Drug Resistance Profile and Clonality of Plasmodium Falciparum Parasites in Cape Verde: The 2017 Malaria Outbreak

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Research
Abstract

Background

Cape Verde is an archipelago located off the West African coast, and is in a pre-elimination phase of malaria control. Since 2010, less than 20 Plasmodium falciparum malaria cases have been reported annually, except in 2017, when an outbreak in Praia before the rainy season led to 423 autochthonous cases. It is important to understand the genetic diversity of circulating P. falciparum to inform on drug resistance, potential transmission networks, and sources of infection, including parasite importation.

Methods

Enrolled subjects involved malaria patients admitted to Dr. Agostinho Neto Hospital at Praia city, Santiago island, Cape Verde, between July and October 2017. Neighbours and family members of enrolled cases were assessed for the presence of anti-P. falciparum antibodies. Sanger sequencing and real time PCR was used to identify SNPs in genes associated with drug resistance (e.g. pf dhfr, pf dhps, pf mdr1, pf k13, pf crt), and whole genome sequencing data was generated to investigate the population structure of P. falciparum parasites.

Results

We analysed 190 parasite samples, 187 indigenous and three from imported infections. Malaria cases were distributed throughout Praia city. There were no cases of severe malaria, and all patients had an adequate clinical and parasitological response after treatment. Anti-P. falciparum antibodies were not detected in the 137 neighbours and family members tested. No mutations were detected in pf dhps. The triple mutation S108N/N51I/C59R in pf dhfr and the chloroquine resistant CVIET haplotype in the pf crt gene were detected in almost all samples. Variations in pf k13 were identified in only one sample (R645T, E668K). The haplotype NFD for pf mdr1 was detected in the majority of samples (89.7%).

Conclusions

Polymorphisms in pf k13 associated with ACTs tolerance in Southeast Asia were not detected, but the majority of the tested samples carried the pf mdr1 haplotype NFD and antimalarial associated mutations in the the pf crt and pf dhfr genes. We performed the first WGS for Cape Verdean parasites that showed that the samples cluster together, have a very high level of similarity and are close to other parasites populations from West Africa.

Introduction

Malaria, caused by Plasmodium parasites, is a global public health problem. Almost half of world population is at risk of malaria, and in 2018 there were 228 million cases and 405,000 deaths, globally (WHO 2019). In Africa, where Plasmodium falciparum infections dominate, six countries (Nigeria, the Democratic Republic of the Congo, Uganda, Côte d'Ivoire, Mozambique and Niger) accounted for more
than half of all malaria cases worldwide. Further, 94% of all malaria deaths occurred in the African continent. Due to malaria control activities, such as improved case management and roll out of insecticide-treated nets and indoor residual spraying, the number of countries moving toward towards disease elimination has increased. In particular, the number of countries with < 100 indigenous cases increased from 17 in 2010 to 27 in 2018 (WHO, 2019).

Cape Verde (population size: ~500,000) is one of the African countries in a pre-elimination phase of malaria control, with < 1 case per 1,000 population per year (DePina et al, 2018). There were 583 indigenous cases and 5 deaths between 2010 and 2018. However, in 2017, 423 (72.6%) cases occurred in an outbreak. This year was an outlier as it corresponds to an increase of 89.9% of cases compared to previous years, and excluding it, the number of cases reported yearly since 2010 has varied between 1 and 48 (average < 20 cases) (WHO, 2019). Despite the control efforts implemented by health authorities, autochthonous cases persist and could delay elimination targets. Malaria prevalence is unstable and autochthonous cases are restricted to the islands of Santiago (96%) and Boavista (4%), while imported cases from countries with disease transmission are recorded in all nine islands. In recent years, local transmission has been restricted to the island of Santiago, especially in Praia city, capital of the country, where it recorded 158 cases, more than 90% of autochthonous cases from 2010 to 2016 (DePina et al, 2018). There is typically low malaria endemicity, but there are fluctuations in morbidity depending on rainfall, with transmission normally occurring between the months of September and November (Arez AP, et al, 1999). However, in 2017, the malaria outbreak occurred before the rainy season, where all the 423 autochthonous P. falciparum cases were reported in Praia city, the capital of the country (WHO, 2018; DePina, et al, 2019). Eighteen patients had at least two relapse episodes in that year, 23 further imported cases were registered (DePina, et al, 2019).

All non-complicated malaria cases in Cape Verde are hospitalized and treated with the artemisin-based combination therapies (ACT; artemether and lumefantrine), which target the parasite erythrocytic asexual stage. All cases also receive the gametocytocidal primaquine drug at the start of treatment, to prevent transmission and interrupt the spread of the disease. Severe cases are treated with intravenous artesunate. Levels of parasitaemia are monitored at the health facilities during the period of the disease and followed-up on multiple occasions up to 42 days (Ministério da Saúde de Cape Verde, 2015). The success in the control and treatment of malaria depends on the clinical efficiency of ACT and avoiding drug resistance (Lubell et al, 2014). Therefore, understanding the epidemiology of drug resistance is vital for an effective drug policy specially within the Cape Verde elimination settings (Khatoon et al, 2009).

The non-temporary movement of people between Cape Verde and malaria endemic countries, particularly to and from West Africa, increases the potential for case importation, and poses a challenge to malaria elimination on the archipelago (WHO, 2012). The circulation of parasites between regions also increases the risk of importing drug resistance, which is underpinned by mutations in the P. falciparum genome. For example, polymorphisms associated with resistance to several anti-malarial drugs have been identified in P. falciparum genes pf dhfr (target for anti-malaria drug pyrimethamine), pf dhps (target for sulphadoxine), pf crt (target for chloroquine), pf k13 (target for artemisin) and pf mdr 1 (target for
mefloquine, chloroquine) Similarly, *P. falciparum* genetics (e.g. *pfama1* gene) also gives the parasite the ability to evade the immune response of the host. Therefore, there is a need to understand the genetic diversity of circulating parasites to inform on drug resistance and transmission networks identified by finding near identical parasite genomic sequences. To support this, we collected epidemiological data and blood samples from close contacts of patients, including neighbours and family members, thereby informing on potential risk factors for malaria susceptibility, transmission, and the emergence and spread of outbreaks. Further, our study on the molecular characterization of *P. falciparum* isolates collected from patients during the 2017 outbreak will provide a baseline assessment of malaria parasite drug resistance profile and genetics in Cape Verde, from which to design population-specific diagnostics, and contribute to strengthening the country’s measures for prevention and control, in order to achieve its elimination targets.

**Material And Methods**

**Participants**

Malaria patients admitted to Dr. Agostinho Neto Hospital at Praia city, Santiago island, Cape Verde, between July and October 2017 were enrolled. A total of 190 (from 446; 187 indigenous, 3 imported) gave informed consent to participate in the present study. The laboratory diagnosis of malaria was performed by rapid diagnostic tests and microscopy.

All 190 patients that agreed to participate remained hospitalized for at least 3 days. Following local guidelines (Ministério de Saúde de Cape Verde, 2015), patients were discharged from hospital, only after laboratory confirmation of *Plasmodium spp.* negative blood smear and clinical evaluation. Follow-up assessments were performed on days 7, 14, 21, 28, 35, and 42, post-start of treatment.

**Data and sample collection**

Data were obtained from laboratorial analysis and from a questionnaire. Of the 190 malaria cases enrolled in this study, 131 (68.9%) cases and their neighbours and family members (n = 137; without malaria symptoms), answered a questionnaire in a community setting. This questionnaire was designed in both Portuguese and Crioulo which captured sociodemographic data. Species identification and parasite quantification was performed by microscopic observations of thick and thin blood smears. Blood drops of malaria cases and their neighbours/family obtained by finger prick were collected on filter paper and stored at -20°C for later molecular characterization of parasites and antibody (AMA1 and MSP1) detection. Parasite DNA was extracted using Chelex (Bereczky et al, 2005) and species confirmation by PCR (Lobo et al, 2014).

**AMA1 and MSP1-19 Indirect ELISA**

Dried blood spot elution was performed as previously described [9]. In brief, 137 bloodspots from samples collected in 2017 were equilibrated to ambient room temperature before opening, and 2.5 mm diameter discs were cut from Whatman 3 MM paper using a leather punch. Each disc was then eluted in
a 96 deep-well plate containing 150 µl PBS/0.05% (v/v) Tween20/0.05% (w/v) Sodium Azide and incubated at ambient room temperature for > 18 hours with gentle rotary agitation ~ 100 rpm. Serum was diluted to a 1:100 dilution. IgG antibodies were detected by indirect ELISA using MSP1-19 (Wellcome Genotype) and AMA1 (3D7) antigens expressed in *E. coli* strain BL21, as described by (Okech et al,2004; Hodder,2001). AMA1 and MSP1-19 antigens were coated on to 96-well ELISA plates (Immulon 4HBX, Thermo) with coating buffer (Carbonate-bicarbonate) in a 50 µl volume at a concentration of 0.5 µg/m and incubated a 40°C overnight. Plates were washed several times using PBS plus 0.05% Tween 20 (PBS/T) and blocked with 1% (w/v) skimmed milk powder in PBS/T (block solution). The previously eluted serum was used at 1:400 working dilution diluted in to block solution. European confirmed negative and pooled hyperimmune positive serum were used as negative/positive control samples. Serum was incubated at 40°C overnight and washed in PBS/T. Rabbit anti-human HRP conjugated secondary antibody (Dako) was used at a concentration of 1:15,000 in PSB/T. TMB substrate was added followed by a 15-minute RT incubation. H$_2$SO$_4$ stop solution was added, followed by plate reading at 450 nm.

**Candidate gene sequencing and analysis**

A total of 38 samples with the highest parasitaemia were selected for sequencing of antimalarial drug resistance loci, including: (i) *pfmdr1* (PF3D7_0523000; including codons 86, 184 and 1246), *pfk13* (PF3D7_1343700; including codons 417 to codon 714), *pfdhfr* (PF3D7_0417200.1; including codons 50, 51, 59, 108 and 164) and *pfdhps* (PF3D7_0810800.1; including codons 431, 436, 437, 540). Genes were PCR amplified and products cleaned using SureClean (Bioline, USA) following manufacturer instructions. The PCR products were analysed by electrophoresis on a 1.5% agarose gel stained with GreenSafe Premium (Nzytech) to confirm amplification. The primer pairs and thermocycling conditions are summarized in Supplementary Table 1. The PCR products were sequenced using Sanger capillary platform, and the resulting sequences were analysed using Geneious software (v4.8.5) and the laboratory adapted strain 3D7 was used as reference. A fragment of the *pfcr* gene (PF3D7_0709000; including codons 72, 74, 75 and 76) was also examined. Three dual labelled probes with sequences complementary to each of the *pfcr* 72-76 haplotypes (denoted as CVIET, CVMNK, SVMNT), were included in the real time PCR reaction, each containing a different fluorescent molecule (Sutherland et al. 2007; Yan He et al, 2019). Two amplicons (479 and a 516 bp) of the gene *pfama1* (PF3D7_1133400) were amplified in 20 samples from 2017 and 4 samples from the 2016, using protocols published elsewhere (Lerch, 2017). Amplicons were amplified with primer pairs and thermocycling conditions summarized in Supplementary Table 1. PCR products were analysed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and sequencing using the same methodology described above.

**Selective whole genome amplification and whole genome sequencing**

Seven samples from 2017 with the highest parasitemias were selected for whole genome sequencing (WGS), together with 4 samples from 2016. Genomic DNA was extracted from dried blood spots using the QIAamp DNA Investigator Kit (QIagen, Germany) as per manufacturer’s directions. DNA concentrations
were measured using the Invitrogen Qubit Fluorometer. A selective whole genome amplification (SWGA) strategy was applied prior to WGS, following previously published protocol (Oyola, 2016). Oligonucleotides that preferentially bind with high frequency to the *P. falciparum* DNA, and rarely bind to the human DNA, were used for SWGA. All SWGA reactions were carried out in a UV Cabinet for PCR Operations (UV-B-AR, Grant-Bio) to eliminate potential contamination. Briefly, a maximum of 60 ng of gDNA (minimum of 5 ng) was added to a total 50 µl reaction alongside with 1X phi-29 buffer (New England Biolabs), 1X bovine serum albumin, 1 mM dNTPs, 2.5 µM of primer mix, 30 U phi-29 DNA polymerase (New England Biolabs) and water. The reaction was carried out on a thermocycler with the following step-down program: 5 minutes at 35 °C, 10 minutes at 34 °C, 15 minutes at 33 °C, 20 minutes at 32 °C, 25 min 31 °C, 16 hours at 30 °C and 10 minutes at 65 °C. The five samples, selected for SWGA, were then whole genome sequenced on a MiSeq (Illumina) at the LSHTM. The QIAseq FX DNA Library Kit (QIAGEN) was used for library preparation according to the manufacturer's protocol, with a 20-minute fragmentation step. Library DNA concentration was analysed using a Qubit 2.0 fluorometer. All sequencing reactions were performed using 2 × 150 bp reads.

**Whole genome sequence data analysis**

Raw fastq les obtained after the MiSeq run were trimmed using trimmomatic set to default parameters (Bolger, 2014), and aligned to the *P. falciparum 3D7* reference genome (PlasmoDB) using bwa-mem software (Li H, 2009). SNPs were identified using samtools software (Li H, 2009) and filtered for quality based on previously described methods (Ravenhall et al, 2016, 2019). The coverage of each nucleotide was analysed using sambamba (Tarasov, 2015), which was set to include SNPs with only a coverage of 5-fold or above. To investigate the population structure of *P. falciparum* parasites, a distance matrix was created which was based on a matrix of pairwise identity calculated from the SNPs present in each sample. Using the distance matrix, a neighbourhood joining tree was produced and visualised in iTOL (Letunic, 2016). WGS data from an extra 400 publicly available samples from Africa, South America, South Asia and Southeast Asia were also used for analysis (Ravenhall et al, 2016, 2019, Manske M, et al, 2012). A neighbourhood-joining tree was used to investigate the clustering of samples from West Africa.

For genetic distance comparisons between samples, a clustering approaches using a Manhattan distance matrix of pairwise identity by state values was calculated from the subset of SNPs available for each pairwise comparison using the R software.

**Statistical analysis**

For sociodemographic characteristics we compared two groups: malaria cases (n = 131) and neighbours/family members (n = 137). Among other, sociodemographic data, present and past about malaria and fever, travel information and signs and symptoms consistent with malaria.

Differences in categorical variables were tested by Chi-Square or Fisher exact tests. Differences in continuous variables were assessed using t-tests (after checking for normality and the homogeneity of variances), or alternatively, Mann-Whitney-Wilcoxon non-parametric test. Unconditional multiple logistic
regression models were used to estimate adjusted odds ratios (OR) with 95% confidence intervals (CIs). Multiple models included all variables with \( p < 0.20 \) in the simple logistic regression models.

## Results

### Characterization of malaria cases

From the 446 cases in the 2017 outbreak, we analysed 190 blood samples collected from malaria patients before treatment administration, including 187 indigenous and three imported infections. All samples were *P. falciparum* positive (PCR confirmed) and one patient presented with a mixed *P. falciparum* and *P. malariae* infection, undetected by microscopy. Considering indigenous cases group, only 27 (14.4%) were children (\( \leq 16 \) years), and the vast majority of participants were adults (\( n = 160, 86.6\%; \) range: 17–91 years old). All imported cases were young adults, two with 33 years and one with 31 years of age.

Malaria cases were distributed throughout Praia city (Fig. 1) and consistent with official statistics. Varzea, Paiol and Achadinha were the neighbourhoods with highest number of cases (\( n = 13 \) (Fig. 1). There were no cases of severe malaria, nonetheless 18 patients presented with hyperparasitemia (>100,000 parasites/µl blood; Wilairatana P et al, 2013; WHO, 1990). Only one fatality was registered, but malaria was not the cause of death. The three most common self-reported symptoms in malaria cases were fever, headache, and chills. At day 3 of hospital treatment, Giemsa stained smears were negative for *Plasmodium spp.* for all patients. The length of hospital stay of malaria cases varied between three days to one month, with a median of four days (Table 1).
Table 1
Characteristics of the malaria patients and their close contacts (family and neighbours).

| Variables                  | Malaria cases (n = 131) | Close contacts (n = 137) | p     |
|----------------------------|-------------------------|--------------------------|-------|
| Age in years (Mean ± SD)   | 32.4 ± 15.3             | 40.3 ± 18.6              | < 0.001 |
| Median (Min-Max)           | 30 (3–86)               | 39 (1–83)                |       |
| Sex n (%)                  |                         |                          | < 0.001 |
| Male                       | 86 (65.6)               | 37 (27.0)                |       |
| Female                     | 45 (34.4)               | 100 (73.0)               |       |
| Marital status n (%)       |                         |                          | < 0.001 |
| Single                     | 96 (73.3)               | 66 (48.9)                |       |
| Married                    | 12 (9.2)                | 25 (18.5)                |       |
| Civil union                | 23 (17.6)               | 38 (28.1)                |       |
| Divorced/widowed           | 0 (0.0)                 | 6 (4.4)                  |       |
| Nationality n (%)          |                         |                          | 1.000 |
| Cape Verde                 | 130 (99.2)              | 135 (98.5)               |       |
| Other                      | 1 (0.8)                 | 2 (1.5)                  |       |
| Professional status n (%)  |                         |                          | 0.150 |
| Unemployed                 | 18(13.8)                | 18 (13.4)                |       |
| Primary sector             | 1(0.8)                  | 0 (0.0)                  |       |
| Secondary sector           | 5(3.8)                  | 11(8.2)                  |       |
| Tertiary sector            | 74(56.9)                | 79(59.0)                 |       |
| Retired                    | 1(0.8)                  | 5(3.7)                   |       |
| Student                    | 31(23.8)                | 21 (15.7)                |       |
| History of malaria n (%)   |                         |                          | 1.000 |
| yes                        | 2 (1.2)                 | 3 (2.2)                  |       |
| no                         | 129 (98.5)              | 133 (97.8)               |       |

* Hospital stay was extended in some patients, based in individual clinical evaluation.
### Variables

| Variables                        | Malaria cases (n = 131) | Close contacts (n = 137) | *p* |
|----------------------------------|-------------------------|--------------------------|-----|
| Length of hospital stay in days  | 4 (3–4)                 | -                        |     |
| Median (P<sub>25</sub>-P<sub>75</sub>) | (3–30)*               | -                        |     |
| (Min-Max)                        |                         |                          |     |

* Hospital stay was extended in some patients, based in individual clinical evaluation.

### Sociodemographic characterization

Table 1 (and Supplementary Table 1) shows epidemiological information of a set of 131 patients and 137 close contacts (family members or neighbours, without malaria symptoms). Almost all participants (265/268; 98.9%) were Cape Verde nationals. Malaria cases were predominantly single (73.3%), male (65.6%) and younger than non-malaria cases (all *p* < 0.001). Malaria cases tended to travel less in the past 6 months (5.6%) than their close contacts (11.8%), but this difference was marginally non-significant (*p* = 0.060). Students and tertiary sector activities accounted 80.2% in malaria cases and 73.0% in non-malaria group. Geographical locations of the cases suggested (Fig. 1) some clusters in Varzea, Paiol, Achadinha, Calabaceira and Pensamento, corresponding to 43.5% of all cases.

Multiple logistic regression models suggested increased risk of malaria from being male (OR = 4.99; 95% CI: 2.90–8.58) and single (OR = 1.92, 95% CI: 1.02–3.58), with potentially lower risk from higher age (OR = 0.98; 95% CI 0.97–1.00). All malaria cases were PCR positive for *P. falciparum*, while all close contacts (n = 137) were negative. Serologic detection of *P. falciparum* anti-AMA1 and MSP-1-19 IgG/IgM was also negative for all 137 neighbours and family members.

### Drug resistance profile of malaria cases

A subset of the malaria cases (n = 38/190) was investigated for the presence of alleles associated with drug resistance. The chloroquine resistant CVIET haplotype (*pfcr* gene) was detected in all samples analysed except one that carried CVMNK (wild type haplotype). No mutations in the *pfk13* gene, associated with artemisinin resistance, were identified. All *pfk13* sequences analysed were identical to that of 3D7 reference strain except one that carried two mutations R645T and E668K, which have not been observed in other isolates (Ravenhall et al, 2016). Sulfadoxine-pyrimethamine (SP) drug resistant associated alleles were also surveyed. For the *pfdhps* gene the wild type (S436/A437/K540) haplotype was and for *pfdfhr* the triple mutation S108N/N51I/C59R was detected in all samples with success PCR (n = 12). The codons 86, 184 and 1246 from the *pfmdr1* gene were also analysed. The majority of samples (89.7%) carried the haplotype NFD (meaning N86/F184/D1246) that is associated with artemether + lumefantrine tolerance and the others were wildtype for the 3 codons (N86/Y184/D1246). No other polymorphisms were detected for the samples and regions analysed in the five genes.

### Population genetic analysis
Whole genome sequencing (WGS) was performed in seven autochthonous samples with the highest parasitaemias (54,000 to 126,690 parasites/µl blood), and four samples from 2016. The resulting data was compared to public available *P. falciparum* global genomic sequences (Ravenhall M et al, 2019, Manske M, et al, 2012), in order to explore their genetic diversity. Prior to WGS, SWGA was used to increase the amount of parasite DNA. The success of the SWGA is very dependent on parasitaemia and the quality of the DNA. After WGS, the samples from 2016 were excluded for analysis due to having less than 1% of genome sequenced. The WGS data confirmed the drug resistance profile results, with all samples having the *pfcrt* CVIET haplotype, the Y184F mutation in *pfmdr1* gene, the triple mutation S108N/N51I/C59R in *pfdhfr* and wild type alleles for *pfdhps*.

A neighbour-joining tree was constructed using SNP data and demonstrates that the Cape Verdean samples group together, and cluster with samples from West African (Fig. 2). To investigate the genetic similarity between samples, an analysis of SNP sharing was performed, including samples from other locations in Africa and Asia. The samples from Cape Verde shared at > 99% of SNPs, while when compared with other samples on average 97% (maximum 97.6%) of SNPS were shared, a value normally observed between samples from different regions (e.g. African and Asian samples share on average 96.6% SNPs). To further explore the genetic diversity two fragments of the *PfAMA1* gene were sequenced for 20 samples, including the samples from 2016. The results show that all amplified samples that were successfully sequenced (n = 18 for 2017; n = 3 from 2006) share the same haplotype in both amplicons.

**Discussion**

**Insights into the epidemiology of Cape Verde malaria**

There is a lack of studies into the genetics and epidemiology of malaria cases in Cape Verde (DePina et al, 2019a; DePina et al, 2019b). Our data contradict the typical profile based on women and children under-five years of age. Official data reports (DePina et al, 2019a; DePina et al, 2019b) have indicated that more males are affected by malaria, and identified high risk occupations including construction or agricultural workers, security, guards, and the homeless. Only one agricultural worker and four security guards appeared in our sample, which was dominated by tertiary sector activities, students and unemployed. The geographical pattern of our samples overlaps with those previous malaria hotspots, including Várzea, Chã de Areia, Fazenda, Achadinha Paiol and Lém Ferreira neighbourhoods (DePina et al, 2019a; DePina et al, 2019b). Further, our results showed that all neighbours and family members tested were negative for malaria (serology and PCR data), which reinforces that infection might be occurring outside of households.

We performed the first WGS for Cape Verdean parasites, and it clearly showed that the analysed samples cluster together and have a very high level of similarity which suggests that possible the outbreak resulted from clonal expansion of local parasites. The Cape Verdean samples also cluster with isolates from West Africa, as expected.

**Drug resistance associated molecular markers**
Artemether + lumefantrine (AL), is the recommended first treatment line for uncomplicated *P. falciparum* malaria in Cape Verde (Ministério de Saúde de Cape Verde, 2015). Apart from therapeutic efficacy studies (TES), the WHO recommends the use of molecular markers to monitor the emergence of mutations associated with resistance to anti-malarial drugs to detect emerging resistance and prevent potential future treatment failure (WHO, 2014). We assessed anti-malarial resistance genes polymorphism by Sanger and Next Generation Sequencing (NGS), the methods were used to screen for mutations in a set of malaria positive blood samples targeting the *pfcr*, *pfdhr1*, *pfk13*, *pfdhps* and *pfdhfr* genes, which have been previously associated with anti-malarial resistance. The prevalence of the different resistance alleles in Cape Verde was not known, either after or before the introduction of ACT in the country. The prevalence of *pfcr* (chloroquine resistance marker) resistant haplotype CVIET remains high, only one of the analysed isolates carried the wildtype haplotype CVMNK. Despite the implementation of ACTs in Cape Verde (since 2006) and the withdrawal of chloroquine, the frequency of the resistant allele 76T remains high, following the trend of continental Africa (Voumbo-Matoumona 2018; Ocan 2019b; Dhingra 2019). Nevertheless, the identification of the wild-type allele K76 is in line with findings in other malaria regions, where AL is the predominant ACT (Sondo P, 2016; Mbogo 2014; Conrad 2014; Tumwebaze 2015). Probably due to lumefantrine regimens tend to select wildtype *pfcr* alleles (Somé et al, 2010; Venkatesan 2014; Sisowath 2009). It is generally reported that the mutant haplotype 51I/59R/108N in *pfdhfr* confers resistance to pyrimethamine while 437G/540E in *pfdhps* confers resistance to sulphadoxine. Combined, known as the quintuple mutant, they confer resistance to SP (Omar 2001; Staedke, 2004; Kublin 2002; Desai, 2016). For the *pfdhps* gene the wildtype (S436/A437/K540) haplotype was identified in all analysed samples and for *pfdhfr* the triple mutation S108N/N51I/C59R was detected also in all samples successfully analysed, consistent with other African populations like Malawi (Ravenhall M 2016) or Senegal (Okell, 2017). SP has never been used as first line of treatment for *P. falciparum* uncomplicated malaria in Cape Verde, nor has it ever been used as intermittent preventive treatment (IPT) in infants and pregnant women.

The *pfmdr1* amino acid mutations that have been implicated in multidrug resistant phenotypes include N86Y, Y184F and D1246Y (Ferreira 2011; Idowu 2019; Muiruri 2018; Gil 2017), where the NFD haplotype is suspected to be involved in parasite tolerance to AL (Nsobya SL, 2010; Nkhoma S, 2009; Uhlemann 2007). In our study there was high prevalence of the 184F (89.7%) allele as well as the NFD haplotype (detected in 80% of the clinical isolates analysed). The NFD haplotype has also been identified as the most commonly reported *pfmdr1* haplotype in continental Africa, were AL is widely used as first line ACT to treat *P. falciparum* malaria (Duah et al, 2013; Lobo et al, 2014; Veiga MI, 2016; Muiruri et al, 2018; Asua V et al, 2019). Regarding the *pfk13* all samples were identical to 3D7 strain, only one sample carried two mutations R645T and E668K, which are not thought to be resistance related. This outcome was expected as the overall prevalence of SNPs in the *pfk13* is reported to have low prevalence outside Southeast Asia and high diversity among African parasites (WHO, 2019; Ocan et al, 2019a). The two SNPs identified in *pfk13* gene in our study, do not coincide either with the most reported or with the non-validated SNPs associated with delayed ART parasite clearance in Africa (Ménard 2016; Ocan et al, 2019a). Although in Africa, artemisinin partial resistance has not yet been confirmed (WHO 2019), there have been
unconvincing reports of treatment failure in travellers returning from Africa (Van Hong et al., 2014; Lu F et al., 2017). Mutations in pfk13, occurring in more than one African region affected by malaria are increasing (Conrad 2014; Kamau 2015; Taylor, 2015; Isozumi 2015; Escobar 2015; Tacoli 2016; Boussaroque 2016; Yang C, 2017), evidencing the need to close monitor this gene.

The Cape Verdean population has low immunity to malaria, 57% live on islands (Santiago and Boavista) where local malaria transmission occurs, and 36% on islands (WHO, 2012; Ministério da Saúde de Cape Verde, 2014). Prevention measures are essential to reduce the risk of malaria transmission and the potential for malaria epidemics. Cape Verde is a country in the elimination phase of malaria, thus, indoor residual spraying with insecticides is the major control strategy. This is supported by good case management with diagnostic tests and inpatient treatment of all cases (WHO, 2015). Other actions recommended by the WHO are chemoprophylaxis and the use of mosquito nets. However, insecticide-impregnated mosquito nets are less used in Cape Verde, where a recent study shows that only 19% of the population use them, but if offered them, 91% would use it (DePina, 2019b). Therefore, there is the potential to reduce the rate of infection, morbidity and mortality, through the very cost-effective mosquito net malaria prevention strategy (Kabir et al. 2014).

**Conclusion**

The majority of the parasite samples analyzed shared the same polymorphisms in the drug resistance associated genes. Polymorphisms in the pfk13 gene associated with ACTs tolerance in Southeast Asia were not detected, but the majority of the tested samples carried the pfmdr1 haplotype NFD. We performed the first whole genome sequencing for Cape Verdean parasites. It showed that all samples cluster together, have a high level of similarity and are close to West African parasite populations.

**Declarations**

**Acknowledgments**

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**Authors’ contributions**
SL, HS, TV, FN and LG were responsible for the study design. SL, DM and IV collected the blood samples. SL, SC, FN, and LG interpreted the data. IV, TC, SL, FN, SC performed the polymerase chain reaction. DW, FN and SC Performed the ELISA test. SC, AI, EDB and TGC performed and analyzed the whole genome sequencing. SL, FN, and HS wrote the first version of the manuscript. EBD, AI, SC, TGC, MLLM, and FN contributed to the writing, review and editing of the manuscript. All authors read and approved the final manuscript.

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**Ethics approval and consent to participate**

The study was reviewed and approved by the Ethical Committee of the Ministry of Health of Cape Verde (Reference no.39/2017) and by the National Data Protection Commission (reference no.30/2019). Informed written consent was obtained for all participants.

**Consent for publication**

Not applicable.

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Figures
Figure 1

Number of malaria cases in Praia city per neighborhood (N). N.1 - São Filipe; N.2 – Fonton; N.3 - Tira Chapéu; N.4 - Monte Vermelho; N.5 - Achadinha Pires; N.6 - Chã de Areia; N.7 - São Pedro Latada; N.8 - Bela Vista; N.27 – Várzea; N.9 - Achada Grande Frente; N.10 - Tahiti Chã de Areia; N.11 - Achada Eugénio Lima; N.12 - Jamaica/Agua Funda; N.13 – Palmarejo; N.14 - Lém Cachorro; N.15 - Achada Mato; N.16 - Achada Santo António; N.17 - Lém Ferreira; N.18 - Ponta de Agua; N.19 – Safende; N.20 - Fazenda
Sucupira; N.21 - Vila Nova; N.22 - Coqueiro Castelão; N.23 – Calabaceira; N.24 – Pensamento. N.25 – Paiol; N.26 – Achadinha. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.
Neighbour-joining trees constructed using SNP data from Cape Verde samples using global samples. Cape Verde (pink) clusters together with other samples from Africa (A) particularly West Africa (B).

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