Molecular surveillance of pfcrt, pfmdr1 and K13-propeller mutations in Plasmodium falciparum isolates imported from Africa to China

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Research

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Abstract

Background

The emergence and spread of multidrug resistance pose a significant risk to malaria control and eradication goals in the world. There has been no indigenous malaria cases reported since 2017, and China is approaching malaria elimination by 2020. Therefore, it is urgent to monitor antimalarial drug resistance and track the emergence and spread of the imported drug resistant malaria cases in China.

Methods

Dried blood samples were obtained from *P. falciparum* infected cases who returned from Africa to China between 2012–2015 prior to antimalarial drug treatment. The known polymorphisms relating to drug resistance of *pfcr*t, *pfmdr1* gene, and the propeller domain of the K13 gene were evaluated by nested PCR and sequencing. The GraphPad prism was used to plot the prevalence of each mutation. The chi-squared test and two-sided *P* value of < 0.05 were used to evaluate differences with statistical significance.

Results

A total of 731 *P. falciparum* isolates were successfully genotyped at the *pfcr*t locus. The wild type haplotype of C_{72}V_{73}M_{74}N_{75}K_{76} was the most prevalent genotype with the prevalence of 62.8% and 29.8% of the isolates showed the triple mutant haplotype C_{72}V_{73}I_{74}E_{75}T_{76}. Total 434 *P. falciparum* isolates were successfully sequenced and *pfmdr1* allelic variants were only observed in codons 86, 184, and 1246. Twelve haplotypes were identified and the prevalence of the wild type variant *pfmdr1* N_{86}Y_{184}D_{1246} was 44.1%. The single mutant *pfmdr1* in codons 86 and 184 was predominant but D1246Y was rare. The double mutant genotype Y_{86}F_{184}D_{1246} was common in Africa. A total of 1381 *P. falciparum* isolates from 33 African countries were sequenced of K13-propeller domain and 1357 were successfully sequenced. The prevalence of K13 mutations was 3.6% and 26 different mutant alleles including 22 nonsynonymous and four synonymous variants (C469C, R471R, G496G, and A627A) were observed. The A578S was the most common haplotype of K13. Three mutation associated with artimisinin resistance (M476I, R539T, and P553L) were observed in three isolates.

Conclusion

The prevalence of mutant *pfcr*t and *pfmdr1* was low or moderate in *P. falciparum* isolates imported from Africa to China between 2012–2015. K13-propeller mutations were highly diverse but most mutations were only found in a few isolates. This study provides evidence for the antimalarial drug resistance level of the imported malaria cases from Africa and the efficacy of antimalarial drug policy in China to treat these imported cases.
Background

Historically, malaria was one of the most serious infectious diseases in China. China has made great contributions towards global malaria control in the past 40 years. In 2010, China launched the National Malaria Elimination Programme (NMEP) 2010–2020 with the goal to interrupt local malaria transmission by 2020. Over the following 5 years, malaria cases decreased dramatically and there has been no indigenous malaria cases reported since 2017 [1]. In 2020, China was close to achieving malaria elimination nationwide under a strong case-based surveillance and response system. However, with increasing globalization, larger numbers of people entering or returning from malaria endemic areas present challenges to malaria elimination in China [2]. According to the national malaria report, there were still more than 2500 imported cases annually including over 100 patients with severe symptoms and approximately 10 deaths in 2017 and 2018 [3].

Over the past 50 years, \textit{P. falciparum} has developed resistance to all antimalarial drugs that have been used including chloroquine (CQ), amodiaquine, sulfadoxine-pyrimethamine (SP), quinine, piperaquine, and mefloquine. Recently, the emergence and spread of multidrug resistance including artemisinin and partner drug resistance of \textit{P. falciparum} in Southeast Asia pose a significant risk to malaria control and eradication goals in the world. World Health Organization (WHO) had implemented a strategy to eliminate \textit{P. falciparum} from the six countries located in the greater Mekong subregion (GMS) by 2025 to respond to the threat of an untreatable multi-drug resistant parasite [4]. Mutations in the \textit{P. falciparum} gene encoding a kelch protein on chromosome 13, Kelch13 (K13) are associated with artemisinin resistance and have arisen multiple times and spread in the GMS. Over 200 nonsynonymous \textit{P. falciparum} K13 mutations have been reported to date, of which nine validated variants (F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H, and C580Y) and over 20 K13 mutations are considered as candidates markers [5]. K13 mutations were detected predominantly in the GMS and were rare in Africa, and their profile was highly heterogeneous [6, 7].

Mutations in \textit{P. falciparum} chloroquine resistance transporter (\textit{pfCRT}), located on the digestive vacuole membrane, were responsible for CQ resistance or treatment failure [8, 9]. Polymorphisms affecting amino acids at \textit{pfCRT} residues 72–76 were observed in CQ resistant field isolates, whereas \textit{pfCRT} C72V73M74N75K76 haplotype was regarded as CQ sensitive isolates [10, 11]. Polymorphisms in the \textit{pfmdr1} gene, the gene encoding the plasmodial homologue of mammalian multidrug resistance transporters, have previously been linked with antimalarial drug resistance [12–15]. The mutations involving \textit{pfmdr1} codons N86Y, Y184F, S1034C, N1042D, and D1246Y have been proven to be associated with mefloquine, lumefantrine, amodiaquine, CQ, and artemisinin, as well [16, 17].

Molecular surveillance of antimalarial drug resistance markers is one of the surveillance tools to monitor and track the emergence and spread of drug resistance in the imported malaria cases in China. This study collected the data of reported malaria cases from the national malaria case report system between 2012–2015, which were used to analyze the epidemic change of the total reported cases and imported cases. Dried blood samples were collected from \textit{P. falciparum}-infected individuals imported from Africa.
at the time of malaria diagnosis in 2012–2015. The prevalence of pfcr, pfmdr1, and K13 mutations were measured by PCR and sequencing. The geographical distribution of the haplotypes of pfcr, pfmdr1 and K13 genes in the imported P. falciparum isolates from Africa were mapped.

Materials And Methods

Reported malaria cases

The data of all reported cases, including indigenous and imported cases, were collected from the Chinese Infectious Disease Report System (CIDRS), a web-based reporting system between 2012 and 2015. Adhering to the “1-3-7” strategy of the NMEP, a patient must be confirmed by microscope, rapid diagnosis test (RDT), or clinical test before the case was reported into the e-data system, and demographic data were recorded, including travel history, and/or imported source countries. The epidemic changes of the imported malaria cases were analyzed, and the main source countries were identified.

Sample collection

Dried blood samples were obtained from P. falciparum infected cases who returned from Africa to China in 2012-2015 prior to antimalarial drug treatment. Microscopic examination of Giemsa-stained thick smears or RDT were used for malaria diagnosis within 24 hours before the case was reported. Nested polymerase chain reaction (PCR), amplifying the small-subunit rRNA gene of Plasmodium spp. [18] was used to confirm the positive samples and the species before the antimalarial drug resistance markers were genotyped.

DNA extraction and PCR

The whole DNA was extracted by a QIAamp DNA mini kit as described by the manufacturer. The known polymorphisms relating to drug resistance at codons 72, 74, 75, 76 of the pfcr gene and codons 86, 130, 184, 1034, 1042, 1109, 1246 of the pfmdr1 gene, and also mutations on the propeller domain of the K13 gene, were evaluated by nested PCR [13, 19-21]. The target amplified fragments covering polymorphic sites were as follows: amino acid position 51-83 (145 bp) for pfcr, amino acid position 69-228 (526 bp) and 1030-1282 (799 bp) for pfmdr1, and amino acid position 433-702 (849 bp) for K13-propeller.

Sequencing

The output sequence data were assembled, edited, and aligned using Sequencher (version 5.1) software. All mutations were assessed by comparing each sequence to the 3D7 reference strain PF3D7_0709000 (pfcr), PF3D7_0523000 (pfmdr1) and PF13_0238 (K13).

Statistical analyses

Data was entered into Microsoft Excel 2017 and analyzed by R software (version 4.0.2). The GraphPad prism 8.3.0 (GraphPad Software Inc., San Diego, CA, USA) was used to plot the prevalence of each
mutation. The chi-squared test and two-sided $P$ value of $< 0.05$ were used to evaluate differences with statistical significance.

**Results**

**Malaria epidemic in China**

The reported cases of malaria decreased to a low level with only thousands of cases in 2012-2015 compared with that was hundreds of thousands cases before 2010 when the NMEP had not been launched. A total of 42 counties in the entire country reported indigenous cases in 2012 and decreased to only nine counties in 2015 (Additional file 1). The proportion of imported cases has remained at more than 90% since 2012, and only 244 indigenous cases were reported in 2012. Since 2013 the number of indigenous cases has dropped below 100 and most cases were reported from Yunnan Province and Tibet in southern China (Additional file 2). In 2017, no indigenous cases were reported in the country for the first time. Nevertheless, the proportion of imported *P. falciparum* cases increased from 2012 ($n=1403, 57.3\%$) to 2015 ($n=1895, 61.6\%$).

The imported cases originated from four continents and more than 70% were from mainly central and west Africa. The main source countries of imported malaria cases in China are shown in Table 1. Ghana, Angola, Equatorial Guinea, and Nigeria have always been main source countries in Africa.

**Polymorphisms of pfcrt**

A total of 731 *P. falciparum* isolates collected from imported cases form Africa between 2012-2015 were successfully genotyped at the *pfcrt* locus. The wild type haplotype $C_{72}V_{73}M_{74}N_{75}K_{76}$ was the most prevalent genotype (62.8%, 459/731) but still 29.8% (218/731) of the isolates showed the triple mutant haplotype $C_{72}V_{73}I_{74}E_{75}T_{76}$. The mixed genotypes $C_{72}V_{73}M/I_{74}N/E_{75}K/T_{76}$ were identified in 35 isolates from west, central, and southern Africa but none from east or north Africa. The prevalence of wild type *pfcrt* was predominant in east Africa (81.1%, 43/53) and lowest in central Africa (58.3%, 140/240). The prevalence of triple mutation haplotype $C_{72}V_{73}I_{74}E_{75}T_{76}$ was also highest in Central Africa (32.9%, 79/240) (Fig. 1). The mutant prevalence of *pfcrt* at codons 74, 75, 76 was showed in Table 2.

**Polymorphisms of pfmdr1**

Total 434 *P. falciparum* isolates were successfully sequenced and *pfmdr1* allelic variants were only observed in codons 86, 184, and 1246. In total, 12 haplotypes were identified including 6 mixed mutation genotypes. The prevalence of wild type *pfmdr1* $N_{86}Y_{184}D_{1246}$ was 44.1%. Comparing the prevalence of *pfmdr1* wild type in the subregion of Africa, the region of highest prevalence was southern Africa and the lowest was east Africa. Three single mutant haplotypes in codons $Y_{86}Y_{184}Y_{1246}$, $N_{86}F_{184}D_{1246}$, $N_{86}Y_{184}Y_{1246}$, and three double mutant haplotypes of $Y_{86}F_{184}D_{1246}$, $Y_{86}Y_{184}Y_{1246}$, and $N_{86}F_{184}Y_{1246}$ were observed. The $Y184F$ of *pfmdr1* was predominant with a prevalence of 24.7% (107/434) and there were 34 mixed mutations with amino acid Y/F in codon 184, which was not included in the calculation of the
The prevalence of different haplotypes of \textit{pfmdr1} were statistically significant ($P<0.001$) except the difference between N86Y and Y184F and the difference among D1246Y, \textit{Y}$_{86}$\textit{Y}$_{184}$\textit{Y}$_{1246}$ and \textit{N}$_{86}$\textit{F}$_{184}$\textit{Y}$_{1246}$. The N86Y variant was at low prevalence and not found in east Africa. The D1246Y variant was only found in two isolates: one was a double mutation with Y184F and the other was a mixed mutation. The \textit{Y}$_{86}$\textit{F}$_{184}$\textit{D}$_{1246}$ haplotype, at 12.67% was more prevalent compared to the other two haplotypes \textit{Y}$_{86}$\textit{Y}$_{184}$\textit{Y}$_{1246}$ and \textit{N}$_{86}$\textit{F}$_{184}$\textit{Y}$_{1246}$. The double mutant haplotype was more common in central Africa and accounted for 38.2% (29/76) of all mutant isolates and had low prevalence in southern Africa (7.0%, 5/71) (Fig. 1).

In total, six mixed mutant haplotypes (\textit{N/Y}$_{86}$\textit{Y}$_{184}$\textit{D}$_{1246}$, \textit{N}$_{86}$\textit{Y/F}$_{184}$\textit{D}$_{1246}$, \textit{Y}$_{86}$\textit{Y/F}$_{184}$\textit{D}$_{1246}$, \textit{N/Y}$_{86}$\textit{F}$_{184}$\textit{D}$_{1246}$, \textit{N/Y}$_{86}$\textit{Y/F}$_{184}$\textit{D}$_{1246}$, and \textit{N/Y}$_{86}$\textit{Y}$_{184}$\textit{D/Y}$_{1246}$) were identified with a combined prevalence of 10.37% (56/434) and the two most common mixed haplotypes were \textit{N}$_{86}$\textit{Y/F}$_{184}$\textit{D}$_{1246}$ and \textit{N/Y}$_{86}$\textit{Y/F}$_{184}$\textit{D}$_{1246}$.

**K13-propeller sequencing**

A total of 1381 \textit{P. falciparum} isolates from 33 African countries were sequenced of K13-propeller domain and 1357 were successfully sequenced. The prevalence of K13 mutations was 3.6% (49/1357) and 26 different mutant alleles including 22 nonsynonymous and four synonymous variants (C469C, R471R, G496G, and A627A) were observed (Table 3 and Fig. 2). There were no K13 mutations isolated from north and south Africa. The prevalence of K13 mutations was highest in east Africa (9.5%, 4/42), followed by central Africa (4.5%, 38/839) and west Africa (1.9%, 7/370). The A578S variant, the most common K13 haplotype in Africa, was identified from 10 isolates (four from Equatorial Guinea, two from Angola, and one each from the Democratic Republic of Congo, Ghana, Guinea, and Uganda, respectively.) The Q613E variant was the second most prevalent haplotype of K13 and was found in Angola, Democratic Republic of the Congo, and Tanzania. Three mutations associated with artemisinin resistance were identified, including M476I, R539T, and P553L. The R539T and P553L variants were found in isolates from Angola and M476I was from Equatorial Guinea.

**Discussion**

This study was part of national antimalarial drug surveillance network and supported by the national malaria diagnosis reference laboratory network and NMEP. China has set up a well-organized network for malaria diagnosis, treatment, and surveillance which is covering national, provincial, and county levels. Nevertheless, there are still several challenges in the post-malaria elimination stage in China. One big challenge is how to maintain the strong surveillance and response capacity in the post-malaria elimination stage because thousands of imported malaria cases are reported in China annually. \textit{P. falciparum} has developed resistance to all antimalarial drugs, even ACTs. This study evaluated the prevalence of \textit{pfcrf}, \textit{pfmdr1}, and K13-propeller mutations of \textit{P. falciparum} isolates returning from Africa and the geographical distribution of these three genes in imported \textit{P. falciparum} isolates were mapped as well.
CQ was the first line antimalarial drug to treat the uncomplicated *P. falciparum* malaria in Africa from the 1940s, and was widely used because of its high efficacy, safety, and low cost [22]. CQ resistance was first identified along the Thai-Cambodian border in the late of 1950s [23, 24], and first reported in Africa in the 1970s [25]. In Africa, CQ was replaced by SP and ACTs for uncomplicated malaria treatment between the late 1990s to the early 2000s. The *pfcr* mutation of codons 72–76 was considered to be the most reliable molecular marker for CQ resistance [19]. The prevalence of the *pfcr* mutation was 37.2% (272/731) in this study and the mutant isolates contained the triple mutant haplotype C_{72}V_{73}I_{74}E_{75}T_{76} with the proportion of 80.2% (218/272). The prevalence of the *pfcr* mutation in Africa decreased significantly in contrast to the late of 1990s. The reduction of the prevalence of the *pfcr* mutation and return of CQ sensitivity was also found in other studies in several malaria endemic countries in Africa [26–28]. The termination of CQ use resulted in the recovery of its efficacy. In addition, 35 isolates from western, central and southern Africa with mixed genotypes C_{72}V_{73}M/I_{74}N/E_{75}K/T_{76} were identified. According to the published study, CQ resistance may have been caused by the drug selective pressure, multiple genomic background of the strains. Resistant mutations selected by antimalarial drugs remove linked neutral variation as they sweep (increase in frequency) through a parasite population [29].

The *pfmdr1* gene was found to be associated with the resistance of multiple antimalarial drugs and the following *pfmdr1* variants: N86Y, Y184F, S1034C, N1042D, and D1246Y have been identified [12–14]. The *pfmdr1* N86Y and *pfcr* K76T variants have been shown to be in strong linkage disequilibrium and it has been reported that they are associated with in vitro responses to CQ, mefloquine, lumefantrine, quinine and dihydroartemisinin [30–32]. This study only identified *pfmdr1* allelic variants in codons 86, 184, and 1246 including 12 haplotypes and six mixed mutant genotypes. The two predominant *pfmdr1* mutations, Y184F and N86Y had prevalences of 24.7% (107/434) and 23.3% (101/434), respectively. One study that genotyped isolates from Angola showed that the prevalence of *pfmdr1* N86Y had decreased to 5.6% in 2017 [33], which was much lower than our average prevalence for that variant. But *pfmdr1* N86Y was not detected in east Africa. *pfmdr1* D1246Y was identified in only two isolates; one was a double mutation with Y184F and the other one was a mixed mutation. D1246Y was rare in Africa compared with previous data [34]. Also, the double mutant haplotype of *pfmdr1* was more common in central Africa (38.2%) and lower prevalence in southern Africa (7.0%).

Mutation in K13-propeller domain was confirmed to be associated with artemisinin resistance in 2014 [20]. This mutation has independently arisen multiple times and spread in the GMS. Until now, nine validated variants and over 20 candidates or associated markers of K13 have been identified [5]. The prevalence of the K13-propeller mutation was 3.6% (49/1357) in this study and 26 different variant alleles were identified including four synonymous (C469C, R471R, G496G, and A627A) and 22 nonsynonymous, which indicated that K13-propeller was highly heterogeneous in Africa, similar to that reported by other studies [35–37]. In addition, three mutations associated with artemisinin resistance were observed, including M476I, R539T, P553L. C580Y, the most common K13 mutation in GMS, and F446I, the predominant mutation in southern China [38] were not found in imported Africa isolates in this study. Another K13 mutation, M579I was identified from one isolate from Equatorial Guinea, which was reported
to be associated with artemisinin resistance in Africa [39]. Nevertheless, this mutation was not observed in our study. The presence of C580Y of the K13 gene was found in malaria parasites (2.7%, 3/113) from migrant Chinese workers returning from Ghana in 2013, but it needed further characterization [40]. Previous studies also reported that R539T was identified from a population returning to China from Africa [41]. In our study, although one isolate carried the R539T variant, it were unable to be proved that this was an artemisinin resistance isolate because there were no treatment failure outcomes associated with the variant. The variant A578S is comprised of two tightly linked SNPs and is the most common haplotype of K13 in Africa and was identified from 10 isolates (four from Equatorial Guinea, two from Angola, and one each from the Republic of Congo, Ghana, Guinea, and Uganda) and A578S might be involved in artemisinin resistance in Africa [42]. More recently, the de novo emergence and clonal expansion of an K13 R561H lineage has been reported in Rwanda and this mutation has been confirmed as a mediator of artemisinin resistance in vitro [43]. Moreover, an imported malaria case from Rwanda to China was reported with this R561H mutation this year [44]. Therefore, molecular surveillance, as a high throughput tool used to monitor the resistance of antimalarial drugs in the national drug policy on imported malaria cases, could provide the early warning and evidence for efficacy of antimalarial drugs to treat these imported cases. China has set up the antimalarial drug surveillance network which is responsible for implementing integrated drug efficacy study (iDES) of antimalarial drugs for the national policy and molecular marker surveillance in the entire country.

**Conclusion**

The target populations in this study were the malaria patients returning from Africa to China. The haplotypes of *pfcr*, *pfmdr1* and K13-propeller indicated the level of associated drug resistance in these imported malaria cases, which provided evidence that the current national antimalarial drug policy in China is suitable for the treatment of imported malaria cases.

**Limitations**

This study only evaluated the prevalence of molecular makers associated with antimalarial drug resistance of imported cases from Africa and the treatment outcome was not analyzed. All the imported malaria cases will be treated according to the national antimalarial drug policy as shown in additional file 3. The iDES, as one component of routine surveillance systems will be considered in malaria elimination stage to provide evidence for updating the guideline of antimalarial drug treatment in China, especially for imported malaria cases.

**Declarations**

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Meetings

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Authors' contributions

FH, XNZ conceived and designed the study. FH, HY and YWC conducted the laboratory work; FH, JBX carried out the data analysis. SSZ, ZGX, RA, and PR proved technique support for the data analysis and reviewing the manuscript. FH drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets analysed in this study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Ethical Review Committee of National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests. PR is a staff member of the World Health Organization. The author alone is responsible for the views expressed in this publication and they do not necessarily represent the decisions, policy or views of the World Health Organization.

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Tables

Table 1: The main source countries of imported cases in China, 2012-2015

| Source country                  | No. of imported malaria cases | Total  |
|--------------------------------|------------------------------|--------|
|                                | 2012 | 2013 | 2014 | 2015 |        |
| 1 Myanmar*                     | 766  | 605  | 495  | 477  | 2343   |
| 2 Ghana                        | 235  | 1349 | 188  | 172  | 1944   |
| 3 Angola                       | 151  | 437  | 272  | 416  | 1276   |
| 4 Equatorial Guinea            | 247  | 300  | 287  | 272  | 1106   |
| 5 Nigeria                      | 207  | 225  | 341  | 283  | 1056   |
| 6 Cameroon                     | 17   | 101  | 175  | 248  | 541    |
| 7 Democratic Republic of Congo| 47   | 64   | 118  | 175  | 404    |
| 8 Ethiopia                     | 38   | 62   | 118  | 150  | 368    |
| 9 Guinea                       | 58   | 75   | 64   | 98   | 295    |
| 10 Indonesia*                  | 36   | 71   | 142  | 35   | 284    |
| 11 Republic of Congo           | 33   | 53   | 83   | 101  | 270    |
| 12 Liberia                     | 44   | 86   | 88   | 34   | 252    |
| **Total**                      | **2474** | **4042** | **3021** | **3077** | **12614** |

* Myanmar and Indonesia were the only two countries in Southeast Asia and all other ten are the countries in Africa.

Table 2: The mutant prevalence of *pfcr, pfmdr1* genes in *P. falciparum* isolates returned from Africa
| Gene (n) | Codons position | No. of mutant isolates | Prevalence of mutations | P    |
|---------|-----------------|------------------------|-------------------------|------|
| **pfcoRT (731)** |               |                        |                         |      |
| M74I    |                 | 249                    | 34.1% (249/731)         | 0.992|
| N75E    |                 | 249                    | 34.1% (249/731)         |      |
| K76T    |                 | 251                    | 34.3% (251/731)         |      |
| **pfmdR1 (434)** |               |                        |                         |      |
| N86Y    |                 | 101                    | 23.3% (101/434)         | 0.000|
| Y184F*  |                 | 107                    | 24.7% (107/434)         |      |
| D1246Y* |                 | 0                      | 0.0%                    |      |
| YFD     |                 | 55                     | 12.7% (55/434)          |      |
| YYY     |                 | 1                      | 0.2% (1/434)            |      |
| NFY     |                 | 1                      | 0.2% (1/434)            |      |

Note: *Total of 34 isolates with mix single mutant haplotypes at 184 Y/F was not included. One isolates with double mutation at 184 and 1246 was included in NFY and the other with mix mutation was not included.

Table 3 K13 mutations identified from the isolates from Africa
| Sub region of Africa | Source countries         | Sample size | Prevalence of K13-propeller mutations | Amino acid of K13 mutation (n) |
|----------------------|--------------------------|-------------|---------------------------------------|-------------------------------|
| Central Africa       | Equatorial Guinea        | 224         | 15                                    | C469C(1) R575K(1) A578S(4) C580F(1) D452N(1) M476I(1) V589I(1) P574L(1) A578T(1) M579I(1) C469F(2) |
|                      | Congo, DRC               | 32          | 1                                     | Q613E (1)                     |
|                      | Republic of Congo        | 35          | 2                                     | I634T (1) A578S (1)           |
|                      | Cameroon                 | 54          | 1                                     | L457S (1)                     |
|                      | Chad                     | 11          | 0                                     |                               |
|                      | Central African Republic | 3           | 0                                     |                               |
|                      | Gabon                    | 14          | 0                                     |                               |
|                      | Angola                   | 466         | 19                                    | P553L(1) A569T(1) A578S(2) Q613E(5) I646K(1) R471R(4) R539T(1) P443R(1) V589I(1) M579I(2) |
| **Sub total**        |                          | **373**     | **19**                                |                               |
| North Africa         | Algeria                  | 1           | 0                                     |                               |
|                      | Egypt                    | 1           | 0                                     |                               |
|                      | Libya                    | 3           | 0                                     |                               |
|                      | Sudan                    | 21          | 0                                     |                               |
| **Sub total**        |                          | **26**      | **0**                                 |                               |
| East Africa          | Ethiopia                 | 8           | 0                                     |                               |
|                      | Kenya                    | 6           | 1                                     | I683R (1)                     |
|                      | Tanzania                 | 20          | 2                                     | Q613E (1) L488V(1)            |
|                      | Uganda                   | 8           | 1                                     | A578S (1)                     |
| **Sub total**        |                          | **42**      | **4**                                 |                               |
| West Africa          | Mali                     | 5           | 0                                     |                               |
| Country        | Count | Subtotal |
|---------------|-------|----------|
| Burkina Faso  | 2     |          |
| Niger         | 4     |          |
| Togo          | 7     |          |
| Togo          | 7     |          |
| Ivory Coast   | 17    |          |
| Benin         | 9     |          |
| Liberian      | 25    |          |
| Sierra Leone  | 29    |          |
| Nigeria       | 152   | 3        |
| Guinea        | 55    | 2        |
| Ghana         | 65    | 2        |
| South Africa  |       | 370 7    |
| Mozambique    | 28    | 0        |
| Zambia        | 39    | 0        |
| Malawi        | 8     | 0        |
| Madagascar    | 3     | 0        |
| Zimbabwe      | 1     | 0        |
| South Africa  | 1     | 0        |
| Subtotal      | 370   | 7        |
| Total         | 1357  | 49       |

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