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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.

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

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%),

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

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.2014.03.004.

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When they don't bite, we smell money: understanding malaria bednet misuse

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SUMMARY

Insecticide-treated nets (ITNs) are a major tool to control malaria. Over recent years increased ITN coverage has been associated with decreased malaria transmission. However, ITN 'misuse' has been increasingly reported and whether this emergent behaviour poses a threat to successful malaria control and elimination is an open question. Here, we use a game theory mathematical model to understand the possible roles of poverty and malaria infection protection by individual and emerging 'community effects' on the 'misuse' of malaria bednets. We compare model predictions with data from our studies in Lake Victoria Islands (LVI), Kenya and Aneityum, Vanuatu. Our model shows that alternative ITN use is likely to emerge in impoverished populations and could be exacerbated if ITNs become ineffective or when large 'community effects' emerge. Our model predicted patterns of ITN use similar to the observed in LVI, where 'misuse' is common and the high ITN use in Aneityum, more than 20 years after malaria elimination in 1990. We think that observed differences in ITN use may be shaped by different degrees of economic and social development, and educational components of the Aneityum elimination, where traditional cooperative attitudes were strengthened with the malaria elimination intervention and post-elimination surveillance.

Key words: *Plasmodium*, poverty, Pareto equilibrium, Nash equilibrium, insecticide treated nets, Kenya, Vanuatu.

INTRODUCTION

The Roll Back Malaria initiative (RBM) was launched in 1998 to tackle malaria, a disease with 3·2 billion people at risk of infection worldwide (World Health Organization, 2000). In 2000, African countries committed to providing proper treatment and insecticide-treated nets (ITNs, which are primarily bednets) to at least 60% of the highest malaria risk population by 2005, a goal raised to 80% by 2010 (RBM-Partnership, 2005). The mass ITN distribution campaign significantly reduced malaria-related morbidity and mortality (Lindblade *et al.* 2004; Fegan *et al.* 2007; O'Meara *et al.* 2008), and further scaling up ITN coverage is ongoing. However, some studies (Minakawa *et al.* 2008; Lover *et al.* 2011; O'Meara *et al.* 2011; Pulford *et al.* 2011) have reported ITN misuse as a potential explanation for partial success to increase net coverage, or the misuse of means, for example subsidized vouchers, to obtain the ITNs (Tami *et al.* 2006). For instance, newly distributed LLINs are often patched together to make a large seine net (Fig. 1A), as old ones with holes are not effective for drying (Fig. 1B) and capturing fish (Fig. 1C) in fishing villages because of the net strength (Minakawa *et al.* 2008). Protecting plant crops (Fig. 1D) or granaries (Fig. 1E) with ITNs are becoming increasingly popular. Residents are now aware of the insecticidal and repellant effects of ITNs on crop pests. It is unclear how widely ITNs are used for other purposes, besides the plain misuse, e.g. as a sleeping mat (Fig. 1F), and a question remains as to whether this phenomenon hampers the ongoing efforts to reduce malaria transmission (Eisele *et al.* 2011).

Community effects may reduce transmission risk for people employing ITNs for purposes other than mosquito bite protection. For example, if some residents sleep under ITNs, in a proportion large enough to significantly decrease mosquito abundance (Howard *et al.* 2000; Hawley *et al.* 2003), that fraction of ITNs used for malaria prevention can shield all individuals in a community independently of the use given by an individual to his/her personal/household ITN(s). The income generated by alternative ITN use may further reduce the risk of malaria

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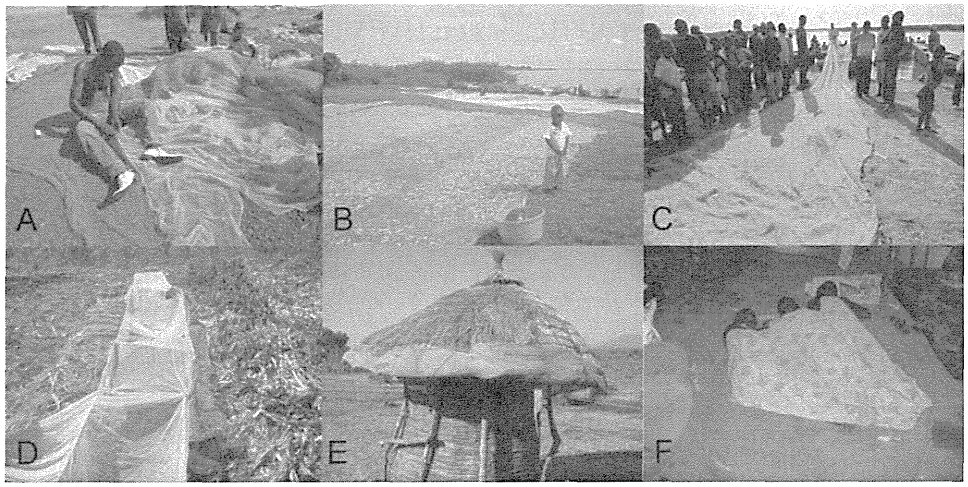

Fig. 1. Examples of alternative ITN uses. (A) Sewing bednets to create larger nets. (B) Drying fish. (C) Fishing. (D) Crop protection. (E) Granary protection. (F) Sleeping mat.

transmission or morbidity and mortality if the income is used for malaria and other infectious disease treatment, housing improvement and socio-economic mobility, all factors associated with the reduction of malaria risk (Ijumba and Lindsay, 2001; Lindsay and Birley, 2004; Chaves and Koenraadt, 2010). Game theory, a conceptual framework widely used to understand behaviour in economics (Nash, 1950; Karlin, 1959) and ecological and evolutionary contexts (Smith and Price, 1973) offers modelling tools to understand the emergence of alternative ITN use by rendering an optimization between the benefits and risks of different ITN use. Game models allow the optimization of a player strategy reward through the derivation of Nash equilibria (Nash, 1950) and also the optimization of the public welfare by combining the strategies of all players in Pareto equilibria (Karlin, 1959). Here, we introduce a two-player game to understand the emergence of alternative ITN use. In the game, each of the two players uses or misuses its ITN for malaria prevention to optimize its own payoff, which we measure as an economic reward. In the model we assume that proper ITN use decreases malaria infection probability, while alternative ITN use increases labour productivity (e.g. income in US $ *per capita*). We derive the Nash and Pareto equilibria to evaluate the individual and social impact of a player strategy. From the distribution of Pareto efficient Nash equilibria in the ITN use game, we found that alternative ITN use can optimize each player reward and public welfare simultaneously which we further illustrate with numerical solutions to our model and field data from Kenya and Vanuatu.

THE MODEL

In the next lines we define a 2-player (which can be also understood as a two strategy) ITN use game. First we define an expected payoff matrix for using/misusing an ITN, i.e. a mathematical set of formulae that are analysed to optimize the individual (Nash equilibrium) and collective (Pareto equilibrium) rewards. We then use the model to explore the economic rationality behind alternative ITN use. Through the model presentation we will use the term player to refer to the residents' use strategy in a malaria-endemic area with freely available ITNs.

Data

To test model predictions we used data on parasite rates (PR) and ITN use for malaria protection (ITNMP) from Aneityum, Vanuatu and Lake Victoria Islands (LVI), Kenya. For the analysis we used (PR) based on blood slide examination, which were about 1/3 of the estimates with a rapid diagnostic test (RDT). Per cent ITNMP was based on ITN self-reported usage by residents, corrected by the percentage of the population covered with ITNs (see Table 1 for further details about coverage, use and PRs using BSE and RDT).

Ethical approval

This study was approved by the Vanuatu Department of Health, the Scientific Steering Committee and National Ethics Review Committee of the Kenya Medical Research Institute (SSC No. 1310 and 2131), and the ethics review committee of Nagasaki University.

Expected payoff matrix

The expected payoff matrix represents the set of strategies and rewards (commonly referred as payoffs in the game theory literature) that players can employ regarding a behaviour in a game model. In the ITN game, the 2 players have a common set of strategies denoted by T and F, which correspond to ITN malaria protection use and misuse, respectively. Each

Table 1. Insecticide-treated net (ITN) self-reported use, coverage and malaria parasite rates in Aneytium, Vanuatu and islands (Nghode, Takawiri, Kibougi, Mfangano) in Lake Victoria, Kenya

Location	Year	Month	Population surveyed*	Parasite rate (per 100 individuals)[a]	ITN coverage[b]	ITN use[c]
Aneityum[19]	1991	January[d]	446	23 (NA)	0	na
Aneityum[19]	1991	October/November[e]	773	0·004 (NA)	94	90
Aneityum	2010	July	1123	0 (0)	100	97
Nghode	2012	February	331	5 (17)	81	59
Takawiri	2012	February	601	4 (15)	79	65
Kibougi	2012	February	130	9 (25)	73	71
Mfangano	2012	February	890	23 (49)	78	50

* All surveys sampled representatively the demographic profile of the islands.
[a] The value outside the parentheses is the estimate based on blood slide examination, the value inside the parentheses is the estimate based on a Rapid Diagnostic test (Paracheck-Pf®) and na indicates not available.
[b] % population owning a bednet.
[c] % population using bednets independent of coverage.
[d] Pre-elimination baseline survey.
[e] Post-elimination baseline survey.

player chooses a strategy (T or F) based on his/her malaria infection risk, labour productivity, and expected payoff. Thus, the ITN game can have 4 profiles, which result from the combination of the strategies by the 2 players: (T, T), (T, F), (F, T) and (F, F).

The relation between the ITN game profiles and the malaria infection risk can be represented by an infection probability (IP) matrix (Fig. 2A). In this matrix we define an IP P, which can take any value between 0 and 1 (i.e. $1 \geqslant P \geqslant 0$). P can be interpreted as the probability of malaria infection by an individual in the setting where she/he resides. To make the connection with epidemiological literature, P could be seen as a function of malaria infection risk factors, i.e. the higher the odds of an individual being infected, the higher the value of P. The use of an ITN by a player is assumed to reduce the individual probability of infection by α_1, as observed in numerous studies (Howard *et al.* 2000; Hawley *et al.* 2003; Lindblade *et al.* 2004; Fegan *et al.* 2007), and the use of ITNs by other residents in the community can lead to an emergent 'community effect' (Howard *et al.* 2000; Kaneko *et al.* 2000; Hawley *et al.* 2003; Fegan *et al.* 2007; Chaves *et al.* 2008) that further reduces P by a factor $(\alpha_2)^n$, where n is the number of players that use the ITN for malaria prevention. Thus, the 'community effect' is null when no players use bednets and the magnitude of its impact increases as more individuals use the ITNs for malaria prevention. The α parameters can take any value above 0 and below 1 (i.e. $0 < \alpha_i < 1$, $i = 1, 2$). We can then define a labour productivity matrix (Fig. 2B) that quantifies the utility of labour in malaria uninfected players, L, and the increased utility β by using bednets for alternative purposes to malaria prevention. Finally, with these two matrices we can define the expected payoff matrix (Fig. 2C) as the

(A)

Player 1 \ Player 2	T	F
T	$\alpha_1\alpha_2^2P, \alpha_1\alpha_2^2P$	$\alpha_1\alpha_2P, \alpha_2P$
F	$\alpha_2P, \alpha_1\alpha_2P$	P, P

(B)

Player 1 \ Player 2	T	F
T	L, L	$L, \beta L$
F	$\beta L, L$	$\beta L, \beta L$

(C)

Player 1 \ Player 2	T	F
T	$(1-\alpha_1\alpha_2^2P)L$, $(1-\alpha_1\alpha_2^2P)L$	$(1-\alpha_1\alpha_2P)L$, $(1-\alpha_2P)\beta L$
F	$(1-\alpha_2P)\beta L$, $(1-\alpha_1\alpha_2P)L$	$(1-P)\beta L$, $(1-P)\beta L$

$(1 - A)_{\circ}(B) = C$

Fig. 2. Deriving an expected payoff matrix for the ITN use game. (A) Infection probability (IP) matrix. P is the malaria infection probability of the players in the absence of ITNs. The parameters α_1 and α_2 denote the individual and community effects of ITN use for malaria protection, respectively. To read this and the subsequent matrices the strategy of player 1 is presented in the rows, and of player 2 in the columns. The matrix value for player 1 is the first entry in a given cell. (B) Labour productivity matrix. L is the labour productivity (which can be measured in US $ per capita) of the players without an ITN, L can take any positive value (i.e. $L > 0$). The parameter β denotes the β-fold increment of L when a player gives an alternative use to his/her ITN, β is assumed to be larger than 1 (i.e. $\beta > 1$). (C) Expected payoff matrix. This matrix is the Hadamard product (i.e. matrix-element-wise product) of the complement of the IP matrix (i.e. 1 – IP matrix) and the Labour productivity matrix for each player.

product of the probability of not being infected with malaria (1-probability of malaria infection) and the labour utility. The model assumes a perfect knowledge of the costs and benefits for different ITN uses. The 2-player model has the advantage of rendering general results independently of whether the impacts of ITN use for malaria protection are density or frequency dependent, since results for small n are independent of density/frequency-dependent pathogen transmission (Antonovics *et al.* 1995) and, in general, 2-player games are useful tools to understand the emergence of different behaviours in a population (Smith and Price, 1973).

Distribution of Nash equilibria

Nash equilibria are the profiles (combination of player strategies) from which any player has no incentive to deviate, because his/her payoff is maximized in response to the other player strategy (Nash, 1950; Smith and Price, 1973). Our model has 3 Nash equilibria that were derived by establishing the conditions when given a profile, none of the players can increase his/her payoff by changing his/her strategy. For example, in the case of the profile (T, T), i.e. when both players use their ITNs for malaria prevention we have that (T, T) is a Nash equilibrium when the following inequality holds:

$$(1-\alpha_1(\alpha_2)^2P)\,L > (1-\alpha_2 P)\,\beta L. \quad (1)$$

This implies a higher payoff for any player if he/she uses the ITN for malaria prevention than if he/she chooses to profit from an alternative ITN use. Here it is worth highlighting that payoffs are independent of labour productivity (L), which cancels out on both sides of Equation (1). Nevertheless, payoffs are proportional to the increase (β) of L by the alternative ITN use. From expression (1) a threshold for IP (P_R) can be derived which ensures that both players will use their ITNs for malaria prevention when $P > P_R$:

$$P_R = \frac{\beta-1}{\alpha_2(\beta-\alpha_1\alpha_2)}. \quad (2)$$

Following a similar procedure, P_L, an IP threshold where (F, F), both players giving alternative uses to their ITNs, is a Nash Equilibrium when $P < P_L$, can be derived:

$$P_L = \alpha_2 P_R. \quad (3)$$

Finally, the profiles (T, F) and (F, T) are Nash equilibria when P follows the following condition:

$$P_L \leqslant P \leqslant P_R. \quad (4)$$

Equation (4) implies the emergence of 'free rider' Nash equilibria, where a player with the strategy F benefits from the alternative use of his/her ITN and from the 'community effect' in malaria protection that emerges by the use of ITNs for malaria prevention by a player with the strategy T.

Distribution of Pareto equilibria

A Pareto equilibrium is a combination of player payoffs that is efficient for the public welfare (Karlin, 1959), in the context of this study meaning it conduces to a reduction of malaria infection risk in a community. To find the Pareto equilibria we solved several inequalities comparing the different profiles of the ITN game (see Supplementary material, online version only, for a detailed and mathematically rigorous derivation). Our analysis showed the Nash equilibria to be Pareto efficient with the exception of the equilibria for the profile (F, F), where a region with a social dilemma (SD), i.e. where the whole community benefits by a player changing his/her strategy, emerges when:

$$P_L^* = \frac{\beta-1}{\beta-\alpha_1\alpha_2^2}. \quad (5)$$

By definition, $P_L^* < P_L$, is the difference between these two thresholds defining the range of malaria infection probability over which a SD emerges, and is expected to be wider as the number of players increases in a community (Nash, 1950).

Model implications

Our model can be used to illustrate the influence of many factors that may underpin patterns of ITN misuse. Malaria is a disease entrenched among the poorest nations in the globe (Chaves and Koenraadt, 2010) and the alternative use of ITNs could represent a significant increase in a household income. Figure 3A illustrates how the range of malaria infection probability (P) where all players prefer to use ITNs for purposes other than malaria protection doubles its width when a 20% increase in the player income is derived by an alternative ITN use, a figure that may be realistic for the poorest of the poor in many developing nations. This is the case even when assuming that ITN use reduces by 40% (i.e. $\alpha_1 = 0{\cdot}60$, e.g. see Killeen *et al.* (2007)) the probability of malaria infection, a value within the range of observed outcomes for ITN trials (Lindblade *et al.* 2004). The free-rider behaviour is expected to become increasingly common as the 'community effect' increases (Fig. 3B and C), in an exacerbated manner as the protection level by individual ITN use diminishes (i.e. α_1 increasing towards 1, Fig. 3C).

ITN use and parasite rates (PR) in Vanuatu and Kenya islands, do they follow the model?

Table 1 shows data from malaria surveys made before (PRE) and after (POST) the 1991 elimination intervention, and 2010, in Aneityum (a Vanuatu island) and from 2012 in several LVI (Ngodhe, Takawiri, Kibuogi, Mfangano). In Aneityum, before

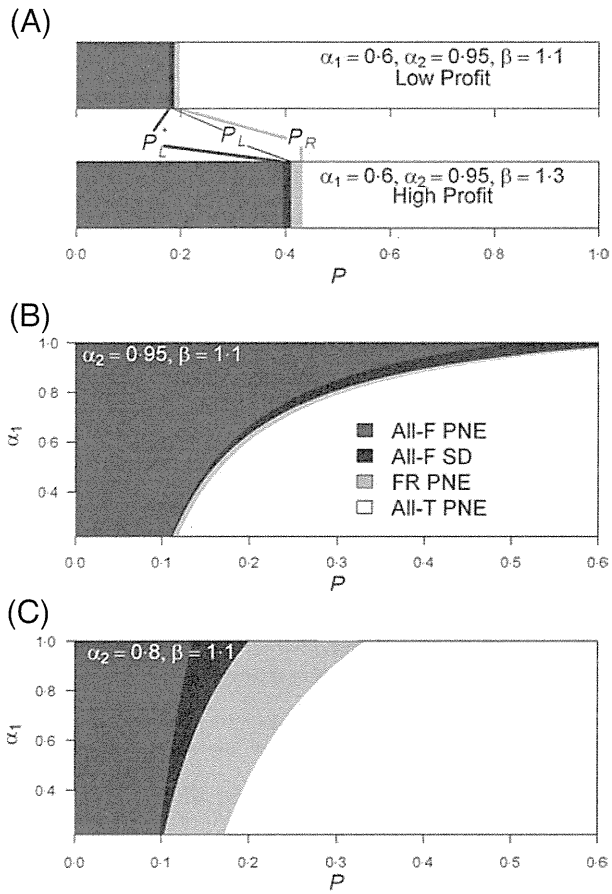

Fig. 3. Pareto efficient Nash equilibria (PNE) and Social Dilemma (SD). (A) Equilibria as function of the infection probability, P, the top panel illustrates a case where profitability for alternative ITN use is low (income increases by 10%, i.e. $\beta = 1{\cdot}1$), the bottom panel represents a case of higher profitability for the alternative bednet use (income increases by 30%, i.e. $\beta = 1{\cdot}3$). In the two panels the thresholds P_R, P_L and P_L^* are indicated (see the main text for an explanation of the thresholds). The legend in panel B applies to panels A, B and C: All-T (All-F) are the equilibria where all players (do not) use the ITN for malaria protection, FR are the free-rider equilibria. (B) Equilibria as function of P and individual bednet protection (α_1) in a setting with a low level of additional protection via a 'community effect' (5% i.e. $\alpha_2 = 0{\cdot}95$). (C) Equilibria as function of P and individual bednet protection (α_1) in a setting with a high level of additional malaria protection via a community effect (20%, i.e. $\alpha_2 = 0{\cdot}80$).

the 1991 elimination trial, PR was slightly above 20% and ITN coverage was null (Table 1). As part of the elimination trial coverage was raised to 94% and use was around 90% (Kaneko *et al.* 2000), and currently coverage is 100% with 97% use. In Vanuatu, current levels of use are in accordance with equation (2) when the parameter $\beta \to 1$, i.e. when there is no significant increase of labour productivity by an alternative ITN use and with a transmission close to 0 all players will use the ITN for malaria protection (i.e. $P_R \to 0$). Nevertheless, our model fails to explain the patterns observed in Vanuatu under the assumption of $\beta > 1$. The situation in LVI reflects a higher coverage and use of ITNs than that observed in Aneityum prior to the elimination trials (Table 1), yet misuse may be higher, since around 20% of the ITNs are not being used for malaria protection (Table 1), and only around 50% of the population use ITNs for malaria protection. ITN use patterns in LVI resemble our model predictions when $\beta > 1$.

DISCUSSION

Our model clearly indicates that ITN use for malaria protection can be thwarted in settings of extreme poverty, where an increase in labour productivity by an alternative ITN use can offset the perceived benefits of avoiding malaria infection. The model also shows that alternative ITN uses are expected to emerge as coverage and concomitant 'community effects' become more common, or if ITNs become unprotective, for example, by the emergence of insecticide-resistant mosquitoes (Kawada *et al.* 2011). We also showed that alternative ITN use is not necessarily detrimental for an endemic community, especially for low and moderate levels of malaria infection risk, since those strategies are Pareto efficient, in the context of this study meaning that alternative ITN use is not detrimental for the community as a whole. Our model also shows that as malaria risk further decreases, social dilemmas, i.e. situations where individual behaviours can improve a situation for a community as a whole, are likely to emerge, especially when the use of ITNs could be crucial to render elimination feasible (Smith *et al.* 2009), because they are not optimal from the perspective of non-cooperative individual residents. However, the only data we have available for a current low malaria risk area, formerly hyperendemic, i.e. Aneityum island in Vanuatu (Kaneko *et al.* 2000), suggest that the non-cooperative behaviour assumed in our model is not likely to interfere with ITN use for malaria protection when there is a likely small proportional increase of labour productivity by alternative ITN use. In Aneityum ITN use is very high (> 95%) well after elimination, probably because of the educational component of the elimination trial aimed at strengthening cooperative practices and promoting community participation of residents during the trial and subsequent malaria freedom (Kaneko, 2010). Also, Vanuatu has a moderate level of human development, where economic development is more sustainable, socially equitable and conducive to a higher standard of living (e.g. better education and access to services) than in most sub-Saharan African nations (UNDP, 2011), which makes unrealistic the scenario of significant increases to individual labour productivity by using an ITN for a purpose other than malaria protection. Thus, high ITN use for malaria protection could also reflect

the relative higher well-being of the Vanuatu population when compared with most nations in sub-Saharan Africa. In fact, although coverage before the 1991 elimination trial of Vanuatu (0%) was far from being as high as it is currently in LVI (> 70%), current ITN use is well over (> 97%) what we can observe in LVI (> 50%), which indicates a proportionally larger ITN misuse in LVI than that which we have observed in Vanuatu over the years. Nevertheless, issues of perceived mosquito annoyance as triggers of ITN use, a topic not explored by our current model, need to be further explored.

Regarding the robustness of our results (Levins, 1968, 2006), i.e. whether our inferences remain the same under different or more elaborated assumptions, we can affirm that our major result, that ITN alternative use is a rational behaviour in impoverished settings, holds when the game is explicitly extended to n players. However, some quantitative differences can be expected in thresholds for social dilemmas and other Pareto equilibria that become a direct function of the n players. Nonetheless, effects of n on ITN use are beyond our research goals in this contribution and will be presented elsewhere.

Finally, results from our model, in addition to common observations on ITN use, where 'misuse' is commonly related to alternative uses aimed at increasing labour productivity (Minakawa *et al.* 2008; Lover *et al.* 2011; Pulford *et al.* 2011), make us believe that malaria elimination efforts will be more likely to achieve success if interventions are embedded within a larger effort aimed at improving the well-being of endemic populations (Chaves and Koenraadt, 2010), since they can improve the adherence to interventions and have indirect effects, such as better access to improved healthcare (Ijumba and Lindsay, 2001), housing and others (Chaves and Koenraadt, 2010), that can further increase the odds of successful malaria control or elimination.

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When they don't bite, we smell money: understanding malaria bednet misuse

Supplementary Online Material

This supplementary document provides detailed theoretical analysis of a two-player ITN (insecticide-treated net) use game. In Sections 2 and 3, the Nash equilibrium and the Pareto equilibrium are defined, and the distributions of the two equilibria are shown. In Section 4, the distribution of Pareto efficient Nash equilibria is shown.

1 Distribution of Nash equilibria

In the ITN use game, the two players $i \in \{1, 2\}$ have the common set of pure strategies $\Sigma := \{\mathrm{T}, \mathrm{F}\}$, and each player chooses a strategy $\sigma_i \in \Sigma$. As shown in Figure 2, the profile $(\sigma_1, \sigma_2) \in \Sigma^2$ determines the players' infection probabilities, labor productivities, and expected payoffs.

The Nash equilibrium is the set of profiles from which any player has no incentive to deviate. At the Nash equilibrium, each player's strategy is the best response against the other player's strategy. Let $\mathcal{B}_i(\sigma_1, \sigma_2)$ be the proposition that σ_i is the best response against σ_j, $i \neq j$, $j \in \{1, 2\}$. The propositions $\mathcal{B}_1(\sigma_1, \sigma_2)$ and $\mathcal{B}_2(\sigma_1, \sigma_2)$ are defined as

$$\mathcal{B}_1(\sigma_1, \sigma_2) \leftrightarrow \forall \sigma_1' \in \Sigma,\ U_1(\sigma_1, \sigma_2) \geq U_1(\sigma_1', \sigma_2),$$
$$\mathcal{B}_2(\sigma_1, \sigma_2) \leftrightarrow \forall \sigma_2' \in \Sigma,\ U_2(\sigma_1, \sigma_2) \geq U_2(\sigma_1, \sigma_2'),$$

where $U_i(\sigma_1, \sigma_2) \in [0, \infty)$ denotes the expected payoff of the i-th player at the profile (σ_1, σ_2). Let $\mathcal{N}(\sigma_1, \sigma_2)$ be the proposition that the profile (σ_1, σ_2) is a Nash equilibrium. The Nash equilibrium is defined as

$$\mathcal{N}(\sigma_1, \sigma_2) \leftrightarrow \mathcal{B}_1(\sigma_1, \sigma_2) \wedge \mathcal{B}_2(\sigma_1, \sigma_2),$$

where the operator $\wedge$ denotes the logical conjunction of propositions.

The all-F profile (F, F) is a Nash equilibrium if

$$[U_1(\mathrm{F}, \mathrm{F}) \geq U_1(\mathrm{T}, \mathrm{F})] \wedge [U_2(\mathrm{F}, \mathrm{F}) \geq U_2(\mathrm{F}, \mathrm{T})].$$

The first and second inequalities correspond to the best responses $\mathcal{B}_1(\mathrm{F},\mathrm{F})$ and $\mathcal{B}_2(\mathrm{F},\mathrm{F})$, respectively. Since $U_1(\mathrm{F},\mathrm{F}) = U_2(\mathrm{F},\mathrm{F})$ and $U_1(\mathrm{T},\mathrm{F}) = U_2(\mathrm{F},\mathrm{T})$, the best responses of the two players are equivalent. Solving $\mathcal{B}_1(\mathrm{F},\mathrm{F})$ with respect to P gives the interval of the all-F Nash equilibrium, that is

$$\mathcal{N}(\mathrm{F},\mathrm{F}) \leftrightarrow P \in [0, P_L],\ P_L := \frac{\beta - 1}{\beta - \alpha_1\alpha_2} \in (0,1).$$

Note that $\alpha_1 \in (0,1)$, $\alpha_2 \in (0,1)$, and $\beta \in (1,\infty)$.

The all-T profile (T,T) is a Nash equilibrium if

$$[U_1(\mathrm{T},\mathrm{T}) \geq U_1(\mathrm{F},\mathrm{T})] \wedge [U_2(\mathrm{T},\mathrm{T}) \geq U_2(\mathrm{T},\mathrm{F})].$$

Similar to the all-F profile, the best responses of the two players are equivalent. Solving $\mathcal{B}_1(\mathrm{T},\mathrm{T})$ with respect to P gives the interval of the all-T Nash equilibrium, that is

$$\mathcal{N}(\mathrm{T},\mathrm{T}) \leftrightarrow P \in [P_R, 1],\ P_R := \frac{P_L}{\alpha_2} \in \left(0, \frac{1}{\alpha_2}\right).$$

Note that $P_L < P_R$. If $\alpha_2 < P_L$, P_R exceeds one, and the all-T Nash equilibrium is crowded out of the domain $[0,1]$.

At the profiles (T,F) and (F,T), the player with the strategy F free rides on the community effect provided by the player with the strategy T, without abandoning the benefit from the alternative use of ITNs. For the free-rider profiles,

$$\mathcal{N}(\mathrm{T},\mathrm{F}) \leftrightarrow [U_1(\mathrm{T},\mathrm{F}) \geq U_1(\mathrm{F},\mathrm{F})] \wedge [U_2(\mathrm{T},\mathrm{F}) \geq U_2(\mathrm{T},\mathrm{T})],$$
$$\mathcal{N}(\mathrm{F},\mathrm{T}) \leftrightarrow [U_1(\mathrm{F},\mathrm{T}) \geq U_1(\mathrm{T},\mathrm{T})] \wedge [U_2(\mathrm{F},\mathrm{T}) \geq U_2(\mathrm{F},\mathrm{F})].$$

$\mathcal{B}_1(\mathrm{T},\mathrm{F})$ and $\mathcal{B}_2(\mathrm{T},\mathrm{F})$ are equivalent to $\mathcal{B}_2(\mathrm{F},\mathrm{T})$ and $\mathcal{B}_1(\mathrm{F},\mathrm{T})$, respectively. Hence, the free-rider Nash equilibria $\mathcal{N}(\mathrm{T},\mathrm{F})$ and $\mathcal{N}(\mathrm{F},\mathrm{T})$ are equivalent. Solving $\mathcal{N}(\mathrm{T},\mathrm{F})$ with respect to P gives the interval of the free-rider Nash equilibria, that is

$$\mathcal{N}(\mathrm{T},\mathrm{F}) \leftrightarrow \mathcal{N}(\mathrm{F},\mathrm{T}) \leftrightarrow P \in [P_L, P_R] \cap [0,1],$$

where the operator $\cap$ produces the intersection of sets.

2 Distribution of Pareto equilibria

The Pareto equilibrium is the set of profiles at which any player cannot increase its payoff without decreasing the other player's payoff. At the Pareto

equilibrium, the vector of the two players' expected payoffs is efficient from the viewpoint of public welfare, which means that any player's strategy is not harmful for the other player. The Pareto equilibrium is not always the Nash equilibrium, and the mismatch between the two equilibria is called a *social dilemma* (SD). In the social dilemma, each player's best response to the other player results in an inefficient expected payoff vector.

Let $\mathbf{u}(\sigma_1, \sigma_2) := (U_1(\sigma_1, \sigma_2), U_2(\sigma_1, \sigma_2))$ be the expected payoff vector at the profile (σ_1, σ_2). A vector $\mathbf{u}(\sigma_1', \sigma_2')$ is Pareto superior to the other vector $\mathbf{u}(\sigma_1, \sigma_2)$ if

$$\mathbf{u}(\sigma_1', \sigma_2') \in \Phi_{\mathrm{S}}(\sigma_1, \sigma_2) := \{[U_1(\sigma_1, \sigma_2), \infty) \times [U_2(\sigma_1, \sigma_2), \infty)\} \setminus \mathbf{u}(\sigma_1, \sigma_2).$$

$\Phi_{\mathrm{S}}(\sigma_1, \sigma_2)$ indicates the Pareto superior region to $\mathbf{u}(\sigma_1, \sigma_2)$. The operator $\times$ produces the Cartesian product of sets, and the operator $\setminus$ produces the relative complement of the right-side set in the left-side set. By moving from $\mathbf{u}(\sigma_1, \sigma_2)$ to $\mathbf{u}(\sigma_1', \sigma_2')$, at least one player can increase its payoff without decreasing the other player's payoff. The negation of the Pareto superiority is the Pareto inferiority. The Pareto inferior region to $\mathbf{u}(\sigma_1, \sigma_2)$, denoted by $\Phi_{\mathrm{I}}(\sigma_1, \sigma_2)$, is expressed as

$$\Phi_{\mathrm{I}}(\sigma_1, \sigma_2) := \{[0, \infty) \times [0, \infty)\} \setminus \Phi_{\mathrm{S}}(\sigma_1, \sigma_2).$$

Figure S1 shows the Pareto superior and inferior regions to $\mathbf{u}(\sigma_1, \sigma_2)$. Let $\mathcal{P}(\sigma_1, \sigma_2)$ be the proposition that the profile (σ_1, σ_2) is a Pareto equilibrium. The vector $\mathbf{u}(\sigma_1, \sigma_2)$ is a Pareto equilibrium if all the other vectors are Pareto inferior to $\mathbf{u}(\sigma_1, \sigma_2)$, that is

$$\mathcal{P}(\sigma_1, \sigma_2) \leftrightarrow \forall(\sigma_1', \sigma_2') \in \Sigma^2,\ \mathbf{u}(\sigma_1', \sigma_2') \in \Phi_{\mathrm{I}}(\sigma_1, \sigma_2).$$

Since $U_1(\mathrm{T}, \mathrm{F}) < U_1(\mathrm{T}, \mathrm{T})$ and $U_2(\mathrm{F}, \mathrm{T}) < U_2(\mathrm{T}, \mathrm{T})$, the free-rider vectors $\mathbf{u}(\mathrm{T}, \mathrm{F})$ and $\mathbf{u}(\mathrm{F}, \mathrm{T})$ are Pareto inferior to the all-T vector $\mathbf{u}(\mathrm{T}, \mathrm{T})$. Hence, the all-T vector is a Pareto equilibrium if the all-F vector $\mathbf{u}(\mathrm{F}, \mathrm{F})$ is Pareto inferior to the all-T vector. Since the two players have the same payoff at the all-T or all-F profile,

$$\mathbf{u}(\mathrm{F}, \mathrm{F}) \in \Phi_{\mathrm{I}}(\mathrm{T}, \mathrm{T}) \leftrightarrow U_1(\mathrm{F}, \mathrm{F}) \leq U_1(\mathrm{T}, \mathrm{T}).$$

Solving this inequality with respect to P gives the interval of the all-T Pareto equilibrium, that is

$$\mathcal{P}(\mathrm{T}, \mathrm{T}) \leftrightarrow P \in [P_L^*, 1],\ P_L^* := \frac{\beta - 1}{\beta - \alpha_1 \alpha_2^2} \in (0, 1).$$

Note that $P_L^* < P_L$.

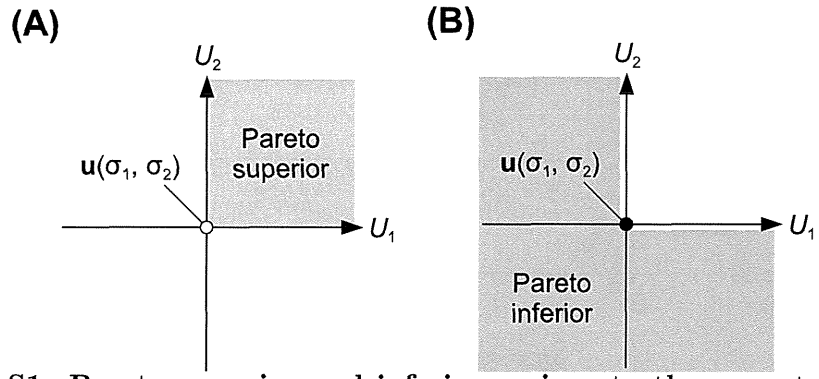

Figure S1: Pareto superior and inferior regions to the expected payoff vector at the profile (σ_1, σ_2). (A) Pareto superior region to the vector $\mathbf{u}(\sigma_1, \sigma_2)$. If the players move from $\mathbf{u}(\sigma_1, \sigma_2)$ to the Pareto superior region, at least one player can increase its payoff without decreasing the other player's payoff. **(B)** Pareto inferior region to the vector $\mathbf{u}(\sigma_1, \sigma_2)$. In the Pareto inferior region, at least one player's payoff decreases compared to $\mathbf{u}(\sigma_1, \sigma_2)$.

Since $U_2(\mathrm{F},\mathrm{F}) < U_2(\mathrm{T},\mathrm{F})$ and $U_1(\mathrm{F},\mathrm{F}) < U_1(\mathrm{F},\mathrm{T})$, the all-F vector is Pareto inferior to the free-rider vectors. The free-rider vectors are Pareto inferior to each other due to the symmetry as follows:

$$U_i(\mathrm{T},\mathrm{F}) \leq U_i(\mathrm{F},\mathrm{T}) \leftrightarrow U_j(\mathrm{T},\mathrm{F}) \geq U_j(\mathrm{F},\mathrm{T}).$$

If a player increases its payoff by moving from one free-rider profile to another, the other player's payoff decreases. The free-rider vectors are Pareto equilibria if the all-T vector is Pareto inferior to the free-rider vectors. Since $U_1(\mathrm{T},\mathrm{F}) < U_1(\mathrm{T},\mathrm{T})$ and $U_2(\mathrm{F},\mathrm{T}) < U_2(\mathrm{T},\mathrm{T})$,

$$\mathbf{u}(\mathrm{T},\mathrm{T}) \in \Phi_{\mathrm{I}}(\mathrm{T},\mathrm{F}) \leftrightarrow U_2(\mathrm{T},\mathrm{T}) < U_2(\mathrm{T},\mathrm{F}),$$
$$\mathbf{u}(\mathrm{T},\mathrm{T}) \in \Phi_{\mathrm{I}}(\mathrm{F},\mathrm{T}) \leftrightarrow U_1(\mathrm{T},\mathrm{T}) < U_1(\mathrm{F},\mathrm{T}).$$

The two inequalities are equivalent, and hence the interval of the free-rider Pareto equilibria is

$$\mathcal{P}(\mathrm{T},\mathrm{F}) \leftrightarrow \mathcal{P}(\mathrm{F},\mathrm{T}) \leftrightarrow P \in [0, P_R) \cap [0, 1].$$

The all-T vector is Pareto inferior to the all-F vector if $U_i(\mathrm{T},\mathrm{T}) \leq U_i(\mathrm{F},\mathrm{F})$. When this inequality holds, the free-rider vectors are also Pareto inferior to the all-F vector because $U_1(\mathrm{T},\mathrm{F}) < U_1(\mathrm{T},\mathrm{T})$ and $U_2(\mathrm{F},\mathrm{T}) < U_2(\mathrm{T},\mathrm{T})$. Hence, the interval of the all-F Pareto equilibrium is

$$\mathcal{P}(\mathrm{F},\mathrm{F}) \leftrightarrow P \in [0, P_L^*].$$

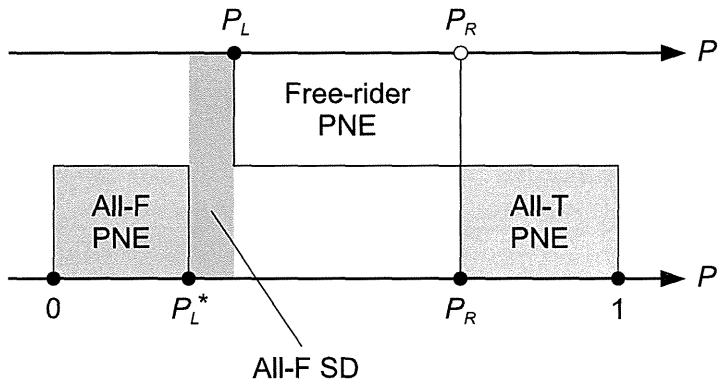

Figure S2: Distribution of Pareto efficient Nash equilibria (PNE) in the ITN use game. At the PNE, the Nash equilibrium and the Pareto equilibrium hold simultaneously. If P lies in the gap between P_L^* and P_L, the players are attracted to the all-F social dilemma (SD) at which the all-F profile is a Nash equilibrium but is not a Pareto equilibrium.

3 Distribution of Pareto efficient Nash equilibria

The Pareto efficient Nash equilibrium (PNE) is the set of profiles at which the Nash equilibrium and the Pareto equilibrium are achieved simultaneously. At the PNE, each player's best response to the other player is also desirable for the public welfare, that is, for the expected payoff vector. For each profile, the interval of the PNE is obtained by taking the intersection of the intervals of the two equilibria. Let $\mathcal{S}(\sigma_1, \sigma_2)$ be the proposition that the profile (σ_1, σ_2) is a PNE. The PNE is defined as

$$\mathcal{S}(\sigma_1, \sigma_2) \leftrightarrow \mathcal{N}(\sigma_1, \sigma_2) \wedge \mathcal{P}(\sigma_1, \sigma_2).$$

The intervals of the all-T, free-rider, and all-F PNE are written as follows:

$$\begin{aligned} \mathcal{S}(\mathrm{T},\mathrm{T}) &\leftrightarrow P \in [P_R, 1] \cap [P_L^*, 1] = [P_R, 1], \\ \mathcal{S}(\mathrm{T},\mathrm{F}) \leftrightarrow \mathcal{S}(\mathrm{F},\mathrm{T}) &\leftrightarrow P \in [P_L, P_R] \cap [0, P_R) \cap [0, 1] = [P_L, P_R) \cap [0, 1], \\ \mathcal{S}(\mathrm{F},\mathrm{F}) &\leftrightarrow P \in [0, P_L] \cap [0, P_L^*] = [0, P_L^*]. \end{aligned}$$

The all-T Nash equilibrium is Pareto efficient everywhere. The free-rider Nash equilibria are also Pareto efficient almost everywhere. The all-F Nash equilibrium is Pareto efficient except for the interval $(P_L^*, P_L]$. This gap is covered by the all-F SD defined as

$$\mathcal{N}(\mathrm{F},\mathrm{F}) \wedge \neg\mathcal{P}(\mathrm{F},\mathrm{F}),$$

where the operator $\neg$ denotes the negation of a proposition.

Figure S2 shows the distribution of PNE in the ITN use game. If $P_R \leq 1$, the domain $[0, 1]$ is covered by the four solutions: the all-F, free-rider, all-T PNE, and the all-F SD. If $P_R > 1$, the all-T PNE is crowded out of the domain $[0, 1]$. The union of the all-F PNE and the all-F SD is equivalent to the all-F Nash equilibrium. Figure 3 in the main manuscript illustrates the PNE for different parameter combinations.