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RESEARCH

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Surveillance of artemether-lumefantrine associated *Plasmodium falciparum* multidrug resistance protein-1 gene polymorphisms in Tanzania

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Abstract

Background: Resistance to anti-malarials is a major public health problem worldwide. After deployment of artemisinin-based combination therapy (ACT) there have been reports of reduced sensitivity to ACT by malaria parasites in South-East Asia. In Tanzania, artemether-lumefantrine (ALu) is the recommended first-line drug in treatment of uncomplicated malaria. This study surveyed the distribution of the *Plasmodium falciparum* multidrug resistance protein-1 single nucleotide polymorphisms (SNPs) associated with increased parasite tolerance to ALu, in Tanzania.

Methods: A total of 687 *Plasmodium falciparum* positive dried blood spots on filter paper and rapid diagnostic test strips collected by finger pricks from patients attending health facilities in six regions of Tanzania mainland between June 2010 and August 2011 were used. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was used to detect *Pfmdr1* SNPs N86Y, Y184F and D1246Y.

Results: There were variations in the distribution of *Pfmdr1* polymorphisms among regions. Tanga region had exceptionally high prevalence of mutant alleles, while Mbeya had the highest prevalence of wild type alleles. The haplotype YFY was exclusively most prevalent in Tanga (29.6%) whereas the NYD haplotype was the most prevalent in all other regions. Excluding Tanga and Mbeya, four, most common *Pfmdr1* haplotypes did not vary between the remaining four regions ($\chi^2 = 2.3$, $p = 0.512$). The NFD haplotype was the second most prevalent haplotype in all regions, ranging from 17% - 26%.

Conclusion: This is the first country-wide survey on *Pfmdr1* mutations associated with ACT resistance. Distribution of individual *Pfmdr1* mutations at codons 86, 184 and 1246 varies throughout Tanzanian regions. There is a general homogeneity in distribution of common *Pfmdr1* haplotypes reflecting strict implementation of ALu policy in Tanzania with overall prevalence of NFD haplotype ranging from 17 to 26% among other haplotypes. With continuation of ALu as first-line drug this haplotype is expected to keep rising, thus there is need for continued pharmacovigilance studies to monitor any delayed parasite clearance by the drug.

Keywords: *Plasmodium falciparum*, *pfmdr1*, Anti-malarial drug resistance, Artemether-lumefantrine, Tanzania, Polymorphisms, Malaria, Molecular markers

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Background

Plasmodium falciparum multidrug resistance protein-1 (*Pfmdr1*) is an adenosine triphosphate-binding cassette protein located on the parasite's food vacuole [1]. Mutations in the *Pfmdr1* coding gene leading to amino acid changes in *Pfmdr1* have different consequences on parasite's sensitivity to anti-malarial drugs. Several *Pfmdr1* single nucleotide polymorphisms have been reported whereby N86Y, Y184F, S1034C, N1042D and D1246Y are the most common. *Pfmdr1* 86Y mutation is associated with chloroquine (CQ) and amodiaquine (AQ) resistance [2-4], while 1034C, 1042D and 1246Y mutations have been reported to confer resistance against quinine (QN) and increased susceptibility to mefloquine (MQ), halofantrine (HF) and artemisinin [5-7]. Furthermore, the 86Y and 1246Y are highly associated with decreased sensitivity to artesunate-amodiaquine (AS-AQ), while the wild types N86 and D1246 are linked to artemether-lumefantrine (ALu) resistance [8-10]. Recent studies have shown that the combination of N86, 184 F, and D1246 forming a haplotype "NFD" lead to decreased susceptibility to ALu and that treatment with ALu selects for such haplotype [11,12]. Furthermore, an increase of asexual parasites and gametocytes harboring *Pfmdr1* NFD haplotype in patients treated with ALu was linked with treatment failure [12].

In Tanzania, ALu was adopted as first-line treatment drug in December 2006 [13]. A recent study in Korogwe, Tanga region reported increase of N86 from 25% to 59% and 184 F from 10% to 30% in 2006 to 2010 [14]. Another study in Igongwe, Mwanza pointed out an increase of N86 in samples collected post ALu treatment as compared with pretreatment samples; from 6.3 to 42.1% [15]. Also in Bagamoyo Pwani region, Malmberg and colleagues reported increase from 10 to 37% of the NFD haplotype from 2006 to 2011 [16]. A similar selection of NFD by ALu was observed in Mozambique [17]. In Kenya the Y184F was associated with high artemisinin IC₅₀ levels in ex-vivo drug sensitivity assays while the wild type N86 was associated with high MQ IC₅₀ [10]. Furthermore, *P. falciparum* parasites carrying NFD haplotype were able to withstand 15-fold higher blood lumefantrine levels than those with YYY (86Y-Y184-1246Y) haplotype [18]. Recently, ACT resistance associated K13 propeller protein mutations selected through increasing drug pressure in laboratory strains and subsequently found in field isolates from South-East Asia were reported [19]. Together with the *Pfmdr1* ALu-associated haplotypes the K13 polymorphism is evidence of emerging tolerance to ACT and calls for continuous monitoring surveillance studies. Following five years of ALu treatment policy implementation in Tanzania there is scarcity of information on current status of ACT markers of resistance. Of the studies reported to-date

most were conducted a few years around the official adoption of ALu in the country (from 2003–2009), while the few most recent had inadequate sample size or did not cover the NFD haplotype with exception of one study [16] conducted in 2010. This study reports on the current status of the *Pfmdr1* NFD haplotype in six regions of Tanzania which can be used as a baseline status for future studies in predicting the trends and for monitoring ALu efficacy.

Methods

Description of study subjects and study sites

Samples used in this study were obtained through collaboration with ongoing studies in six regions of mainland Tanzania between June 2010 and August 2011. Except for the Coastal region where the samples involved pregnant women attending the Kibiti health centre for antenatal care, all other samples were collected from all-age groups. Finger prick blood on filter paper (Whatman-3) or malaria rapid diagnostic test (RDT) (Paracheck, Orchid Biomedical Systems, India) (Mwanza samples only) from febrile patients attending to various health facilities in the respective regions were collected after patient's or children's guardians had consented for use of their blood samples for malarial genetic studies. The study sites (with their respective number of samples in brackets) include Mwanza (Misungwi district, n = 107) and Kagera (Muleba district, n = 129) around Lake Victoria in the north-western zone, Tanga (Bondo village, n = 94) in north-eastern zone, Mtwara (Tandahimba and Mtwara-Urban, n = 70) and Coastal Region (Kibiti-Rufiji, n = 144) in south-eastern zone and Mbeya (Kyela and Rungwe districts, n = 143) in the south-western zone.

DNA extraction and genotyping of the *Pfmdr1* gene

Malaria-positive RTDs or dried filter paper blood spots from microscopically confirmed cases were stored in desiccants at room temperature. Malaria parasite DNA was extracted using chelex-100 method as described previously [20]. Genotyping for *Pfmdr1* was performed using PCR-RFLP-methods described elsewhere [7,21]. In brief, PCR products were digested with *ApoI* and *Afl-III* which recognize the 86 N and (86Y) respectively, *Dra-I* which recognises the 184Y and *EcoRV* which recognises 1246Y. Endonuclease digest products were eluted on 2.5% agarose gel (Amasham Biosciences, Sweden) stained with ethidium bromide (Sigma Aldrich, USA) and visualized under ultraviolet light. PCR reagents and restriction endonucleases were purchased from New England Biolabs (NEB inc., Ipswich, MA, USA). Primers were purchased from Biologio (Biologio Inc., The Netherlands). Prevalence was calculated by adding the number of samples carrying mixed infections to both wild-type allele and mutant allele, thereby obtaining a new 'n' (which includes the mixed

Table 1 Distribution of *Pfmdr1* single nucleotide polymorphisms in Tanzania

Regions	<i>Pfmdr1</i> polymorphisms											
	N86Y				Y184F				D1246Y			
	N n (%)	Y n (%)	N/Y n (%)	Y Prevalence (%)	Y n (%)	F n (%)	Y/F n (%)	F Prevalence (%)	D n (%)	Y n (%)	D/Y n (%)	Y Prevalence (%)
Tanga	36 (38.3)	56 (59.6)	2 (2.1)	58 (60.4)	32 (34.8)	60 (65.2)	0 (0)	60 (65.2)	45 (52.9)	39 (45.9)	1 (1.2)	40 (46.5)
Coastal	93 (72.1)	33 (25.6)	3 (2.3)	36 (27.3)	85 (63.0)	50 (37.0)	0 (0)	50 (37.0)	134 (93.7)	7 (4.9)	2 (1.4)	9 (6.2)
Mtwara	49 (74.2)	16 (24.2)	1 (1.5)	17 (25.4)	43 (64.2)	23 (34.3)	1 (1.5)	24 (35.3)	55 (78.6)	15 (21.4)	0 (0)	15 (21.4)
Kagera	90 (72.0)	31 (24.8)	4 (3.2)	35 (27.1)	82 (67.8)	38 (31.4)	1 (0.8)	39 (32.6)	112 (88.2)	13 (10.2)	2 (1.6)	15 (11.6)
Mbeya	129 (95.6)	4 (3.0)	2 (1.5)	6 (4.4)	111 (88.0)	13 (10.3)	2 (1.6)	15 (11.7)	119 (89.5)	13 (9.8)	1 (0.8)	14 (10.4)
Mwanza	72 (77.1)	23 (22.9)	0 (0)	23 (24.2)	64 (64.0)	35 (35.0)	1 (1.0)	36 (35.6)	73 (83.0)	15 (17.0)	0 (0)	15 (23.6)
Total	469 (72.8)	163 (25.3)	12 (1.8)		417 (65)	219 (34.2)	5 (0.7)		538 (83)	102 (15.7)	6 (0.9)	

infections twice). Prevalence of wild-type and mutant allele was then calculated as the percentage of wild-type plus mixed infection or mutants plus mixed infection out of the new 'n'. For the haplotype analysis the mixed infections were however excluded.

Statistical analysis was performed using Pearson Chi-square (SPSS version 16) and Fisher's exact (FE) test. The study received ethical approval from the Kilimanjaro Christian Medical University College ethical board subsequent to the National IRB (NIMR) approval obtained in the collaborating projects.

Results

Out of the 687 samples, 644 (93.7), 641 (93.3) and 646 (94%) were successfully genotyped for *Pfmdr1* N86Y,

Y184F and D1246Y SNPs respectively. There was statistically significant difference in the distribution of individual *Pfmdr1* polymorphisms among the regions; N86Y ($\chi^2 = 91.0$, $p < 0.0001$), Y184F ($\chi^2 = 68.4$, $p < 0.0001$) and D1246Y ($\chi^2 = 73.7$, $p < 0.0001$). Tanga region had the highest prevalence of mutant alleles in all codons while Mbeya had the highest prevalence of wild type alleles for N86Y and Y184F (Table 1 and Figure 1).

Haplotype analysis

When the SNPs were constructed into codon 86-184-1246 haplotypes, eight haplotypes were detected among 578 of the samples, omitting samples that had mixed genotype infections (Table 2) and those that could not be genotyped for all the three SNPs. Of these haplotypes,

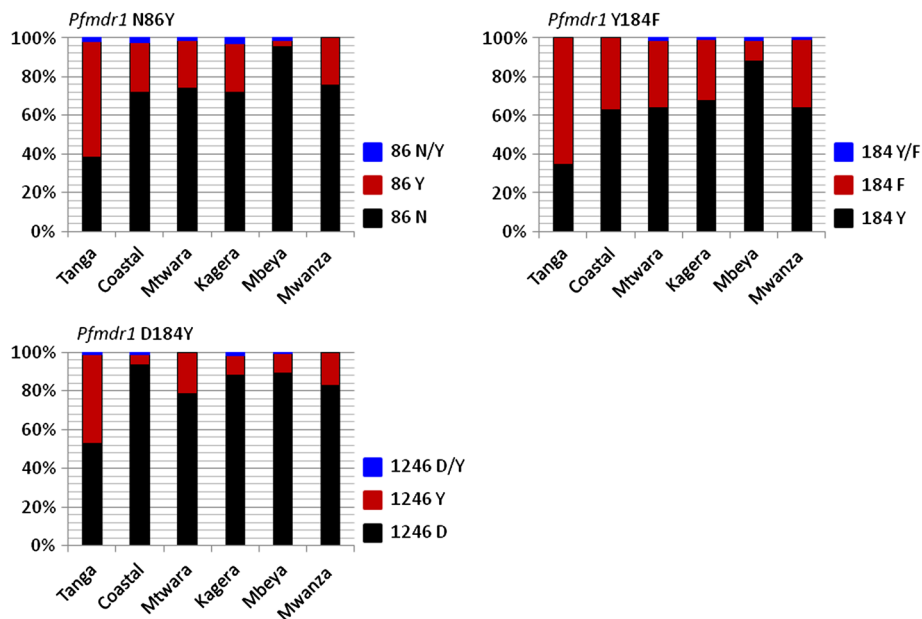


Figure 1 Prevalence of the *Pfmdr1* N86Y, Y184F, and D1246Y polymorphisms by region in Tanzania. Shown in black, Wild-types; brick-red: Mutants and Blue: mixed genotypes.

Table 2 Prevalence of the *Pfmdr1* haplotypes in six regions of Tanzania

Regions	<i>Pfmdr1</i> haplotypes								Total (N)
	NYD	NYI	NFY	NFD	YYY	YYD	YFD	YFY	
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	
Tanga	9 (11.1)	7 (8.6)	2 (2.5)	14 (17.3)	4 (4.9)	8 (9.9)	13 (16.0)	24 (29.6)	81
Coastal	53 (44.9)	2 (1.7)	2 (1.7)	28 (23.7)	1 (0.8)	17 (14.4)	14 (11.9)	1 (0.8)	118
Mtwara	25 (39.7)	5 (7.9)	3 (4.8)	16 (25.4)	2 (3.2)	10 (15.9)	1 (1.6)	1 (1.6)	63
Mbeya	86 (77.5)	10 (9.0)	0 (0.0)	11 (9.9)	1 (0.9)	3 (2.7)	0 (0.0)	0 (0.0)	111
Kagera	46 (41.4)	5 (4.5)	2 (1.8)	29 (26.1)	5 (4.5)	19 (17.1)	5 (4.5)	0 (0.0)	111
Mwanza	33 (36.7)	9 (10.0)	5 (5.6)	24 (26.7)	2 (2.2)	14 (15.6)	1 (1.1)	2 (2.2)	90
Total N (%)	252 (43.6)	38 (6.6)	14 (2.4)	122 (21.1)	15 (2.6)	71 (12.3)	34 (5.9)	28 (4.8)	578

the most common were NYD (43.6%), NFD (21.1%) and YYD (12.3%) haplotypes (Figure 2). A minor haplotype YFY (4.8%), was almost exclusively present in Tanga region (85.7% of total YFY haplotypes) compared to other regions and was the most prevalent (29.6%) of the eight haplotypes in that region. Conversely, the NYD was the most prevalent in all other regions, with a markedly high prevalence in Mbeya (77.5%) compared to the other regions (Figure 2). When comparing individual haplotypes against the regions, each haplotype varied significantly between the regions ($p < 0.05$). However, when Mbeya with exceptionally high wild-type haplotype (77.5%) was excluded, the NFD distribution did not vary between the regions ($\chi^2 = 2.3$, $p = 0.512$). Furthermore, when both Mbeya and Tanga were excluded from the analysis, all the common haplotypes did not vary significantly among the regions (YYD: $\chi^2 = 0.32$, $p = 0.952$; NYD: $\chi^2 = 1.498$,

$p = 0.683$; NFD: $\chi^2 = 0.28$, $p = 0.964$ and YFY: FE = 2.77, $p = 0.462$). Mbeya and Tanga regions were, therefore, exceptional with Tanga having the most mutant alleles at the three codons while Mbeya had the most wildtypes in two of the three.

Discussion

Molecular markers are useful predictors of emerging or existing levels of resistance to anti-malarial drugs. The surveillance of these markers have proven important during recent years where reports on the molecular marker for chloroquine (CQ) resistance; *Pfprt* have shown recovery of CQ sensitivity in Mozambique and Tanzania [22-24]. Furthermore, accumulation of mutations in the genes *Pfdhfr* and *Pfdhps* associated to sulphadoxine-pyrimethamine (SP) resistance have recently been shown to culminate with the emergence of

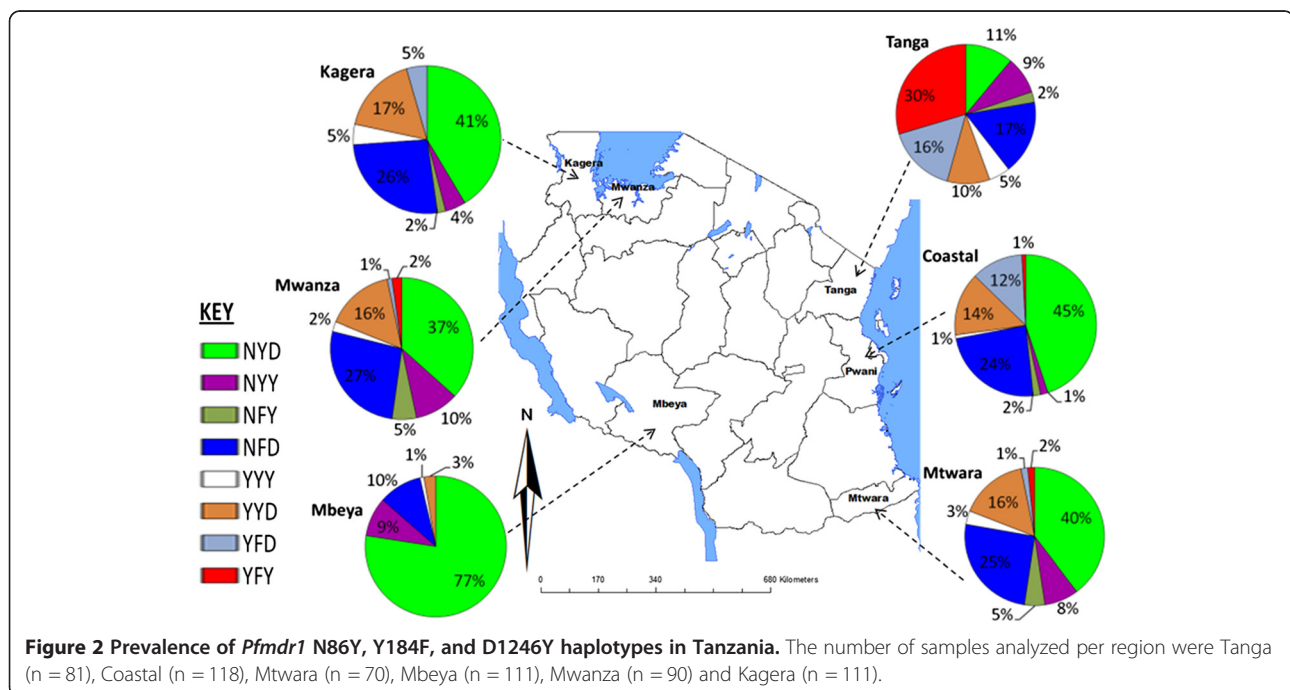


Figure 2 Prevalence of *Pfmdr1* N86Y, Y184F, and D1246Y haplotypes in Tanzania. The number of samples analyzed per region were Tanga (n = 81), Coastal (n = 118), Mtwara (n = 70), Mbeya (n = 111), Mwanza (n = 90) and Kagera (n = 111).

sextuple *Pfdhfr* and *Pfdhps* mutants [25,26]. These super-resistant mutants render intermittent preventive treatment of pregnant women (IPTp) using SP redundant in places such as in Tanga where high prevalence of such mutants have been documented [27]. In this study, variation in the distribution of *Pfmdr1* polymorphisms among regions in Tanzania is reported. The overall prevalence of single SNPs and as well, the resulting triple 86-184-1246 haplotype YFY haplotype was highest in Tanga. Interestingly, this coincides with highest prevalence of SP resistance markers also documented in Tanga region [25,28,29]. The haplotype YFY is linked to AQ and CQ resistance [21]. On the other hand high prevalence of NYD haplotype was highest in Mbeya region. This made Tanga and Mbeya regions different from the rest of the studied regions. While there is no clear explanation for Mbeya, a general very high malaria transmission thus high use of anti-malarials especially in early 1980s and 1990s may have led to a particularly high selection pressure for resistant parasites in Tanga relative to other places in Tanzania [30,31]. In a recent survey on availability of anti-malarials in Muheza Tanga, AQ and SP were still available in private shops and used by the local population for malaria self-medication [32]. Continued use of AQ in the study area or neighbourhood may account for the observed high YFY haplotype. Also these findings point to a possible low adherence to the ALu treatment policy in Tanga relative to other regions.

The NFD did not vary between five of the regions. These results show homogeneity in *Pfmdr1* haplotypes distribution, which suggests similar selection pressure throughout the country, indicative of homogeneity in ALu policy implementation in Tanzania. ALu has been shown to select for the NFD haplotype, where the prevalence of 86Y and 1246Y mutations has been decreasing while the 184 F has been increasing [11,14,17]. In this study, low prevalence of mutations 86Y and 1246Y were observed relative to 184 F. Similar findings elsewhere in East and West Africa have been reported where ALu is the treatment policy [33-35]. In recent in-vitro studies done using parasite isolates in Senegal and South East Asia, the 86Y and 1246Y were associated with high CQ, AQ and MQ inhibitory concentrations (IC_{50}) whereas the 184 F was associated with high artemisinin IC_{50} values [36,37]. Furthermore, in Cambodian samples the prevalence of the 184 F mutation selectively increased after ACT pressure [38]. These reports are suggestive of some overlap in mechanism of ACT resistance between South-East Asia and Africa and that these molecular markers can serve as universal tools for ACT resistance monitoring.

Conclusions

This is the first country-wide survey on *Pfmdr1* mutations associated with ACT resistance. Distribution of

Pfmdr1 mutations at codons 86, 184 and 1246 varies throughout Tanzanian regions. There is homogeneity in distribution of common *Pfmdr1* haplotypes in four out of six regions of Tanzania which may reflects homogeneity in countrywide implementation of ALu policy. The overall prevalence of NFD haplotype claimed to be associated with emerging ALu tolerance ranges from 17 to 26% among other haplotypes. With continuation of ALu as first-line drug and in the absence of CQ and AQ, this haplotype is expected to keep rising. There is need for continued pharmacovigilance studies in order to predict early parasite tolerance to the drug.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RAK conceived the idea, designed the study, analysed the data and wrote the manuscript. PP participated in study design, performed the experiments, participated in interpreting the data and drafted the manuscript. RDK participated in performing the experiments and in manuscript writing. AK supervised sample collection in the field and revised the manuscript. MvS and JC participated in analysing the data and revised the manuscript. CR and MA participated in overall interpretation of the results and in writing the manuscript. All authors read and approved the final version of the manuscript.

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