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Single nucleotide polymorphisms in *Plasmodium falciparum* V type H^+ pyrophosphatase gene (*pfvp2*) and their associations with *pfcrt* and *pfmdr1* polymorphisms

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ABSTRACT

Background: Chloroquine resistance in *Plasmodium falciparum* malaria has been associated with *pfcrt* 76T (chloroquine resistance transporter gene) and *pfmdr1* 86Y (multidrug resistance gene 1) alleles. *Pfcrt* 76T enables transport of protonated chloroquine out of the parasites digestive vacuole resulting in a loss of hydrogen ions (H^+). V type H^+ pyrophosphatase (PfVP2) is thought to pump H^+ into the digestive vacuole. This study aimed to describe the geographic distribution of single nucleotide polymorphisms in *pfvp2* and their possible associations with *pfcrt* and *pfmdr1* polymorphisms.

Methods: Blood samples from 384 patients collected (1981–2009) in Honduras (n = 35), Colombia ($n = 50$), Liberia ($n = 50$), Guinea Bissau ($n = 50$), Tanzania ($n = 50$), Iran ($n = 50$), Thailand ($n = 49$) and Vanuatu ($n = 50$) were analysed. The *pfcrt* 72–76 haplotype, *pfmdr1* copy numbers, *pfmdr1* N86Y and *pfvp2* V405I, K582R and P711S alleles were identified using PCR based methods.

Results: *Pfvp2* was amplified in 344 samples. The *pfvp2* allele proportions were V405 (97%), 405I (3%), K582 (99%), 582R (1%), P711 (97%) and 711S (3%). The number of patients with any of *pfvp2* 405I, 582R and/or 711S were as follows: Honduras (2/30), Colombia (0/46), Liberia (7/48), Guinea-Bissau (4/50), Tanzania (3/48), Iran (3/50), Thailand (1/49) and Vanuatu (0/31). The alleles were most common in Liberia ($P = 0.01$) and Liberia + Guinea-Bissau ($P = 0.01$). The VKP haplotype was found in 189/194 (97%) and 131/145 (90%) samples harbouring *pfcrt* 76T and *pfcrt* K76 respectively ($P = 0.007$).

Conclusions: The VKP haplotype was dominant. Most *pfvp2* 405I, 582R and 711S SNPs were seen where CQ resistance was not highly prevalent at the time of blood sampling possibly due to greater genetic variation prior to the bottle neck event of spreading CQ resistance. The association between the *pfvp2* VKP haplotype and *pfcrt* 76T, which may indicate that *pfvp2* is involved in CQ resistance, should therefore be interpreted with caution.

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1. Introduction

Plasmodium falciparum has persisted as a major cause of human suffering and death despite the deployment of antimalarial drugs. A contributing factor has been the development of resistance to antimalarial drugs such as chloroquine (CQ). For example, CQ resistance was associated with 2–6-fold increase in malaria attributed mortality (Trape, 2001). Thus, reports of developing tolerance to currently recommended artemisinin based combination therapies are of major concern (Dondorp et al., 2009; Noedl et al., 2008) and an understanding of the mechanisms of drug resistance in the malaria parasite is crucial.

Resistance to CQ appears to have developed independently in Colombia, Venezuela, Thai–Cambodian border, Papua New Guinea and the Philippines (Mita et al., 2009; Wootton et al., 2002). The *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene appears to be the main determinant of CQ resistance. Specific haplotypes at positions 72–76 are linked to the regional evolution of CQ resistance (Awasthi and Das, 2013; Mita et al., 2009; Wootton et al., 2002) and the 76T allele is essential for resistance (Djimde et al., 2001; Plowe, 2003). Resistance has also been linked to the N86Y allele of the multidrug resistance gene 1 (*pfmdr1*) (Babiker et al., 2001). Resistance to CQ is associated with a loss of inherent fitness (Ord et al., 2007). It is therefore probable that compensatory mutations have evolved in *P. falciparum* over time as have been shown to occur in drug resistant bacteria (Jiang et al., 2008; Levin et al., 2000).

Pfcrt is located in the membrane of the digestive vacuole (DV) (Valderramos and Fidock, 2006) and transports protonated CQ down its electrochemical gradient out of the DV (Martin et al., 2009; Sanchez et al., 2007). This result in a loss of Hydrogen ions (H^+) that must be replaced if the pH is to be maintained. Thus, the transport of H^+ into the DV most probably increases when CQ is being removed. In line with this, a 10-fold increased transcription of a putative H^+ pump located in the DV membrane has been observed in *P. falciparum* exposed to CQ and a 2-fold increase when exposed to lumefantrine (Jiang et al., 2008; Mwai et al., 2012). The putative pump is a V type H^+ pyrophosphatase (PfVP2), which constitutes a novel class of H^+ pump found in plants and some protozoa (Luo et al., 1999; McIntosh et al., 2001; McIntosh and Vaidya, 2002; Saliba et al., 2003). The aim of this study was to explore the role of the *pfvp2* gene in antimalarial drug resistance by analysing single nucleotide polymorphisms (SNPs) in *pfvp2* and their prevalence in eight different countries and possible association with polymorphisms in *pfcrt* and *pfmdr1*.

2. Materials and methods

2.1. Biological material

Blood samples were collected from children and adults with symptomatic or asymptomatic *P. falciparum* mono infections, verified by microscopy, as part of clinical studies or community based cross sectional surveys. Details of these studies are reported elsewhere (Bjorkman et al., 1986; Jovel et al., 2011; Kofoed et al., 2007; Malmberg et al., 2013; Ursing et al., 2006; Veiga et al., 2011) and submitted for publication (Colombia study). Samples were chosen from available regions representing several origins of CQ resistance and the situation prior to the arrival of CQ resistance. The studies were conducted in the following countries; Honduras (2004–2009), Colombia (1999–2001), Liberia (1978–1981), Tanzania (2008), Guinea Bissau (2001–2004), Iran (2001–2002), Thailand (2002–2008) and Vanuatu (2002). During the collection of the samples the official drug policy for each country was as follow; Honduras CQ + primaquine (PQ), Colombia sulphadoxine–pyrimethamine (SP) + amodiaquine, Liberia CQ, Guinea-Bissau CQ, Tanzania artemether + lumefantrine, Iran CQ + PQ, Thailand artesunate + mefloquine and Vanuatu CQ + SP. Fifty samples were randomly selected from each country except Honduras ($N = 35$) and Thailand ($N = 49$) where all available samples were analysed. Q5

2.2. Ethics

All clinical studies had regional ethical approval as follows: Ethical Review Committee of Cardio Pulmonary National Institute in Tegucigalpa, Honduras (Jovel et al., 2011), Liberian Institute of Biomedical Research (Bjorkman et al., 1986), Ministério da Saúde Pública in Guinea-Bissau No. 019/DHE/2004 (Kofoed et al., 2007), National Institute for Medical Research Tanzania No. NIMR/HQ/R.8A/Vol. IX/344 (Malmberg et al., 2013), Institute Pasteur, No. 502 in Iran (Ursing et al., 2006), and Ethical Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (Veiga et al., 2011), Ethical Review Committee of the Centro Internacional de Entrenamiento en Investigaciones Médicas (CIDEIM), Cali, Colombia. Studies in Vanuatu were approved by the Ethical Committee in Tokyo Women's Medical University, Tokyo, Japan. Molecular analyses were approved by the Stockholm regional ethical review board (reference number 2013/836-3).

2.3. Sample storage, DNA extraction

DNA from samples collected in Honduras, Guinea-Bissau, Tanzania, Iran, Vanuatu countries was extracted from the filter papers. DNA from samples collected in Liberia was extracted from frozen whole blood. DNA from samples collected in Thailand and Colombia were extracted from culture adapted parasites. DNA extraction was done using an ABI Prism® 6100 Nucleic Acid Prep Station (Applied Biosystems, Fresno, CA) and QIAamp DNA mini kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions with minor modifications (Dahlstrom et al., 2008; Sakihama et al., 2001). Extracted DNA was stored at 20 °C.

2.4. Pfvp2 molecular analysis

Pfvp2 SNP's were identified by searching the PlasmoDB version 7.0 in October 2009 (Accession No: PF3D7_1235200) (Aurrecoechea et al., 2009). Laboratory strains from Honduras (HB3), El Salvador (Santa Lucia), Brazil (7G8), Ghana (RO-33 and GHANA_1), Sierra Leone (D6), Senegal (Senegal_3404), Africa (3D7), Indochina/Laos (Dd2), Thailand (K1), Vietnam (V1_S), China (FCC-2), Papua New Guinea (D10), were aligned and SNPs identified. SNPs 405I, 582R and 711S were found in 7G8, Senegal_3404 and 3D7, respectively. All other strains had alleles V405, K582 and P711. A first set of primers were used to amplify nucleotide 1112 to 2260 to include all 3 SNPs. Three primer pairs were then used to amplify fragments that included codons 405 (nt. 1182–1291), 582 (1484–1929) and 711(2094–2297). Primers were designed using Primer Express software (Applied Biosystems, Fresno, CA, USA) based on published sequence of *P. falciparum* (PlasmoDB Accession No. PF3D7_1235200). Primers and PCR thermocycler conditions are shown in Table 1. A 20 μL reaction volume for the first reaction contained 0.4 mM dNTPs, 2.5 mM $MgCl_2$, 1.4 U of GoTaq® DNA polymerase, 0.8 μM of the first set of primers and 3 μL DNA template. Nest PCR was performed with a final volume of 25 μL containing 0.5 mM dNTPs, 2 mM $MgCl_2$, 1 U of GoTaq® DNA polymerase, 0.5 μM of nest primers and 2 μL of 1st amplification product. PCR-RFLP (restriction fragment length polymorphism) method was used to identify the SNPs 405 and 711 using restriction enzymes (New England Biolabs) AseI and DpnI respectively. Enzyme AseI and DpnI cleaved codons 405I (Ile) and 711S (Ser), respectively. Cleaved products sizes were 78 and 32 bp for AseI

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Table 1
Primers and thermocyler conditions for amplification of *pfvp2* SNPs.

Primer		Sequence 5′–3′	Size (bp)	PCR
1st amplification	VP2 1F	TGT TGC TGT ACG TGC TAA TGT AAA AGT	1148	94 °C, 3′ followed by 45 cycles (94 °C, 30″; 55 °C, 30″; 72 °C, 1′20″); 72 °C, 7′
	VP2 1R	TGT GAT CTC CTG TTA TAT TAC TCT TTA ATC CT		
Nest for SNP 405	VP2 405F	TGC TTT AGA AGC GGT GCT GTT A	110	94 °C, 3′ followed by 45 cycles (94 °C, 30″; 55 °C, 30″; 72 °C, 30″); 72 °C, 7′
	VP2 405R	GAA AAG GCT AAA GTT GGA TAT AGG ATA TTA A		
Nest for SNP 582	VP2 582F	TGG AGA TTG TGC AGG ACA ATG T	465	94 °C, 3′ followed by 45 cycles (94 °C, 30″; 50 °C, 30″; 72 °C, 45″); 72 °C, 7′
	VP2 582R	CCC ACA ACT CCA AGT GAG CA		
Nest for SNP 711	VP2 711F	AAA AGT TAA AAA AAT AGC TCA TGC TTC TT	104	94 °C, 3′ followed by 45 cycles (94 °C, 30″; 55 °C, 30″; 72 °C, 30″); 72 °C, 7′
	VP2 711R	TTA CAA TGA CTG GGA AAA AAG TAG ATT C		

(405I) and 67 and 37 bp for DpnI (711S). SNPs at codon 582 were identified by PCR amplification followed by sequencing. The sequencing primer VP2 582F (5′-GTG CTG AAA TTA TTG CA-3′) was used to sequence 582 codon. The sequenced fragment was 465 base pairs representing 15% of the *pfvp2* gene (3174 base pairs).

2.5. Pfcrt and pfmdr1 molecular analysis

A previously described multiplex PCR-RFLP method was used to identify *pfcrt* K76T and *pfmdr1* N86Y alleles (Veiga et al., 2006). *Pfcrt* 72–76 haplotypes were identified by PCR amplification followed by sequencing (Echeverry et al., 2007). *Pfmdr1* copy numbers were determined using real time PCR (ABI Prism® 7000 Sequence Detection System) as previously described (Price et al., 2004). Real time PCR reactions were run in triplicate for each sample. Laboratory strains 3D7, D10 and K1 with single copies of the *pfmdr1* gene were used as calibrators and FCB and Dd2 laboratory strains with multiple copies of the gene were used as controls. The sample copy numbers were calculated using a comparative threshold method ($\Delta\Delta C_t$). Assays were repeated if the following results were obtained: copy number 1.3–1.6 and 2.3–2.6 or Ct value > 35 or standard deviation value > 0.5.

PCR and restriction products were resolved on 2% agarose gels (Amresco, Solon, OH). All gels were stained with ethidium bromide and visualised under UV transillumination (GelDoc®, Biorad, Hercules, CA, USA).

2.6. Sequencing

PCR products were purified and sequenced commercially (Macrogen Inc. Seoul, Korea). The Sequencher™ software version 4.6 (Gene Codes Corporation, Ann arbor, MI) was used for sequencing analysis. The *pfvp2* and *pfcrt* reference sequences were taken from *P. falciparum* 3D7 clone obtained from PlasmoDB version 7.0 (Accession No: PF3D7_1235200) and NCBI database (Gen-Bank Accession No. NC_004328), respectively.

2.7. Statistics

Data were entered and analysed using Microsoft Excel 2003. Allele proportions were calculated by dividing the number of samples with a certain allele by the number of samples with an identifiable allele at that position. Thus mixed infections contributed to the proportion of both alleles. When the association between *pfvp2* alleles and alleles in *pfcrt* and *pfmdr1* were assessed patient samples with mixed *pfcrt* K76T and/or *pfmdr1* N86Y alleles were excluded. When the association between the number of patient samples with SNPs in *pfvp2* and *pfcrt* K76T and *pfmdr1* N86Y were assessed (Table 3 and Table S1) only patient samples in which all alleles had been successfully identified were used. Associations were determined using Fishers Exact test using StataCorp 12. Linkage disequilibrium between SNPs in *pfvp2* and *pfcrt* or *pfmdr1* were calculated. Absolute linkage was indicated by a value $D = 1$ whereas $D = 0$ indicated no linkage. A samples size of 50 was chosen due to the limited number of available samples from Honduras, Colombia, Liberia and Thailand.

3. Results

Pfvp2 was successfully amplified by PCR in 344/384 (90%) patient samples. Frequencies and geographic distribution of *pfvp2* V405I, K582R and P711S are shown in Table 2. The VKP alleles were predominant with frequencies >85% in all countries. Alleles 405I + 711S were found together in 6/344 (1.7%) patient samples. All other *pfvp2* 405I, 582R and 711S SNPs were identified in separate patient samples.

Pfcrt K76T and *pfmdr1* N86Y alleles were successfully amplified in 367/385 (95%) and 358/385 (93%) patient samples, respectively. Allele haplotypes and frequencies in each country are presented in Table 2. Mixed K76 + 76T and/or N86 + 86Y were found in 8 samples. The proportion of *P. falciparum* with *pfcrt* K76 was significantly higher in Liberia 50/50 (100%) and Honduras 30/30 (100%) compared to all other countries ($P < 0.001$). The proportion of *pfcrt* K76 was also higher in Guinea-Bissau 36/50 (72%) and Tanzania compared to Colombia, Iran, Thailand and Vanuatu ($P < 0.001$). Irrespective of whether Liberia was included (119/150, 79%) or not (69/100, 69%), the proportion of *pfcrt* K76 was significantly higher ($P < 0.001$) in African countries compared to Asia 2/99 (2%) or South America 0/50 (0%).

There was no statistically significant association between any *pfvp2* allele alone and any allele in *pfcrt* or *pfmdr1*. The haplotype *pfvp2* V405, K582 and P711 occurred more frequently with *pfcrt* 76T ($P = 0.007$). Conversely, *Pfvp2* 405I and/or 582R and/or 711S (i.e. not the VKP haplotype) occurred more frequently with *pfcrt* K76 ($P = 0.007$) as shown in Table 3. *Pfvp2* alleles are tabulated against *pfcrt* K76T and *pfmdr1* N86Y haplotypes in supplementary material (Table S1). There were no statistically significant associations.

Linkage disequilibrium analyses were of most interest from Guinea-Bissau and Tanzania as *pfcrt* 76K and 76T alleles showed greatest variability there. The *pfvp2* VKP haplotype was moderately strongly linked to *pfcrt* 76T in Guinea-Bissau ($D = 0.65$) and Tanzania ($D = 0.68$). Similarly, having *pfvp2* 405I and/or 582R and/or 711S (i.e. not the VKP haplotype) was moderately strongly linked to *pfcrt* 76K in Guinea-Bissau ($D = 0.72$) and Tanzania ($D = 0.65$). When data for all countries was pooled, *pfvp2* 405I and/or 582R and/or 711S (i.e. not the VKP haplotype) were linked with *pfcrt* 76K ($D = 0.5$) but not with *pfcrt* 76T ($D = 0$). There was similar and moderate linkage between the *pfvp2* VKP haplotype and *pfcrt* 76T ($D = 0.5$) and *pfcrt* 76K ($D = 0.4$). No significant linkages were found between *pfvp2* alleles and *pfmdr1* N86Y.

In the respective countries, the number of patient samples with any *pfvp2* 405I, 582R and/or 711S SNP(s) was as follows; Honduras 2/30 (7%), Colombia 0/46 (0%), Liberia 7/48 (15%), Guinea-Bissau 4/50 (8%), Tanzania 3/48 (6%), Iran 3/50 (6%), Thailand 1/49 (2%),

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Table 2
Frequencies of polymorphisms in *pfvp2*, *pfcrt* 76 and *pfmdr1*.

Country	*pfvp2*				*pfcrt*		*pfmdr1*		*pfmdr1* CN[e]	
	405I	582R	711S	VKP	K76[a]	76T	N86	86Y	1	>1
Liberia	3/48	1/48	4/49	40/47	50/50	0/50	46/47	2/47	30/30	
Guinea Bissau	3/50	1/50	3/50	46/50	36/50	16/50[b]	28/50	24/50	50/50	
Tanzania	0/49	1/50	2/48	45/48	33/50	17/50[b]	34/50	16/50	46/46	
Iran	1/50	1/50	1/50	47/50	2/50	49/50[c]	13/50	37/50	36/36	
Thailand	1/49	0/49	1/49	48/49	0/49	49/49[b]	49/49	0/49	26/49	23/49
Vanuatu	0/31	0/32	0/38	28/28	1/38	38/38[c]	0/32	32/32	9/9	
Honduras	2/30	0/30	1/30	28/30	30/30	0/30	30/30	0/30	28/28	
Colombia	0/46	0/46	0/50	44/44	0/50	50/50[d]	50/50	0/50	44/44	

[a] *Pfcrt* 72–76 haplotype was CVMNK.
[b] *Pfcrt* 72–76 haplotype was CVIET.
[c] *Pfcrt* 72–76 haplotype was SVMNT.
[d] *Pfcrt* 72–76 haplotype was CVMNT.
[e] CN: copy number.

Table 3
The frequency of *pfvp2* alleles in *P. falciparum* with varying *pfcrt* K76T and *pfmdr1* N86Y alleles.

Pfvp2	*Pfcrt*		*Pfmdr1*	
	K76	76T	N86	86Y
V405	95% (138/145)	99% (198/201)[a]	97% (235/242)	98% (99/101)
K582	98% (143/146)	99% (199/200)[b]	99% (240/243)	99% (101/102)
P711	95% (138/146)	99% (204/207)[c]	96% (236/246)	99% (103/104)
VKP haplotype	90% (131/145)	97% (191/196)[d]	93% (224/240)	97% (95/98)
405I	5% (7/145)	1% (3/201)	3% (7/242)	2% (2/101)
582R	2% (3/146)	1% (1/200)	1% (3/243)	1% (1/102)
711S	5% (8/146)	1% (3/207)	4% (10/246)	1% (1/104)
I and/or R and/or S*	10% (14/145)	97% (5/196)	7% (16/240)	3% (3/98)

Patients with both *pfcrt* K76 and 76T and patients with both *pfmdr1* N86 and 86Y were excluded.
[a] V405 occured non significantly more often with 76T $P = 0.1$.
[b] K582 occured non significantly more often with 76T $P = 0.3$.
[c] P582 occured non significantly more often with 76T $P = 0.06$.
[d] The *pfvp2* V405 + K582 + P711 haplotype was significantly more common with *pfcrt* 76T ($P = 0.007$).
* i.e. not the VKP haplotype.

and Vanuatu 0/31 (0%). The proportion of patient samples with any of *pfvp2* 405I, 582R and/or 711S was significantly more common in Liberia ($P = 0.01$), African countries (Liberia + Guinea-Bissau + Tanzania, $P = 0.004$), and countries where CQ resistance had not been described at the time of blood sampling (Liberia + Honduras, $P = 0.001$) compared to the other countries studied.

Frequencies of *pfmdr1* with multiples copies are shown in Table 2. None of the samples with amplification was found to have any of *pfvp2* 405I, 582R and/or 711S SNPs.

4. Discussion

Studies have previously indicated that *PfVP2* may be involved in resistance to CQ and lumefantrine (Jiang et al., 2008; Mwai et al., 2012). We therefore assessed the proportion of SNPs in *pfvp2* and their association to polymorphism in *pfcrt* and *pfmdr1*. This is, to our knowledge, the first such report. The most striking result was the lack of variation of the *pfvp2* alleles studied. Only 26 SNPs in 20 samples were found among 344 samples (including sequencing of approximately 15% of the gene) collected in 8 countries with varying origins and proportions of CQ resistant *P. falciparum* at the time of blood sampling. The results thus suggest that the parts of the *pfvp2* gene that were analysed are conserved.

Despite the lack of variation, the *pfvp2* V405, K582 and P711 haplotype was found to be associated with and linked to *pfcrt* 76T. As *pfcrt* 76T is essential for CQ resistance these results suggest that the *pfvp2* V405, K582 and P711 haplotype might be associated with the development of CQ resistance. Previously, *pfvp2* up-regulation was shown to occur in *P. falciparum* under CQ pressure (Jiang et al., 2008). This was proposed to be due to increased H^+ transport into the parasite DV to compensate for H^+ loss when CQ was transported out (Martin et al., 2009; Sanchez et al., 2007). Assuming that *pfvp2* functions as suggested by Jiang et al., the results of this study indicate the *pfvp2* V405, K582 and P711 haplotype provides a more efficient H^+ pump than the IRS haplotype in *P. falciparum* with the *pfcrt* 76T genotype. However, given the high frequency of VKP in Liberia and Honduras where *pfcrt* K76 prevalences were 100% and CQ resistance had not been described (when blood samples were collected), the association between the VKP haplotype and *pfcrt* 76T should not be over emphasised.

There was also an association and linkage between *pfvp2* 405I, 582R and/or 711S and *pfcrt* K76. These alleles were significantly more common in Liberia in patient samples collected before CQ resistance reached the country (Bjorkman et al., 1985). Though not significant, the only *pfvp2* 405I, 582R and/or 711S alleles found in the Americas were detected in Honduras from where indigenous CQ resistant *P. falciparum* have to date not been reported (Jovel et al., 2011). The presence of these alleles in CQ sensitive settings in both Africa and the Americas suggests that there was a larger variation in the *pfvp2* gene prior to the spread of CQ resistance, a bottleneck event for *P. falciparum* that reduced its genetic diversity (Wootton et al., 2002).

Fourteen of 20 patient samples with *pfvp2* 405I, 582R and/or 711S came from African countries of which, 11/20 came from West Africa. This might suggest that the association between *pfvp2* and *pfcrt* is incidental possibly due to geographical variation. However the *pfvp2* SNPs were also linked to *pfcrt* 76K in Tanzania. An alternative explanation for the relatively common occurrence in Africa

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is that CQ resistance had not reached Liberia at the time of sampling and the proportion of CQ resistant *P. falciparum* had remained relatively low ~25% in Guinea-Bissau (Ursing et al., 2009). There had thus been less selective pressure on *pfvp2* in these two countries and *P. falciparum* had not passed through the parasite population bottle neck of CQ resistance spreading.

Sequencing of the *pfcrt* 72–76 haplotype identified the expected region specific haplotypes. There was no association between CQ resistance associated *pfcrt* 72–76 haplotypes CVIET or SVMNT and the *pfvp2* alleles suggesting that the association between *pfvp2* alleles and *pfcrt* 76T was independent of the origin of CQ resistant *P. falciparum*. However, we did not have access to samples representing all origins of CQ resistance.

To conclude, the *pfvp2* V405, K582 and P711 alleles were predominant throughout the eight countries studied. An association between the *pfvp2* 405 V, 582 K and 711P haplotype and *pfcrt* 76T was detected. These observations are in line with previous data indicating that PfVP2 may have a role in CQ resistance (Jiang et al., 2008). However, *pfvp2* SNPs were only found in 20/385 patient samples. The correlations found should therefore be interpreted with caution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.20 14.0 3.004.

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