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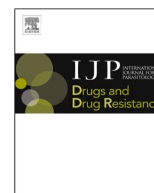

Surveillance of molecular markers of *Plasmodium falciparum* artemisinin resistance (*kelch13* mutations) in Papua New Guinea between 2016 and 2018

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ABSTRACT

Plasmodium falciparum resistance to artemisinin-based combination therapy (ACT) is a global threat to malaria control and elimination efforts. Mutations in the *P. falciparum kelch13* gene (*Pfk13*) that are associated with delayed parasite clearance have emerged on the Thai-Cambodian border since 2008. There is growing evidence of widespread *Pfk13* mutations throughout South-East Asia and they have independently emerged in other endemic regions. In Papua New Guinea (PNG), *Pfk13* "C580Y" mutant parasites with reduced *in vitro* sensitivity to artemisinin have been isolated in Wewak, a port town in East Sepik Province. However, the extent of any local spread of these mutant parasites in other parts of PNG is unknown. We investigated the prevalence of *Pfk13* mutations in multiple malaria-endemic regions of PNG. *P. falciparum* isolates (n = 1152) collected between 2016 and 2018 and assessed for *Pfk13* variation by sequencing. Of 663 high quality *Pfk13* sequences a total of five variants were identified. They included C580Y, a mutation at a previously documented polymorphic locus: N499K, and three previously undescribed mutations: R471C, K586E and Y635C. All variants were found in single isolates, indicating that these *Pfk13* mutations were rare in the areas surveyed. Notably, C580Y was absent from Maprik district, which neighbours Wewak where C580Y mutant parasites were previously identified. The single C580Y isolate was found in the port town of Lae, Morobe Province, a potential entry site for the importation of drug resistant parasites into PNG. Although sample size in this location was small (n = 5), our identification of a C580Y mutant in this second location is concerning, highlighting the urgent need for further surveillance in Lae. Other *Pfk13* mutants were rare in PNG between 2016 and 2018. Continued surveillance for molecular markers of drug resistance is critically important to inform malaria control in PNG.

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1. Introduction

Resistance of the human malaria parasite, *Plasmodium falciparum* to antimalarial drugs is a public health concern for malaria endemic countries. The parasite's ability to develop resistance to antimalarial drugs presents significant challenges for malaria treatment, control and elimination (World Health Organisation, 2019). Historically, South-East Asia has been the epicentre of single and multi-drug resistant malaria and remains a hotspot for emerging antimalarial resistance (Ashley et al., 2014; Mita et al., 2009). Currently, the World Health Organisation (WHO) recommends the use of artemisinin-based combination therapy (ACT) as first-line treatment for uncomplicated malaria (World Health Organisation, 2015). While it remains the most effective and widely used treatment in many malaria endemic countries, the growing number of countries of emerging artemisinin resistant parasites is a great concern (Leang et al., 2015; Mathieu et al., 2020; Menard et al., 2016; Uwimana et al., 2020).

Artemisinin resistance (ART-R) is characterised clinically by a delay in parasite clearance following an artemisinin-based therapy (Dondorp et al., 2009). This phenotype was first observed on the Thai-Cambodian border between 2006 and 2008 following both artemisinin mono- and combination therapy and has been linked to non-synonymous single nucleotide polymorphisms (NS-SNPs) found in the *P. falciparum* chromosome 13 *kelch* gene (*Pfk13*) (Ariey et al., 2014; Dondorp et al., 2009; Noedl et al., 2008; Straimer et al., 2015). Genetic analysis of *P. falciparum* isolates shows that *Pfk13* mutations Y493H, R539T, I543T and C580Y were associated with increased ring-stage parasite survival rates in *in vitro* drug resistance assays and prolonged parasite clearance times *in vivo* (Ariey et al., 2014; Witkowski et al., 2013). Several *Pfk13* mutations, some of which are associated with ART-R, have emerged independently in multiple regions along the Thai-Cambodian and Thai-Myanmar border and have spread throughout the Greater Mekong Region (Miotto et al., 2013; Takala-Harrison et al., 2015; Talundzic et al., 2015). Initially, a soft-selective sweep influenced the emergence and spread of these *Pfk13* mutant alleles (Miotto et al., 2015; Takala-Harrison et al., 2015), however this evolved to a hard-sweep in which the *kelch13* Haplotype Group 1 (KEL1) lineage, within which C580Y is commonly found, became the dominant lineage in artemisinin-resistant parasite populations (Amato et al., 2018). Consequently, C580Y appears to be outcompeting other mutants and reaching near-fixation in the South-East Asian population (Imwong et al., 2017). The continued use of ACTs in South-East Asia and its cumulative drug pressure on these artemisinin-resistant parasite populations has also resulted in parasite resistance to partner drugs.

PNG carries the highest burden of malaria in the WHO Western Pacific region, accounting for more than 70% of clinical cases (World Health Organisation Regional Office for the Western Pacific, 2017). In 2011, the PNG National Department of Health implemented ACTs into its treatment regimen with artemether-lumefantrine (AL) and DHA-PPQ as first- and second-line treatments respectively, for uncomplicated malaria infections (Papua New Guinea National Department of Health: National malaria treatment protocol, 2009). This was a result of high treatment failure rates associated with a high prevalence of drug resistance mutations limiting the efficacy of the previous treatment regimen of chloroquine plus SP (Casey et al., 2004; Genton et al., 2005; Karunajeewa et al., 2008; Marfurt et al., 2008, 2010). The existing ACT regimen has so far remained efficacious (Tavul et al., 2018), with no evidence of treatment failures in ongoing therapeutic efficacy studies by the PNG National Department of Health and PNG Institute of Medical Research (PNGIMR) (World Health Organisation, 2020). However, two recent reports have identified the *Pfk13* C580Y mutation in a traveler returning to Australia from West New Britain Province, PNG in 2012 (Prosser et al., 2018), and in three malaria patients in presenting to a clinic in East Sepik Province in 2017 (Miotto et al., 2020). Whole genome sequence analyses showed haplotype sharing with both Indonesian and PNG parasite populations, thus indicating continuous recombination events between imported and local parasites (Miotto et al., 2020). This raises important questions regarding the presence of artemisinin resistance associated *Pfk13* mutations in the broader PNG parasite population, their prevalence and implications for the existing ACT first line treatment regimen.

The PNG Institute of Medical Research together with the National Department of Health has conducted *Pfk13* surveillance in PNG for many years. Previous analysis of 43 *P. falciparum* isolates collected in 2013 and 2014 from 12 locations across the country did not identify any *Pfk13* mutations (Menard et al., 2016). In addition, genotyping of clinical infections from therapeutic efficacy studies conducted in the Milne Bay (Alotau) and East Sepik (Maprik) Provinces in 2011–2014 identified only wild type *PfK13* alleles (unpublished data). Here we have investigated the presence of *Pfk13* mutations in PNG by screening a large number of previously collected *P. falciparum* samples from cross-sectional and nationwide surveys conducted between 2016 and 2018. We establish the prevalence of *Pfk13* mutations in a number of locations throughout PNG during this time frame and screen for the presence of *Pfk13* mutations that may reduce artemisinin efficacy.

2. Materials and methods

2.1. Ethics and informed consent

Approval for the use of human blood samples for investigating molecular markers of antimalarial drug resistance was provided by the PNG Medical Research Advisory Committee (MRAC) and the PNGIMR Institutional Review Board (IRB) (2016-17 MIS: MRAC 15.21, IRB 1512, International Centre for Excellence in Malaria Research (ICEMR MRAC:11.12, IRB:1116), WHO Tropical Disease Research Residual Malaria Call 2015 (TDR MRAC:16.08, IRB:1517), Australia-PNG-Trilateral Malaria Project (TMP MRAC:17.11, IRB:1711)) and by the Walter and Eliza Hall Human Research and Ethics Committee (HREC: 13.14 and 12.10). All samples were collected by voluntary consent from participants or their parents or guardians.

2.2. Study sites and samples

Samples were compiled from four studies (Table 1) conducted in collaboration between the PNGIMR and the National Department of Health (NDOH) in malaria-endemic provinces across PNG. These included: (i) Malaria positive RDT (mRDT) from the national malaria indicator survey (MIS) in 2016/17, (ii) Dried blood spots (DBS) from consenting febrile and mRDT-positive individuals attending four health facilities in malaria sentinel site surveillance from 2017 to 2018, Australia-China-PNG Trilateral Malaria Project), (iii) Capillary blood (200–350 μL) from individuals > 6months in two cross-sectional surveys in 2016 in the highly malaria endemic East Sepik (ICEMR) and Madang (TDR) provinces. Due to low DNA concentration of extracted samples from RDTs (MIS), 8 of 13 provinces in the malaria indicator survey could be assessed.

2.3. Laboratory procedures

2.3.1. Genomic DNA extraction

Genomic DNA was extracted from the DBS and red cell pellets samples using the Favorgen 96-Well Genomic DNA Kit (Favrogen Biotech Corp. Taiwan) as per the manufacturer's instructions. With the RDT samples, each RDT cassette was disassembled to access the internal cellulose membrane strip where two 3-4 mm-wide pieces were cuts from the sample pad area. Genomic DNA was extracted from these pieces using the Chellex-Saponin method (Plowe et al., 1995) as well as the QIAamp DNA Mini Kit (QIAGEN N.V) according to the manufacturer's instructions.

Table 1
Samples genotyped for *Pfk13* mutations.

Study[1]/Year of collection	Survey Type/Population	Sample Type	*Pf* qPCR positive	*Pfk13* PCR positive	Sequencing (% *of Pf* isolates screened)
MIS 2016/17	Community Survey/General population)	RDT Strips	469	58	38 (8.1)
ICEMR Cross sectional 2016	Clinical (>6 months)	Capillary blood/ Microtainer	192	181	178 (92.7)
TDR Cross sectional 2016	Cross-Sectional Survey (>6months)	Capillary blood/ Microtainer	384	360	353 (91.9)
TMP 2017/18	4 Sentinel Sites, Clinical (General population)	Dried Blood spot/Filter paper	107	107	94 (87.8)
Total			1152	706	663 (57.5)

- [1]National malaria indicator survey (MIS 2016/18) from 13/22 province. Malaria RDT (available from 13/22 provinces; general population sample, all ages.
- ICEMR East Sepik cross-sectional survey 2016.
- Tropical Disease Research (TDR) Grant cross-sectional survey in Madang Province in 2016.
- Australia-China-PNG Trilateral Malaria Project (TMP): Sentinel site surveillance 2017/2018.

2.3.2. Identification and quantification of Plasmodium parasite DNA

Parasite DNA detection and quantification was determined using a previously described *Plasmodium* species-specific real-time PCR TaqMan assay where parasite density within the sample was determined by using a DNA template of known starting concentration (copy number) serially diluted from 1×10^5 down to 5 copies/μL and assayed in the same run. A standard curve in linear regression was generated using the log of the starting copy number and unknown sample densities were interpolated from the standard curve (Rosanas-Urgell et al., 2010).

2.3.3. Amplification and sequencing of Pfk13 gene

DNA samples positive for *P. falciparum* by quantitative PCR were used as template in a nested PCR reaction to amplify the *BTB-POZ* domain of the *Pfk13* gene known to be associated with artemisinin resistance. The protocol was adapted from a previously published protocol (Menard et al., 2016) with slight modifications with the reaction conditions. In brief, 2–4 μL of genomic DNA was combined with 2x Reaction Buffer (Solis Bio Dyne), 0.5 μM of each primer, 2.5 mM $MgCl_2$, 0.25 mM dNTP and 1.25 U Hot Start Taq Polymerase (Solis Bio Dyne, Denmark) in a 20 μL volume for both primary: PF 5′-CGGAGTGACCAAATCTGGGA-3′ and PR 5′-GGAATCTGGTGGTAACAGC-3′) and nested reactions: NF 5′-GCCAAGCTGCCATTCATTTG and NR 5′-GCCTTGTTGAAAGAAGCAGA-3′). Primary reaction thermocycling conditions included a hot start of 95°C (to activate the Taq Polymerase) for 15 minutes, followed by 30 cycles at 95°C for 30 seconds, 58°C for 1 minute, 65°C for 1 minute 40 seconds, and final extension for 10 minutes at 65°C. Secondary cycling conditions were similar to that of primary reaction except the annealing and extension temperatures were 60°C for 30 seconds and 1 minute respectively for 35 cycles. Final PCR reactions were assessed for *Pfk13* amplification by gel-electrophoresis in a 1% Agarose gel stained with 1x ethidium bromide DNA staining dye and visualised using the Bio-Rad XR + Gel Electrophoresis Documentation System. Reactions showing successful DNA amplification of 850–900 bp fragments were sent to Macrogen Inc. (Korea) for Sanger sequencing. Sequence electropherograms were trimmed and aligned to the *Pfk13* 3D7 clone reference PF3D7_1343700 (PlasmoDB) using Geneious Version 6.1.8 software (Biomatters LTD, New Zealand).

3. Results

A total of 1152 *P. falciparum* positive samples were assessed from eight of the thirteen locations (Table 1). The five locations not assessed were included in the RDT sample set that overall performed poorly, due to low DNA quality. *P. falciparum* positive samples were screened by PCR to amplify an 850 (Talundzic et al., 2015) base pair fragment of *Pfk13*. From this, 706 (61%) samples that were successfully amplified were subjected to traditional Sanger sequencing. Sequencing returned 663 high quality sequences and when aligned to the PF3D7_1343700 reference revealed six NS *Pfk13* SNPs resulting in variations in the protein product. These included C580Y and in a previously described mutant position N499D (Ouattara et al., 2015) we identified a novel allele: N499K. Three additional newly identified mutants were found: R471C, K586E and Y635C (Fig. 1). In addition, three synonymous substitutions were found at codons numbers 491, 536 and 628 (Table 2). All NS mutations were identified in single isolates. This was also the case for synonymous mutations, except for the substitution in codon 491, which was found in two isolates from Madang and Milne Bay (Table 2).

4. Discussion

The decreasing sensitivity of *P. falciparum* to first-line malaria treatments is a continuing global health problem with the emergence and expansion of an artemisinin resistant C580Y population (KEL/PLA1) originating from Western Cambodia in 2008 (Amato et al., 2017, 2018; Dondorp et al., 2009; Noedl et al., 2008). The discovery of C580Y mutant parasites in PNG is a major concern to the national malaria control programme since AL is first line treatment (Pulford et al., 2013). By screening a large number of archived *P. falciparum* samples from epidemiological surveys and sentinel surveillance conducted in PNG between 2016 and 2018, we were able to examine the prevalence of *Pfk13* mutations in eight endemic regions of the country. The results suggest that between 2016 and 2018 there were *Pfk13* mutations in geographically distinct endemic regions of the country, including one isolate in Lae, Morobe Province found to be carrying the C580Y mutation.

Whilst all *Pfk13* mutations were found to be present in only one isolate each and therefore considered rare, the C580Y mutation was detected in a population with a very small sample (n = 5). Due to the low quality of the DNA extracted from stored RDTs for Morobe, only a small number of samples were successfully genotyped. The prevalence of this mutation therefore may not be accurate, with further sampling at this site an urgent priority. Nevertheless, the presence at all of the C580Y mutation in isolates in Morobe in 2018 (this study) is concerning, with previous studies identifying the mutation several hundred kilometres away in Wewak, East Sepik Province in 2017 (Miotto et al., 2020). Firstly, due to its known phenotypic association with *in vitro* artemisinin resistance and secondly, its identification in a densely populated urban port town Lae (Morobe), that is a staging point for transport into other provinces, a hub for frequent human movement. Of note, Lae's locality is highly favourable for parasite importation either from the north coast region (Wewak) or West New Britain where one other C580Y mutant is believed to have originated (Prosser et al., 2018). In terms of cross-border (PNG-Indonesia) importation, there is no evidence to date of C580Y mutations in Indonesian parasites, suggesting this is unlikely. However, samples have so far only been surveyed in a single location (Timika) on the Indonesian side of New Guinea (Miotto et al., 2020).

Our discovery of a C580Y mutant in Morobe, but lack of C580Y mutants in other sites, including the area surrounding Maprik, only 50

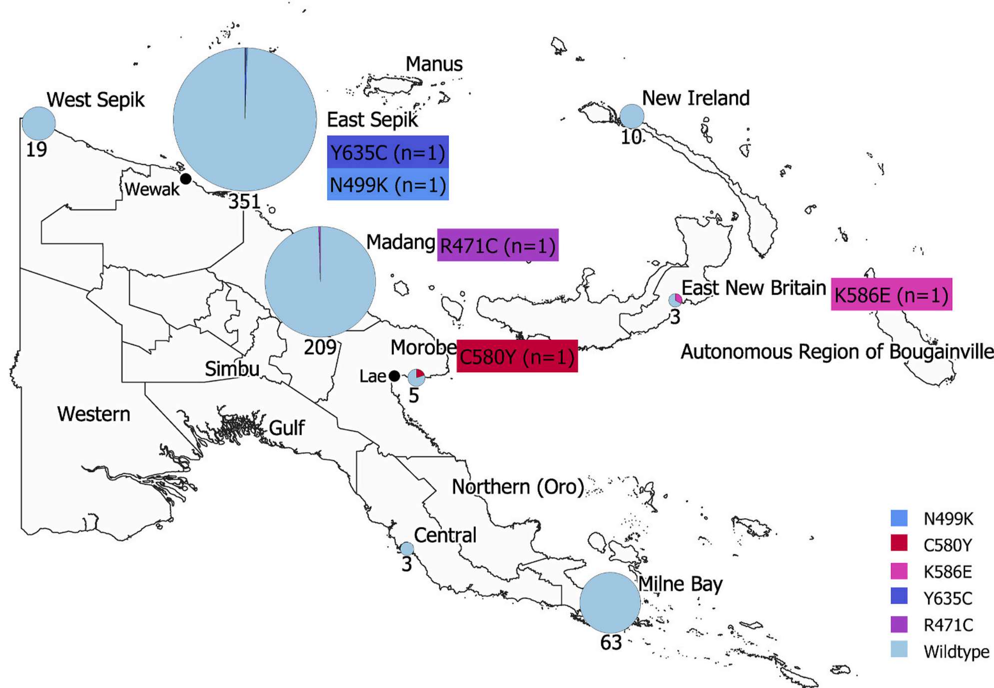

Fig. 1. Map showing the prevalence of the *Pfk13* mutations in samples that were successfully sequenced (n = 633). The size of the pie graph represents the number of samples successfully sequenced per locations. Five locations, Western, Northern, Simbu, Manus and Bougainville had RDT samples that performed poorly therefore were not assessed.

Table 2
Pfk13 mutations identified by year and location. Mutations showing amino acid changes and proportion of mutations in the 663 samples that were successfully sequenced (n; number of mutants, N; samples successfully sequenced per site).

Location	Year sample collected			Nucleotide coordinate	Frequency (n/N)
	2016	2017	2018		
Non-synonymous mutation (amino acid change)					
Madang		R471C		1411_C/T	0.48 (1/209)
East Sepik	N499K*			1497_C/A	0.28 (1/351)
Morobe		C580Y*		1739_G/T	20 (1/5)
East New Britain	K586E			1756_G/A	33.3 (1/3)
East Sepik	Y635C			1904_A/T	0.28 (1/351)
Synonymous mutations (nucleotide change)					
Milne Bay		†		1473_T/C	0.16 (1/63)
Madang		†		1608_A/G	0.28 (1/209)
Madang	†	†		1473_T/C	0.48 (1/209)
East Sepik	†			1884_T/C	0.28 (1/351)

*Previously described *Pfk13* mutations.
†Indicating the synonymous mutation at that particular year.

km inland from Wewak where C580Y mutants have been detected (Miotto et al., 2020), suggests that ports may represent an important entry point into PNG for imported drug resistant parasites. It is also important to factor in other influences that may contribute to the emergence and potential spread of C580Y mutants in PNG. One such factor could be the influence of low malaria transmission on emergence of drug resistance. Malaria transmission in PNG had dropped significantly after the nationwide distribution of long-lasting insecticide treated mosquito nets (LLIN) and later implementation of ACT (Hetzel et al., 2015, 2017) but has resurged in recent years (PNGIMR, 2018). During low transmission periods, polyclonal infections are less frequent in PNG (Koepfli et al., 2017) and linkage disequilibrium between distant loci increases (Anderson et al., 2000; Kattenberg et al., 2019). Immunity also decreases, whereby inadequate immunity prevents the elimination of parasite strains that may have survived drug treatment and therefore increases their chance for selection (Ataide et al., 2017). This may provide ideal conditions for the emergence of *Pfk13* C580Y mutant parasites in PNG. Whatever the case, it will be important to characterise any new C580Y mutants that are found through surveillance programs in terms of their genomic relationships with previously identified mutants, to establish whether these mutations are spreading or emerging independently in different locations.

Several other *Pfk13* mutations were identified in the genetic surveillance conducted in this study. The N499K mutation is located at a documented polymorphic position found in *Pfk13* in African parasites, where an alternative allele, namely N499D, has been observed (Fairhurst, 2015; Taylor et al., 2015), although there is no evidence to date of its association with artemisinin resistance. The remaining *Pfk13* mutations, R471C, K586E and Y635C are novel mutations and polymorphic positions that have not been described elsewhere, to our knowledge. While all *Pfk13* mutations observed in these 2016-18 samples were rare, identified in only one sample each, they are indicative of the selective pressure occurring within the local parasite population. Evaluation of any mutants for their association with phenotypic resistance will be important to establish whether they pose any risk to artemisinin effectiveness in PNG.

To date, there is no clinical evidence of resistance to ACTs in PNG, since ACTs remain efficacious in therapeutic efficacy studies (World Health Organisation, 2019). However, the occurrence of *Pfk13* mutations in PNG's parasite population indicates underlying genetic changes, potentially as a result of ACT exposure. Continued drug exposure in

malaria parasites has been known to induce genomic variations in the organism that enable drug tolerance and survival (Hamilton et al., 2019). However, not all *Pfk13* mutations will be associated with delayed parasite clearance and resistance to artemisinin. For example, in Africa the most common allele A578S, which occurs in low frequency in the African parasite populations and also present in South-East Asian artemisinin resistant parasites, shows no association with delayed parasite clearance or artemisinin resistance in African parasites (Amaratunga et al., 2019; Kamau et al., 2015; MalariaGEN, 2016). While this may be the case for the rare novel mutations found in this study, C580Y is a well-validated and extremely common artemisinin resistance allele throughout South-East Asia (Ariey et al., 2014) and has now emerged independently in multiple locations (Mathieu et al., 2020; Miotto et al., 2020).

The findings from this study are critical for the control of malaria and demonstrate the need for ongoing genetic surveillance to determine the emergence and spread of drug resistant parasites and rigorous therapeutic efficacy studies (TES) to establish possible impacts on treatment efficacy. These studies are particularly urgent in and around regions where C580Y mutants circulate as well as among populations of frequent immigration, while parasite genotyping for drug resistance mutations will require a robust and highly sensitive methods that can be routinely conducted in PNG.

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2021.06.004.

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