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Multiple Origins of Mutations in the \textit{mdr1} Gene—A Putative Marker of Chloroquine Resistance in \textit{P. vivax}

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Abstract

Background

Chloroquine combined with primaquine has been the recommended antimalarial treatment of \textit{Plasmodium vivax} malaria infections for six decades but the efficacy of this treatment regimen is threatened by chloroquine resistance (CQR). Single nucleotide polymorphisms (SNPs) in the multidrug resistance gene, \textit{Pvmdr1} are putative determinants of CQR but the extent of their emergence at population level remains to be explored.

Objective

In this study we describe the prevalence of SNPs in the \textit{Pvmdr1} among samples collected in seven \textit{P. vivax} endemic countries and we looked for molecular evidence of drug selection by characterising polymorphism at microsatellite (MS) loci flanking the \textit{Pvmdr1} gene.

Methods

We examined the prevalence of SNPs in the \textit{Pvmdr1} gene among 267 samples collected from Pakistan, Afghanistan, Sri Lanka, Nepal, Sudan, São Tomé and Ecuador. We measured and diversity in four microsatellite (MS) markers flanking the \textit{Pvmdr1} gene to look evidence of selection on mutant alleles.
Results
SNP polymorphism in the Pvmdr1 gene was largely confined to codons T958M, Y976F and F1076L. Only 2.4% of samples were wildtype at all three codons (TYF, n = 5), 13.3% (n = 28) of the samples were single mutant MYF, 63.0% of samples (n = 133) were double mutant MYL, and 21.3% (n = 45) were triple mutant MFL. Clear geographic differences in the prevalence of these Pvmdr mutation combinations were observed.

Significant linkage disequilibrium (LD) between Pvmdr1 and MS alleles was found in populations sampled in Ecuador, Nepal and Sri Lanka, while significant LD between Pvmdr1 and the combined 4 MS locus haplotype was only seen in Ecuador and Sri Lanka. When combining the 5 loci, high level diversity, measured as expected heterozygosity (H_e), was seen in the complete sample set (H_e = 0.99), while H_e estimates for individual loci ranged from 0.00–0.93. Although Pvmdr1 haplotypes were not consistently associated with specific flanking MS alleles, there was significant differentiation between geographic sites which could indicate directional selection through local drug pressure.

Conclusions
Our observations suggest that Pvmdr1 mutations emerged independently on multiple occasions even within the same population. In Sri Lanka population analysis at multiple sites showed evidence of local selection and geographical dispersal of Pvmdr1 mutations between sites.

Author Summary
Chloroquine combined with primaquine has been the recommended antimalarial treatment for Plasmodium vivax malaria infections for sixty years but the efficacy of this treatment regimen is threatened by chloroquine resistance. In this study we describe the prevalence of mutations in the P. vivax gene, Pvmdr1 among samples collected in seven endemic countries. The mutations are thought to be associated with chloroquine resistance and here we looked for evidence of drug selection by characterising polymorphism in DNA repeat regions (microsatellite (MS) loci) flanking the Pvmdr1 gene. Mutations in the Pvmdr1 gene were mainly identified at codons T958M, Y976F and F1076L. Just 2.4% of samples were wildtype at all three codons, while 63% were single mutants (MYF). Clear geographic differences in the prevalence of these Pvmdr mutation combinations were observed. At the flanking MS loci, we found high levels of diversity, and significant differentiation between geographic sites. This pattern of variation could indicate directional selection through local drug pressure. In summary, our observations suggest that Pvmdr1 mutations and thus, chloroquine resistance has emerged independently on multiple occasions even within the same population.

Introduction
Malaria is one of the world’s leading causes of mortality and morbidity. Since late in the 1940s the antimalarial drug chloroquine (CQ) has been the primary chemotherapeutic for prophylaxis and treatment of malaria because of its good safety profile, low cost and high efficacy
against the blood stages of CQ sensitive (CQS) Plasmodium parasites, causing the disease. Since the 1950’s, CQ treatment of P. vivax infections has been combined with the hypnozoitocidal drug primaquine (PQ) for clearance of the latent P. vivax liver stages, responsible for later relapses of the disease [1–3]. Compared to P. falciparum, development of CQ resistance (CQR) in P. vivax has been relatively slow with the first reports emerging in 1989 in Papua New Guinea (PNG) [4]. Since then, CQR has spread and today it is considered to be present in vivax-malaria endemic countries all over the world (reviewed in [5] and more recently in [6]). Development of CQR has been slower in P. vivax than P. falciparum and this is sometimes attributed to the use of combined treatment (CQ with PQ), where PQ acts synergistically with CQ against CQR parasites [7]. It is also proposed that CQR in P. vivax has a different CQR mechanism than P. falciparum [8].

Knowledge of the mode of action of CQR in P. vivax is limited. In P. falciparum, reduced CQ sensitivity is strongly associated with single nucleotide polymorphisms (SNPs) in the chloroquine resistance transporter-gene, PfCRT [9;10]. However, studies of the PfCRT orthologue in P. vivax, PvCrt, have not been able to find an association to CQR [8;11]. In P. falciparum is the P-glycoprotein–like molecule Pgh-1 encoded by Pfmdr1, is also associated with CQR though it may only modulate the effects of the PfCRT gene [12;13]. In 2005, Brega et al. characterized the mdr-like gene Pvmdr1 in P. vivax isolates [14], and evidence suggests that SNPs in the Pvmdr1 gene are a possible genetic determinant of CQR [11;14;15]. Cross-species comparisons led the focus in P. vivax to be primarily on the mdr-codons orthologous to codons implicated in P. falciparum CQR namely 86, 184, 1034, 1042 and 1246 [14;16]. However codons 91 and 189 which are homologous to codons 86 and 184 in P. falciparum [14;17] and codons 1071 and 1079 which are homologous to codons 1034 and 1042 in P. falciparum [14] are rarely polymorphic in Pvmdr1. Instead SNPs at codons, 976 and 1076, have been detected multiple times [11;14;15;18;19]. Suwanarusk et al. observed an association between the Y976F mutation and increased CQ IC50 in samples from Thailand and Papua province of Indonesia, and stated that the Y976F mutation is a useful tool to indicate foci of chloroquine resistance [11]. Others detect the mutations, but doubt their association with CQR [15;18;20–23].

Studies of P. falciparum drug resistance loci have used flanking microsatellite (MS) variation to describe selective sweeps around PfDhfr [24] and PfDhps [25] and PfCRT [26]. This approach has revealed lineages of drug resistance mutant alleles which are derived from a single emergence event. Notably, in P. falciparum some resistance lineages were found to have spread across vast geographical distances [26;27]. When the same approach was repeated antifolate drug targets in P. vivax Pvdhfr [28] and Pvdhps [29] contrasting results were found. In Pvdhfr and Pvdhps there was evidence of multiple independent mutation events with little selective sweep around mutant alleles at those loci. This result may reflect the limited antifolate drug selection pressure that has historically been applied to P. vivax, or it may point to differences in transmission and selection dynamics in the two species. In this study we looked for evidence of drug selection on the CQR candidate Pvmdr using MS flanking the Pvmdr1 gene.

We analysed samples collected from Pakistan, Afghanistan, Sri Lanka, Nepal, Sudan, São Tomé and Ecuador. In Pakistan approximately 83% of the malaria cases are caused by P. vivax and in Afghanistan, 95% are P. vivax. In both countries P. vivax infections are still being treated with CQ + PQ [30]. Around 83% of reported malaria infections in Sri Lanka were caused by P. vivax and CQ with PQ were still efficient and recommended treatment of P. vivax infections on the island until autochthonous cases of both P. falciparum and P. vivax in Sri Lanka fell to zero [30]. In Nepal 88% of the malaria cases are caused by P. vivax and treated with CQ + PQ [30]. In Ecuador, P. vivax accounts for 86% of all malaria infections and is treated with CQ + PQ [30]. To the best of our knowledge no cases of CQR have been reported from either Nepal or Ecuador. Only, 5% of the malaria infections in Sudan are caused by P. vivax and these
are treated with artemether-lumefantrine + PQ [30]. No reports of P. vivax CQR have been published from Sudan. Falciparum malaria is the main cause of malaria in São Tomé and no recommendations are provided regarding treatment of P. vivax [30].

The objectives of the present study were to 1) Determine the diversity of SNPs in the Pvmdr1 gene, a putative marker of CQR, in P. vivax samples collected from Pakistan, Afghanistan, Sri Lanka, Nepal, Ecuador, Sudan and São Tomé and 2) Characterise flanking MS variation and use this to explore the evolutionary origin of Pvmdr mutations.

**Methods**

**Sample collection and description of collection sites**

The total number of P. vivax samples analysed in this study was 267. The samples originated from 7 countries: Pakistan (n = 36), Afghanistan (n = 13), Nepal (n = 55), Sri Lanka (n = 136), Ecuador (n = 17), São Tomé (n = 4) and Sudan (n = 6). The samples were all (with the exception of São Tomé, and Sudan) derived from larger sets of PCR positive samples and the subset selected by computer randomisation. The DNA from a total of 263 P. vivax samples selected were already extracted as part of a P. vivax microsatellite study described previously [31].

**Pakistan and Afghanistan:** Forty-nine samples from a cluster of neighbouring sites in Pakistan and Afghanistan were analysed. Thirty-six were from Pakistan (n = 36); Ashagroo refugee camp in Azizai from 2003 (n = 10), Adizai Refugee Village in Peshawar from 2004–2005 (n = 3) sampled as part of another study [32] and lastly Adizai, Baghicha and Khagan villages located near Peshawar 2005–2006 (n = 23) described in [33]. Thirteen samples were from Afghanistan collected at the Malaria Reference Center in Jalalabad in 2004–2005 [32]. The samples from all these sites were grouped together because of similar study designs and close geographical distance between the sites.

**Sri Lanka:** The samples from Sri Lanka were collected in 9 malaria endemic districts during 2002–2007, see [34]. For this study, the samples were divided into 9 groups of districts, where after randomised computerisation was used to select samples from each district (N = 136).

**Nepal:** Samples from two separate studies in Nepal were grouped together. Thirty-eight samples collected in 2009–2010 from the districts of Jhapa (N = 34) and Banke, Chitwan and Dang (N = 5) have been previously described in [35]. The other study collected samples in the districts of Kanchanpur (N = 5) and Jhapa (N = 12) in 2005–2006 as a part of a cross-sectional prevalence survey estimating the malaria burden and risk behaviour in two endemic districts of Nepal (S. Hewitt, personal communication). The Kanchanpur samples were grouped with Banke, Chitwan and Dang.

**Ecuador:** Twenty-one P. vivax samples were collected from 2007–2009 in the Province of Sucumbios through the network of laboratories of the Ministry of Health.

**Sudan:** Six P. vivax samples from Sudan were collected in the village of Asar in Gedaref state in 2006 as a part of an artemether-lumefantrine efficacy trial community based survey [36]. The amount of available P. vivax DNA was small, and only limited analysis was possible.

**São Tomé:** The island São Tomé is a part of the Democratic Republic of São Tomé and Príncipe in the western equatorial coast of Central Africa. Four samples were available. As with the samples from Sudan, limited analysis was possible because only a small amount of extracted DNA-solution was available.

**Amplification and sequence analysis of the Pvmdr1 gene**

A fragment spanning nucleotide 2596 and 3532 (amino acids 865–1177) of the Pvmdr1 gene and was amplified using semi-nested primers Pvmdr1-4F [11], Pvmdr1-AS and Pvmdr1-S [14]. Primer sequences are shown in Table 1. Cycling conditions were as follows: 94°C for 15
min, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min, and finally 72°C for 10 min. The amplified *Pvmdr1* fragments were sequenced on an ABI Prism 377 (Perkin-Elmer) using the Big Dye terminator reaction mix (Perkin-Elmer). After sequencing, the individual haplotypes were aligned and analysed by use of the DNASTAR-Lasergene software.

**Identification of microsatellites**

The *Pvmdr1* gene is located on chromosome 10, and sequences flanking the gene were screened for suitable microsatellite marker loci in the Salvador-1 (Sal-1) reference strain (accessed through the European Bioinformatics Institute homepage (www.ncbi.nlm.nih.gov/)). Multiple repeats were identified using the software Tandem Repeats Finder [37] and semi-nested primers designed (Table 1). The primary reaction comprised of 1μl template, 0.5 unit Taq polymerase, 1.1μl Thermopol Reaction buffer (New England Biolabs Inc, Glostrup, Denmark), and 0.4μM dNTPs, 0.1μM of forward (F) and reverse primers (R) with cycling conditions as follows; 2 min at 94°C and then 25 repeated cycles of 30 s at 94°C, 30 s at 42°C, 30 s at 40°C and 40 s at 65°C followed by 2 min at 65°C and a minimum of 10 min at 15°C. In the secondary PCR, the same concentrations of reagents were added, but with 0.15μM of reverse primers (R) and fluorescent-labelled inverse primers (I). The cycling conditions were initiated with 2 min at 94°C followed by 25 repeated cycles of 20 s at 94°C, 20 s at 45°C, 30 s at 65°C, and finished with 2 min at 65°C and 10 min at 15°C.

A panel of four MS were selected for further analysis on the basis of their successful amplification and were named according to their distance to the *Pvmdr1* gene: m9.5 (9,489 bp downstream of *Pvmdr1*), m10.1 (10,120 bp downstream), m10.4 (10,420 upstream) and m43.1 (43,168 bp upstream). (Fig 1). PCR amplified fragments were run with LIZ-500 size standard on an ABI 3730XL genetic analyzer (Applied Biosystems), and analysed using the GeneMapper software (Applied Biosystems). Samples that were negative by PCR were repeat amplified with 2μl template in the first PCR.

### Table 1. Primers for amplification and sequencing of the *Pvmdr1* gene and four flanking polymorphic repeat regions.

| Locus     | Primer     | Use          | Sequence (5'-3')                  |
|-----------|------------|--------------|-----------------------------------|
| *Pvmdr1*  | Pvmdr1-4F  | 1st PCR (F)  | CCCTCTACATCTTAGTCATCG             |
|           | Pvmdr1-AS  | 1st and 2nd PCR (R) | ACGTTTGCTCCTGCAAGATATC         |
|           | Pvmdr1-S   | 2nd PCR (F)  | ATAGTCATGCCCCAGAGTTG             |
| MS        | Repeat     | Size range   |                                    |
| m9.5      | AAT        | 199–207      |                                    |
|           | m9.5F      | 1st and 2nd PCR (F) | TATGTGGAGAAGGGGAACG           |
|           | m9.5R      | 1st PCR, (R) | TCTCGTTATTGCTGACACT              |
| m10.1     | A, AT      | 266–322      |                                    |
|           | m10.1F     | 1st and 2nd PCR (F) | GCTGCGCTATAAAGTTGC         |
|           | m10.1R     | 1st PCR, (R) | GACCTGGAAATCCACTGCTA            |
|           | m10.1I-FAM | 2nd PCR, (R) | FAM-GCACTCCATTTGCGACTG          |
| m10.4     | GCATTAT    | 230–308      |                                    |
|           | m10.4F     | 1st and 2nd PCR (F) | GCAGTCTTTTGTCTCCTCAC        |
|           | m10.4R     | 1st PCR, (R) | TCAAACTCAAAGCGCTTGC            |
| m43.1     | TA, GT     | 419–472      |                                    |
|           | m43.1F     | 1st and 2nd PCR (F) | GACATGACACCGCAAGTGC         |
|           | m43.1R     | 1st PCR, (R) | GTAAACGCGAGGCCACAGTA            |
|           | m43.1-I-FAM| 2nd PCR, (R) | FAM-CGTTGTGCATACGCAGACATA       |

(F): forward primer, (R): reverse primer. MS: Microsatellites. FAM: primer labeled with FAM in 5’-end.

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The number and length of the repeats in each of the four MS is shown in Table 1. In the cases where multiple (≥ 2) microsatellite alleles were detected in a single sample the major/predominant allele chosen, (‘predominant’ is defined by the electropherogram peak height which had to be twice that of the minor allele).

Data analysis

Linkage disequilibrium (LD) was calculated to test for a non-random association of \( P v m d r1 \) allele and the MS alleles. Only the non-mixed samples were used in the calculations. LD was measured by the formula \( D' = \frac{D}{D_{\text{max}}} \), where \( D \) equals derivation of random association between alleles at different loci, and \( D' \) measures \( D \) standardized by the maximum value (\( D_{\text{max}} \)), given the observed allele frequencies. LD values range from -1 to +1, where the value +1 refers to a complete non-random association between the alleles. Values of gene diversity were calculated by expected heterozygosity by the formula \( H_e \) = \( \frac{n}{n-1} \left(1-\sum p_i^2\right) \), by use of the Arlequin software [38], where \( H_e \) is expected heterozygosity, \( n \) the number of samples, and \( p_i \) the frequency of the \( i \)-th allele in the sample set.

Ethical statement

Clearance for analysis of Plasmodium genes were approved by London School of Hygiene Tropical Medicine and Hygiene Ethics Board, locally by Bioethics Committee, Pakistan Medical Research Council and Directorate of Public Health, Jalalabad, Nangahar, Comitee de Bioetica Universidad San Francisco de Quito, Committee on Research and Ethical Review at the Faculty of Medicine, Peradeniya, Kandy and the Nepal Health Research Council. All data analysed were anonymized.

Results

Analysis of the \( P v m d r1 \) gene

The analysis of \( P v m d r1 \) in 39 samples from Nepal was previously published [35]. Of the remaining 228 samples, sequencing was successful in 173 (75.9%); Pakistan (n = 24), Nepal (n = 4), Sri Lanka (n = 120), São Tomé (n = 3), Sudan (n = 4) and Ecuador (n = 17). Combined with the 39 samples from Nepal, 212 \( P v m d r1 \) sequence fragments were available for further analysis (Table 2, Fig 2).

SNP variation in fragment 2 was largely confined to three codons; T958M (ACG→ATG), Y976F (TAC→TTC) and F1076L (TTT→CTT), (Fig 1). Three novel SNPs were detected in two samples from the Jhapa district in Nepal. These were sequenced twice to confirm the results. One of the samples carried a SNP at c1080 (S1080N, AGT→AAT), while the other
sample possessed SNPs at c979 (F979S, TTT→TCT) and c980 (M980V, ATG→GTG) (Table 3). These two samples and the SNPs were described by Ranjitkar et al. [35].

The substitutions at codons 958, 976, and 1076 were found in various configurations. TYF (the wild-type) was found only in Ecuador in 5 of 17 samples (Fig 2, S1 Table). All the remaining samples carried one of three mutant haplotypes, MYF (single mutant, T958M), MYL (double mutant, T958M+F1076L) and MFL (triple mutant T958M+Y976F+F1076L). The SNPs were described by Ranjitkar et al. [35].

Table 2. Allelic diversity measured by expected heterozygosity (He).

| Origin           | Pvmdr1 | m9.5 | m10.1 | m10.4 | m43.1 | 5-loci haplotype |
|------------------|--------|------|-------|-------|-------|-----------------|
| Pakistan/Afghanistan | 0.00 (1/24) | 0.10 (2/20) | 0.87 (8/17) | 0.74 (6/39) | 0.44 (2/29) | 0.96 (9/11) |
| Nepal            | 0.32 (3/43) | 0.56 (4/32) | 0.93 (17/37) | 0.82 (10/45) | 0.40 (3/34) | 1.00 (24/24) |
| Sri Lanka        | 0.54 (3/120) | 0.34 (2/115) | 0.82 (12/17) | 0.82 (8/122) | 0.47 (2/97) | 0.97 (39/73) |
| Ecuador          | 0.44 (2/17) | 0.00 (1/17) | 0.68 (4/16) | 0.68 (3/16) | 0.49 (2/17) | 0.83 (8/15) |
| Sao Tomé         | 0.00 (1/3) | 0.00 (1/2) | 1.00 (2/2) | 0.00 (1/2) | 1.00 (1/1) | —— |
| Sudan            | 0.67 (2/4) | 0.50 (2/4) | 0.50 (2/4) | 0.80 (3/5) | 0.67 (2/3) | 1.00 (1/1) |
| Mixed infections (%) | —— | 2 (1.1) | 17 (8.7) | 27 (11.8) | 22 (12.2) | 33 (26.4) |
| He (alleles/n)   | 0.54 (4/211) | 0.43 (4/190) | 0.90 (26/196) | 0.83 (13/229) | 0.47 (3/181) | 0.99 (78/125) |

He is shown for every locus and for the combined 5 locus haplotype at each study site. In brackets are the number of alleles detected and the sample size.

The number (and percentage) of mixed infections detected at each locus is shown. The 5-loci haplotype calculations included samples with mixed infection and used the majority allele for each locus.

Fig 2. Pvmdr1 polymorphism in Plasmodium vivax samples collected at 8 geographical sites. Four Pvmdr1 alleles were detected in the study abbreviated as TYF, MYF, MYL and MFL. The TYF wild-type is shown in blue, MYF (single mutant, T958M) in yellow, MYL (double mutant, T958M+F1076L) in green, and MFL (triple mutant T958M+Y976F+F1076L) in red. The number of positive samples from each site is shown on the map in brackets, and the number of alleles and percentage frequency overall are shown in the legend.

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Table 3. Plasmodium vivax mdr1 alleles TYF, MYF and MYL and flanking microsatellite alleles.

| Origin | 5-loci haplotypes number | Upstream | Downstream |
|--------|--------------------------|----------|------------|
|        |                          | M43.1    | M10.4      | Pvmdr     | m9.5 | m10.1 | No. of samples with identical haplotype |
| Sal-1  | ---                      | 441      | 293        | TYF       | 204  | 270   | ---                                   |
| Ecuador| 1                        | 419      | 261        | TYF       | 201  | 266   | 3                                     |
| Nepal  | 2                        | 437      | 266        | MYF       | 204  | 298   | 1                                     |
| Ecuador| 3                        | 437      | 266        | MYF       | 201  | 300   | 1                                     |
| Ecuador| 4                        | 437      | 266        | MYF       | 201  | 301   | 1                                     |
| Ecuador| 5                        | 437      | 266        | MYF       | 201  | 303   | 1                                     |
| Sri Lanka | 6                      | 419      | 259        | MYF       | 201  | 283   | 4                                     |
| Sri Lanka | 7                       | 419      | 252        | MYF       | 201  | 283   | 1                                     |
| Ecuador | 8                        | 419      | 252        | MYF       | 201  | 300   | 6                                     |
| Ecuador | 9                        | 419      | 252        | MYF       | 201  | 301   | 1                                     |
| Nepal  | 10                       | 419      | 233        | MYL       | 204  | 308   | 1                                     |
| Sri Lanka | 13                     | 419      | 252        | MYL       | 204  | 282   | 4                                     |
| Sri Lanka | 14                     | 419      | 252        | MYL       | 204  | 289   | 1                                     |
| Sri Lanka | 15                     | 419      | 252        | MYL       | 204  | 300   | 1                                     |
| Nepal  | 16                       | 419      | 252        | MYL       | 207  | 289   | 1                                     |
| Sri Lanka | 17                     | 419      | 255        | MYL       | 204  | 289   | 2                                     |
| Nepal  | 18                       | 419      | 259        | MYL       | 201  | 289   | 1                                     |
| Nepal  | 19                       | 419      | 259        | MYL       | 201  | 286   | 1                                     |
| Nepal  | 20                       | 419      | 259        | MYL       | 201  | 290   | 1                                     |
| Nepal  | 21                       | 419      | 259        | MYL       | 204  | 266   | 1                                     |
| Nepal  | 22                       | 419      | 259        | MYL       | 207  | 277   | 1                                     |
| Pakistan | 23                     | 419      | 259        | MYL       | 204  | 280   | 1                                     |
| Nepal  | 24                       | 419      | 259        | MYL       | 204  | 289   | 1                                     |
| Sri Lanka | 25                     | 419      | 259        | MYL       | 204  | 297   | 1                                     |
| Sri Lanka | 26                     | 419      | 259        | MYL       | 204  | 298   | 1                                     |
| Sri Lanka | 27                     | 419      | 259        | MYL       | 204  | 299   | 3                                     |
| Sri Lanka | 28                     | 419      | 259        | MYL       | 204  | 300   | 1                                     |
| Nepal  | 29                       | 419      | 259        | MYL       | 204  | 300   | 1                                     |
| Nepal  | 30                       | 419      | 259        | MYL       | 204  | 308   | 1                                     |
| Sri Lanka | 31                     | 419      | 266        | MYL       | 204  | 289   | 4                                     |
| Sri Lanka | 32                     | 419      | 266        | MYL       | 204  | 298   | 1                                     |
| Sri Lanka | 33                     | 419      | 266        | MYL       | 204  | 311   | 2                                     |
| Pakistan | 34                     | 419      | 266        | MYL       | 204  | 287   | 1                                     |
| Nepal  | 35                       | 419      | 266        | MYL       | 201  | 315   | 1                                     |
| Nepal  | 36                       | 419      | 266        | MYL       | 201  | 289   | 1                                     |
| Sri Lanka | 37                     | 419      | 270        | MYL       | 204  | 289   | 4                                     |
| Sri Lanka | 38                     | 419      | 270        | MYL       | 204  | 299   | 1                                     |
| Sudan  | 39                       | 419      | 276        | MYL       | 207  | 303   | 1                                     |
| Sudan  | 40                       | 419      | 308        | MYL       | 204  | 293   | 1                                     |
| Sri Lanka | 41                     | 437      | 252        | MYL       | 201  | 297   | 1                                     |
| Sri Lanka | 42                     | 437      | 252        | MYL       | 204  | 297   | 5                                     |
| Sri Lanka | 43                     | 437      | 255        | MYL       | 204  | 311   | 1                                     |
| Pakistan | 44                     | 437      | 255        | MYL       | 204  | 283   | 1                                     |
| Sri Lanka | 45                     | 437      | 259        | MYL       | 201  | 289   | 1                                     |
| Sri Lanka | 46                     | 437      | 259        | MYL       | 204  | 289   | 1                                     |

(Continued)
double mutant MYL was present in 63.0% of the samples (n = 133), the triple mutant MFL in 21.3% (n = 45), and the single mutant MYF in 13.3% (n = 28). Their relative abundance at the different sites is shown in Fig 2. In Pakistan only the MYL double mutant haplotype was detected, while the Ecuador samples (apart from the wild-type TYF) possessed the single mutant MYF (n = 12). The Sudan P. vivax samples a mix of MYL (n = 2) and MFL (n = 2) haplotypes were found, while the three samples from São Tomé all possessed the MFL haplotype (Fig 2).

The heterozygosity of \( P_{\text{vmdr1}} \) measured as \( H_e \) is shown in Table 2. Measured over all samples \( H_e = 0.54 \). When divided by collection site, Sri Lanka was the most diverse (\( H_e = 0.54 \)), and Pakistan the least diverse (\( H_e = 0 \)) with only one allele-the MYL haplotype. Although the \( H_e \) value for Sudan was high (0.67), the sample size was small (n = 4), and the broad variation in sample size between sites precluded further in-depth analysis of difference between the sites.

District level analysis was possible for Sri Lanka (Fig 3). In Sri Lanka, samples were collected in 9 districts, and despite the small sample size per district, the distribution of alleles was similar at district level when compared to the pooled sample set, with a dominating MYL haplotype, followed by MFL and MYF. The exception was Kurunegala district where the MFL haplotype was most common (n = 15), followed by the MYL haplotype (n = 5).

**Analysis of the four microsatellites**

Four MS from \( P_{\text{vmdr1}} \) flanking genomic regions were genotyped in the 267 samples although these were amplified with varying successes; for m9.5 (n = 190), m10.1 (n = 196), m10.4 (n = 229) and m43.1 (n = 181) alleles (Table 2). The m10.1 locus was the most polymorphic with 26 alleles identified among 196 samples (\( H_e = 0.90 \)), while m9.5 had 4 alleles, m10.4 had

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**Table 3. (Continued)**

| Origin | 5-loci haplotypes number | M43.1 | M10.4 | Pvmdr | m9.5 | m10.1 | No. of samples with identical haplotype |
|--------|--------------------------|-------|-------|-------|------|-------|------------------------------------------|
| Nepal  | 47                       | 437   | 259   | MYL   | 201  | 286   | 1                                        |
| Sri Lanka | 48                      | 437   | 266   | MYL   | 204  | 303   | 1                                        |
| Sri Lanka | 49                      | 437   | 270   | MYL   | 204  | 289   | 2                                        |
| Sri Lanka | 50                      | 419   | 244   | MFL   | 204  | 305   | 1                                        |
| Sri Lanka | 51                      | 419   | 252   | MFL   | 204  | 283   | 1                                        |
| Sri Lanka | 52                      | 419   | 255   | MFL   | 201  | 283   | 1                                        |
| Sri Lanka | 53                      | 419   | 255   | MFL   | 204  | 297   | 1                                        |
| Sri Lanka | 54                      | 419   | 266   | MFL   | 204  | 299   | 4                                        |
| Sri Lanka | 55                      | 419   | 270   | MFL   | 204  | 289   | 1                                        |
| Nepal  | 56                       | 419   | 276   | MFL   | 204  | 298   | 1                                        |
| Sri Lanka | 57a                     | 437   | 255   | MFL   | 204  | 291   | 5                                        |
| Sri Lanka | 58                      | 437   | 270   | MFL   | 204  | 299   | 1                                        |

The samples from Ecuador (n = 13), Nepal (n = 16), Pakistan (n = 3), Sri Lanka (n = 58) and Sudan (n = 2) a shown together with Sal-1 which is a wild-type chloroquine sensitive haplotype (GenBank accession number AY571984.1). The number of samples per geographical origin, which possessed each distinct haplotype are shown in the last column where there was more than one the row is highlighted dark gray. Excluded in the table are all the polyclonal samples.

a5-loci haplotypes calculated to be in significantly linkage disequilibrium (\( D' = 1, \text{P}<0.0001 \)).

A sample from Nepal contained two further mutations at c979 (F979S, TTT→TCT) and c980 (M980V, ATG→GTG).

This sample from Nepal was also mutated at c1080 (S1080N, AGT→AAT).

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Fig 3. Genetic diversity of the *Plasmodium vivax mdr1* gene in *P. vivax* samples collected at 9 malaria endemic districts of Sri Lanka. Three different genotypes were detected in the samples from Sri Lanka named MYF (yellow), MYL (green) and MFL (red). MYF (single mutant, T958M), MYL (double mutant, T958M)
and m43.3 had 3 different alleles ([Table 2, and S1 Table]). The number of mixed samples (those containing more than one allele) detected using each locus differed; only 1% were mixed at the m9.5 locus, while 12% were mixed at the m43.3 locus. When combining all 5 loci, 26% were mixed among 125 samples ([Table 2]).

The m10.1 locus had a mono-A-repeat as well as an AT dinucleotide repeat, and this was reflected in the high number of different alleles (Hc = 0.90). Ecuador was an exception, only possessing 4 different m10.1 alleles among the 16 positive samples (Hc = 0.68). Allele size variation is shown in [S1 Table], the highest number of observed MS alleles were generally of intermediate size.

The combination of all 5 loci resulted in 57 different 5-loci haplotypes among the monoclonal samples ([Table 3]) and 78 when majority alleles in the mixed genotype samples were included. All 5-locus haplotypes differed from the CQS wild-type Sal-1 haplotype. The most commonly observed 5-locus haplotype was detected in Ecuador (n = 6, haplotype number 8 in [Table 3]). The other frequently observed 5-locus haplotypes were all from Sri Lanka. The distribution of the Pvmdr1 5-locus haplotypes among the sub-populations sampled in Sri Lanka is shown in [Fig 4]. A large number of haplotypes occurred only once and these are indicated by grey segments in the pie charts. Haplotypes which occurred multiple times are indicated each by a different colour. The sample collections with the greatest degree of haplotype sharing were from Trincomalee and Anuradhapura (6 haplotypes) and Anuradhapura and Polonaruwa (5 haplotypes of which only 1 was found in Trincomalee). Other sites appear more isolated, for instance all the haplotypes found in the district of Batticaloa were unique.

Unfortunately, the small amount of DNA-solution available from Sudan and São Tomé prevented reanalysis of these samples, and no 5-loci haplotype from Sao Tomé could be created.

We tested for LD, between Pvmdr1 alleles and flanking MS alleles. Significant associations are shown in [S2 Table]. Strong linkage associations were seen in Ecuador where MS alleles occurred in association with the MYF single mutant allele and also with the wildtype allele TYF. Other population level LD associations were observed in Sri Lanka, and Nepal. In each case different MS alleles were associated with the Pvmdr1 allele. When the 4 MS loci were combined, significant LD between certain 4-loci haplotypes and either the TYF, MYF or MFL haplotypes was found in Ecuador and Sri Lanka, ([S2 Table]). When samples from all sites were pooled the LD analysis found significant associations of MS alleles with TYF and MFL which are likely attributable to admixture.

**Discussion**

The aim of this study was to characterise SNPs in the Pvmdr1 gene, to examine whether the putative CQR mutations have one, few or many origins, and to determine whether there has been geographical spread of certain Pvmdr1 haplotypes. SNPs were found at three codons, T958M, Y976F and F1076L among 267 samples from Pakistan, Nepal, Sri Lanka, Ecuador, Sudan and Sao Tomé. Polymorphism in the last two codons has been described in multiple studies [11;14;15;18;19;22;23], but the high prevalence of 958M which we observed (206/211, 97.6%) was surprising since this SNP has only been mentioned in two earlier studies; in Madagascar (with a 100% fixation of the 958M) [18] and in a few samples from Indonesia and Brazil [39]. Besides these studies, all others either report the presence of the wild-type T958 allele, or do not mention the locus [11;20–23]. Since the 958M mutation was present in countries with both high and low level of reported CQR over a wide time span, we hypothesize that the
T958M is an allelic variant of the wildtype and most likely not associated with CQR. In the present study the T958 wild-type was only detected in Ecuador. It is also seen in the Sal-1 reference sample which originates from El Salvador, so it is possible this allele might be a characteristic of American samples while the 958M is characteristic of Asia and Africa.

Rare mutations, F979S (TTT→TCT), M980V (ATG→GTG) and S1080N (AGT→AAT) were found in two samples from the Jhapa district of Nepal, both possessing the MYL double mutant \textit{Pvmdr1} haplotype (Table 3); One of these samples was mutated at codon 1080 while the other was mutated at codon 979 and codon 980. These results have been previously published by Ranjitkar in 2011 [35]. Thus, in total only five \textit{Pvmdr1} SNPs were detected which was an unexpected result. Orjuela-Sanchez \textit{et al.} (2009) [23] reported up to 24 \textit{Pvmdr1} mutations in a study of only 7 samples from Brazil, while Barnadas \textit{et al.} (2008) [18] reported 21 mutations among 105 samples from Madagascar. However, both studies amplified longer fragments of the \textit{Pvmdr1} gene than the present study, which might be a part of the explanation.

Genotyping of microsatellites flanking the \textit{Pvmdr1} gene revealed high levels of diversity around single, double and triple mutant alleles. There were too few wildtype TYF alleles to meaningfully compare the MS heterozygosity surrounding wildtype and mutant alleles for evidence of selective sweeps on the mutant alleles. However our finding that all 3 wildtype TYF alleles were flanked by an identical microsatellite haplotype would not support the view that reduced diversity among microsatellite haplotypes is attributable to selective sweeps, but rather suggests a tendency to clonal population structure in some populations.

The evidence for association of MS alleles with particular mutations was patchy. TYF and MYF \textit{Pvmdr1} haplotypes occurred together with the “201” m9.5 allele, while the “204” allele at m9.5 was more commonly seen with MFL and MYL. No obvious pattern of distribution was seen for the other 3 MS, suggesting this association was caused by greater representation of certain mutant alleles in particular geographic localities rather than a selective sweep.

The combination of the 5 \textit{Pvmdr1} loci into a 5-loci haplotype revealed 57 different haplotypes among the 125 samples positive at all loci, many of them unique. Country-wise, Nepal was the most diverse, when analysed by locus and for the combined 5-loci haplotype, whereas Ecuador was more conserved. The diminished diversity within the Ecuadorian samples is consistent with the general finding of little diversity amongst \textit{P. vivax} samples from the Americas, although this is not a hard and fast rule [40]. Just 10 samples of African origin were available for this study but both double and triple \textit{Pvmdr1} mutant alleles were present, and their microsatellite fingerprint was distinct from that associated with the same alleles in Asia. Likewise, our South American sample from Ecuador (n = 17) was distinct from the other populations being less diverse, and unique in having the wild-type \textit{Pvmdr1} allele.

Studies of \textit{P. falciparum} have revealed a contrasting pattern of resistance evolution in which relatively few resistance mutants emerge but then become globally disseminated. The pattern is consistent for both CQR [26;41] and high levels of resistance to SP [24;27;42–44]. Hawkins \textit{et al.} [28;29] analysed the origin and dissemination of SPR in \textit{P. vivax} by analysing SNPs in and surrounding the \textit{Pvdhfr} and \textit{Pvdhps} genes. They concluded that the genes are considerably more diverse than seen in \textit{P. falciparum} [28;29] and that highly pyrimethamine-resistant
Pvdhfr alleles arose three times in Thailand, Indonesia and PNG/Vanuatu, and that sulfadoxine resistance associated SNPs had evolved independently on multiple occasions. This is consistent with our findings on Pvmdr and may be explained by comparisons of total genomic diversity among P. vivax and P. falciparum isolates. A study by Neafsey et al. [45] reported twice as much SNP diversity, significantly higher MS diversity and a far deeper divergence among P. vivax geographic isolates than among a comparable set of P. falciparum isolates. The higher level of diversity in P. vivax can explain the multiple origin pattern of resistance emergence in of Pvmdr1, Pvdhfr and Pvdhps.

Our findings can indicate three things. First, Pvmdr1 mutant alleles have developed on multiple haplotype backgrounds by convergent evolution in Pakistan, Nepal, Sri Lanka, Ecuador, Sao Tomé and Sudan. Second, assuming that Pvmdr1 is a reliable CQR marker, there is little evidence that the variation around mutant haplotypes has been subject to a selective sweep, (the result of positive natural selection causing diminished diversity in sequences flanking the selected marker). Third, the historical pattern of drug resistance emergence in P. falciparum is not repeated in P. vivax. The time-delay of around 30 years between initial reports of CQR in the two malaria species, and the two different treatment regimens (mono-in P. falciparum and usually combined CQ/primaquine treatment in P. vivax) might explain this, and may suggest that it is just a matter of time before the effect of selection of the markers can be seen since high grade treatment failure has not yet been reported in any of the sites sampled in this study. P. vivax is generally a chronic disease with low parasitaemia causing mild symptoms compared to P. falciparum, therefore there is less selective drug pressure on the parasite. Furthermore, the fast gametocytogenesis in P. vivax enables uptake of gametes by vectors before clinical symptoms arise and antimalarial treatment is initiated which balances the spread of sensitive and resistant P. vivax parasites.

Equally, these differences may lie with the biology and transmission dynamics of the two species. Our district level analysis of Pvmdr and linked MS variation in Sri Lanka found evidence of exchange of genotypes between districts which may or may not be linked to their resistance phenotype. Latent P. vivax infections cause relapses of the disease, which may increase the possibility of migration of parasites within the human host while the broader temperature tolerance by P. vivax parasites compared to P. falciparum might increase the likelihood of gene flow between sites with varying temperature or microclimate.

Finally CQR may be a complex trait including other genes in addition to Pvmdr1 and future research will hopefully illuminate the genomic-level change underpinning changes in CQ sensitivity. Meanwhile, monitoring and research of CQR is of highest importance for the public health in the afflicted areas.

Supporting Information

S1 Table. The number of detected alleles per collection site and locus; the Pvmdr1 gene and the four microsatellites m9.5, 10.1, 10.4, and 43.1. The Pvmdr1 alleles are named TYF (wild-type allele), MYF, MYL and MFL, corresponding to the codes of the amino acids encoded by the polymorphic codons 958, 976 and 1076, respectively.

S2 Table. Linkage disequilibrium analysis between Pvmdr1 alleles and alleles of flanking microsatellites. Significant LD ($D' = 1, P < 0.05$) between Pvmdr1 alleles (TYF, MTF, MYL and MFL) and alleles of each of three flanking microsatellites (MS); m9.5, m10.1, and m10.4. Significant LD was not found between the Pvmdr1 alleles and m43.1 alleles. LD calculations were carried out for the total pooled sample (T) and for individual populations in Pakistan, Nepal, Sri Lanka, Ecuador, Sao Tomé and Sudan. Significant LD could only be detected in...
Ecuador (E), Sri Lanka (SL) and Nepal (N) and in the total sample set (T). In brackets is the number of alleles in complete LD detected per study site. Excluded in the table are haplotypes with frequencies >1 and mixed infection samples. Pairwise significance levels are as follows: “**” significance at the 0.1% nominal level, “*” significance at the 1% nominal level and “+” significance at the 5% nominal level.

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(AUTHOR CONTRIBUTIONS)

Conceived and designed the experiments: MLS ICB CR MA. Performed the experiments: MLS SR. Analyzed the data: MLS SR CR MA. Contributed reagents/materials/analysis tools: MLS SR RSR PHA FM RP RO TL MR NBG FK ICB CR MA. Wrote the paper: MLS SR RSR PHA FM RP RO TL MR NBG FK ICB CR MA.

(REFERENCES)

1. ALVING AS, HANKEY DD, COATNEY GR, JONES R Jr., COKER WG, GARRISON PL, et al. Korean vivax malaria. II. Curative treatment with pamaquine and primaquine. Am J Trop Med Hyg 1953 Nov; 2 (6):970–6. PMID: 13104805
2. ALVING AS, JOHNSON CF, TARLOV AR, BREWER GJ, KELLERMEYER RW, CARSON PE. Mitigation of the haemolytic effect of primaquine and enhancement of its action against exoerythrocytic forms of the Chesson strain of Plasmodium vivax by intermittent regimens of drug administration: a preliminary report. Bull World Health Organ 1960; 22:621–31. PMID: 13793053
3. Lopez-Antunano FJ. Is primaquine useful and safe as true exo-erythrocytic merontocidal, hypnozoitocidal and gametocidal antimalarial drug? Salud Publica Mex 1999 Sep; 41(5):410–9. PMID: 11142837
4. Rieckmann KH, Davis DR, Hutton DC. Plasmodium vivax resistance to chloroquine? Lancet 1989 Nov 18; 2(8673):1183–4. PMID: 2572903
5. Baird JK. Resistance to therapies for infection by Plasmodium vivax. Clin Microbiol Rev 2009 Jul; 22 (3):506–34. doi: 10.1128/CMR.00008-09 PMID: 19597012
6. Price RN, von SL, Valecha N, Nosten F, Baird JK, White NJ. Global extent of chloroquine-resistant Plasmodium vivax: a systematic review and meta-analysis. Lancet Infect Dis 2014 Oct; 14(10):982–91. doi: 10.1016/S1473-3099(14)70859-2 PMID: 25213732
7. Bray PG, Deed S, Fox E, Kalkanidis M, Munghin M, Deady LW, et al. Primaquine synergises the activity of chloroquine against chloroquine-resistant *P. falciparum*. Biochem Pharmacol 2005 Oct 15; 70 (8):1158–66. PMID: 16139253
8. Nomura T, Carlton JM, Baird JK, del Portillo HA, Fryauff DJ, Rathore D, et al. Evidence for different mechanisms of chloroquine resistance in 2 Plasmodium species that cause human malaria. J Infect Dis 2001 Jun; 183(11):1653–61. PMID: 11343215
9. Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diouf Y, et al. A molecular marker for chloroquine-resistant falciparum malaria. N Engl J Med 2001 Jan 25; 344(4):257–63. PMID: 11172152
10. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, et al. Mutations in the P. falciparum digestive vacuole transmembrane protein PICT and evidence for their role in chloroquine resistance. Mol Cell 2000 Oct; 6(4):861–71. PMID: 11090624
11. Suwanarusk R, Russell B, Chavchich M, Chalfein F, Kenangalem E, Kosaisavee V, et al. Chloroquine resistant Plasmodium vivax: in vitro characterisation and association with molecular polymorphisms. PLoS ONE 2007; 2(10):e1089. PMID: 17971853
12. Duraisingh MT, Cowman AF. Contribution of the pfmdr1 gene to antimalarial drug-resistance. Acta Trop 2005 Jun; 94(3):181–90. PMID: 15876420
13. Duraisingh MT, Jones P, Sambou I, von SL, Pinder M, Warhurst DC. The tyrosine-86 allele of the pfmdr1 gene of Plasmodium falciparum is associated with increased sensitivity to the anti-malarials mefloquine and artemisinin. Mol Biochem Parasitol 2000 Apr 30; 108(1):13–23. PMID: 10802315
14. Brega S, Meslin B, de Monbrison F, Severini C, Gradoni L, Udomsangpetch R, et al. Identification of the Plasmodium vivax mdr-like gene (pvmdr1) and analysis of single-nucleotide polymorphisms among isolates from different areas of endemicity. J Infect Dis 2005 Jan 15; 191(2):272–7. PMID: 15609238
15. Imwong M, Pukrittayakamee S, Pongtavornpinyo W, Nakeesathit S, Nair S, Newton P, et al. Gene amplification of the multidrug resistance 1 gene of Plasmodium vivax isolates from Thailand, Laos, and Myanmar. Antimicrob Agents Chemother 2008 Jul; 52(7):2657–79. doi: 10.1128/AAC.01459-07 PMID: 18443118
16. Foote SJ, Kyle DE, Martin RK, Oduola AM, Forsyth K, Kemp DJ, et al. Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in Plasmodium falciparum. Nature 1990 May 17; 345(6272):255–8. PMID: 2185424
17. Brega S, de Monbrison F, Severini C, Udomsangpetch R, Sutanto I, Ruckert P, et al. Real-time PCR for dihydrofolate reductase gene single-nucleotide polymorphisms in Plasmodium vivax isolates. Antimicrob Agents Chemother 2004 Jul; 48(7):2581–7. PMID: 15215112
18. Barnadas C, Ratsimbason A, Tichit M, Boucher C, Jahevitra M, Picot S, et al. Plasmodium vivax resistance to chloroquine in Madagascar: clinical efficacy and polymorphisms in pvmdr1 and pvcrt-o genes. Antimicrob Agents Chemother 2008 Dec; 52(12):4233–40. doi: 10.1128/AAC.00578-08 PMID: 18809933
19. Suwanarusk R, Chavchich M, Russell B, Jaidee A, Chalfein F, Barends M, et al. Amplification of pvmdr1 associated with multidrug-resistant Plasmodium vivax. J Infect Dis 2008 Nov 15; 198(10):1558–64. doi: 10.1086/592451 PMID: 18808339
20. Gama BE, Oliveira NK, Souza JM, Daniel-Ribeiro CT, Ferreira-da-Cruz MF. Characterisation of pvmdr1 and pvdfr genes associated with chloroquine resistance in Brazilian Plasmodium vivax isolates. Mem Inst Oswaldo Cruz 2009 Nov; 104(7):1099–11. PMID: 20027469
21. Kim YK, Kim C, Park I, Kim HY, Choi JY, Kim JM. Therapeutic efficacy of chloroquine in Plasmodium vivax and the pvmdr1 polymorphisms in the Republic of Korea under mass chemophylaxis. Am J Trop Med Hyg 2011 Apr; 84(4):532–4. doi: 10.4269/ajtmh.2011.10-0486 PMID: 21460004
22. Lu F, Lim CS, Nam DH, Kim K, Lin K, Kim TS, et al. Genetic polymorphism in pvmdr1 and pvcrt-o genes in relation to in vitro drug susceptibility of Plasmodium vivax isolates from malaria-endemic countries. Acta Trop 2011 Feb; 117(2):69–75. doi: 10.1016/j.actatropica.2010.08.011 PMID: 20933490
23. Orjuela-Sanchez P, de Santana Filho FS, Machado-Lima A, Chehuan YF, Costa MR, Alemcrim M, et al. Analysis of single-nucleotide polymorphisms in the crt-o and mdr1 genes of Plasmodium vivax among chloroquine-resistant isolates from the Brazilian Amazon region. Antimicrob Agents Chemother 2009 Aug; 53(8):3561–4. doi: 10.1128/AAC.00045-09 PMID: 19451296
24. Nair S, Williams JT, Brockman A, Paiphun L, Mayxay M, Newton PN, et al. A selective sweep driven by pyrimethamine treatment in southeast asian malaria parasites. Mol Biol Evol 2003 Sep; 20(9):1526–36. PMID: 12832643
25. Pearce RJ, Pot H, Evehe MS, Ba e, Mombo-Ngoma G, Malisa AL, et al. Multiple origins and regional dispersal of resistant dhps in African Plasmodium falciparum malaria. PLoS Med 2009 Apr 14; 6(4): e1000055. doi: 10.1371/journal.pmed.1000055 PMID: 19365539
26. Wootton JC, Feng X, Ferdig MT, Cooper RA, Mu J, Baruch DI, et al. Genetic diversity and chloroquine selective sweeps in Plasmodium falciparum. Nature 2002 Jul 18; 418(6895):320–3. PMID: 12124623
27. Roper C, Pearce R, Nair S, Sharp B, Nosten F, Anderson T. Intercontinental spread of pyrimethamine-resistant malaria. Science 2004 Aug 20; 305(5687):1124. PMID: 15326348
28. Hawkins VN, Auliff A, Prajapati SK, Rungsihirunrat K, Hapuarachchi HC, Maestre A, et al. Multiple origins of resistance-conferring mutations in Plasmodium vivax dihydrofolate reductase. Malar J 2008; 7:72. doi: 10.1186/1475-2875-7-72 PMID: 18442404
29. Hawkins VN, Suzuki SM, Rungsihirunrat K, Hapuarachchi HC, Maestre A, Na-Bangchang K, et al. Assessment of the origins and spread of putative resistance-conferring mutations in Plasmodium vivax dihydropteroate synthase. Am J Trop Med Hyg 2009 Aug; 81(2):348–55. PMID: 19635897
30. World Health Organization. World Malaria Report 2014—Country Profiles: http://www.who.int/malaria/publications/world_malaria_report_2014/wmr-2014-profiles.pdf?ua=1. 2014. Ref Type: Online Source
31. Schousboe ML, Ranjitkar S, Rajakaruna RS, Amaresinghe PH, Konradsen F, Morales F, et al. Global and local genetic diversity at two microsatellite loci in Plasmodium vivax parasites from Asia, Africa and South America. Malar J 2014; 13:392. doi: 10.1186/1475-2875-13-392 PMID: 25277367
32. Leslie T, Mayan MI, Hasan MA, Safi MH, Klinkenberg E, Whitty CJ, et al. Sulfadoxine-pyrimethamine, chlorproguanil-dapsone, or chloroquine for the treatment of Plasmodium vivax malaria in Afghanistan and Pakistan: a randomized controlled trial. JAMA 2007 May 23; 297(20):2201–9. PMID:17519409

33. Leslie T, Mayan I, Mohammed N, Erasmus P, Kolaczinski J, Whitty CJ, et al. A randomised trial of an eight-week, once weekly primaquine regimen to prevent relapse of plasmodium vivax in Northwest Frontier Province, Pakistan. PLoS ONE 2008; 3(8):e2861. doi:10.1371/journal.pone.0002861 PMID:18682739

34. Schousboe ML, Rajakaruna RS, Salanti A, Hapuarachchi HA, Galappaththy GN, Bygbjerg IC, et al. Island-wide differences in single nucleotide polymorphisms of the Plasmodium vivax dihydrofolate reductase (Pvdhfr) and dihydropteroate synthetase (Pvdhps) genes in Sri Lanka. Malar J 2007; 6(28).

35. Ranjitkar S, Schousboe ML, Thomsen TT, Adhikari M, Kapel CM, Bygbjerg IC, et al. Prevalence of molecular markers of anti-malarial drug resistance in Plasmodium vivax and Plasmodium falciparum in two districts of Nepal. Malar J 2011; 10:75. doi: 10.1186/1475-2875-10-75 PMID: 21457533

36. Gadalla NB, Adam I, Elzaki SE, Bashir S, Mukhtar M, Ogulke M, et al. Increased pfmdr1 copy number and sequence polymorphisms in Plasmodium falciparum isolates from Sudanese malaria patients treated with artemether-lumefantrine. Antimicrob Agents Chemother 2011 Nov; 55(11):5408–11. doi: 10.1128/AAC.05102-11 PMID: 21896916

37. Benson G. Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Res 1999 Jan 15; 27(2):573–80. PMID: 9862982

38. Excoffier L, Laval G, Schneider S. Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evol Bioinform Online 2005; 1:47–50.

39. Sa JM, Nomura T, Neves J, Baird JK, Wellemes TE, del Portillo HA. Plasmodium vivax: allele variants of the mdr1 gene do not associate with chloroquine resistance among isolates from Brazil, Papua, and monkey-adapted strains. Exp Parasitol 2005 Apr; 109(4):256–9. PMID: 15755424

40. Taylor JE, Pacheco MA, Bacon DJ, Beg MA, Machado RL, Fairhurst RM, et al. The evolutionary history of Plasmodium vivax as inferred from mitochondrial genomes: parasite genetic diversity in the Americas. Mol Biol Evol 2013 Sep; 30(9):2050–64. doi: 10.1093/molbev/mst104 PMID: 23733143

41. Chen N, Wilson DW, Pasay C, Bell D, Martin LB, Kyle D, et al. Origin and dissemination of chloroquine-resistant Plasmodium falciparum with mutant pfcrt alleles in the Philippines. Antimicrob Agents Chemother 2005 May; 49(5):2102–5. PMID: 15855538

42. Cortese JF, Caraballo A, Contreras CE, Plowe CV. Origin and dissemination of Plasmodium falciparum drug-resistance mutations in South America. J Infect Dis 2002 Oct 1; 186(7):999–1006. PMID: 12232841

43. Pearce R, Malisa A, Kachur SP, Barnes K, Sharp B, Roper C. Reduced variation around drug-resistant dhfr alleles in African Plasmodium falciparum. Mol Biol Evol 2005 Sep; 22(9):1834–44. PMID: 15917494

44. Roper C, Pearce R, Bredenkamp B, Gumedze J, Drakeley C, Mosha F, et al. Antifolate antimalarial resistance in southeast Africa: a population-based analysis. Lancet 2003 Apr 5; 361(9364):1174–81. PMID: 12686039

45. Neafsey DE, Galinsky K, Jiang RH, Young L, Sykes SM, Saif S, et al. The malaria parasite Plasmodium vivax exhibits greater genetic diversity than Plasmodium falciparum. Nat Genet 2012 Sep; 44(9):1046–50. doi: 10.1038/ng.2373 PMID: 22863733