Chloroquine Resistant *Plasmodium vivax*: In Vitro Characterisation and Association with Molecular Polymorphisms

Rossarin Suwanarusk1,*, Bruce Russell1,*, Marina Chavchich2, Ferryanto Chalfein3, Enny Kenangalem2,4, Varakorn Kosaisavee5, Budi Prasetyorini6, Kim A. Piera1, Marion Barends8, Alan Brockman1, Usa Lek-Uthai5, Nicholas M. Anstey1, Emiliiana Tjitra6, François Nosten7,8, Qin Cheng2, Ric N. Price1,9

1International Health Program, Infectious Diseases Division, Menzies School of Health Research and Charles Darwin University, Darwin, Australia, 2Department of Drug Resistance and Diagnostics, Australian Army Malaria Institute, Brisbane, Australia, 3National Institute of Health Research and Development and Menzies School of Health Malaria Research Program, Timika, Indonesia, 4District Ministry of Health, Timika, Papua, Indonesia, 5Department of Parasitology, Faculty of Public Health, Mahidol University, Bangkok, Thailand, 6National Institute of Health Research and Development, Ministry of Health, Jakarta, Indonesia, 7Faculty of Tropical Medicine, Mahidol University, Bangkok Thailand, 8Shoklo Malaria Research Unit, Mae Sod, Tak Province, Thailand, 9Centre for Vaccinology and Tropical Medicine, Nuffield Department of Clinical Medicine, John Radcliffe Hospital, Oxford, United Kingdom

**Background.** Treatment failure of chloroquine for *P. vivax* infections has reached high levels in the eastern provinces of Indonesia, however, *in vitro* characterization of chloroquine resistance and its associated molecular profile have yet to be determined. **Methods.** Using a modified schizont maturation assay we investigated the *in vitro* chloroquine susceptibility profile and molecular polymorphisms of *P. vivax* isolates collected from Papua, Indonesia, where high levels of clinical chloroquine treatment failure have been reported, and from Thailand, where chloroquine treatment is generally effective. **Results.** The geometric mean chloroquine IC₅₀ for *P. vivax* isolates from Papua (n = 145) was 312 nM [95%CI: 237–411 nM] compared to 46.8 nM [95%CI: 34.7–63.1 nM] from Thailand (n = 81); p < 0.001. Correlating with the known clinical efficacy of the area, a cut off for chloroquine resistance was defined as 220 nM, a level exceeded in 13.6% (11/81) of Thai isolates and 65% (94/145) of Papuan isolates; p < 0.001. Several sequence polymorphisms in *pvcrtn* and *pvmdr1*, and difference in pvmdr1 copy number were identified. A Y976F mutation in pvmdr1 was present in 96% (123/128) of Papuan isolates and 25% (17/69) of Thai isolates; p < 0.001. Pvmdr1 amplification occurred in 23% (15/66) of Thai isolates compared to none (0/104) of Indonesian isolates (p < 0.001), but was not associated with increased chloroquine resistance after controlling for geographical location. **Conclusions.** In *in vitro* susceptibility testing of *P. vivax* discriminates between populations with differing levels of clinical efficacy of chloroquine. The pvmdr1 polymorphism at Y976F may provide a useful tool to highlight areas of emerging chloroquine resistance, although further studies defining its clinical correlates are needed.

Citation: Suwanarusk R, Russell B, Chavchich M, Chalfein F, Kenangalem E et al (2007) Chloroquine Resistant *Plasmodium vivax*: In Vitro Characterisation and Association with Molecular Polymorphisms. PLoS ONE 2(10): e1089. doi:10.1371/journal.pone.0001089

**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 affecting 40% of the worlds population and causing between 147–436 million clinical infections each year [3]. Although associated with less mortality than *P. falciparum* it exerts a considerable morbidity particularly in children and pregnant women. Control measures are confounded by two major factors: firstly, the presence of dormant hypnozoite stages in the liver, which result in relapse infections weeks after the cure of the initial episode, and secondly the emergence of chloroquine resistance.

In most of the world chloroquine remains the first line of treatment for patients with vivax malaria. Not only is it well-tolerated and affordable, but its long half-life provides protection from early relapses following treatment. The first cases of chloroquine resistant *P. vivax* were reported in 1989 from PNG [4] and northern Papua (formerly Irian Jaya), Indonesia [5,6,7,8]. Chloroquine monotherapy is now virtually ineffective in Papua Indonesia [8,9,10] with significant clinical resistance apparent throughout the Indonesian archipelago [5,11]. More recently sporadic cases have been reported from Myanmar [12], South America [13,14], Viet Nam [15], and Turkey [16].

Despite these clinical reports, the global prevalence of chloroquine resistant *P. vivax* remains poorly defined. Clinical studies are difficult to carry out and subject to individual variations in patient immune status, reinfection and frequent relapses. *In vitro* susceptibility assays provide an alternative means of assessing drug susceptibility of *Plasmodium* spp. Although these tests have been well established for *P. falciparum*, their application in *P. vivax* has
been more difficult to develop due to limitations of in vitro culture methods in this species. Recently several centres have reported methods for conducting in vitro P. vivax drug susceptibility which are generally based on the P. falciparum WHO microtest using quantification of schizont maturation [17,18,19].

The mechanism of P. vivax chloroquine resistance is unknown and as yet no genetic markers have been identified. In P. falciparum, polymorphisms in pfcr and pfmdr1 have been shown to confer resistance [20,21]. However, no associations have been found between point mutations in the orthologue genes, pvcrt-o (pvcrt) and pfmdr1 and the clinical response of vivax malaria to chloroquine [22,23]. Heterologous systems investigating the effect of pvcrt-o expression on chloroquine response showed a 2.2-fold decrease in susceptibility to chloroquine in P. falciparum transformed with pvcrt-o, suggesting a possible role of pvcrt-o in chloroquine resistant P. vivax [24].

In this study, we investigated the in vitro chloroquine susceptibility profile and molecular polymorphisms of P. vivax isolates collected from Papua, Indonesia, where high levels of clinical chloroquine resistance have been reported [10,25] and from Thailand where chloroquine treatment is generally effective [26,27].

MATERIALS AND METHODS
Field location and sample collection
Clinical isolates were collected between 2003 and 2006 from two sites, one in Indonesia and the other in Thailand. Timika, located in the southern region of Papua Province, Indonesia, has documented clinical chloroquine resistance with day 28 failure rates following chloroquine monotherapy exceeding 65% and 16% of patients having early treatment failure [10]. At the second site at the Shoklo Malaria Research Unit, Mae Sod, Tak Province on the western border of Thailand, P. vivax remains clinically sensitive to chloroquine [27].

Patients with symptomatic infections of pure P. vivax presenting to an outpatient facility were recruited into the study and 5 ml blood samples collected by venepuncture. After removal of host white blood cells using a CF11 column, 2 ml of packed infected RBC blood cells (IRBC) were divided as follows: 1 ml was cryopreserved in glycerolyte, 200 μl of BMM per well instead of 50 μl of genomic DNA. PCR was performed under the following conditions: a total volume of 50 μl containing 5 μl of 10×PCR buffer, 2.5 mM MgCl₂, 0.20 mM each dNTP, 1 μl of primer and 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). Polymorphisms which were identified in the core products were sequenced using the BigDye terminator 3.1 (Applied Biosystems).

Determining pvmdr1 and pvcrt-o genes
In order to identify relevant polymorphisms in the pvmdr1 and pvcrt-o genes in our parasite population 25 Indonesian and 7 Thai P. vivax isolates (“core” samples), were fully sequenced for both genes using primers listed in Table 1, comparing the sequences to those of the pvmdr1 (GenBank Acc. No. AY618622) and pvcrt-o (GenBank Acc. No. AF314649) of the Sal 1, a chloroquine sensitive strain from Salvador used as a reference strain in this study. All core isolates were single species, monoclonal infections. PCR conditions were as follows: a total volume of 50 μl containing 5 μl of 10×PCR buffer, 2.5 mM MgCl₂, 0.20 mM each dNTP, 1 μl each primer and 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 1 μl of genomic DNA. PCR was performed under the following conditions: 95°C for 10 minutes and 40 cycles of 94°C for 40 seconds, 55°C for 1 minute and 72°C for 2 minutes. PCR products were sequenced using the BigDye terminator 3.1 (Applied Biosystems). Polymorphisms which were identified in the core samples were then examined in the complete sample set.
The assay was optimised to achieve equal amplification efficiencies for the pvmdr1 and aldolase gene fragments within the range of DNA concentrations from 100 ng/µl to 10 pg/µl, thus the ΔΔCt method ([Applied Biosystems User Bulletin N2 (P/N 4303859B)] could be used and the pvmdr1 copy number (N) was calculated as follows: \(N = \frac{2^{\Delta \Delta Ct}}{S_{\text{aldolase}} - \Delta \text{Ct}_{\text{pvmdr1}} - \Delta \text{Ct}_{\text{aldolase}}}\), where \(\Delta \text{Ct} = (\Delta \text{Ct}_{\text{aldolase}} - \Delta \text{Ct}_{\text{pvmdr1}} - \Delta \text{Ct}_{\text{aldolase}})\). The Ct_{aldolase} and Ct_{pvmdr1} used above are threshold cycle values for the pvmdr1 and aldolase gene respectively, whereas Ct_{aldolase} is an average difference between Ct_{aldolase} and Ct_{pvmdr1} obtained for the positive control containing a single copy of pvmdr1 and aldolase gene fragments. The SD is a standard deviation calculated as follows: \(SD = \sqrt{S_{\text{aldolase}}^2 + S_{\text{pvmdr1}}^2 + S_{\text{aldolase}}^2}\) where \(S_{\text{aldolase}}\) and \(S_{\text{pvmdr1}}\) are the standard deviations from the average Ct calculated for 3 or 4 replicates in the pvmdr1 and pvmdr1 amplifications and \(S_{\text{aldolase}}\) is an average standard deviation of the ΔCt values for the calibrator. Assessment of copy number was repeated at least twice for all isolates and the repeatability coefficient determined as 0.30 (viz 95% of repeated estimates of pvmdr1 copy number were within 0.15 of the first).

### Table 1. Primers and sequences used to study mutations in pvmdr1 and pvcr-o and in the pvmdr1 copy number assay.

| A. pvmdr1                  | Cm |
|----------------------------|----|
| Pvmr1-1F                   | S' 5'-CTT TTA TGC CTC TCC CCC |
| Pvmr1-1Fb                  | S' 5'-AGA TGG TCC TGG AGC CGT |
| Pvmr1-1R                   | S' 5'-GCG TAA GCT GAT AAA ATG AACC |
| Pvmr1-2F                   | S' 5'-ATT TAA CCT TCC AGA AAA GCT G |
| Pvmr1-2R                   | S' 5'-CCA CAC GAC AAC TTA GAT GC |
| Pvmr1-3F                   | S' 5'-CTG ATA CAA GTG AGG AAC AAC TAC |
| Pvmr1-3R                   | S' 5'-ACT ATC CTG GTG AAA AAA G |
| Pvmr1-4F                   | S' 5'-CCC TCT ACA TCT TAG TCA TCG |
| Pvmr1-4R                   | S' 5'-TGG TCT GGA CAA GTA TCT AAAA |
| Pvmr1-5F                   | S' 5'-GGA AGT TGA TGT CCC TAA AGG |
| Pvmr1-5R                   | S' 5'-CCT GCC GGC TCT ACT TAG |
| B. pvcr-o                  | Cm |
| Pvcg10-1F                  | S' 5'-GCC TGT CGAAG GCC |
| Pvcg10-1R                  | S' 5'-AGT TCC CCT CTA CAC CGG |
| Pvcg10-2F                  | S' 5'-GCC CGG GTA GAA GC |
| Pvcg10-2R                  | S' 5'-GCT GAG GCG ACA TGG |
| Pvcg10-3F                  | S' 5'-GCT AGG GCC ACA TTT CC |
| Pvcg10-3R                  | S' 5'-GTA GTC CTG AAA AGA CAC ACA TC |
| Pvcg10-4F                  | S' 5'-TAT GAA GCA AAT CGC AAC AA |
| Pvcg10-4R                  | S' 5'-CTT GAG AGT AAG GCA GGC AA |
| Pvcg10-5F                  | S' 5'-TCA TCC AGA GAG CAA ACT TCT TA |
| C. pvmdr1 976             | Cm |
| Pvmr1 976 F                | S' 5'-GGA TAG TCA TGC CCC CCG ATG G |
| Pvmr1 976 R                | S' 5'-CAT CAA CCT CCC GGC GTA GC |
| pvmdr1 976 internal        | S' 5'-CGG TGC TAC TGA CCG GAG C GT A |
| D. pvmdr1 copy number      | Cm |
| Pvmr1 R                    | S' 5'-CTG ATA CAA GTG AGG AAG AAC TAC G |
| pvmdr1 R                   | S' 5'-GTC CAC CTG ACA ACT TAG ATG C |
| pvmdr1 R                   | S' 5'-CAT CAA CCT CCC GGC GTA GC |
| pvmdr1 R                   | S' 5'-GAG TGC CTC TCA CCG AAG G |
| pvmdr1 R                   | S' 5'-GTC CAC CTG ACA ACT TAG ATG C |

List of primers and their sequences used to amplify and sequence pvmdr1 (A), pvcr-o (B) and identification of the pvmdr1 Y976F mutation (C). Primers used to amplify the fragment of the pvmdr1 and P. vivax aldolase reference gene in the pvmdr1 copy number assay (D). 

The geometric mean chloroquine IC50 for P. vivax isolates from Indonesia was 312 nM [95% CI: 237–411 nM] significantly higher than that for Thai isolates (46.8 nM [95% CI: 34.7–63.1 nM]; p<0.001). After ranking the 226 P. vivax isolates in order of decreasing chloroquine susceptibility, a continuous non-linear curve of chloroquine IC50 was observed (Figure 2). The 35th percentile of isolates from this region was significantly shorter in Indonesian isolates (26 hours [Range: 22–48]) compared to 36 hours [Range: 21–48] for isolates from Thailand; p<0.001.

Between April 2003 and December 2006, 247 isolates were assayed for in vitro susceptibility of which acceptable chloroquine susceptibility data could be derived in 226 (91%). Further analysis was restricted to these isolates (145 from Indonesia and 81 from Thailand); see figure 1. In total 51% (74/145) of Indonesian isolates began the assay with more than 40% ring stages prior to culture, compared to 81% (65/81) of isolates from Thailand; p<0.001. The time to reach 40% schizonts, and thus the duration of the assay, was significantly shorter in Indonesian isolates (26 hours [Range: 22–48]) compared to 36 hours [Range: 21–48] for isolates from Thailand; p<0.001.

The assay was optimised to achieve equal amplification efficiencies for the pvmdr1 and aldolase gene fragments within the range of DNA concentrations from 100 ng/µl to 10 pg/µl, thus the ΔΔCt method ([Applied Biosystems User Bulletin N2 (P/N 4303859B)] could be used and the pvmdr1 copy number (N) was calculated as follows: \(N = \frac{2^{\Delta \Delta Ct}}{S_{\text{aldolase}} - \Delta \text{Ct}_{\text{pvmdr1}} - \Delta \text{Ct}_{\text{aldolase}}}\), where \(\Delta \text{Ct} = (\Delta \text{Ct}_{\text{aldolase}} - \Delta \text{Ct}_{\text{pvmdr1}} - \Delta \text{Ct}_{\text{aldolase}})\). The Ct_{aldolase} and Ct_{pvmdr1} used above are threshold cycle values for the pvmdr1 and aldolase gene respectively, whereas Ct_{aldolase} is an average difference between Ct_{aldolase} and Ct_{pvmdr1} obtained for the positive control containing a single copy of pvmdr1 and aldolase gene fragments. The SD is a standard deviation calculated as follows: \(SD = \sqrt{S_{\text{aldolase}}^2 + S_{\text{pvmdr1}}^2 + S_{\text{aldolase}}^2}\) where \(S_{\text{aldolase}}\) and \(S_{\text{pvmdr1}}\) are the standard deviations from the average Ct calculated for 3 or 4 replicates in the pvmdr1 and pvmdr1 amplifications and \(S_{\text{aldolase}}\) is an average standard deviation of the ΔCt values for the calibrator. Assessment of copy number was repeated at least twice for all isolates and the repeatability coefficient determined as 0.30 (viz 95% of repeated estimates of pvmdr1 copy number were within 0.15 of the first).
Polymorphisms in pvmdr1, pvcrt-o and pvama1 in core samples

Using SalI as the reference strain, sequence analysis of 32 core isolates revealed single nucleotide polymorphisms (SNP) at 5 loci of pvmdr1, two non-synonymous mutations resulting in amino acid changes at Y976F and L1076F (Table 3) and three synonymous SNPs (at codons 493, 908 and 1396). In these core isolates the Y976F mutation was significantly more prevalent in Indonesian isolates (96%, 24/25) compared to Thai isolates (43%, 3/7); \( p = 0.004 \).

Analysis of three loci (pvama1, pvmsp1 and pvmsp3) revealed 26 combined haplotypes in the 32 core isolates indicating that the isolates are of a diverse genetic background. Clustal-c analysis of partial pvama1 sequences from 89 isolates (Figure 3) showed no evidence that sequence diversity was less among isolates from within each field site compared to between locations.

P. vivax chloroquine susceptibility and pvmdr1 and pvcrt-o polymorphisms

Polymorphisms were assessed for the pvmdr1 SNP at codon 976 in an additional 165 isolates with in vitro susceptibility data and for the pvcrt-o insertion at amino acid position 10 in an additional 55 isolates (Figure 1). After combining these with the core isolates the Y976F allele was found in 96.1% (123/128) of Indonesian isolates compared to 25% (17/69) of Thai isolates \( p < 0.001 \). Overall, the geometric mean chloroquine IC50 in isolates with the Y976F mutation was 283 nM [95%CI: 211–379], significantly higher than that in isolates with the wild type allele (geometric mean = 44.5 nM [95%CI: 31.3–63.4]; \( p < 0.001 \)). In Thailand, isolates with the Y976F mutation had a mean IC50 of 65.6 nM [95%CI: 29.9–144] compared to 39.0 nM [95%CI: 27.8–54.8] in those with the wild type allele (\( p = 0.008 \), after controlling for assay duration and percentage of rings pre incubation). The trend was similar in Indonesian isolates, however the proportion of Y976F mutation almost reached fixation and thus prohibited analysis of the correlation between the polymorphism and the phenotype (see figure 4).

The pvcrt-o AAG insertion occurred in 76% (31/41) of Thai isolates, but only 2.2% (1/46) of the Indonesian isolates \( p < 0.001 \). Overall the pvcrt-o AAG insertion was associated with a significant reduction in chloroquine IC50, (geometric mean 47.6 nM [95%CI: 29.7–76.1] vs 261 nM [95%CI: 172–396]; \( p < 0.001 \)). After stratifying by geographical location, the AAG insertion of pvcrt-o was not linked to the pvmdr1 Y976F mutation and was not significantly associated with reduced chloroquine IC50.

P. vivax chloroquine susceptibility and pvmdr1 copy number

The pvmdr1 copy number was successfully quantified in 86% (170/198) of isolates tested. In total 23% (15/66) of isolates from Thailand had an increased pvmdr1 copy number (13 with 2 copies and two with 3 copies) and none (0/104) from Indonesia; \( p < 0.001 \). In Thailand all (15/15) of the isolates with increased copy number were wild type at 976, compared to 67% (34/51) of those with a single copy number of pvmdr1 (\( p = 0.007 \)). Although isolates with increased pvmdr1 copy number had significantly lower chloroquine IC50, (geometric mean = 39.6 [95%CI: 24.5–64.1]) compared to isolates with single copies of pvmdr1 (geometric mean = 184 [95%CI: 137–247]; \( p < 0.001 \)); this was not apparent after stratification by country.

DISCUSSION

In clinical studies, P. vivax remains predominantly sensitive to chloroquine in Thailand, whereas in Papua, Indonesia high grade clinical resistance is already established [10,25,26]. In 2004 a chemotherapeutic study at the Papuan field site demonstrated that 65% of patients failed treatment within 28 days of chloroquine monotherapy, 16% of whom had early high grade failures. Treatment guidelines were changed accordingly to an Artemisinin combination therapy for both P. falciparum and P. vivax.
precluding further clinical studies on the use of chloroquine monotherapy in this region. In the present study we have continued our analysis of chloroquine resistance \textit{P. vivax} using an identical \textit{in vitro} methodology in both Indonesia and Thailand and correlating our results with the known data on the clinical efficacy of chloroquine in these regions. The Indonesian \textit{P. vivax} isolates tested had a significantly higher median chloroquine IC$_{50}$ and a higher proportion above the resistance threshold compared with that of Thai isolates.

The determination of chloroquine susceptibility in \textit{P. vivax} using the schizont maturation method is more complicated than the same method in \textit{P. falciparum}, due to the asynchrony of the vivax parasites and possible differential responses to the drug by parasites at different development stages. Patient samples with higher percentage of trophozoites and late rings require less incubation time to reach maturation (unpublished data). The decreased susceptibility to chloroquine in these samples provides a plausible explanation for our observation of the negative correlations between IC$_{50}$ and culture

![Figure 2. Distribution of isolate chloroquine IC$_{50}$](image)

Thai (closed diamonds) and 141 Indonesian (open diamonds) \textit{P. vivax} isolates ranked in order of increasing chloroquine IC$_{50}$. doi:10.1371/journal.pone.0001089.g002

| Table 2. \textit{In vitro} chloroquine sensitivity (nM) of isolates from Thailand and Indonesia. |
|---------------------------------------------------------------|
| N | Geometric Mean IC$_{50}$ | 95% Confidence Intervals | Range | P |
|---|---------------------------|--------------------------|-------|---|
| ALL | | | | |
| Indonesia | 145 | 312 | 237–411 | 4.6–5637 | P<0.001 |
| Thailand | 81 | 46.8 | 34.7–63.1 | 6.7–2231 | |
| >30 Hours Duration | | | | |
| Indonesia | 48 | 113 | 67.9–188 | 4.6–3024 | P<0.001 |
| Thailand | 69 | 33.2 | 26.0–42.5 | 6.7–430 | |
| >40% parasites at ring stage prior to culture | | | | |
| Indonesia | 74 | 208 | 139–312 | 4.6–3506 | P<0.001 |
| Thailand | 65 | 33.7 | 25.8–44.1 | 6.7–1264 | |

Values given overall and after selecting cultures with greater than 30 hour duration of assay or a starting with more 40% of parasites at ring stage. Criteria for duration of assay and percentage of rings in initial culture taken from Tasanor et al 2002 [19]

doi:10.1371/journal.pone.0001089.t002
duration as well as with percentage of rings at the start of culture. Although differentiating between these possibilities is difficult, we attempted to control for these confounding factors by stratifying our results according to culture duration and the percentage of rings at the start of culture; the differences in IC50 between isolates from Indonesia and Thailand remained.

The *in vitro* cut-off defining clinically relevant chloroquine resistance has yet to be defined. Using the clinical failure rate (65%) observed in the same area, we defined this from the 35th percentile as 220 nM, almost double the 100nM cut-off value for chloroquine resistance in *P. falciparum*. However clinical failures may have included some relapses that occur within the 28 day follow up period, and the true rate of recrudescence may be lower. Hence this threshold is likely to be the minimum value associated with resistance.

The chloroquine IC50 of Thai isolates were significantly lower than the Indonesian isolates (Geometric mean = 46.8 vs 312 nM), although the difference was less after controlling for the duration of assay (33.2 vs 113 nM) or initial stage of parasite prior to culture (33.7 vs 208 nM). Interestingly 13.6% (11/81) of Thai isolates had a chloroquine IC50 over 220 nM. Although clinical studies in Thailand in the 1990s have repeatedly demonstrated the continued efficacy of chloroquine monotherapy for *P. vivax* [26,27], our *in vitro* results raise the possibility that clinically relevant chloroquine resistance may now be present at low prevalence along the western border of Thailand. This is corroborated by a recent clinical study from the Thai-Myanmar border demonstrating 34% *P. vivax* recurrence rates within 28 days of chloroquine monotherapy [32].

### Table 3. Mutations in *pvmdr1* and *pvcrt-o* among 32 core *Plasmodium vivax* isolates from Indonesian and Thailand and the reference strains SAL1.

| Sample | Origin   | Genotype groups | Polymorphisms in *pvmdr1* and *pvcrt-o* | *pvmdr1* | *pvcrt-o* exon |
|--------|----------|-----------------|----------------------------------------|----------|----------------|
|        |          | Msp1 LP Msp3 RFLP AMA1 Sequence Combined Genotype Copy number Y976F SNP F1076L SNP K10 Insert H3M SNP |
| SAL 1  | Central America | a a a a | 1 Y F F - I |
| ANV20  | Indonesia   | b a b b | 1 F L - I |
| VI21   | Thailand    | a b c c | 1 Y L K - I |
| VI20   | Thailand    | a a d d | 1 F L K - I |
| VI32   | Thailand    | a a d d | 2 Y L K - I |
| VI5    | Thailand    | a a c e | 2 Y L K - I |
| VRP21  | Indonesia   | b a e f | 1 F L - I |
| VI1    | Thailand    | a a f g | 1 F L - I |
| PV14   | Thailand    | b c d h | 1 F L K - I |
| VI23   | Thailand    | a a g i | 2 Y L K - I |
| VP63   | Indonesia   | b a h j | 1 F L - I |
| FC1010 | Indonesia   | b a i k | 1 F L - I |
| UVT27  | Indonesia   | b a i k | 1 F L - I |
| FC1232 | Indonesia   | b a c l | 1 F L - I |
| VPS56  | Indonesia   | b a c l | 1 F L - I |
| FC1108 | Indonesia   | b a c l | 1 F L - I |
| VP23   | Indonesia   | b a c l | 1 F L - I |
| VP59   | Indonesia   | a a c m | 1 F L - I |
| FC1158 | Indonesia   | a a c m | 1 F L - I |
| ANV15  | Indonesia   | b d c n | 1 F L - I |
| FC1248 | Indonesia   | b d c n | 1 F L - I |
| VPR20  | Indonesia   | a c j o | 1 F L - I |
| UVT22  | Indonesia   | a a i p | 1 Y L - I |
| ANV16  | Indonesia   | c a i q | 1 F L - I |
| FC1083 | Indonesia   | a c k r | 1 F L - I |
| UVT44  | Indonesia   | a a b s | 1 F L - I |
| ANV18  | Indonesia   | c a i t | 1 F L - I |
| ANV12  | Indonesia   | c d l u | 1 F L - I |
| UVT70  | Indonesia   | c a m v | 1 F L - I |
| FC269  | Indonesia   | b d n w | 1 F L - I |
| FC1290 | Indonesia   | a a o x | 1 F L - I |
| FC10122| Indonesia   | b a l y | 1 F L - I |

Isolates are grouped and ordered alphabetically according to the combined *pvmsp1*, *pvmsp3* and *ama1* genotype. SNP positions and corresponding amino acid changes relative to the SAL1 reference strain are in bold.

doi:10.1371/journal.pone.0001089.t003
Figure 3. Sequence relatedness among *P. vivax* isolates from different locations according to clustal-c analysis of *pvama1* sequence. Stars indicate Thai isolates, doi:10.1371/journal.pone.0001089.g003
The correlation between in vitro susceptibility and clinical efficacy at our two study sites validates our in vitro susceptibility test and suggests that the adapted schizont maturation method may be usefully applied to investigate the emergence of drug resistance in P. vivax in other locations. Furthermore, the ability to define parasite susceptibility free from the confounding factors of host and environment provides a useful framework from which to investigate putative molecular markers of drug resistance. We used our carefully defined sample set to test for associations between the in vitro response to chloroquine and polymorphisms of the orthologues of two genes (pvmdr1 and pvcr1-o) known to be important determinants of chloroquine resistance in P. falciparum. Although previous studies have not established a link between these genes and chloroquine resistant P. vivax, these generally used a relatively small number of clinical isolates in which the phenotypic definition was possibly confounded by patient immunity, re-infection and relapses [22,23]. Brega et al identified the pvmdr1 Y976F and 1076 mutation in a small number of Thai and Indonesian isolates, although in vitro and clinical correlates were not presented [33].

In the present study we found two polymorphisms which were correlated with in vitro chloroquine susceptibility: the pvmdr1 Y976F mutation and an insertion in the 1st exon (amino acid position 10) of pvcr1-o. Overall both polymorphisms were associated with a significant increase in chloroquine IC50. In Papua, Indonesia, where the Y976F mutation has reached fixation and the AAG insertion was almost absent it was not possible to test the relevance of these markers. However in Thailand, the Y976F mutation was present in 25% (17/69) of isolates and associated with 1.7 fold increase in IC50 to chloroquine.

To rule out the possibility that the polymorphisms were related to geographical isolation of the samples, we performed phylogeny analyses to compare the samples from two locations on pvama1, a marker unrelated to chloroquine pressure. The results did not show clustering of samples with location. In addition, we analysed pvmdr1 sequence including all synonymous changes in pvmdr1 which are presumed not to be selected by drug pressure. Again we did not see clustering of the samples with location. These analyses suggest that the Y976F is unlikely to be geographically associated with the Papua location per se, and provide further evidence for its selection by chloroquine selective pressure.

Notably a small number of isolates with high IC50 values were observed from both sites in the absence of the 976 mutation, and vice versa, suggesting that other major molecular determinants are likely to be involved. However a role of pvmdr1 in modulating...
chloroquine susceptibility is supported by the almost ubiquitous selection of the Y976F allele in Papua, where high grade chloroquine resistance is known to predominate.

Gene amplification of the **pfmdr1** gene has been shown to be a major determinant of multidrug resistance in *P. falciparum*. Furthermore, on the Thai–Myanmar border widespread deployment of mefloquine has been associated with high prevalence of *P. falciparum* isolates with increased **pfmdr1** copy number and an associated decrease in susceptibility to mefloquine, quinine, lumefantrine, halofantrine and the artemisinin derivatives in *P. falciparum*. In this study we report that amplification of **pfmdr1** copy number occurs in *P. vivax* in Thailand, but not Papua, where mefloquine has not been used. Our data raise the prospect of similar molecular mechanisms of multi drug resistant phenotype as found in *P. falciparum*, although further work is needed to confirm this.

In conclusion, using an *in vitro* susceptibility assay, we have been able to define a spectrum of chloroquine susceptibility in *P. vivax* and discriminate between populations with differing levels of clinical efficacy following chloroquine monotherapy. Although the molecular mechanism underlying chloroquine resistance *P. vivax* may involve multigenic loci, the **pfmdr1** polymorphism at Y976F may provide a useful tool to monitor the emergence of chloroquine resistance.

**ACKNOWLEDGMENTS**

We are grateful to Lembaga Pengembangan Masyarakat Amungme Ramblero and Dr Paulus Sugiyono, the director of the Rumah Sakit Mitra Masyarakat Hospital, for their support in conducting this study. We thank Prof. Dennis Shanks for his review of our draft manuscript.

**Author Contributions**

Conceived and designed the experiments: RP FN NA QC BR MC UL ET. Performed the experiments: MB BR RS MC FC EK VV KP AB. Analyzed the data: RP MB BR RS MC FC VV KP AB. Contributed reagents/materials/analysis tools: QC BR MC. Wrote the paper: RP FN NA QC BR RS MC EK UL ET.