In vivo therapeutic efficacy of chloroquine alone or in combination with primaquine in vivax malaria in Kolkata, West Bengal, India and polymorphism in \textit{pvmdr1} and \textit{pv crt-0} genes

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Running Title: Efficacy of CQ in vivax malaria in Kolkata, India

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pvmdr1
pvcrt-0
Malaria
India
Abstract:

Plasmodium vivax malaria though benign, has now become a matter of concern due to recent reports of life threatening severity and development of parasite resistance against different antimalarial drugs. The magnitude of the problem is still undetermined. The present study was undertaken to determine the \textit{in vivo} efficacy of chloroquine and chloroquine plus primaquine in \textit{P. vivax} malaria in Kolkata and polymorphisms in \textit{pvmdr1} and \textit{pvcrt-0} genes.

A total of 250 patients with \textit{P. vivax} monoinfection were recruited and randomised into two groups A and B, treated with chloroquine and chloroquine plus primaquine respectively and followed up for 42 days as per WHO protocol (2009). Data were analysed using per protocol analyses. We assessed polymorphisms of \textit{pvmdr1} and \textit{pvcrt-0} genes by DNA sequencing method.

Out of 250 recruited patients, 204 cases completed 42 days follow up period, 101 in group A and 103 in group B. In group A, PCR uncorrected efficacy of CQ was 99\% (95\% CI 0.944-1.00) and in group B all cases were classified as ACPR. Day 3 positivity was observed in 11 (5.3\%) cases. No specific mutation pattern was recorded in \textit{pvcrt-0} gene. Eight non-synonymous mutations were found in \textit{pvmdr1} gene, of which three were new. Y976F mutation was not detected in any isolate.

Chloroquine either alone or in combination with primaquine is still effective against \textit{P. vivax} malaria in the study area.
1. Introduction

The burden of malaria caused by Plasmodium vivax has been greatly under-appreciated both in terms of its clinical spectrum and incidence of disease (1,2). P. vivax is the most widely distributed cause of malaria in the world with approximately 2.6 billion people at risk and ten countries including India are at highest risk of infection (3,4,5,6).

Plasmodium vivax infections have been associated with mild symptoms such as fever, headache, fatigue, chills, and musculoskeletal pain, in particular paroxysms. Recently, however, severe complications, including renal failure, jaundice, acute respiratory distress syndrome, cerebral malaria, seizures, anaemia, hyperparasitaemia, thrombocytopenia, pulmonary edema, splenic rupture and death, have been reported in exclusive association with P. vivax (7,8). The situation is further complicated by the emergence of resistant power of the parasite against chloroquine.

In most of the world chloroquine (CQ) remains the first line of treatment for patients with vivax malaria. In India, CQ has been replaced by Artemisinin Combination Therapy (ACT), a combination of Artesunate plus Sulphadoxine-Pyrimethamine in 2010 for P. falciparum malaria but in case of P. vivax malaria CQ remains the first line agent along with primaquine (PQ, 0.25 mg/kg base) for 14 days under supervision or by detecting G6PD level. The first case of P. vivax resistance to CQ was reported in 1989, from Papua New Guinea (9). A higher rate of CQ resistant P. vivax malaria is reported from different regions of Indonesia which exceeded 50% (10,11,12,13,14). Further sporadic cases were subsequently observed in the Philippines, Myanmar, Vietnam, Colombia, Guyana, and Turkey (15).
are also available from Madagascar (16) and Ethiopia (17). Despite these reports, it remains difficult to estimate the worldwide prevalence of \textit{P. vivax} resistance to CQ. In India, first case of CQ resistant \textit{P. vivax} malaria was reported from Assam in 1995 (18) and then in Bombay (19) and Gujarat (20).

The molecular mechanisms underlying CQ resistance in \textit{P. vivax} malaria remain unknown and may involve multigenic loci, but two genes orthologous to the \textit{pfmdr1} and \textit{pfcrt} genes, which encode putative transporters, \textit{pvmdr1} (21) and \textit{pvcrt-0} (22), have been suspected as possible genetic markers of CQ resistance.

Information about prevalence of CQ resistance \textit{P. vivax} malaria and distribution of possible genetic markers of CQ resistance in India are very poor particularly from North-East India. The present study was designed to study the therapeutic efficacy of chloroquine and chloroquine plus primaquine in \textit{P. vivax} malaria and polymorphisms of \textit{pvmdr-1} and \textit{pvcrt-0} genes associated with it.

2. Materials and methods

The study was randomized, double-arm, open-label, interventional trial for evaluation of clinical and parasitological responses of CQ and CQ plus PQ for treatment of uncomplicated \textit{P. vivax} malaria, based on the therapeutic efficacy protocols "Methods for surveillance of antimalarial drug efficacy" of WHO 2009 (23).

2.1 Study site

The study was conducted in the Malaria Clinic attached with Protozoology Unit, Calcutta School of Tropical Medicine during December 2011 – August 2012. In Kolkata, vivax malaria is perennial, and falciparum malaria is seasonal with a peak during August to December each year. \textit{Anopheles stephensi} is the principal vector. \textit{P. falciparum} and \textit{P. vivax} are the two predominant species with an incidence of
almost 1:1. The study was approved by the Institutional Ethics Committee of the
Calcutta School of Tropical Medicine.

2.2 Patient recruitment and follow-up
All patients attending the clinic were screened for presence of malaria parasite by
examining thick and thin smears of peripheral blood followed by Giemsa staining, to
identify patients who might meet the inclusion criteria as per WHO protocol (WHO,
2009) (23). A total of 250 patients with P. vivax mono-infection, confirmed by
presence of negative HRP-II test, aged above 6 months were included in the study.
Inclusion criteria included a parasitaemia of 1000–100000 parasites/μl blood,
absence of severe disease, without any anti-malarial treatment during the preceding
four weeks, and no history of hepatic or kidney diseases. Written informed consent
for study participation was obtained from all patients or their guardians.

On enrolment, each patient was clinically examined thoroughly. A questionnaire
documenting fever, body weight, past history of malaria etc. was also completed. An
amount of 3 - 5 ml of blood was collected from each patient for bio-chemical and
molecular biological studies.

All recruited patients were requested to return for follow-up on days 1, 2, 3, 7, 14, 21,
28, 35 and 42 after initiation of treatment and were examined both clinically and
parasitologically. Members of the study team paid home visits to patients who
missed the scheduled clinic visits for clinical examination and collection of blood
samples for parasitological assessment.

2.3 Treatment
Before commencement of the treatment, G6PD level of all recruited patients were
determined qualitatively using G6PDH Hemopak kit (Apin Diagnostics & Chemicals,
Vadodara, India). All the recruited patients were treated with standard dose of CQ (25 mg/kg of base over 3 days) in group A and CQ (25 mg/kg of base over 3 days) plus PQ (0.25 mg/kg daily for 14 days) in group B and followed-up for 42 days. Patients were directly observed for 30 minutes after treatment, and the dose was re-administered, if vomiting occurred. Patients who repeatedly vomited their first dose of study medication were excluded from the study. In the present study, PQ therapy was supervised for first 7 days and then again on Day 14 by examining the empty blister packs.

2.4 Study end points and statistical analysis

Therapeutic outcomes were classified according to WHO (WHO, 2009) (23) guidelines into early treatment failure (ETF), late clinical failure (LCF), late parasitological failure (LPF) and adequate clinical and parasitological response (ACPR). Therapeutic efficacy was determined by per protocol method using WHO software. The data was entered into a standard data entry programme designed by Global Malaria Programme and analyzed by Kaplan-Meier survival curve according to WHO standard procedures (http://www.who.int/malaria/resistance). The 95% confidence interval was calculated by Dimension Research calculator (http://www.dimensionresearch.com/resources/resources overview.html).

2.5 LABORATORY EXAMINATION

2.5.1 Blood smear for malaria diagnosis and parasite count

Giemsa-stained thick smear slides were read independently by two microscopists and diagnosed as negative on initial review, if no parasites were seen in 100 oil immersion fields. The number of parasites per 200 WBC was counted. Assuming a WBC count to be 8000/μL, parasitaemia was calculated and expressed as per μL of
2.5.2 Rapid Diagnostic Test

All microscopically positive *P. vivax* cases were screened for *P. vivax* specific pLDH and HRP-II (SD Bio Standard Diagnostics Pvt. Ltd., Gurgaon, India) to detect any mixed infection.

2.5.3 Preparation of DNA template from blood samples

Genomic DNA of *P. vivax* from all blood samples collected into EDTA coated vials was extracted by using QIAamp DNA blood Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions, with minor modifications - the incubation time with proteinase K was increased to 20 minutes at 56°C, to improve the yield of the extraction. Extracted parasite genomic DNA of all the samples were preserved at -20°C and an aliquot was used as the DNA source for further study.

2.5.4 Genus and species specific PCR

To ensure *P. vivax* mono-infection genus and specific PCR method was applied as described elsewhere (24).

2.5.5 Sequencing of *pvmdr 1* and *pvcrt-0* gene

The complete DNA sequence of *pvmdr 1* gene is 4606-basepairs (bp) and is located on chromosome 10. The full *pvmdr 1* gene was amplified by polymerase chain reaction (PCR). Six pairs of different primers, as described earlier (25), were used for PCR amplification. All the PCR reactions were performed in total volume of 35 µl. The reaction mixture consisted of 3 µl of genomic DNA, 0·3 µM of each primer pair, 0·2 mM of each deoxynucleotide triphosphate (dATP, dTTP, dGTP, dCTP), 2·5 mM MgCl₂, PCR buffer, and 2 unit of Taq DNA Polymerase (Perkin Elmer, Branchburg, NJ, USA). Amplification was performed using a Veriti 96 well Thermal Cycler (Perkin
Elmer, Branchburg, NJ, USA) under the following conditions: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 94°C for 40 seconds, annealing at 55°C (57°C for primer 1 & 2) for 1 minute, and extension at 72°C for 2 minutes and a final extension period at 72°C for 10 minutes.

The complete DNA sequence of *pvcrt-0* gene is 4281 bp and is located on chromosome 1. The gene contains 14 exons and 13 introns. Amplification of full length *pvcrt-0* gene by PCR was performed with similar PCR mixture as for *pvmdr1* and five pairs of synthetic oligonucleotides, as described earlier (25). PCR was performed under the following conditions: 95°C for 10 minutes and 45 cycles of 95°C for 40 seconds, 55°C (57°C for primer 3 & 4 and 59°C for primer 1) for 1 minute, 72°C for 2 minutes and final extension at 72°C for 10 minutes.

The quality and concentration of PCR products were ascertained by agarose gel electrophoresis. They were subsequently purified using a commercial Kit (QiAquick PCR Purification Kit; Qiagen) and used as template for sequencing. Sequencing reactions were carried out with the ABI Prism Big Dye Terminator cycle sequencing ready reaction kit on a 3730 XL genetic analyzer (Perkin Elmer, Branchburg, NJ, USA).

The sequences were analyzed using the free software Bioedit Sequence Alignment Editor version 7.0.5.2. The sequences were then aligned with the *pvmdr1* (GenBank Acc. No. AY618622) and *pvcrt-0* (GenBank Acc. No. AF314649) of the Sal 1 using the online sequence alignment tool ClustalW (available at: http://www.ebi.ac.uk/clustalw).
3. Result:

3.1 Study population

During the study period, 7439 patients, attending the Malaria Clinic presenting with fever were examined for presence of malaria parasite of whom 1695 were positive for malaria (148 \textit{P. falciparum}, 1544 \textit{P. vivax}, and 3 mixed infections of both \textit{P. falciparum} and \textit{P. vivax}). Of the 1544 \textit{P. vivax} positive cases, 250 patients were enrolled for the study. All the recruited patients were normal for the G6PD test. Their baseline characteristics are described in Table -1.

3.2 In vivo drug efficacy

Total 250 recruited patients were divided into 2 study groups – A and B, each group comprising of 125 patients. Patients in study arm A received only chloroquine and those in study group B received both chloroquine and primaquine.

In study arm A, 24 patients were lost to follow-up, 101 patients completed the CQ treatment course as well as the 42 day follow-up schedule and reached one of the study end-points. In the present study arm there was one case of LCF on day 42 and rest 100 (99%, 95% CI 0.96-1.00) patients were classified as ACPR (Figure 1). Subsequently we could not classify the LCF case as either relapse or re-infection as we were unable to amplify the parasite DNA of day 42 sample for \textit{pvcsp} and \textit{pvmsp-3α} genotypes.

In study group B, 21 patients were lost to follow-up and 104 patients completed the CQ treatment course as well as the 42 day follow-up schedule thus reaching one of the study end-points. In this study arm, one case was classified as ETF, but subsequently after parasite DNA amplification by PCR it was found to be a case of mixed infection of both \textit{P. vivax} and \textit{P. falciparum}. Hence this case was excluded.
from the study. Remaining 103 (100%, 95% CI 0·96-1·00) patients were classified as ACPR. There was no case of therapeutic failure (Figure1).

In all subjects, fever subsided by Day 2. In group A parasite was cleared in 63 (57.8%), 34 (31.2%) and 6 (5.5%) cases by Day1, 2 and 3 respectively. In group B it was 66 (60%), 24 (21.8%) and 5 (4.5%) cases by Day1, 2 and 3 respectively. No significant difference was observed on day 1 (Z = 1·74, P = 0·08), day 2 (Z = 0·465, P = 0·641) and day 3 (Z = 0·337, P = 0·735) positivity in both the study groups. But in eleven cases (6 in group A and 5 in group B) parasite was detected in peripheral blood smears on Day 4 which was cleared on Day5 without any additional anti-malarial drug. Ten patients with long parasite clearance were adults, age ranging from 20-60 years and 1 patient belonged to the age group 5-15 years.

During the entire study, no significant adverse side effects were recorded apart from headache (n = 55), nausea (n = 34), vomiting (n = 4), abdominal pain (n = 8), diarrhoea (n = 6), pruritis (n = 15) during the course of medication and were treated symptomatically.

3.3 Polymorphism of *pvcrt-0* and *pvmdr1* gene

As we did not find any significant proportion of therapeutic failure cases, we attempted to sequence 10% of the total isolates including those eleven cases with long parasite clearance time for *pvcrt-0* and *pvmdr1* genes.

Sequences of *pvcrt-0* gene of all 25 isolates were of mutant type. But no specific pattern of polymorphism was detected. Four synonymous (L122L, P565P, Q782Q and K807K) and four non-synonymous mutations (N706P, P1201L, R1224P and R1278K) were detected. Among the synonymous mutations, L122L, P565P, and K807K were located in exon 1, 4 and 7 respectively and Q782Q in intron 6. Among
the non-synonymous mutations N706P, P1201L, were located in intron 5 and 10 respectively, and R1224P, R1278K were located in exon 11 and 12 respectively (Table 2).

The \textit{pvmdr1} gene was fully sequenced in 25 isolates, among them 5 (20%) isolates were of wild type. Four synonymous (K68K, G172G, L310L, L697L) and eight non-synonymous (R88Q, A296V, E478G, S513R, G698S, M908L, T958M and F1076L) point mutations were recorded. However, the Y976F substitution, thought to be involved in CQ resistance in \textit{P. vivax}, was not recorded in the present study. Five different haplotypes with two [G478S698 (2, 8%)], three [(S698L908M598 (7, 28.0%), L908M958L1076 (4, 16.0%) and four [Q88R513L908M958 (4, 16.0%)] amino acid substitutions were identified (Table 2). Distribution of point mutations in \textit{pvcrt-0} and \textit{pvmdr1} and haplotypes of \textit{pvmdr1} were equally prevalent among the cases with long parasite clearance time and other cases.

4. Discussion

\textit{In vivo} studies remain the gold standard for assessment of efficacy of different anti-malarial drugs. However there are some operational difficulties associated with the follow-up of recruited patients for 42 days and differentiation of ‘recrudescence from ‘re-infection’ in endemic areas. In case of \textit{P. falciparum}, \textit{msp 1}, \textit{msp 2} and \textit{glurp} genotyping can address the problem (26). Relapse in \textit{P. vivax} malaria caused by hypnozoite reactivation makes interpretation of recurrences in drug efficacy trials complicated. Genotyping cannot distinguish relapse from re-infection when the parasites causing the relapse arise from a dormant subset of the inoculated sporozoites that caused the primary infection. \textit{P. vivax} parasites causing primary infections as well as relapses have been compared using molecular markers \textit{pvcs} and \textit{pvmsp1}, and \textit{pvmsp1} alone, respectively (27,28).
In the present study one patient treated with chloroquine returned with fever and his peripheral blood smear showed *P. vivax* infection on day 42. We could not classify it either as re-infection or recrudescence or relapse as we were unable to amplify the parasite DNA for *pvcsp* and *pvmsp-3α* genotyping. In the other study group, patients treated with chloroquine plus primaquine, all cases were categorised as ACPR. So, in Kolkata CQ alone was 99% and CQ plus PQ was 100% effective in *P. vivax* malaria. Similar observation was also made during 1998-2001 (29) and 2003-2004 (30). So during past 15 years efficacy of CQ remained unchanged in *P. vivax* malaria but in case of *P. falciparum* malaria CQ resistance level reached to 76.3% (31). However, day 3 parasite positivity was noted in 11 (5.3%) patients. Ten of them were in adult group and one in 5-15 years. The mean Day 0 parasite count of those eleven patients was 10825 (Range: 2200-31680; SD: ±7946.7), which was higher than those patients whose parasite cleared on Day 3 with mean Day 0 parasite count was 4210 (Range: 1000-25600; SD: ±3961.9). All eleven cases were classified as ACPR as per WHO 2009 protocol (23). Perhaps, long parasite clearance time is an indication of diminished sensitivity of the parasite to chloroquine alone as well as chloroquine plus primaquine. G6PD is an important issue related to *P. vivax* malaria. Mediterranean type of G6PD deficiency significantly protects *P. vivax* infection among Afghan refugees in Pakistan (32). Similar report is also available for the G6PD Mahidol variant from South East Asia (33).

Molecular markers seem to be useful for monitoring the drug resistance of malarial parasites. Two genes *pfcr* and *pfmdr1* were found to be associated with efficacy of CQ in *P. falciparum* malaria. The K76T mutation in the *pfcr* gene is known to be involved in CQ resistance. But little information is available regarding the possible relationship between the *pvcrt*-0 and *pvmdr1* genes and CQ resistance. Only a few
studies have been carried out and associations between treatment failure and non-synonymous mutations in isolates obtained before treatment have not yet been clearly established (21,34,35). However, the Y976F substitution in the \textit{pvmdr1} gene is thought to be involved in CQ resistance in \textit{P. vivax} (25,36). Suwanarusk et al., (2007) observed that the geometric mean 50\% inhibitory concentration of CQ was significantly higher in \textit{P. vivax} isolates carrying the Y976F mutation than in isolates with the wild-type allele.

In the present study no definite mutation pattern was noticed in \textit{pvmdr1} gene. Out of 25 isolates studied, 13 isolates were of wild type. Four synonymous and eight non-synonymous mutations were observed in \textit{pvmdr1} gene, three of which (R88Q, A296V and E478G) were not identified previously. But no mutation at codon 976 was observed. In \textit{pvcrt-0}, four non-synonymous mutations were found, two ((N706P and P1201L) in intron 5 and 10 and two (R1224P and R1278K) in exon 11 and 12.

Frequent multiple mutant \textit{pvmdr1} haplotypes (quadruple, sextuple and septuple) were reported from Madagascar, but none of the mutant haplotypes was found to be associated with CQ resistant \textit{P. vivax} malaria (16). In the present study, only double \([\text{G478S}_{598} (8.0\%)\)], triple \([(\text{S}_{698}\text{L}_{908}\text{M}_{598} (28.0\%), \text{L}_{908}\text{M}_{958}\text{L}_{1076} (16.0\%)\)] and quadruple \([\text{Q}_{88}\text{V}_{296}\text{L}_{908}\text{M}_{958} (12.0\%), \text{Q}_{88}\text{R}_{513}\text{L}_{908}\text{M}_{958} (16.0\%)\]) mutant haplotypes along with wild type allele (20.0\%) were observed. Though the sample size was small but the absence of Y976F point mutation and multiple mutant haplotypes (sextuple or septuple) of \textit{pvmdr1} along with persistence of significant proportion of wild type gene justified our \textit{in vivo} therapeutic outcomes.

In the study area, CQ alone and CQ plus PQ (99\% and 100\%) were effective against \textit{P. vivax} malaria. Day 3 parasite positivity in 11 (5.3\%) cases needs further
evaluation for development of resistance of the parasite against the drugs. Neither
definite pattern of polymorphisms of \textit{pvcrt-0} and \textit{pvmdr1} nor mutation at \textit{pvmdr1}
Y976F was observed. Hence, it is difficult to correlate any association of genetic
marker with therapeutic outcomes. Periodical monitoring may elucidate the changing
pattern of the susceptibility of the parasite to CQ and CQ plus PQ.

\textbf{Clinical trial registration:} The study protocol was registered in CTRI (Clinical trial
registry-India) of Indian council of Medical Research. Registration
No.CTRI/2011/09/002031

\textbf{Conflicts of interest:} The authors have no conflicts of interest concerning the work
reported in this paper.

\textbf{Authors' contributions:} AKM and SKG conceptualized and designed the study
protocol; SG, PS, SD,BS, PKK, DKB and AB performed the clinical assessment and
the in-vivo therapeutic efficacy study; SG and PS performed the PCR and
sequencing analysis and interpretation of data; AKM,SG, SKG, PS, BS, KR and DKB
drafted the manuscript. All authors read and approved the final manuscript.

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References:


genotyping protocols for *Plasmodium vivax* using *Pvcs* and *Pvmsp1*. Malar. J. 4:20.


### Table 1: Baseline Characteristics of the Study Patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>CQ (n = 125)</th>
<th>CQ + PQ (n = 125)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex: no. (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>110 (88)</td>
<td>113 (90.4)</td>
</tr>
<tr>
<td>Female</td>
<td>15 (12)</td>
<td>12 (9.6)</td>
</tr>
<tr>
<td><strong>Weight: kg</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>49.3</td>
<td>49.38</td>
</tr>
<tr>
<td>Range</td>
<td>11-87</td>
<td>16-95</td>
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<tr>
<td>SD</td>
<td>±12.703</td>
<td>±13.56</td>
</tr>
<tr>
<td>95% CI</td>
<td>47.07-51.53</td>
<td>41-51.76</td>
</tr>
<tr>
<td><strong>Age category: no. (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-15</td>
<td>26 (20.8)</td>
<td>24 (19.2)</td>
</tr>
<tr>
<td>Adult</td>
<td>99 (79.2)</td>
<td>101 (80.8)</td>
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<tr>
<td><strong>Age: year</strong></td>
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<td></td>
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<tr>
<td>Mean</td>
<td>25.21</td>
<td>25.16</td>
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<tr>
<td>Range</td>
<td>5-65</td>
<td>6-60</td>
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<tr>
<td>SD</td>
<td>±12.86</td>
<td>±12.82</td>
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<tr>
<td>95% CI</td>
<td>22.96-27.47</td>
<td>22.91-27.41</td>
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<tr>
<td><strong>Temperature: °C</strong></td>
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<td></td>
</tr>
<tr>
<td>Mean</td>
<td>37.79</td>
<td>37.72</td>
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<tr>
<td>Range</td>
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<tr>
<td>SD</td>
<td>±0.28</td>
<td>±0.17</td>
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<tr>
<td>95% CI</td>
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<tr>
<td><strong>Haemoglobin: g/dl</strong></td>
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<td></td>
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<tr>
<td>Mean</td>
<td>12.61</td>
<td>12.60</td>
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<tr>
<td>Range</td>
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<tr>
<td>SD</td>
<td>±1.95</td>
<td>±2.07</td>
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<tr>
<td>95% CI</td>
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<td>12.24-12.97</td>
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<tr>
<td><strong>Parasite count: no/µl</strong></td>
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<tr>
<td>Mean</td>
<td>4744</td>
<td>4285</td>
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<tr>
<td>Range</td>
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<td>1000-25600</td>
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<tr>
<td>SD</td>
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<td>±4214</td>
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<tr>
<td>95% CI</td>
<td>3938-5549</td>
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Table 2: Mutation profile of *pvmdr1* and *pvcrt-0* gene in study isolates

<table>
<thead>
<tr>
<th>Candidate Gene</th>
<th>Occurrence of mutation in study population</th>
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<tr>
<td><strong>pvmdr 1</strong> (n = 25)</td>
<td></td>
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<tr>
<td>Synonymous Mutation</td>
<td></td>
</tr>
<tr>
<td>K68K</td>
<td>2</td>
</tr>
<tr>
<td>G172G</td>
<td>4</td>
</tr>
<tr>
<td>L310L</td>
<td>3</td>
</tr>
<tr>
<td>L697L</td>
<td>5</td>
</tr>
<tr>
<td>Non-synonymous Mutation</td>
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<td>M908L</td>
<td>18</td>
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<tr>
<td>T958M</td>
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<tr>
<td>F1076L</td>
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<td>Haplotypes</td>
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<td>Wild type</td>
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<tr>
<td>Double mutants</td>
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<td>S598</td>
<td>8</td>
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<tr>
<td>L598</td>
<td>10.4 – 45.6</td>
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<tr>
<td>L908M</td>
<td>16.0 – 30.37</td>
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<tr>
<td>Quadruple mutants</td>
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<tr>
<td>Q88V</td>
<td>12.0 – 24.74</td>
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<td>16.0 – 30.37</td>
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<td><strong>pvcrt-0</strong> (n = 25)</td>
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<tr>
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<td>R1278K</td>
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* Non-synonymous mutations are shown in bold faces
Figure 1 CONSORT FLOW CHART

Total Screened: 7439

Negative Cases: 5744
P. vivax: 1544
P. falciparum: 148
Mixed Infection: 3

Total Enrolled: n = 250

CQ: n = 125
- WTH: 0
- LFU: 24
- Evaluable by Day 42: n = 101

CQ + PQ: n = 125
- Mixed infection: 1
- WTH: 0
- LFU: 21
- Evaluable by Day 42: n = 103

*WTH: Withdrawn, LFU: Loss to Follow-up*