analysis of *P. falciparum* infected samples in Mozambique showed a mixed temporal trend in the prevalence of WT 86N, 184Y and 1246D alleles. Between the 2003–2005 and 2010–2012 periods, *pfmdr*1 86N prevalence rose from 19.5% to 73.2%, while 184Y WT allelic prevalence remained stable (from 19.6% to 22.7%), and the WT 1246D alleles showed marginal increase from 74.4% to 96.7%, in tandem with ACT use in the 2010–2012 period (Dokomajilar et al., 2006; Lobo et al., 2014). Thus, recovery to CQ susceptible *pfmdr*1 alleles shows a variable temporal trend, with AL

being a major influence. Findings of *pfmdr1 86N, 184F,* and *1246D* allelic selection by treatment with AL raise concerns of a possible alteration to AL drug sensitivity by these alleles (Baliraine & Rosenthal, 2011). The relative contribution of CQ cessation and ACT scale-up on *pfmdr1* epidemiology requires further exploration.

Similar to previous studies in Southern Rwanda (Zeile et al., 2012), Tanzania (Matondo et al., 2014), Kenya (Shah et al., 2015, Iriemenam et al., 2012) and Uganda (Mbogo et al., 2014), our studies also detected high levels (>92%) of *pfdhps* 437G, 540E and *pfdhps* double (437G-540E) mutants. In contrast, declines in *pfdhfr* and *pfdhps* resistance imparting polymorphisms after SP withdrawal have been reported in studies from Ethiopia (Hailemeskel et al, 2013; Tessema et al., 2015), Tanzania (Gesase et al., 2009; Matondo et al., 2014) and Mozambique (Raman et al., 2008). Overall, as seen at 6 Tanzanian sites, SP resistance continuing to increase, with emergence and dispersal of "super resistant" mutants in east Africa (Baraka et al., 2015). The sustained high prevalence of *pfdhps* mutants in spite of the reduced or absent SP selection pressure in Rwanda may be due to: (1) high malaria endemicity in the study areas, (ii) the continued use of PfDHFR/PfDHPS inhibitors like trimethoprim-sulfamethoxazole (TS) for the treatment of and prophylaxis against bacterial infections among HIV infected individuals, and (iii) the continued use of IPTp-SP especially in the East and Central African regions.

In our study, intense levels of 85% *pfdhfr* triple mutants were seen, and other studies have likewise shown increased *pfdhfr* triple mutant prevalence in other sites in southern, eastern and western Rwanda, in spite of the presumed 7 years absence of SP drug pressure (Zeile et al., 2012, Karema et al., 2010). Similar to the high levels of *pfdhp* mutants seen in this study, a possible source of *pfdhfr* mutants may be the use of TS. Indeed, in vitro *P. falciparum* culture studies have demonstrated a TS cross-resistance with SP that may lead to the development of *pfdhfr* and *pfdhps* mutants (Khalil et al., 2003; Iyer et al., 2001). Additionally, our studies also detected high prevalence of quintuple (*pfdhfr* triple and *pfdhps* double) mutants similar to those reported in Kenya and Uganda even >10 years of SP drug pressure (Iriemenam et al., 2012; Mbogo et al., 2014). High levels of the quintuple mutants have been associated with reduced efficacy of SP-IPTp (Allen et al., 2009), and the fact that IPT-Sp continues to be is use in Kenya and Uganda, unlike in Rwanda, may account for high prevalence of quintuple mutants in Kenya and Uganda.

High polymorphism at *pfdhfr* 164L and *pfdhps* 581G have been associated with increased therapeutic failure of SP (Karema et al., 2010; Lynch et al., 2008; Gesase et al., 2009; Gasasira et al., 2010; Spalding et al., 2010), however in our studies the prevalence was ~24%. A possible reason for high *pfdhps* 581G levels may be the continued IPTp-SP use (Harrington et al. 2009) and resulting reduced effectiveness of IPT—Sp has been attributed to significant reduction in birth weight of newborns (Minja et al., 2013). In our study, 581G levels varied by study area with a 26.8% declined at Ruhuha, eastern Rwanda vs. 60% decline reported at Rukara, eastern Rwanda (Karema et al., 2010) in the period 2005–2006 to 2016. In contrast, 581G allelic prevalence remained comparable for the Mubuga (29%) and Masheshe (21.7%) sites in western Rwanda in the same periods, respectively (Karema et al., 2010). Differences in malaria transmission intensity between the eastern and western regions may partly account for the differential 581G allelic temporal effects noted. Isolates

collected from Ruhuha, eastern Rwanda revealed 5% prevalence of *pfdhfr* 164L mutant similar to (Karema et al., 2010), and these were found only in association with the *pfdhps* double (100%) but with ~95% (18/19) of alleles concurrently seen alongside the triple *pfdhfr* mutant. The 164L mutants are preferentially concentrated in the Kenya and Uganda eastern Africa, albeit at variable prevalence (Lobo et al., 2014; Spalding et al., 2010).

We also identified additional site-specific genotype differences. The proportion of *pfcrt* 76T mutant infection was significantly higher at the lower-endemic Mubuga site compared to the higher-endemic Ruhuha site. A number of factors may account for this variability including differences in access to AL external sources of *pfcrt* 76T mutants introduced by individuals from neighbouring countries due to high frequency of cross border movements. On the other hand the *pfdhfr* 164L mutants were found exclusively in the high malaria endemic Ruhuha site, while the same has been reported to vary between low (Braun et al., 2015; Alifrangis et al., 2009) and concentrated local hotspots in Rwanda and southwest Uganda (Karema et al., 2010; Lynch et al., 2008). The other difference was higher prevalence of the *pfdhfr* 59R mutants observed at Mubuga, western Rwanda compared to Ruhuha, eastern Rwanda. The western Rwanda borders the Democratic Republic of Congo and Burundi and hence may be more influenced by drug resistance pressure from across the border due to highly dynamic human populations, relative to the other sites. Finally, samples analysed in this study were collected from two sites located in the low and high malaria intensities zones where as malaria risk in Rwanda is categorised into four ecologic zones. Therefore, study findings may not be generalizable to all Rwandan sites. That notwithstanding, our findings provide the most recent accurate surveillance data for key CQ and SP resistance- mediating polymorphisms at two sites of variable malaria transmission intensities.

Conclusions

Overall, sustained high levels of SP resistance and a slow recovery of CQ susceptible parasites were found in our study conducted after 7 and 14 years after complete SP and CQ drug withdrawal, respectively. Most likely, the high prevalence of SP resistant parasites is due to the continued selection by use of Pfdhfr/Pfdhps inhibitors like TS (among HIV infected individuals) and IPTp-SP (for malaria prevention among pregnant women). Interestingly, the prevalence for the two high-level SP resistance imparting *pfdhfr* 164L and *pfdhps* 581G mutants were observed to decline with the *pfdhfr* 164L mutant noted to be restricted to the Eastern Rwanda site Continued surveillance of *P. falciparum* polymorphisms and characterization of the determinants of anti-malarial drug sensitivity epidemiology is recommended for guiding future rational drug policy-making and mitigation of future risk of anti-malaria drug resistance development.

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Highlights

- Antimalarial drugs chloroquine and sulphodoxine – pyrimethamine resistance was a setback to malaria control.
- Studies reveal that withdrawal of there can lead to recovery of sensitivity of parasites over time.
- In Rwanda chloroquine susceptibility recovery appears slow but sulphodoxine – pyrimethamine resistance remains high after years of withdrawal.
- Elucidating the determinants of these drug-related genomic adaptations over time and exposure to varying drug pressures in Rwanda is needed

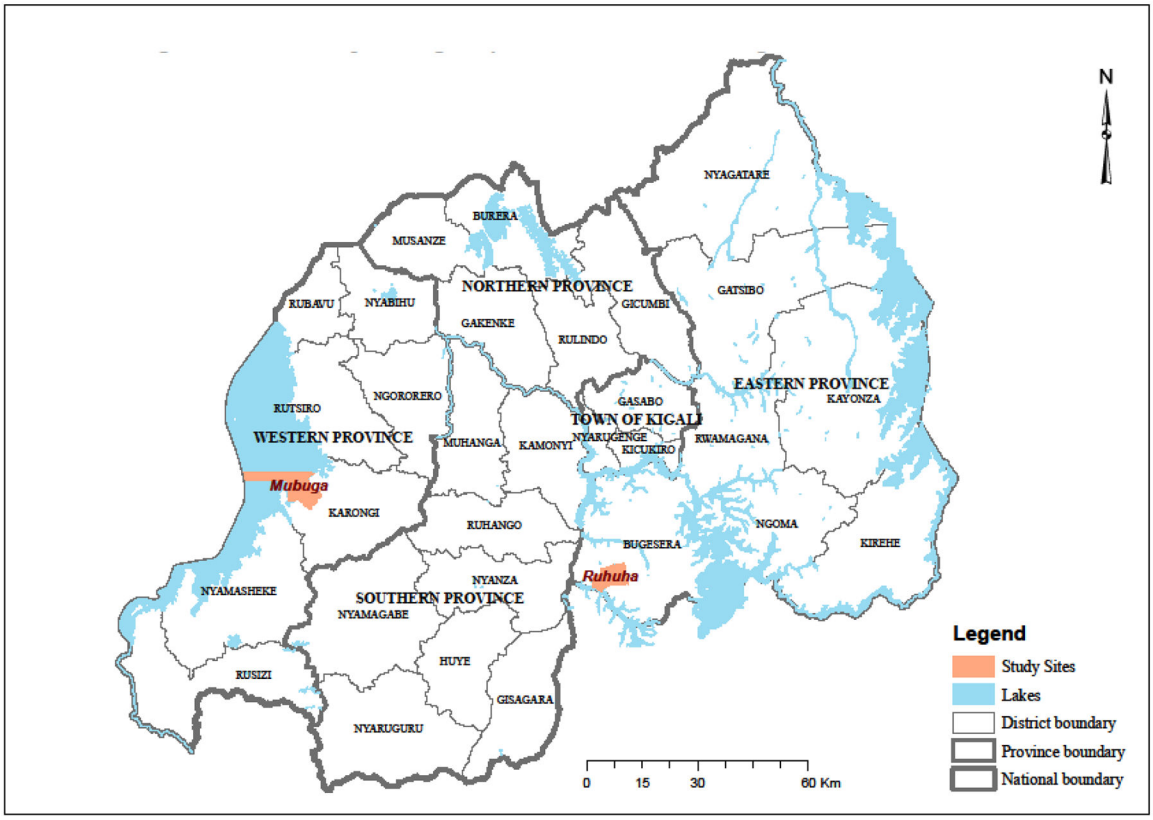

Figure 1.
Location map showing study sites of Ruhuha and Mubuga in Rwanda.

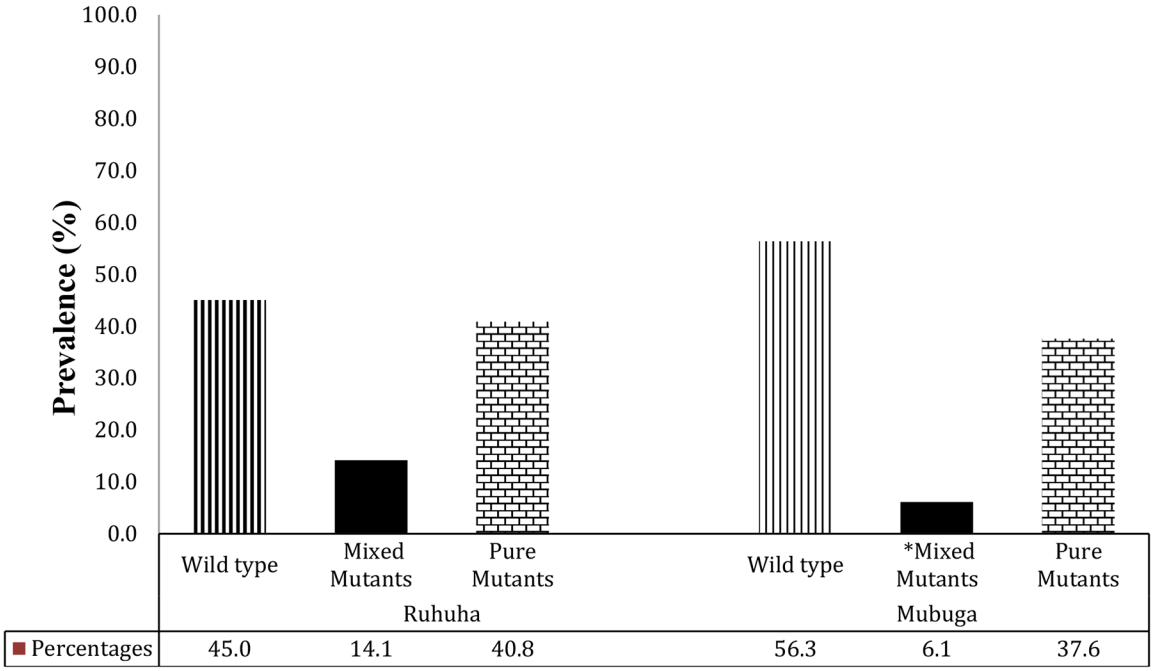

Figure 2.

Prevalence of *pfcrt* K76T genotypes among isolates collected at Ruhuha vs. Mubuga sites, Rwanda.

*Mixed infections denote isolates in which both WT and mutant genotypes were detected in the same individual.

Table 1.

Demographic characteristics at enrolment for 399 study participants from Ruhuha and Mubuga sites, Rwanda. *

Variable	Variable sub-group	Mubuga n =205	Ruhuha n=194	Overall N=399
Age (Mean ± SD)	-	17.7 ± 14.0 *	13.1 ± 12.7	15.5 ± 13.5)
Age Group	0 – 5 years	26 (12.7)	51 (26.3)	77 (19.3)
	6 – 15 years	96 (46.8)	94 (48.4)	190 (47.6)
	> 16 Years	83 (40.5)	49 (25.3)	132 (33.1)
Sex	Male	100 (48.8)	79 (40.7)	179 (44.9)
	Female	105 (51.2)	115 (59.3)	220 (55.1)
Geometric Mean Parasite / µl blood	-	599.5 (95% CI#: 457.2 – 786.0)	2190.7 (95% CI#: 1649.1 – 2910.2)	1125.7 (95% CI#: 916.7 – 1382.4)

* Shows Mean + standard deviation (SD);

shows 95% Confidence Interval (CI

Table 2.

A comparison of *pfmdr1* genotype proportions by study site Mubuga and. Ruhuha

	Ruhuha			Mubuga			All sites
***pfmdr1* genotypes**	**Wild Type n (%)**	**Mixed* n (%)**	**Mutant n (%)**	**Wild type n (%)**	**Mixed n (%)**	**Mutant n (%)**	**Total Mutants (Mixed + pure mutants) n (%)**
N86Y	146 (78.5)	31 (16.7)	9 (4.8)	163 (83.2)	28 (14.3)	5 (2.6)	73 (19.1)
Y184F	64 (37.4)	54 (31.6)	53 (31.0)	75 (42.9)	58 (33.1)	42 (24.0)	207 (59.8)
N1042C	188 (100)	0 (0)	0 (0)	201 (100)	0 (0)	0 (0)	0 (0)
S1034C	188 (100)	0 (0)	0 (0)	190 (100)	0 (0)	0 (0)	0 (0)
D1246Y	141 (79.2)	29 (16.3)	8 (4.5)	162 (82.2)	19 (9.6)	16 (8.1)	72 (19.2)
N86Y, D1246Y	177 (91.2)	17 (8.8)		190 (92.7)	15 (7.3)		32 (8.0)

*Mixed infection denotes an isolate in which both WT and mutant genotypes were detected.

Table 3.

Prevalence of *pfdhrf* and *pfdhps* genotypes by study site Mubuga and Ruhuha

	Ruhuha			Mubuga			All sites
***pfdhfr* alleles**	**Wild Type n (%)**	**Mixed* n (%)**	**Mutant n (%)**	**Wild type n (%)**	**Mixed n (%)**	**Mutant n (%)**	**All Mutants (Mixed + pure mutants) (%)**
N51I	0 (0)	2 (1.1)	188 (98.9)	1 (0.5)	0 (0)	185 (99.5)	375 (99.7)
C59R	30 (15.8)	33 (17.4)	127 (66.8)	7 (3.7)	24 (12.6)	160 (83.8)	344 (90.3)
S108N	0 (0)	0 (0)	190 (100)	0 (0)	0 (0)	189 (100)	379 (100.0)
I164L	168 (89.8)	9 (4.8)	10 (5.3)	190 (100)	0 (0)	0 (0)	19 (5.0)
N51I, C59R, S108N	34 (17.5)	160 (82.5)		26 (12.7.)	179 (87.3)		339 (90.2)
N51I, C59R, S108N, I164L	194 (100)	0 (0)		187 (91.2)	18 (8.8)		18 (4.5)
***pfdhps* alleles**							
A437G	15 (8.2)	15 (8.2)	154 (83.7)	11 (6.1)	28 (15.6)	141 (78.3)	338 (92.9)
K540E	10 (5.5)	1 (0.5)	172 (94.0)	10 (5.6)	1 (0.6)	169 (93.9)	343 (94.5)
A613S	100.0 (100)	0.0 (0)	0.0 (0)	100.0 (100)	0.0 (0)	0.0 (0)	0 (0.0)
A581G	134 (73.2)	16 (8.7)	33 (18.0)	141 (78.3)	14 (7.8)	25 (13.9)	88 (24.2)
Double *pfdhfr* (A437G-K540E)	39 (19.0)	166 (81.0)		26 (13.4)	168 (86.6)		334 (83.7)
Triple *pfdhfr* (A437G-K540E-A581G)	34 (17.5)	160 (82.5)		29 (12.7)	179 (87.3)		339 (85.0)
Quintuple *pfdhfr* E540-G437 / *pfdhfr* 51I-59R-108N)	51 (26.3)	143 (73.7)		52 (25.4)	153 (74.6)		296 (74.2)
Sextuple *pfdhfr* E540-G437 / *pfdhfr* 51I-59R-108N + *pfdhps* A581)	152 (78.4)	42 (21.6)		174 (84.9)	31 (15.1)		73 (18.3)

*Mixed infection denotes an isolate in which both WT and mutant genotypes were detected.

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Table 4.

Comparisons in proportional distributions of Chloroquine and Sulphadoxine – Pyrimethamine polymorphisms by study sites. [C]

Allele	**Polymorphism**	**Number (%) of mutant alleles**		**Pearson's X^2 test**	**P value***
		Ruhuha - n (%)	**Mubuga - n (%)**		
pfcrt	76T	105 (55.0)	86 (43.7)	4.971	**0.026**
pfmdrl	86Y	40 (21.5)	33 (16.8)	1.346	0.246
	184Y	107 (62.6)	100 (57.1)	1.0611	0.303
	1042C	0	0	-	-
	1034C	0	0	-	-
	1246Y	37 (20.8)	35 (17.8)	0.5497	0.458
pfdhps	613S	0	0		
	581G	49 (26.8)	39 (21.7)		
	437G	169 (91.9)	169 (93.9)	0.572	0.450
	540E	173 (94.5)	170 (94.4)	0.001	0.970
pfdhfr	51I	190 (100)	185 (99.5)	2.982	0.225
	59R	160 (84.2)	184 (96.3)	19.510	< 0.0001
	108N	190 (100)	189 (100)	-	-
	164L	19 (10.2)	0 (0)	20.329	< **0.0001**
Grouped alleles	Double *pfdhps* (540E-437G)	168 (91.8)	166 (92.2)	2.311	0.129
	Triple *pfdhfr* (51I-59R-108N)	160 (84.2)	179 (96.2)	1.830	0.176
	Quadruple (51I-59R-108N-164L)	18 (8.8)	0 (0.0)	17.839	< **0.0001**
	Quintuple *pfdhfr* 540E-G437G */pfdhfr* 51I-59R-108N)	143 (73.7)	153 (74.6)	0.044	0.833
	Sextuple *pfdhfr* E540-G437–581G */pfdhfr* 51I-59R-108N)	42 (21.7)	31 (15.1)	2.8411	0.092

** P* value comparing proportions of mutant parasites were based on a 2-sample t-test.

Significant values are in boldface.