Identification of chloroquine resistance *Pfcrt*-K76T and determination of *Pfmdr1*-N86Y copy number by SYBR Green I qPCR

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PEER REVIEW

ABSTRACT

Objective: To identify prevalence of chloroquine resistance point mutation at (*Pfcrt*, K76T) and (*Pfmdr1*, N86Y) copy number variation.

Methods: SYBR Green I based real time PCR was used. One hundred and thirty-three samples were analyzed for (*Pfcrt*, K76T) and (*Pfmdr1*,N86Y) copy number from dried blood spot. Parasite DNA was extracted using high pure DNA preparation kit. The amplification of DNA was done by using AccuPower 2× GreenStar[TM] qPCR Master mix. For quantification purpose a new primer pair was designed for 178 base pair template from complete genome sequence of *Plasmodium falciparum* strain 3D7 at NCBI. Absolute quantification method was used to determine the *Pfmdr1*-N86Y copy number variations. Standard curve was built from strain 3D7 gDNA since it has single copy of *Pfmdr1* per haploid genome. The known positive controls with single and multi-copy number of *Pfmdr1* gene were included in each experiment. The copy number ratio of the samples to the standard calibrator was made to obtain the fold difference among the samples with respect to copy number variation.

Results: Out of 133 samples 73 (54.89%) were confirmed as mutant (*Pfcrt,* 76T) and the remaining 60 (45.11%) were genotyped as wild type (*Pfcrt,* K76). The (*Pfmdr1*, N86Y) copy number variation was determined for 133 clinical samples. Out of these samples 61 (45.86%) had single copy and the remaining 72 (54.14%) had multi-copy numbers higher than 1.5 copies per genome. Thirty-four (25.56%) multi-copies were between 1.5 and 2.5 copies per genome while 38 (28.57%) were more than 2.5 copies per genome. The minimum and maximum copies per genome were 0.474 and 4.741, respectively.

Conclusions: The study showed high prevalence level and fixation of *Pfcrt*, 76T mutation after chloroquine withdrawal. The prevalence of *Pfmdr1* copy number variant suggested that the presence of modulating factor for emergence of *Plasmodium falciparum* strains with higher copy numbers. However, the prevalence level was not statistically significant.

1. Introduction

Plasmodium falciparum (*P. falciparum*) has a potential for drug resistance after any drug introduction to treat malaria. The parasite evolves to a new evolutionary line against anti-malaria drugs. Combination drugs are effective for treating chloroquine resistance *P. falciparum* malaria, but the parasite could develop a resistance including combination regimens with or without artemisinin[1].

Drug resistance malaria is a major challenge to control *P. falciparum* malaria in developing and low income countries particularly sub-Saharan Africa. It is the leading cause of morbidity and mortality[2].

Structurally chloroquine is a 4-aminoquinoline which was introduced in the late 1940s. Chloroquine was used as a massive scale for malaria treatment and prophylaxis. Due to its efficacy, affordability and safety, chloroquine is a kind of standard treatment drug against malaria for a long time[3,4]. Chloroquine is one of the cheapest and safest drugs ever used for malaria treatment[5]. It has one of the longest half-lives among antimalarials with approximately 60 d, which provides a chemoprophylactic effect during the drug elimination phase. It also exposes parasites to an extended time period after which chloroquine has fallen below the therapeutic concentration, which may select for drug-resistant parasites[4].

There are two main mutations which characterize chloroquine resistance in *P. falciparum* such as the *Pfcrt* and *Pfmdr1* mutations. The (*Pfcrt*, K76T) and (*Pfmdr1*, N86N) are the most well-known markers for chloroquine resistance *P. falciparum*. *P. falciparum* chloroquine resistance at digestive food vacuole membrane has an association with each other. *Pfcrt*, K76T mutation occurs first and then another mutation followed. The (*Pfcrt*, K76T) point mutation yields more resistance than other *Pfcrt* point mutation[6]. The association of K76T mutation with other mutations in *Pfcrt* gene gives different levels of chloroquine resistance[7]. The K76T mutation is selected first followed by its association with other mutations in *Pfcrt* gene.

The (*Pfmdr1*, N86Y) copy number and codon mutations are important factors for drug resistance *P. falciparum*. An increase in copy number of a gene enhances the level of drug resistance. The high copy number of a target gene found together with a codon mutation, and then the parasite possesses enhanced anti-malaria drug resistance level. A study showed that an increased *Pfmdr1* copy number with wild type codon (N86) had mefloquine sensitivity. On the other hand, increased *Pfmdr1* copy number with codon mutation (86Y) had a higher resistance level[8]. However, other recent studies showed that higher copy number of *Pfmdr1* could cause resistance in mefloquine, artesunate and lumefantrine while low copy number was sensitive to *in vitro* test regardless of codon mutation[9]. *Pfmdr1* polymorphism showed no relationship in *in vitro* susceptibility of these drugs[10]. *Pfmdr1* copy number variation is more frequent event than *Pfmdr1* codon mutation[11].

Strong association was reported between K76T and N86Y mutations for chloroquine resistance[6,12]. When (*Pfcrt*, K76T) and *Pfmdr1* wild type (N86) occur together, the parasite shows resistance to chloroquine. On the other hand, when *Pfcrt*-K76T occurs with *Pfmdr1* mutant type (86Y), the parasite shows resistance to chloroquine, but (*Pfcrt*, K76) and (*Pfmd1*, N86) do not have resistance to chloroquine. This implies that the presence of (*Pfcrt*, K76T) mutation is pre-condition for the *Pfmdr1* parasite to develop multi-drug resistance property against chloroquine and also *Pfmdr1* increases the level of chloroquine resistance having synergetic effect with (*Pfcrt*, K76T). High parasite population with (*Pfcrt*, K76T) mutation favours the emergence of (*Pfmdr1*, N86Y) mutation[13]. Chloroquine resistance reached at fixation stage after the development of resistance[14,15]. The fixed population of chloroquine resistant *P. falciparum* will be continued in areas where *P. falciparum* and *Plasmodium vivax* (*P. vivax*) are co-endemic[16].

Chloroquine, primaquine and quinine were the most popular anti-malarial drugs for malaria treatment in Ethiopia before 1998. The Federal Ministry of Health, Ethiopia in 2004 recommended the use of quinine tablets and injection to treat severe malaria. The widespread chloroquine treatment failure by *P. falciparum* in 1997–1998 led to the introduction of sulfadoxine/pyrimethamine for the treatment of *P. falciparum* malaria. Sulfadoxine/pyrimethamine has been in use until the adoption of Coartem in 2004[17].

After the banning of sulfadoxine/pyrimethamine, Coartem is recommended to treat complicated malaria and mixed infections. Since 2005 up to date chloroquine, artesunate, quinine and Coartem are in use to treat malaria[17,18]. Chloroquine is still in use to treat uncomplicated malaria particularly *P. vivax*[17,18].

In the study area *P. falciparum* is common *Plasmodium* species and self-treatment is also common, so there is a danger that parasites develop resistance to Coartem and chloroquine[18]. The treatment of malaria in Ethiopia is done by chloroquine and Coartem nationwide. Coartem is given for complicated malaria and chloroquine is given for uncomplicated malaria especially for *P. vivax* infections. The treatment of *P. vivax* by using chloroquine as first-line drug might increase the prevalence of chloroquine transporter gene point mutation and *P. falciparum* multi-drug resistance. Chloroquine is incompletely withdrawn from the study area. Hence the (*Pfcrt*, K76T) and (*Pfmdr1*, N86Y) mutation would have been fixed in *P. falciparum* population. The prevalence of chloroquine resistance molecular markers for *P. falciparum* has not been established in the study area. Therefore, the prevalence level of chloroquine resistance point mutation at (*Pfcrt*, K76T) and (*Pfmdr1*, N86Y) copy number variants were determined as critical factors for future malaria treatment in the study area.

2. Method and materials

2.1. Description of the study area

The study was conducted in six health centres in Dembia District and one hospital in Gendawuha District. Dembia District is located at an altitude range of 1750 m to 2100 m above sea level; latitude and longitude of 12°36'N 37°28'E and 729 Km from the capital

Figure 1. Map of Ethiopia showing the study sites.

Addis Ababa. Dembia district covers an area of 1270 km^2 with a total population of about 263000. This area is endemic to malaria and *P. falciparum* and *P. vivax* are the most commonly reported species from microscope diagnosis of febrile patient. The hospital is located at Gendawuha District which is found in latitude and longitude of 12°58'N 36°12'E with an elevation of 685 m above sea level and approximately 950 km from the capital Addis Ababa. The area has approximate population of 5502. The two areas have severe malaria transmission seasons such as October to December (Figure 1).

2.2. Ethical clearance

The research proposal was submitted to University of Gondar Natural and Computational Science College Ethical Review Committee for Ethics approval. The Committee approved the proposed research is ethically cleared after thorough consideration of the proposed research proposal. Consent form was prepared for study participants. The purpose of this study was explained for participants and parents/guardians in their mother tongue before being recorded in the study. Those who did not consent were not included in the study. In agreement with clinics and hospital the cost of drug for treating positive patients were covered by the clinics and hospital.

2.3. Study design

The study was cross sectional. It was conducted during malaria transmission season (September to December, 2013). Malaria transmission is seasonal in Ethiopia, which is peak during autumn, September to December, and spring seasons, April to May. Six clinics (Tseda, Makisegnit, Chuahit, Aymba, Kola Diba and Sankisa) and one hospital (Metemma Hospital) were used as the study sites to collect samples from outpatients.

2.4. Study participants

Patients who attended the health institutions during study period were recruited for the study. Participants, who were between six months and seventy years old, were included in the study. Infants, under six months of age and pregnant women were excluded. Patients, who were reported as *P. falciparum* positive during health service, were asked for consent to participate in the study by health professionals. Finger pricks were done, by health professionals, for those patients who consented to participate in the study.

2.5. Sampling

After finger pricking, two spots of blood samples were prepared on

Whatman 3MM filter paper from each study participant. The blood spots were prepared by dropping three to four blood drops per spot on a piece of filter paper. In total, 168 blood samples were collected from all study sites (Tseda, $n=14$; Makisegnit, $n=14$; Sankisa, $n=33$, Chuahit, $n=6$; Kola Diba, $n=32$, Aymba, $n=31$; Metemma Hospital, $n=38$). The spots were properly labelled, air dried. Silica gel was added inside each plastic bag to prevent humidity. In order to avoid cross contamination one plastic bag was used for one filter paper. The cards were collected in sealed plastic bag and transported to Kenya Medical Research Institute for molecular analysis.

2.6. Parasite DNA extraction

A piece of dried blood spot of approximately 2-3 mm in size was cut out with sterile scissors. The pieces were placed in sterile 1.5 mL extraction tubes using flamed forceps. High pure PCR template preparation kit (Version 20; Roche diagnostics, GmbH, Germany) was used to extract parasite DNA from dried blood spots. The extraction was done according to the manufacturer's instructions. The extracted DNA was stored in -20 ℃ freezer until used for PCR.

2.7. Real-time PCR condition and amplification

Real-time PCR is an advanced PCR type over convetional PCR. It is assisted by software for monitoring of the progress of DNA amplification. It does not depend on end product analysis. The progress of DNA amplification can be easily observed during the amplification reaction. In addition, real-time PCR is helpful for exclusion of primer-dimers and non-specific amplifications and, melting curve analysis is applied in real-time PCR. Real-time PCR helps to quantify the target gene and takes less time than convetional PCR. Therefore, in order to achieve the three objectives real-time PCR was used in the study.

One step PCR was done using AccuPower 2× GreenStar™ qPCR Master mix in Exicycler 96 ™ (Bioneer South Korea). Exicycler™ Version 3.0 Software was used for programming the Exicycler Thermal Block (Exicycler 96) and data analysis was made based on operating manual from Bioneer. The reaction condition was done according to manufacturers' instruction from the AccuPower 2× GreenStar™ qPCR Master mix. It was done as first initiation step at 95 °C for 10 min; denaturation step at 95 °C for 20 seconds; combined annealing and extension at 55 °C for 30 seconds followed by scanning the amplification product. This one step PCR runs for continuous 40 cycles. The PCR products were immediately subjected for melting to generate melting curves.

The melting process occurs when double stranded DNA formed after PCR amplification is dissociated at high temperature and as it melts the SYBR Green I dye fluorescent gradually decreases because the dye no longer stays being attached to the double stranded DNA. This phenomenon detected by the Exicycler software and the software gave melting curves which had sharp turning point at the maximum melting temperature of each double stranded PCR amplification products. Therefore, each PCR product has specific "melting point" and hence temperature profiling is used for identification of PCR products.

2.8. Melting curve analysis and primer-dimers

Primer-dimers occur when two PCR primers either same sense primers or sense and anti-sense primers bind to each other instead of to the target. Melting curve analysis identified the presence of primer-dimers because PCR amplicon is usually higher in length than primer-dimers. Therefore it has higher "melting temperature" than primer-dimers. The presence of primer-dimers is not desirable in samples that contain template, as it decreases PCR efficiency and obscures analysis. The formation of primer-dimers most often occurs in no-template controls (NTCs), where there is an abundance of primers and no template. The presence of primer-dimers in the NTC served as an alert that they were also found in reactions that included template. If there were primer-dimers in the non-template control tube, the Ct values of the reactions were used for confirmation because Ct values of primer-dimers and Ct values of template were different. Primer-dimers have higher Ct values because primers are lower in concentration than template except the non-template control tube. Therefore, amplification curves with lower Ct values than NTC Ct value were taken as template reactions. Melting curve analysis of NTCs discriminated between primer-dimers and spurious amplification due to contaminating nucleic acids in the reagent components.

2.9. Real-time PCR amplification for (Pfcrt, K76T) identification

The amplification of (*Pfcrt*, K76T) gene was employed using published primer pairs CRT-F (5'-TGA CGA GCG TTA TAG AG-3') and CRT-R (5'-GTT CTT TTA GCA AAA ATT G-3')[19]. The amplification was done at 25 μL reaction volume. AccuPower 2x GreenStar™ qPCR Master mix (12.5 μL), 2 μL (10 pmole) forward primer, 2 μL (10 pmole) reverse primer, 3.5 μL DEPC-distilled water and 5 μL DNA template were used. NTC was included to control formation of primer-dimers.

Chloroquine resistant and sensitive *P. falciparum* strains (W2, D6 and 3D7) obtained from Kenya medical research institute malaria laboratory were used as controls in order to identify wild type and mutant type clinical samples. These controls were included in each PCR run throughout the experiment. An artificial mix of DNA

extracted from these strains were made in different ratio in order to determine the efficiency and sensitivity of the assay to identify the wild type (*Pfcrt*, K76) and mutated type (*Pfcrt*, 76T) at different concentration levels. The ratios of strain W2 to strain D6 were (10 μL:10 μL, 5 μL:10 μL) and strain W2 to strain 3D7 were (10 μL:10 μL, 5 μL:10 μL). The mixing of the two strains was made to obtain the optimum detection threshold of the assay for genotyping (*Pfcrt*, K76T). The melting curves obtained from artificial mixed resistant strain with sensitive strain, and melting curves obtained from clinical samples were compared for confirmation purpose.

2.10. Melting curve analysis for (Pfcrt, K76T) identification

Melting curve analysis is useful for identification of wild and mutant genotypes of (*Pfcrt*, K76T). Since the melting curve shapes and peaks are different in these two genotypes, the curve and specific temperature profile enable to discriminate K76 and 76T genotypes. Therefore, after amplification of the template DNA the PCR product was immediately subjected for melting at 70 °C to 83 °C at ramping rate of 1.0 °C for 1 second. The known positive controls were included in order to identify the wild type and mutant type clinical samples. Chloroquine sensitive strains (D6, 3D7) and resistant strain (W2) were used as positive controls. NTC was also included for each run. The samples were identified as wild type and resistant type based on respective temperature profile and the shape of the melting curve graphs. The primary differential (F') was selected from melting curve window for identification of the graph shape and peaks. The shape and peaks of the melting curves were carefully observed. The dual and single melting curve peaks were observed from 70 °C to 83 °C.

2.11. (Pfmdr1, N86Y) and copy number determination by qPCR

Real-time PCR quantification of the target gene is better quantified when the template is short usually less than 200 bp. Therefore, in this study the *Pfmdr1* amplification and copy number variation were determined from 178 bp long template DNA. In order to obtain short template newly designed primer pairs (forward 5'-GAT GGT AAC CTC AGT ACT-3' and reverse 5'- CTC CTG ATA ATA CAG CAC-3') were used with dual labelled single probe (5'-TET-ACC TAA ATA CAT GTT CTT T-3'-BHQ1). The probe was used to differentiate the mutant and wild type of the samples at (*Pfmdr1*, N86Y) codon. The labelled probe complements to the codon mutation specific region of the (*Pfmdr1*, N86Y). Fluorescence can be detected from the complementation of the probe with template because the quencher and reporter dyes fluoresce only when there is complementation between the target and the probe sequences. The probe sequence was obtained from published paper[20].

In order to determine the copy number variation targeted in (*Pfmdr1*, N86Y) position, these newly designed qPCR primer pairs

flanking in the complete mRNA (coding DNA sequence) regions of *P. falciparum* (3D7 strain) at chromosome five were used. *Pfmdr1* or Polyglycoprotein 1 is coded by the gene located at chromosome five in *P. falciparum* genome and is responsible for multidrug resistance development in *P. falciparum*. The 3D7 strain sequence was selected for primer design because this strain is fully sequenced and representative for *P. falciparum* genome study. *Pfmdr1* develop due to functional Polyglycoprotein1, therefore, non-functional sequences were excluded during the primer design.

The FASTA sequence was browsed from NCBI (www.ncbi.nlm. nih.gov/) at GenBank accession number (XM_001351751) and pasted in the Integrated DNA Technology Primer Quest (www. idtdna.com). After copy-pasting the FASTA sequence format, the parameters such as primer length, product length, GC content, Tm of primer, Tm of product and salt concentration were adjusted from drop down menu of the software. After designing the new primer pair, it was analyzed for Tm mismatch, self-dimer formation (homo-dimer), hairpin formation, and hetro-dimerization. The analysis was done by IDT Oligo analyser online (www.idtdna.com) and finally, "blastn" was done for the new primer pair from NCBI. After these all process the primer pairs were validated to be used for absolute quantification of the target gene for copy number variation.

The amplification of (*Pfmdr1*, N86Y) was done at 25 μL reaction volume of which 12.5 μL AccuPower 2× GreenStar™ qPCR Master mix, 2 μL (10 pmole) forward primer, 2 μL (10 pmole) reverse primer, 2 μL single probe, 1.5 μL DEPC-distilled water and 5 μL DNA template were used. Non-template control was included to control formation of primer-dimers.

Chloroquine resistant and sensitive *P. falciparum* strains (Dd2, W2, D6 and 3D7) obtained from Kenya medical research institute malaria laboratory were used as controls in order to determine copy number variation in clinical samples. These controls were included in each PCR run throughout the experiment in triplicated.

2.12. Absolute quantification of Pfmdr1 (N86Y) copy number variation

The standard curve was built from *P. falciparum* gDNA. The supporting manual from (www6.appliedbiosystems.com/support/ tutorials/pdf/quant_pcr.pdf) was obtained and used for preparation of the standard curve from strain 3D7 gDNA. *P. falciparum* (strain 3D7) was used as reference copy number. This strain is known to have single copy of *Pfmdr1* gene in the haploid *P. falciparum* genome. In order to build the standard curve the concentration of DNA extracted from 3D7 was measured by NanoDrop 2000 spectrophotometer. The average concentration of the DNA was 15.75 ng/μL which is equal to 15750 pg/μL. Absorbance of the DNA sample was measured at A260, A280, A260/280, A260/230. Derivation of DNA mass formula is as follows:

$$m=[n]\left[\frac{1}{6.023e^{23}\ \text{Moleculules (bp)}}\right]\left[\frac{660\ \text{g}}{\text{mole}}\right]=[n]\left[1.096\ e^{-21}\ \frac{\text{g}}{\text{bp}}\right]$$

$$m=[n]\left[1.096\ e^{-21}\frac{g}{bp}\right]$$

Where n refers to genome size (bp), while m refers to mass.

The Avogadro's number is equal to 6.023×10^{23} molecules per mole and Average molecular weight of a double-stranded DNA molecule is 660 g/mole. The size of *P. falciparum* genome is around 23 Mbp which is equal to 23000000 bp[21]. In order to obtain the mass of *P. falciparum* haploid genomic DNA from the whole genome size, 23000000 bp was inserted for n- value in the above formula. The mass of *P. falciparum* haploid genome is calculated as follows:

$$m=[23\times10^6\ bp]\left[\frac{1.096\times10^{-21}\ g}{bp}\right]=0.025\times10^{-15}\ g$$

$$m=[0.025\times10^{-15}\ g]\left[1\times10^{12}\frac{picogram}{g}\right]=0.025\ pg$$

Dividing the mass of the haploid genome by the copy number of the gene of interest per haploid, genome gives the copy number of the gene in 0.025 pg. The 3D7 *P. falciparum* strain has the target gene that exists as a single copy gene per haploid genome (or two copies per *P. falciparum* cell). This implies that:

$$\frac{0.025\ pg}{Haploid\ genome}\div\frac{One\ copy\ pfmdr1}{Haploid\ gemone}=\frac{0.025\ pg}{One\ copy\ of\ Pfmdr1}$$

Therefore, 0.025 pg of *P. falciparum* haploid genome contains one copy of *Pfmdr1* (N86Y) gene. Then, calculations of the mass of gDNA containing the copy numbers of interests, which are 800000 to 0.8 copies, were done. If one copy of *Pfmdr1* gene is obtained from 0.025 pg of haploid genomic DNA, then 800000 copies of *Pfmdr1* gene can be obtained from 20000 pg.

Copy number of interest×mass of haploid genome=mass of gDNA needed

The following calculation is used to obtain mass of haploid genomic DNA needed and the concentration of the DNA samples in five microliter template DNA in the ranges of copy numbers of interest from 800000 to 0.8 copies. The multiplication of copy number of interest by 0.025 pg gives the mass of haploid genome needed to get the concentration of DNA needed at five microliter template. The division of mass of haploid genome to five microliter DNA template gives final concentration of DNA for each dilution (Table 1).

Table 1

Mass of genomic DNA needed and final concentration of input DNA (stock DNA) at five microliter template are calculated from copy number of interests.

Copy number of interest	Haploid genome of P. falciparum (pg)	Mass of needed (pg)	Volume of template (μL)	Final concentration (pg/μL)
800000		20000		4000
80000		2000		400
8000		200		40
800	×0.025	20	/5	4
80		2		0.4
8		0.2		0.04
0.8		0.02		0.004

Ten-fold serial dilution of the stock DNA was obtained by using the following formula based on the above concentration of template DNA.

$$C_1V_1=C_2V_2$$

Where: C_1 refers to stock concentration (15750 pg/μL); V_1 is required volume; V_2 refers to 100 μL; C_2 refers to 4000 pg/μL

$$V_1=\frac{C_2V_2}{C_1}$$

Then the value of V_1 is 25.4 μL. In order to obtain the 100 μL dilution a difference of 25.4 μL from 100μL diluent is needed. The volume of diluent is 74.6 μL. Ten-fold serial dilution was prepared from the stock DNA (Table 2) and standard curve was built from the diluted DNA sample.

Table 2

Serial dilutions from DNA stock solution.

Serial number	Initial concentration (pg/μL)	Volume 1 (μL)	Final concentration (pg/μL)	Volume 2 (μL)
Stock	15750	25.4	4000	100
1	4000	10	400	100
2	400	10	40	100
3	40	10	4	100
4	4	10	0.4	100
5	0.4	10	0.04	100
6	0.04	10	0.004	100
7	0.004	10	0.0004	100

The standard was included in each run. The average amplification efficiency was 106% including at least three points from the serially diluted standard DNA template on the linear regression line. The PCR efficiency value is acceptable in the range 100+/-10%. Outlier points were omitted from the reaction plate in order to get appropriate efficiency. The samples were laid on the linear regression line and there was no any outlier points omitted from the sample. There was no PCR efficiency difference between the standard and clinical samples, because the primer pairs were the same and designed for the same target (*Pfmdr1*, N86Y) region in the calibrator strain 3D7 and *P. falciparum* positive samples. Input DNA concentration was determined automatically for each sample by the Exicycler software based on the standard concentration. The following formula was used in order to calculate the copy number of samples from the concentration of the input DNA per microliter (www.scienceprimer.com).

$$DNA\ copy\ number=\frac{[6.023\times10^{23}\ copy/mol]\times[DNA\ amount(g/\mu l)]}{[amplicon\ length(bp)]\times[660g/mol\ 1/bp]}$$

$$DNA\ copy\ number=\left[\frac{6.023\times10^{23}\ copy/mol}{117480\ g/mol}\right]\times[DNA\ amount(g/\mu l)]$$

The copy numbers were calculated using the above formula from DNA input concentration which was determined by the PCR. Molecular weight of DNA is 660 g per mole per base pair and the amplicon length is 178 in base pair. Finally, the copy number ratio of positive controls (Dd2, W2 and D6) to 3D7 and samples to 3D7 were calculated manually in order to obtain copy number variation or fold difference among the calibrator 3D7 strain, positive controls and clinical samples. In other words the ratio of input template

concentration of the samples and positive controls to the input template concentration of the 3D7 strain was determined. The ratio gives the fold difference between the sample and the reference strain 3D7. The 3D7 strain has single copy of *Pfmdr1* gene per haploid genome. Therefore, if the sample had more copies than 3D7 it would be considered as multi-copy. In contrast, if the sample had less or equal copy with 3D7, the sample would be considered as single. For instance, the copy number of 3D7 strain at 4000 pg/µL input concentration of DNA template was calculated as follows:

$$\text{3D7 strain DNA copy number} = \frac{\left[6.023 \times \frac{10^{23}\ \text{copy}}{\text{mol}}\right] \times \left[4000\left(\frac{\text{pg}}{\text{uL}}\right)\right]}{[178\ (\text{bp})] \times \left[\frac{660\ \text{g}}{\text{mol}} \times \frac{1}{\text{bp}}\right]}$$

$$= 2.05 \times 10^{10}\ \text{copies/µL}$$

The ratio 3D7 to 3D7 $\gg \dfrac{3D7}{3D7} = \dfrac{2.05 \times 10^{10}}{2.05 \times 10^{10}} = 1$ copy/µL

The copy number of the positive control Dd2 strain at 694 pg/µL input concentration of DNA template was also calculated. Then, the ratio of Dd2 to 3D7 was obtained by dividing the copy number of Dd2 to copy number of 3D7 strain in order to get the fold difference between the two *P. falciparum* strains.

$$\text{Dd2 strain DNA copy number} = \frac{\left[6.023 \times \frac{10^{23}\ \text{copy}}{\text{mol}}\right] \times \left[694\left(\frac{\text{pg}}{\text{uL}}\right)\right]}{[178(\text{bp})] \times \left[\frac{660\ \text{g}}{\text{mol}} \times \frac{1}{\text{bp}}\right]}$$

$$= 3.56 \times 10^{10}\ \text{copies/µL}$$

The ratio Dd2 to 3D7 $= \dfrac{Dd2}{3D7} = \dfrac{3.56 \times 10^{10}}{2.05 \times 10^{10}}$ 1.736 fold

The remaining values for clinical samples are computed in the same way in order to get the copy number variations.

3. Results

3.1. Pfcrt (K76T) identification and Pfmdr1 (N86Y) copy number determination

In the study 133 samples were re-confirmed by SYBR Green I-based real-time PCR as *P. falciparum* positive samples. Those samples were analysed further for (*Pfcrt*, K76T) genotyping and (*Pfmdr1*, N86Y) copy number determination.

The *P. falciparum* chloroquine transporter gene was targeted for (*Pfcrt*, K76T) genotyping. The amplification of the target gene was recorded from log scale form of the amplification curves. This form of graph helps to exclude the non-specific amplifications, primer-dimers and other artefacts to the left bottom side of the graphs (Figure 2). The negative and positive controls were included in the experiment in each and every PCR runs.

Melting curve analysis was made for (*Pfcrt*, K76T) genotyping. The shapes of the melting curves indicated the wild type (*Pfcrt*, K76) as flat curves and mutant type (*Pfcrt*, 76T) as raised curves with dual peaks. The strains (3D7 and D6) were positive controls for the wild type and showed flat curves and strain (W2) was
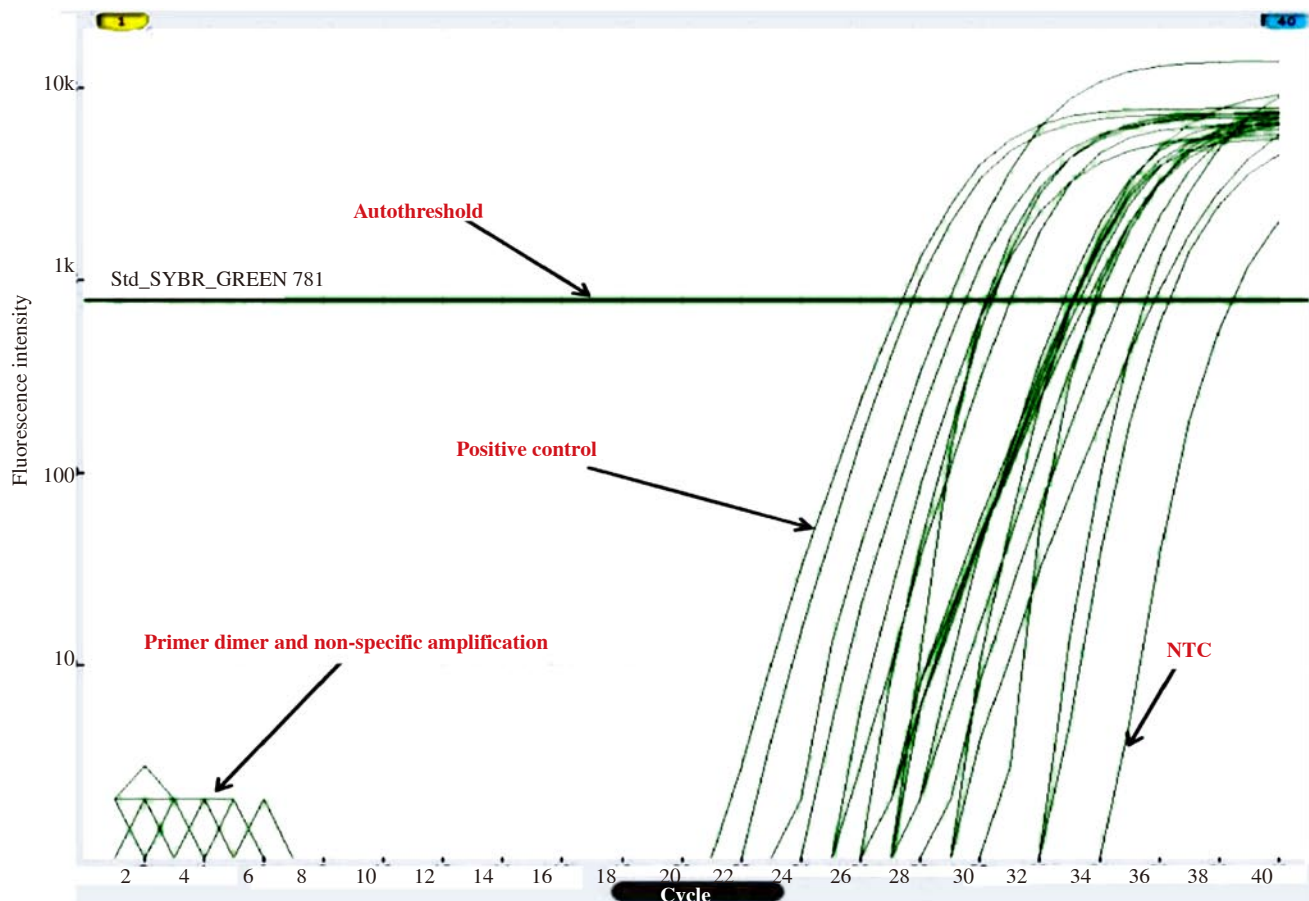

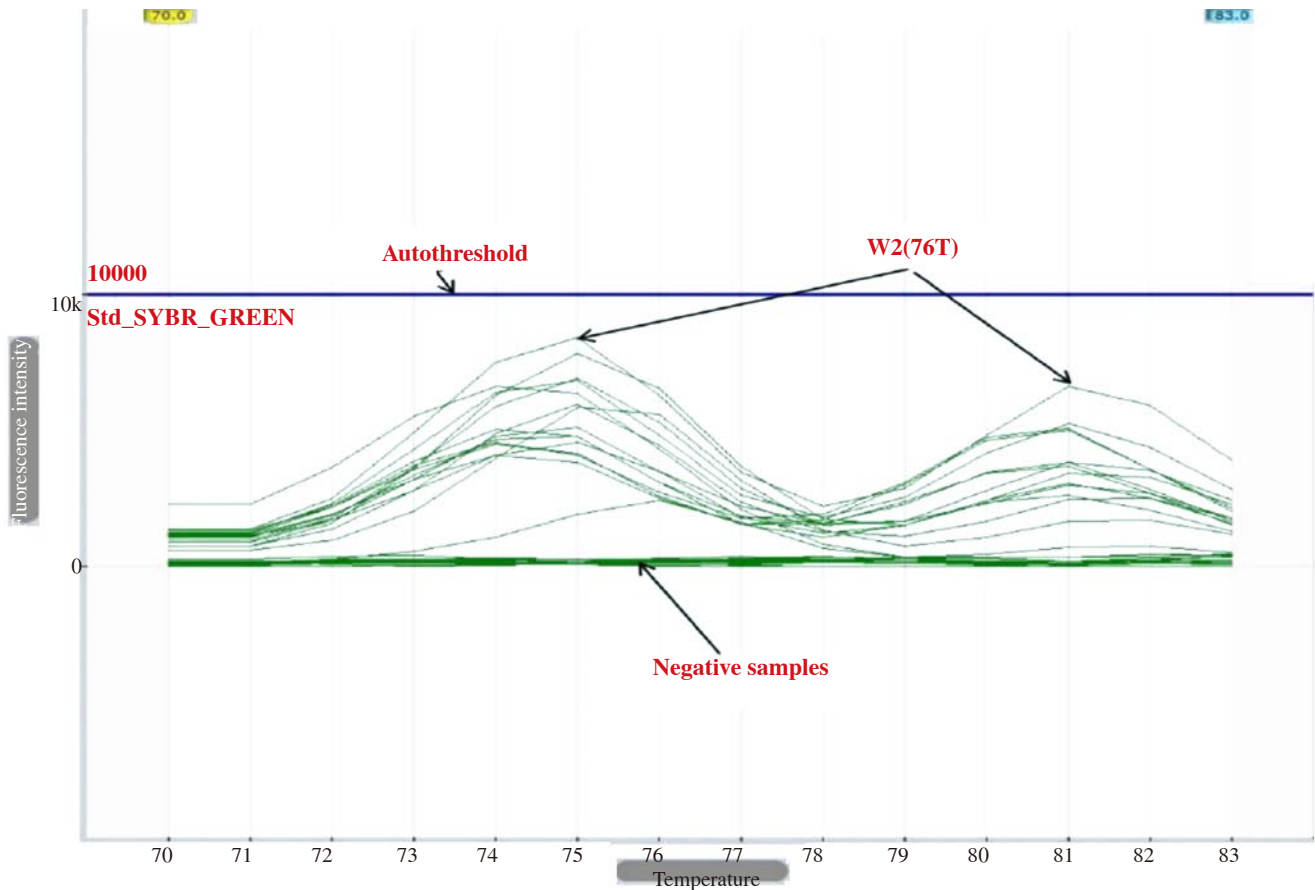**Figure 2.** Amplification of (*Pfcrt*, K76T) in log scale form.

Figure 3. Melting curves for wild type and mutant type (*Pfcrt*, K76T) gene.

positive control for mutant type and showed raised melting curve with dual peaks (Figure 3).

Among the 133 samples 73 (54.89%) were genotyped as resistant type (*Pfcrt*, 76T) and the remaining 60 (45.11%) were genotyped as wild type (*Pfcrt*, K76). In this study high prevalence of mutant genotype (*Pfcrt*, 76T) was found (Figure 4).
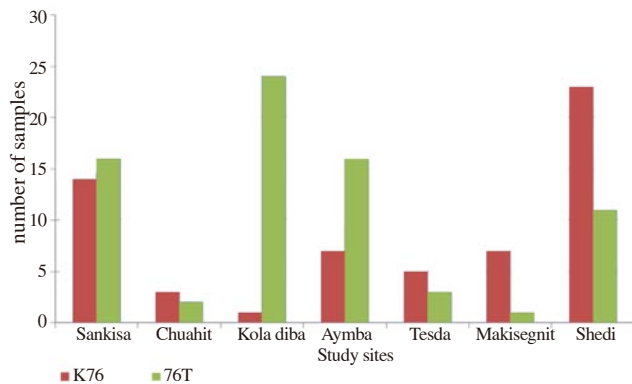

Figure 4. The prevalence of (*Pfcrt*, K76T) point mutation.

3.2. Pfmdr1 (N86Y) amplification and copy number variation

The genotyping of wild type (*Pfmdr1*, N86) and mutant type

(*Pfmdr1*, 86Y) single amino acid polymorphs was done by using the dual labelled single probe, which was specifically designed to complement the mutant genotype (*Pfmdr1*, 86Y) codon mutation, and SYBR Green I dye. The yellow graphs generated by the TET-labelled probe fluorescent were represented as mutant genotype (*Pfmdr1*, 86Y) and the green graphs generated by SYBR Green I dye were represented as wild type (*Pfmdr1*, N86) (Figure 5). The positive controls from resistant strains such as W2 and Dd2 were included and showed as yellow coloured graphs in the amplification graphs which were confirmed as positive for mutant genotypes in the assay throughout the experiment. In contrast, sensitive strains such as 3D7 and D6 showed as green in the amplification graph were confirmed as positive for wild type genotypes. Therefore, clinical samples were genotyped based on the positive controls and the colour of the graph. A few samples were categorized as the mutant genotype (*Pfmdr1*, 86Y) and the remaining of the samples were represented as wild type genotype (*Pfmdr1*, N86) (Figure 5).

The melting curve analysis was not successful for further analysis of these polymorphisms. The failure of melting curve analysis could be due to loose complementation between the probe and the template hence it melted rapidly before formation
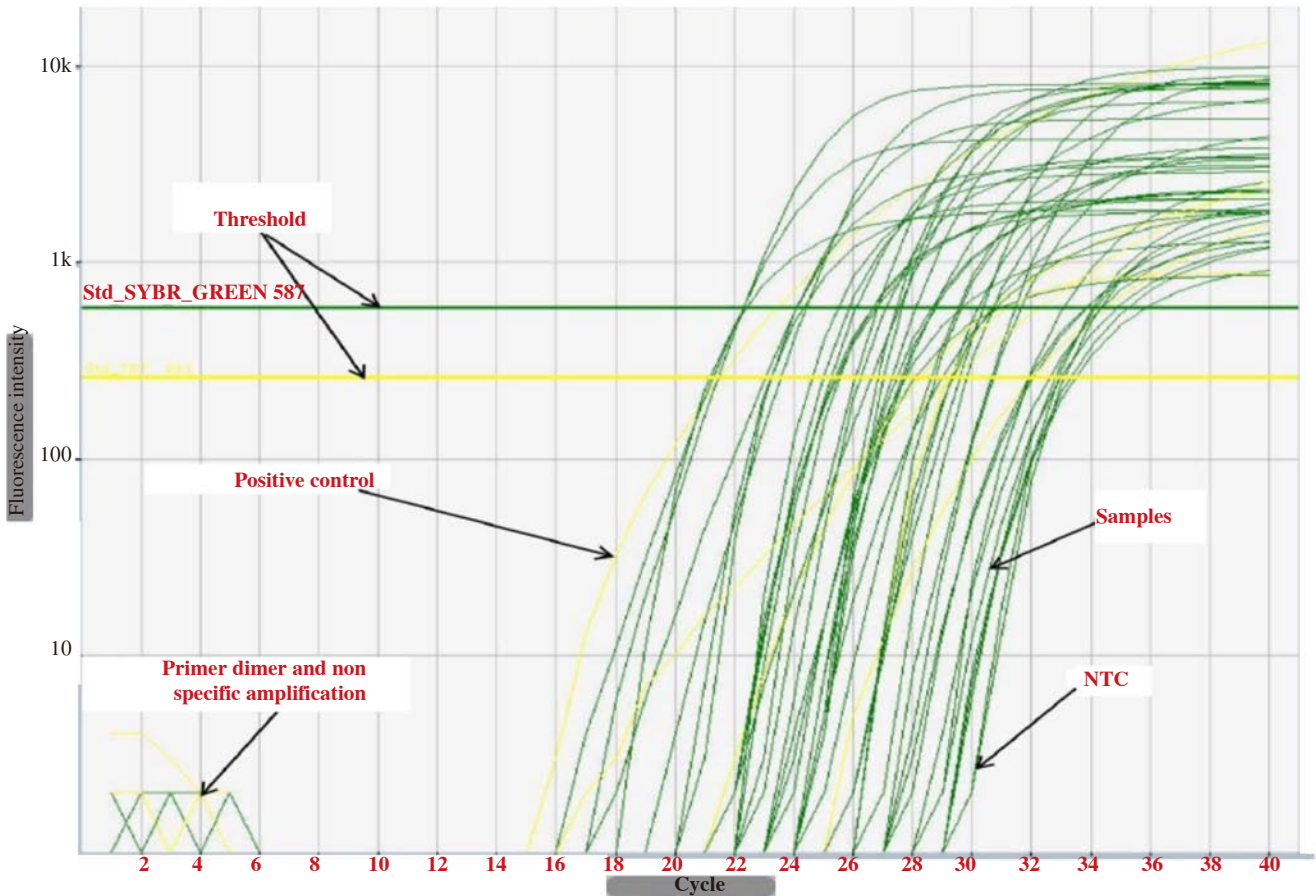
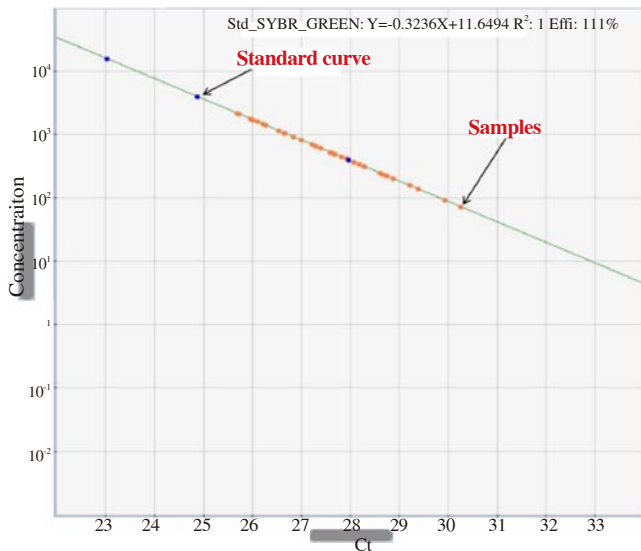**Figure 5.** Amplification curves of (*Pfmdr1*, N86Y) gene in log scale form.

Figure 6. Standard curve built from serially diluted 3D7 *P. falciparum* genomic DNA for absolute quantification.
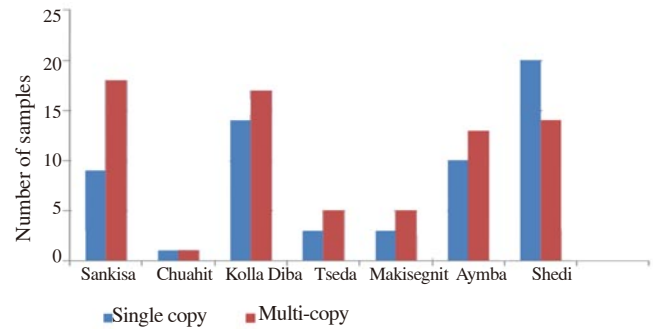

Figure 7. Copy number variation prevalence of (*Pfmdr1*, N86Y) gene.

Figure 8. Both medium and higher copy number variants of (*Pfmdr1*, N86Y).

of the sharp melt curve peaks.

In order to determine the copy number of the target gene, the standard curve was made from a known concentration of genomic DNA which was serially diluted and having the same target gene with single copy per haploid genome. Then the input

DNA concentration of each clinical sample was determined by the software automatically based on the concentration of serially diluted DNA from the standard curve (Figure 6). The blue points on the regression line represents the concentration of the standard sample. In other words, these points were obtained from the 3D7

strain genomic DNA and the yellow points on the regression line represented the concentration of the clinical samples.

A total of 133 samples were analysed further for determination of the (*Pfmdr1*, N86Y) copy number variation. Among the 133 samples 73 (54.89%) samples were confirmed as multi-copy variants of (*Pfmdr1*, N86Y) having copy numbers of the target gene from 1.5 copies to 4.471 copies per genome and the remaining 60 (45.11%) were confirmed as single copy variants of (*Pfmdr1*, N86Y) having copy numbers less than 1.5 copies per genome (Figure 7).

The multi-copy number variants of (*Pfmdr1*, N86Y) were categorized as medium copy number variants and higher copy number variants. In this category 34 (25.56%) were medium copy number variants of (*Pfmdr1*, N86Y) having copy numbers less than 2.5 copies per genome and 38 (28.57%) were with higher copy number variants of (*Pfmdr1*, N86Y) having 2.5 copies or more copy numbers per genome (Figure 8).

The minimum and maximum copy numbers per genome were 0.497 (single copy) and 4.741 (multi-copies), respectively. Positive controls obtained from Kenya medical research institute malaria laboratory were included in the assay. The controls were Dd2 *P. falciparum* strain which is known to have multiple copy numbers of (*Pfmdr1*, N86Y), and W2 and D6 *P. falciparum* strains which possess single copy number of (*Pfmdr1*, N86Y). The copy number variants of the positive controls were consistently found as single and multi-copy in each experimental runs.

4. Discussion

This is the first study conducted to determine prevalence of chloroquine transporter gene point mutations at (*Pfcrt*, K76T) and (*Pfmdr1*, N86Y) copy number variation in the study area. The (*Pfcrt*, 76T) point mutation is the marker which shows the status of chloroquine resistant malaria. The high prevalence level of this mutation is an indicator for the spread of drug resistant *P. falciparum* population in the study area. There are no related data done for the prevalence level of chloroquine resistant malaria in the study area for comparison the result obtained from this study showed high prevalence of (*Pfcrt*, 76T) mutation, but not statistically significant (P>0.05). The (*Pfcrt*, 76T) point mutation is well established molecular marker for chloroquine resistance level and the prevalence rate of the resistant parasite (*Pfcrt*, 76T) is the first mutation to be occurred and shows high level of resistance compared to the rest point mutations at *Pfcrt* gene[22]. Therefore, the higher the prevalence of this mutation, the more risk of malaria and challenging for drug resistance malaria control.

In addition, high prevalence of (*Pfcrt*, 76T) suggests that the presence of drug pressure or other factors which favours (*Pfcrt*, 76T) mutation.

Chloroquine was withdrawn from the study area since 2004 for treatment of *P. falciparum* malaria[17]. The parasite population with (*Pfcrt*, 76T) point mutation was expected that it would have been reduced. Change in chloroquine with other drug for *P. falciparum* treatment might disadvantage the mutant parasite survival. However, the parasite with mutant (76T) haplotype was fixed. The following reasons are contributing factors for fixation of (76T) mutation. The first is the incomplete withdrawal of chloroquine in the study area. Chloroquine is still in use for treatment of *P. vivax*. The second is high prevalence of mixed species infection. *P. falciparum* and *P. vivax* are endemic to the study area; therefore, mixed infection is common. The third is poor clinical diagnosis. The facilities and the skills of the laboratory technicians are critical for accurate detection of *Plasmodium* species. The fourth is poor management of malaria drugs. Patients purchase drugs from the shop and treat themselves without clinical diagnosis confirmation[18].

If chloroquine was completely withdrawn, the parasite would have been reverted to (*Pfcrt*, K76) and chloroquine could be used for treatment of *P. falciparum*. Studies in Kenya, Tanzania and Malawi showed the conversion of (76T) to (K76) and reduction of (*Pfcrt*, 76T) prevalence[23,24]. The use of artemesinin combination therapy (ACT) increased throughout Africa[25]. Therefore, the prevalence of 76T point mutation was expected to be reduced in Africa. However, in Southern Ethiopia one study showed high prevalence of (*Pfcrt*, K76T) and (*Pfmdr1*, N86Y) point mutations[26]. The drug chloroquine could be used again as combination or single treatment drug to treat malaria caused by *P. falciparum* where chloroquine mutation prevalence is very low or disappeared[22]. The current study and another study done in Ethiopia showed a high prevalence level of chloroquine resistant *P. falciparum* point mutation and hence re-use of chloroquine for *P. falciparum* treatment in the Ethiopian context is not advisable.

Malaria parasite population dynamically evolves along with anti-malarial drug pressure. The change of treatment pressure or drug affects the parasite evolutionary process. The drugs of malaria can be reused after a long period of withdraw. Both *Pfcrt* and *Pfmdr1* have synergic effect on chloroquine resistance level. The prevalence of *P. falciparum* with (76T) point mutation and high copy number variability at (*Pfmdr1*, N86Y) gene could trigger resistance for the new drug Coartem and impair the re-introduction of the previous drugs in the study area. The high prevalence of (*Pfcrt*, 76T) point mutation with either (*Pfmdr1*,

N86) or (*Pfmdr1*, 86Y) high copy can affect the treatment of malaria.

In this study high prevalence of *P. falciparum* multidrug resistance   copy number variants were found, but it was not significant statistically (*P*>0.05). Copy number variation exists among *P. falciparum* strain regardless of codon mutation. Parasites do have coping mechanism against drug pressure and the mechanism is highly dynamic across *P. falciparum* population. When chloroquine was a treatment drug for *P. falciparum* the parasite was able to survive and become fully resistant to chloroquine. Codon mutation and/or copy number variation is/ are the most common means of resistance development against the most used treatment drugs. Studies showed that copy number variations occur first and the *P. falciparum* becomes tolerant for many drugs. The higher the copy numbers of (*Pfmdr1*, N86Y) gene is, the more tolerant against treatment drugs. Even the uses of combination drugs become risky for effective treatment. High population of multidrug resistance mutation and copy number variation in *P. falciparum* develop resistance for many drugs like mefloquinine, lumefantrine and/or artemisinin derivative combination drugs such as Coartem (artemether/lumefantrine).

An *in vitro* study done in Ghana showed that nascent clones of *P. falciparum* manifested by reduced susceptibility to artemisinin derivatives[27]. Since lumefantrine and mefloquinine have physiochemical similarity, therefore, resistance to mefloquinine can be modulated by lumefantrine. A study done in Kenya showed that (*Pfmdr1*, N86Y) has association with mefloquinine response[28]. Another *in vitro* study in Cambodia-Thailand border showed that parasites with higher copy number showed significantly reduced susceptibility to mefloquinine, lumefantrine and artesunate. However, *Pfmdr1* polymorphism showed no relationship in *in vitro* susceptibility of these drugs[10].

Pfmdr1 copy number variation is more frequent event than *Pfmdr1* codon mutation[11]. A knockdown study done on *Pfmdr1* gene copy number showed reduced susceptibility to mefloquinine, lumefantrine and artesunat in an *in vitro* susceptibility test. In contrast, this knockdown study showed that parasite line with low copy numbers had heightened susceptibility to artesunate and lumefantrine[9]. Parasites with single copy number are sensitive for mefloquinine, lumefantrine and artesunate. These drugs can be used for combination therapy where the population of *P. falciparum* with single copy number of *Pfmdr1* gene are fixed[15].

The study of copy number variation in *P. falciparum* population is very vital for monitoring of resistance malaria particularly where mefloquine and lumefantrine are used for combination

therapy (ACT). *Pfmdr1*, are promising tools for the surveillance of drug resistance[29]. *Pfmdr1* copy number is not only strongly associated with recrudescence to artesunate-mefloquine but also with Coartem failure[8].

In the Ethiopian context Coartem is the treatment drug for uncomplicated malaria especially for *P. falciparum*. Since Coartem is an ACT drug which has lumefantrine component the presence of strains with high copy number variation in *P. falciparum* seems inevitable. The current study found high prevalence of multi-copy number variants in the study area. The parasite shifts from high *Pfmdr1* copy number to low copy number or vice versa depending on the drug pressure in the parasite population. *P. falciparum* with higher copy number is spread and favoured when mefloquinine, mefloquinine combination or drugs physiochemical similarities to mefloquinine are used for treatment of *P. falciparum*. In contrast, *P. falciparum* population with higher copy shifts to population of parasites with low copy because withdrawal of mefloquinine pressure has fitness disadvantage for continuation of mefloquinine resistant *P. falciparum*[11].

In this study a high prevalence of *P. falciparum* with a higher *Pfmdr1* copy number variants were found. This showed that fixation of the *P. falciparum* population with fitness advantage with possessing multiple copy of (*Pfmdr1*, N86Y). It is, indirectly, an alert that there is drug pressure which favours these populations in the study area. The reasons could be incomplete withdrawal of chloroquine, use of lumefantrine combination drug (Coartem), misdiagnosis of single infections and mixed infections. It was found a high prevalence of mixed infection and misdiagnosed *P. falciparum* which would have been treated by undesired drug that could contribute for fixation of the resistant *P. falciparum*.

Although, it is not statistically significant, a high prevalence level of chloroquine resistant *P. falciparum* was obtained. The (*Pfcrt*, K76T) point mutation showed fixation of previous chloroquine resistant *P. falciparum* population after withdrawal of chloroquine in the study area. The prevalence of (*Pfmdr1*, N86Y) copy number variant was not statistically significant, but it was highly spread and found to be an indicator of the evolutionary path shift from resistant *P. falciparum* for monotherapy to resistant *P. falciparum* population against combination drug therapy. Therefore, a high copy number variation is shown to be a risk factor for use of combination drugs for treatment of malaria.

Further, studies should be done to determine the prevalence level of *P. falciparum* chloroquine resistance point mutations and its fixation. It is critical for continuation or discontinuation of chloroquine use at a national level. Comprehensive molecular

surveillance is needed in order to control the spread of *Pfmdr1* copy number variants in *P. falciparum* population and it could be useful indicator of resistance development against combination therapy because *Pfmdr1* copy numbers are increased when mefloquinine and related drugs are used for treatment of *P. falciparum*.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

Comments

Background

This study was conducted in the endemic area of *P. falciparum* where self-treatment is common, so it is danger that parasite develop resistance to Coartem and chloroquine (a first line drug). The (*Pfcrt*, K76T) and (*Pfmdr1*, N86N) are the most known markers for chloroquine resistance *P. falciparum*. Prevalence level of chloroquine resistance point mutation at (*Pfcrt*, K76T) and (*Pfmdr1*, N86Y) copy number variants were determined as marker for this study area.

Research frontiers

The present research work depicts prevalence of chloroquine resistance point mutation at (*Pfcrt* ,K76T) and (*Pfmdr1*, N8Y) copy number determination by using SYBR Green I qPCR. The method has been publish but this is the first report of prevalence of (*Pfcrt*, K76T) and (*Pfmdr1*, N86Y) in this area after chloroquine was withdrawn from this area.

Related reports

There is another study report in 2014 for the first time the return of chloroquine sensitive *P. falciparum* in Southern and Eastern Ethiopia (not the same area of this study). The *Pfcrt* K76 CQ-sensitive allele was observed in 84.1% of the investigated *P. falciparum* clinical isolates.

Innovations and breakthroughs

The present study gives a chloroquine drug resistance surveillance report with drug resistance warning message for chloroquine drug pressure.

Applications

This surveillance study has found the prevalence point mutation at (*Pfcrt*, K76T) and high copy number of *Pfmdr1*, N86Y in the study area. Although the result showed that it was not statistically significant. It might be alert that chloroquine was incompletely withdraw from this area and it could be a risk of drug resistance.

Peer review

This is the first study conducted to determine the chloroquine transporter gene point mutations at (*Pfcrt*, K76T) prevalence and (*Pfmdr1*, N86Y) copy number variation in the study area. In this study high prevalence of *P. falciparum* with a higher *Pfmdr*1 copy number variants were found. Surveillance studies to investigate resistance markers of *Pfmdr1* and *Pfcrt* genes of *P. falciparum* in the same geographic area may help to assess the prevalence of antimalarial resistance.

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