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A review of the frequencies of *Plasmodium falciparum* Kelch 13 artemisinin resistance mutations in Africa

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ABSTRACT

Artemisinin resistance (AR) emerged in South East Asia 13 years ago and the identification of the resistance conferring molecular marker, *Plasmodium falciparum* Kelch 13 (*Pfk13)*, 7 years ago has provided an invaluable tool for monitoring AR in malaria endemic countries. Molecular *Pfk13* surveillance revealed the resistance foci in the Greater Mekong Subregion, an independent emergence in Guyana, South America, and a low frequency of mutations in Africa. The recent identification of the R561H *Pfk13* AR associated mutation in Tanzania, Uganda and in Rwanda, where it has been associated with delayed parasite clearance, should be a concern for the continent. In this review, we provide a summary of *Pfk13* resistance associated propeller domain mutation frequencies across Africa from 2012 to 2020, to examine how many other countries have identified these mutations. Only four African countries reported a recent identification of the M476I, P553L, R561H, P574L, C580Y and A675V *Pfk13* mutations at low frequencies and with no reports of clinical treatment failure, except for Rwanda. These mutations present a threat to malaria control across the continent, since the greatest burden of malaria remains in Africa. A rise in the frequency of these mutations and their spread would reverse the gains made in the reduction of malaria over the last 20 years, given the lack of new antimalarial treatments in the event artemisinin-based combination therapies fail. The review highlights the frequency of *Pfk13* propeller domain mutations across Africa, providing an up-to-date perspective of *Pfk13* mutations, and appeals for an urgent and

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concerted effort to monitoring antimalarial resistance markers in Africa and the efficacy of antimalarials by re-establishing sentinel surveillance systems.

1. Introduction

Efficacious antimalarial drugs are a critical component of malaria control. They rapidly clear the parasite biomass following early detection of malaria infections, thus reducing the burden of disease. Chloroquine (CQ), was a low cost treatment that was the mainstay of uncomplicated *Plasmodium falciparum* malaria treatment for decades. CQ resistance and clinical failure was initially observed in South East (SE) Asia in the late 1950s along the Thai-Cambodia border, that later spread into Africa in the late 1970s and an independent emergence of resistance was described in Colombia, South America also in the late 1950s (Payne 1987; Wellems and Plowe 2001). Thereafter, Sulphadoxine-Pyrimethamine (SP) became the preferred, affordable alternative to CQ. However resistance to SP quickly emerged, mirroring the spread of CQ resistance, emerging in Thailand in the mid 1960s and then spreading into Africa (Roper et al., 2004) in the 1990s, with the independent emergence of SP resistance in Africa (Pearce et al., 2009) and South America (Cortese et al., 2002; Mita et al., 2007). The widespread resistance to SP by the mid 1990s rendered the drug ineffective, prompting a policy change in the treatment of uncomplicated malaria to artemisinin-based combination therapies (ACTs). Though SP was ineffective as a radical cure, it was in use until recently in combination with artesunate (WHO Report on antimalarial efficacy, 2020) and is still in use as a chemoprophylatic agent in Intermittent Preventive Treatment in pregnancy (IPTp) (ter Kuile et al., 2007), IPT in infants (Naidoo and Roper 2011) and seasonal malaria chemoprevention (SMC) in the Sahel region (WHO, SMC, 2012). The malaria infection prevalence and disease burden significantly increased in Africa between 1985 and 2004, and although it is not possible to determine the exact contributions, it seems plausible that drug resistance was a factor (Snow et al., 2001; Snow et al., 2017). In the last 2 decades, there has been a significant decline in the malaria disease burden in Africa (WHO Malaria Report, 2020), which may be partially attributed to the successful roll out of ACTs from 2001 onwards.

2. Molecular markers of resistance

The ~40 year delay between observing CQ clinical failure to defining the molecular marker of resistance meant that tracking resistance was initially based on clinical phenotype, limiting monitoring capacities of antimalarial resistance to the laborious therapeutic efficacy studies (TES) and a >25% treatment failure by day 14 (WHO Report on antimalarial efficacy, 2020). Nevertheless, these clinical data provided valuable data that prompted the change in antimalarial treatment policy in Africa. The subsequent identification of the *Plasmodium falciparum* chloroquine resistance transporter (*Pfcrt*) gene as the marker for CQ resistance in 2000 (Fidock et al., 2000; Djimde et al., 2001), made tracking the extent of CQ resistance possible. More recently in East (Mwai et al., 2009; Wamae et al., 2019) and Southern (Frosch et al., 2014) Africa it highlighted a reversion to CQ sensitive parasite populations. In Malawi specifically, CQ clinical efficacy has also been demonstrated (Laufer et al., 2006). In contrast, SP treatment failure was observed in combination with the molecular characterization of resistance conferring mutations (i.e. *Plasmodium falciparum* dihydrofolate reductase (*Pf*dhfr) triple and *Plasmodium falciparum* dihydropteroate synthase (*Pf*dhps) double mutants (Dieckmann and Jung 1986; Cowman et al., 1988), and when both treatment failure and molecular markers of resistance were found to be widespread this prompted the change in treatment policy to ACTs. Molecular markers are therefore a valuable tool for strengthening resistance monitoring, confirming resistance, providing an early warning signal and for assessing resistance trends (WHO Report on antimalarial efficacy, 2020).

3. Artemisinin resistance

The rapid identification of the artemisinin resistance (AR) molecular marker, *Plasmodium falciparum* kelch 13 (*Pfk13*) (Ariey et al., 2014), only 6 years after the initial observation of ACT clinical failure once again in SE Asia along the Thai-Myanmar and Thai-Cambodia borders (Noedl et al., 2008; Dondorp et al., 2009), changed the course of surveillance in SE Asia. . The *Pfk13* molecular marker made it possible to define the foci and origins of the emergence of resistance and its spread in the Greater Mekong subregion (Miotto et al., 2015). Initially, C580Y and three other loci (Y493H, R539T, I543T) were identified as the ART resistance conferring mutations associated with delayed parasite clearance (Ariey et al., 2014).

The *Pfk13* gene encodes a protein containing a conserved N-terminal domain followed by a BTB/POZ domain and a 6-blade propeller domain at the C-terminal end (Adams et al., 2000; Straimer et al., 2015). The validated mutations in the propeller domain associated with ART resistance, includes F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, P574L, C580Y and A675V (WWARN Genotype-Phenotype Study Group, 2019). These mutations arose independently along the Thai-Myanmar and Thai-Cambodia border regions and have since spread widely across SE Asia (Tun et al., 2015; Wang et al., 2015). Outside SE Asia, Papua New Guinea (Miotto et al., 2020) and Guyana (Mathieu et al., 2020) have reported the independent emergence of *Pfk13* mutations, notably the C580Y mutation.

In South America, where ACT clinical failure has not yet been confirmed, monitoring the *Pfk13* gene has allowed for the early identification of mutations that could potentially result in resistance to artemisinin. For instance, in Guyana an indegenious C580Y mutation, has been observed at a low frequency (Mathieu et al., 2020). In Africa, several studies have identified a number of low frequency *Pfk13* propeller domain mutations that are the focus of this brief review that leads to a plea to mount a concerted and deliberate approach to monitor *Pfk13* mutations across the continent.

4. Molecular epidemiology

Molecular epidemiology is an effective tool for the monitoring and tracking of parasite susceptibility to antimalarials, which in combination with drug efficacy trials enables prompt changes in treatment policy before clinical treatment failure negatively impacts control efforts. Given the immense success in the tracking the emergence and spread of CQ and SP resistant parasite across the globe, we have a robust framework to understand and monitor the spread of AR parasites from SE Asia. Furthermore, with a relatively small number of antimalarials in use (WHO Report on antimalarial efficacy, 2020) and the importance of ACTs to malaria control efforts in Africa, *Pfk13* mutants should be closely and systematically monitored in Africa. Numerous studies have been published examining *Pfk13* mutations across the continent (Kamau et al., 2015; Kayiba et al., 2020). A recent study conducted by Uwimana et al. (2020), between 2012 and 2015 in Rwanda, highlighted the local emergence of an ART resistance validated mutation, R561H. They also demonstrated through CRISPR-Cas genome editing that the 561H mutation conferred *in vitro* resistance to ART. In a reversal of fortune, molecular surveillance (the detection of the resistance mutation) preceded the clinical evidence of an association of the R561H resistance mutation with delayed parasite clearance (Uwimana et al., 2021). Thus, demonstrating that the utility of molecular surveillance is rapid and can be conducted at a much larger spatial scale than therapeutic efficacy

studies, allowing clinical studies to be targeted in high risk locations. Unfortunately, the *de novo* emergence of ART resistance markers in Africa is a concern, since the spread of resistant parasites across Africa would be catastrophic.

This brief review includes a collation of data from Africa, following two recent publications that reported the R561H mutation in Rwanda (Uwimana et al., 2020) and Tanzania (Moser et al., 2020). The R561H mutation is one of the 10 validated SE Asian AR associated mutations (WWARN Genotype-Phenotype Study Group, 2019) (Table 1), raising the concern that it may spread and lead to clinical impacts. Fortunately, it currently appears at a low frequency, and is limited to three separate studies from Rwanda, Tanzania and Uganda (Table 1). We therefore examined other African studies with parasite samples collected from 2012 to date, to determine whether these studies in the continent have also identified the R561H mutation or other propeller domain mutations that would give an indication of its spread.

5. Study selection criteria

We searched the PubMed database for peer reviewed articles from malaria endemic African countries published between January 1, 2016 and November 11, 2020, that had genotyped the *Pfk13* gene, focusing on propeller domain mutations. The restriction on publication date was to enable the review of recent publications that would encompass parasite samples overlapping with the Rwandan (Uwimana et al., 2020) and Tanzanian (Moser et al., 2020) studies and recent samples, i.e. from 2012 to date. The following search terms were used: "*((malaria OR falciparum) AND (molecular marker OR kelch13 OR Pfkelch13 OR K13 OR PfK13)) AND ((Africa OR Afrique OR country name [focusing on malaria endemic sub-Saharan African countries])* AND (("2016/01/01" [Date - Publication]: "2020/11/11" [Date - Publication]))". Three individuals independently screened the articles and excluded studies based on genetically modified parasites, reviews, conference proceedings, letters of correspondence and modelling studies. Additionally, six individuals extracted the following details from the articles: the year samples were collected, country of origin, age of study participants, first line ACT recommended in the country of origin, genotyping assay, study population (asymptomatic or symptomatic), sample size and polymorphisms across the propeller domain of the *Pfk13* gene.

6. Summary of the *Pfk13* mutations from the extracted literature

Recently, a systematic review identified *Pfk13* mutations across Africa that have been associated with ART resistance, though at low frequencies (Kayiba et al., 2020). Here we present data of *Pfk13* propeller domain mutations from >70 studies based on the PRISMA guidelines (Fig. 1) (Page et al., 2021) undertaken in Africa from samples obtained from 2012 to 2020, across 30 countries (Table S1, Full table accessible at Harvard Dataverse: https://doi.org/10.7910/DVN/EUPXCF). The *Pfk13* genotypes detected across many African countries were primarily wild-type with only Ghana reporting the C580Y mutation (Matrevi et al., 2019; Aninagyei et al., 2020) associated with the majority of ART resistance in SE Asia. There were no *Pfk13* mutations reported in Benin (2014), The Gambia (2012–2014) and Liberia (2014–2017) (Table S1). Only 3 other countries, apart from Rwanda, have reported the R561H mutation. In Tanzania, two parasites sampled from 764 were identified in 2017 (0.3%) (Moser et al., 2020) and a single parasite from 422 parasites sampled in 2019 (0.2%) (Bwire et al., 2020), similarly in Uganda one parasite was identified from 796 samples in 2018/2019 (0.1%) (Asua et al., 2020), no clinical failure was observed (consistent with the findings in Rwanda). In addition to the R561H mutation, the A675V mutation was also identified in four separate studies conducted in Uganda, showing an increase in its frequency to 5% in sample sets obtained in 2018 and 2019. The A675V mutation has also been described in a Rwandan and Nigerian study (Table 1). Only one high frequency propeller domain mutation was identified (i.e. A578S), which has been detected in 14 countries at frequencies of up to 11%. Another high frequency (>5%) *Pfk*13 mutation outside the propeller domain, K189T, has been identified in 8 countries. These high frequency mutations have not been associated with ART resistance or delayed parasite clearance.

The collated data provides an Africa-wide perspective on the current status of the prevalence of *Pfk13* mutations. The mutations known to be associated with delayed parasite clearance (i.e. M476I, P553L, R561H, P574L, C580Y and A675V) were observed at low frequencies (<5%) in four countries, Ghana, Rwanda, Uganda and Tanzania, indicating that we cannot be complacent regarding the potential spread of such mutations in Africa and that data on early signs of potential resistance to ACTs are essential. Notably, the data presented in this review is from 30 (67%) countries (Table S1) across 45 malaria endemic continental Africa and the islands of Madagascar, Sao Tome & Principe and Comoros (WHO Malaria Report, 2020), highlighting the paucity of molecular surveillance data and countries to target for future molecular surveys.

7. Conclusions

In comparison to the late identification of *Pfcrt*, *Pfk13* was identified swiftly after artemisinin clinical failure was observed, due to the rapid advances in whole genome sequencing technologies that has revolutionized our ability to detect important mutations.

Research teams in many African countries are using a wide range of techniques from the gold standard Sanger sequencing method through to amplicon deep sequencing, molecular inversion probes (MIPs) and whole genome sequencing to provide important data on the current state of *Pfk13* mutation prevalence. Molecular surveillance capacity is present in Africa to allow for the roll out of molecular surveillance tools, and African institutions have capacity to undertake the laboratory work and bioinformatics analyses across the continent. However, these studies are largely opportunistic and linked to specific research projects, rather than programmatic and linked to public health surveillance initiatives.

Based on historical evidence of antimalarial resistance to CQ and SP emerging in SE Asia and spreading to Africa, it was expected that ART resistance was likely to follow the same trajectory and hence the numerous studies to genotype *Pfk13*. However, the data on the *de novo* emergence of resistance mutations emphasize that such studies (Table S1) are important to provide a quick overview of mutation frequencies to inform policy decision making. With no new antimalarials immediately available, if ACTs fail, this threatens to reverse the significant gains made in the global reduction in malaria over the last 20 years (World Malaria report, 2020). Consequently, this recent detection of ART resistance mutations at codons R561H (recently associated with delayed parasite clearance), P574L, C580Y and A675V, serves as an early warning signal of potential resistance.

Currently, only 4 countries across malaria-endemic Africa have shown evidence of low frequency AR associated mutations, with an early indication of indigenous *Pfk13* mutations in the East Africa region, suggesting that the threat of independent acquisition of resistance should be taken as seriously as the threat of imported resistance. The data generated come from independent research projects, and hence a mechanism to report regularly on findings and interact with national and international policy makers has not been defined. There is therefore an urgent need for increased, standardized and prospective antimalarial resistance molecular surveillance across Africa.

How to intergrate molecular surveillance and clinical effectiveness to support decision making by National Malaria Control Programmes needs defining and needs investment in order to stay ahead of the ART resistance curve. Taking lessons from historically successful networks, twenty years ago, the East African Network for Monitoring Antimalarial Treatment (EANMAT) served as a flagship collaboration between sub-regional research scientists and national ministry of health case-management implementing and policy staff (EANMAT, 2001; EANMAT, 2003). The network successfully lobbied governments and donors

Table 1
Frequencies of *k13* validated mutations across Africa from 2012 to date.

Year	Study country	Reference	Assay	*N*	PfK13 mutations frequency % [n]									
					F446I	M476I[a]	Y493H[a]	R539T[a]	I543T[a]	P553L	R561H[a]	P574L	C580Y[a]	A675V
2019	Angola	Dimbu et al., 2021	Sanger	103										
2014	Benin	Ogouyèmi-Hounto et al., 2016	Sanger	78										
2012–2016	Botswana	Tawe et al., 2018	Sanger	127										
2014–2017	Cameroon	Moukoko et al. 2019	Sanger	175										
2017–2019	Central African Republic	Nzoumbou-Boko et al., 2020	Sanger	187										
2015–2016	Congo	Mayengue et al., 2018	Sanger	127										
2017	Democratic Republic of Congo	Yobi et al., 2020	Sanger	717										
2013–2014	Equatorial Guinea	Li et al., 2016	Sanger	98										
2014–2017	Eritrea	Pacheco et al., 2019	Amp-Seq	117										
2014	Ethiopia	Lo et al., 2017	Sanger	226										
2017–2018	Gabon	Adegbite et al., 2019	Sanger	100										
2012–2014	Gambia	Amambua-Ngwa et al., 2017	Taq-man/Sanger	184										
2014–2017	Ghana	Mensah et al. 2020	MIPs	619						0.2 [1]				
2007–2016	Ghana	Matrevi et al. (2019)	Sanger	854		0.1 [1]				0.1 [1]			0.1 [1]	
2017–2018	Ghana	Aninagyei et al. (2020)	WGS	84									3.6 [3]	
2016	Guinea	Beavogui et al., 2020	ND	411										
2016	Guinea-Bissau	Nag et al., 2019	Amp-seq	97										
2014–2019	Kenya	Omedo et al.,. Unpublished data	Amp-Seq	284										
2014–2017	Liberia	Pacheco et al., 2019	Amp-Seq	21										
2015–2016	Mali	Kone et al., 2020	WGS	216										
2015–2017	Mozambique	Gupta et al., 2020	Sanger	206										
2013	Niger	Laminou et al. 2017	Sanger	366										
2014–2017	Nigeria	Pacheco et al., 2019	Amp-Seq	29										3.4 [1]
2013–2015	Rwanda	Uwimana et al. (2020)	Sanger	466							4.1 [19]	0.2 [1]		
2014–2015	Rwanda	Tacoli et al. 2016	Sanger	147							0.7 [1]	0.7 [1]		0.7 [1]
2015–2019	Senegal	Delandre et al. 2020	Sanger	327										
2016–2017	Somalia	Warsame et al. 2019	Sanger	138										
2018	South Africa	Raman et al. 2019	Sanger	532										
2015–2017	Sudan	Hussien et al., 2020	Amp-Seq	176										
2019	Tanzania	Bwire et al. (2020)	Amp-Seq	422							0.2 [1]			
2017	Tanzania	Moser et al. (2020)	MIPs	764							0.3 [2]			
2012–2013	Togo	Dorkenoo et al. 2016	Sanger	500										
2012–2016	Uganda	Conrad et al. 2019	Sanger	716								0.1 [1]		0.1 [1]
2016–2017	Uganda	Asua et al. 2020	*ND*	412										1.7 [7]
2014–2016	Uganda	Ikeda et al., 2020	ND	194										0.5 [1]
2018–2019	Uganda	Asua et al. (2020)	MIPs	796							0.1 [1]			5.5 [44]
2017	Zambia	Sitali et al. 2020	Sanger	70										

[a] Represents validated SE Asian artemisinin resistance mutations. N is the number of samples that were successfully genotyped per study. n is the number of samples harbouring the respective mutation. ND = could not be determined. Assay represents the genotyping assay used in the respective study i.e. Sanger - Sanger sequencing, MIP - molecular inversion probes, WGS - whole-genome sequencing, Amp-Seq - amplicon sequencing. DR Congo stands for the Democratic Republic of Congo.

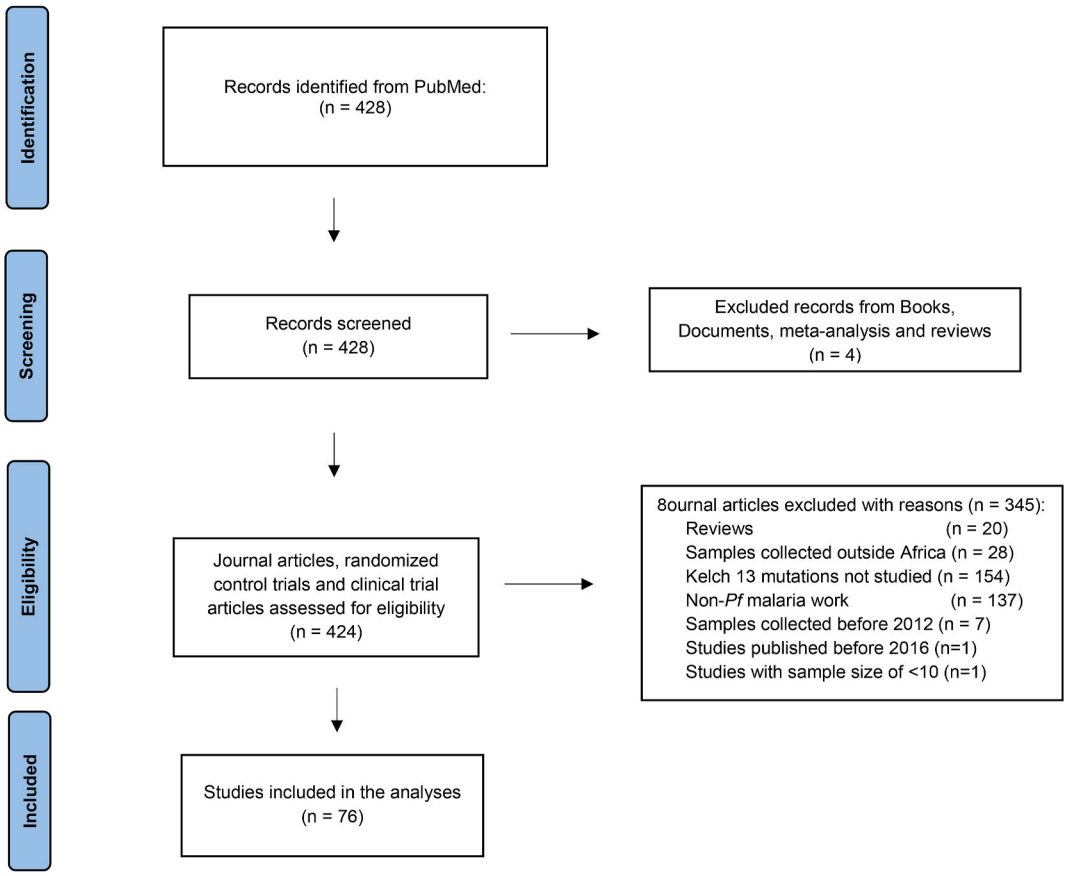

Fig. 1. Flow diagram of study selection criteria. The diagram indicates the numbers of publications identified in PubMed, manually screened and excluded to settle on a list of 86 publications reviewed for this study. An initial screen to exclude reviews, documents, books and meta-analyses was conducted. A further selection process was done based on the following non-eligibility criteria to exclude studies that were: reviews, from outside Africa, not examining Kelch 13, non-*Plasmodium falciparum* (Pf), parasite samples collected before 2012, published before 2016 and a low sample size <10 to obtain 76 publications. The 86 publications that met the criteria, focusing on kelch 13 mutations in Africa in either a standard research or clinical trial article.

to switch to ACTs that were expected to last for a long time, in 2004, however EANMAT has been inactive since 2004. Re-establishing sentinel surveillance sites to formulate evidence-based antimalarial drug policies through sub-regional technical networks for monitoring antimalarial resistance such as EANMAT, West African Network for Monitoring Antimalarial Treatment (WANMAT) and Horn of Africa Network for Monitoring Antimalarial Treatment (HANMAT) (EANMAT, 2000; EANMAT, 2001; Talisuna et al., 2006; HANMAT Report 2013), would provide an effective framework to combine longitudinal, sentinel site surveillance and clinical therapeutic efficacy studies (TES). In addition, including the new recommendation of a change in drug policy if day 28 or day 42 efficacy falls below 90% in TES (WHO Report on antimalarial efficacy, 2020) to transform policy and provide a holistic view of antimalarial resistance in Africa.

Author contributions

L.N., K.M.K. and K.W. conducted the literature review and drafted the manuscript. I.O. V.O. and M.A. conducted the literature review and reviewed the manuscript. B.A., A.G., D.S.I., A.A-N., N.D-Q., S.K.T., C.K., S.T., A.A.D., and A.D. reviewed the manuscript. J.R. and R.W.S. contributed to the literature review and reviewed the manuscript. P.B. and L.I.O–O. conceived, drafted and reviewed the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

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