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journal homepage: www.elsevier.com/locate/ijidSubpatent *Plasmodium* with mutant *pfmdr1*, *pfprt*, and *pvmdr1* alleles from endemic provinces in Mindanao, the Philippines: implications for local malaria elimination

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ABSTRACT

Objectives: This study was performed to identify and characterize circulating *Plasmodium* species in three provinces of Mindanao approaching malaria elimination.

Methods: Rapid diagnostic tests (RDT), microscopic examination, and PCR were used to detect malaria parasites. PCR-positive isolates were genotyped for polymorphisms in loci of interest.

Results: A total of 2639 participants were surveyed in Mindanao between 2010 and 2013. Malaria prevalence by PCR was 3.8% (95% confidence interval (CI): 2.7–5.2%) in Sarangani, 10% (95% CI: 7.7–12.7%) in South Cotabato, and 4.2% (95% CI: 3.2–5.6%) in Tawi-Tawi. *P. falciparum* and *P. vivax* were identified in all three provinces, and there was one case of *P. malariae* in South Cotabato. RDT was inferior to PCR for detecting asymptomatic *P. falciparum* and *P. vivax*. In Tawi-Tawi, microscopy failed to identify 46 PCR-positive malaria infections. The presence of *pfprt* haplotypes CVMNK, CVIET, and SMNT (codons 72–76), *pfmdr1* haplotype NFSND (codons 86, 184, 1034, 1042, 1246), and *pvmdr1* haplotype NFL (codons 91, 976, 1076) was confirmed in Mindanao.

Conclusions: Asymptomatic *Plasmodium* infections persisted in local communities between 2010 and 2013. PCR successfully identified subpatent malaria infections, and can better characterize malaria epidemiology in communities seeking malaria control and elimination at the local level.

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1. Introduction

To achieve the goal of malaria elimination by 2030, the Department of Health (DOH) in the Philippines is following a devolved malaria elimination strategy in all endemic provinces (Espino et al., 2004). The Philippines scaled up vector control and has deployed artemether-lumefantrine (AL) with primaquine (PQ) as the first-line treatment for *Plasmodium falciparum* since 2009. Chloroquine

(CQ) and PQ remained the approved regimen for treating *Plasmodium vivax* malaria. These measures resulted in an 80% reduction in malaria cases, with 27 provinces declared malaria-free in 2013 (Department of Health Philippines 2011, The Global Health Group 2013). By 2019, 42 of the country's 81 provinces, including South Cotabato in Mindanao, had been declared malaria-free. The Philippines is aiming to further reduce local malaria incidence by 90% in 2022 and has moved its target year for malaria elimination from 2020 to 2030 (Cortez, 2019, Wen et al., 2016).

Challenges for elimination in the Philippines include that strategies aimed at eliminating *P. falciparum* may not reduce *P. vivax* transmission, sustained by relapse of hepatic hypnozoite stages, the lack of information on the distribution of *P. falciparum* and *P. vivax* in the remaining endemic provinces of Mindanao, where political instability can threaten programme security, and inadequate data on *Plasmodium malariae*, *Plasmodium ovale* spp., and *Plasmodium knowlesi*. The latter species has been reported on Palawan Island and recent information suggests its public health impact in that province has been underestimated (Luchavez et al., 2008, Fornace et al., 2018). *P. knowlesi* has not been investigated in Mindanao. Finally, there is limited knowledge of the prevalence of variant alleles of the *P. falciparum* and *P. vivax* chloroquine resistance transporter (*cr*) and multidrug resistance (*mdr1*) genes in Mindanao. These variants may limit the efficacy of currently used antimalarials in the country (Chen et al., 2005, Chen et al., 2003, Hatabu et al., 2003).

A sustained reduction in the total number of malaria cases can result in a heterogeneous pattern of malaria transmission localized to geographic foci, as in the Solomon Islands (Harris et al., 2010). Microscopy conducted on peripheral blood samples from febrile individuals, which remains the operational standard for malaria diagnosis in the Philippines (Department of Health Philippines 2010), may not be sensitive in detecting these, and smaller foci with higher capacity for malaria transmission may occur at the household (hotspots) or individual (hotpops) level (Bousema et al., 2012). In these malaria foci, infected people may be asymptomatic such that they harbour blood-stage *Plasmodium* without clinical signs of malaria and thus, will not actively seek diagnosis and treatment. These infectious reservoirs can remain hidden as microscopy and rapid immunochromatographic diagnostic tests (RDT) both fail to detect parasites at low density in peripheral blood (Snounou and Singh, 2002, World Health Organization 2000). Some studies have already explored the usefulness of RDT in remote and low transmission settings of the Philippines (Bell et al., 2005, Fung et al., 2012), but more sensitive methods may be required to support effective elimination.

This study examined the epidemiology of malaria in three endemic provinces of Mindanao using RDT or microscopy as the primary parasite detection method, with later validation by PCR as the reference standard. The prevalence and distribution of circulating *Plasmodium* species were also determined. Genetic polymorphisms in *pfmdr1*, *pfcr*, and *pvmdr1* were identified, and the likely impact of these on malaria elimination in the region is discussed.

2. Methods

2.1. Study sites

Three malaria endemic provinces in Mindanao, Philippines were selected for this study between 2010 and 2013. Each province is home to vulnerable indigenous communities and people displaced by local armed conflicts. There have been no previous studies on the genetic background of circulating malaria parasites in these provinces. All three provinces are regarded as experiencing a 'type IV' climate under the rainfall classification in use in the Philippines

where a type IV climate is describe as having evenly distributed rainfall throughout the year.

Sarangani Province stretches across a 230-km coastline of southeastern Mindanao between latitude 5° 33' 41" to 6° 32' 4" N and longitude 124° 21' 39.6" to 125° 35' 11" E (Figure 1A). The Sarangani Bay separates its four municipalities in the eastern part of the province from its three municipalities in the western part of the province. The population of Sarangani Province was estimated as 498 904 in 2010. It was described as experiencing 'stable low' malaria transmission with fewer than 100 malaria cases confirmed in 2010 (Philippines National Malaria Program 2013).

South Cotabato Province is located at latitude 6° 15' N and longitude 125° 00' E in southern Mindanao (Figure 1A). It has 10 municipalities and a component city. Twenty-one (19%) of its villages are classified as geographically isolated and disadvantaged areas. There were 1.3 million people in South Cotabato in 2013. It was described as an area of 'unstable malaria', with no endogenous cases of malaria reported since 2010 (Philippines National Malaria Program 2014).

Tawi-Tawi Province is an archipelago located at latitude 5° 10' N and longitude 125° 00' E south of the main island of Mindanao. This province is among the five provinces composing the Autonomous Region in Muslim Mindanao (ARMM). It has 307 islands and islets grouped into the Tawi-Tawi island group, the Tawi-Tawi de Cagayan island group, and the Turtle Islands. This province shares sea borders with Sabah, Malaysia, and North Kalimantan, Indonesia (Figure 1B). It has been classified as having 'stable high' malaria with more than 1000 cases reported in 2013 (Philippines National Malaria Program 2014).

2.2. Study design

This study was conducted in the municipalities of Kiamba, Maasim, and Glan in Sarangani Province in 2010, in the municipalities of Bongao, Languyan, Panglima Sugala, and Tandubas in Tawi-Tawi Province in 2012, and in the municipalities of T'Boli and Lake Sebu in South Cotabato Province in 2013. These municipalities were selected based on the recommendations of the Provincial Malaria Control Program in each province. This study estimated that a sample size of 263 participants was required per municipality to detect at least a malaria prevalence by PCR of 1% at 80% statistical power and 5% level of significance, assuming an average malaria prevalence by microscopy of 0.1% in the target population, and an approximate 10-fold higher prevalence by PCR.

2.3. Data collection

Local residents were invited to participate in the malaria survey through their rural health unit. People across all ages were gathered at a common place, usually the rural health centre. Participants who provided signed informed consent and who had been residents of the municipality for at least 6 months prior to the study were selected. Those who self-reported taking other medication were excluded to remove drug pressure aside from currently used antimalarial drugs. Information on age, sex, tribal affiliation, occupation, and habit of sleeping under a bed net were collected from participants. *P. falciparum* and *P. vivax* infections were diagnosed on site from finger-prick blood of participants by point-of-care (POC) tests. In Sarangani and South Cotabato, the RDT Falcivax (Zephyr Biochemicals, India) test was used following the manufacturer's instructions. This RDT detects *P. falciparum* histidine-rich protein 2 (HRP2) in blood and *P. vivax*-specific lactate dehydrogenase (PvLDH). The RDT kits were unavailable for surveys in Tawi-Tawi Province and microscopy diagnosis was used as the POC test. Two local expert microscopists independently examined the

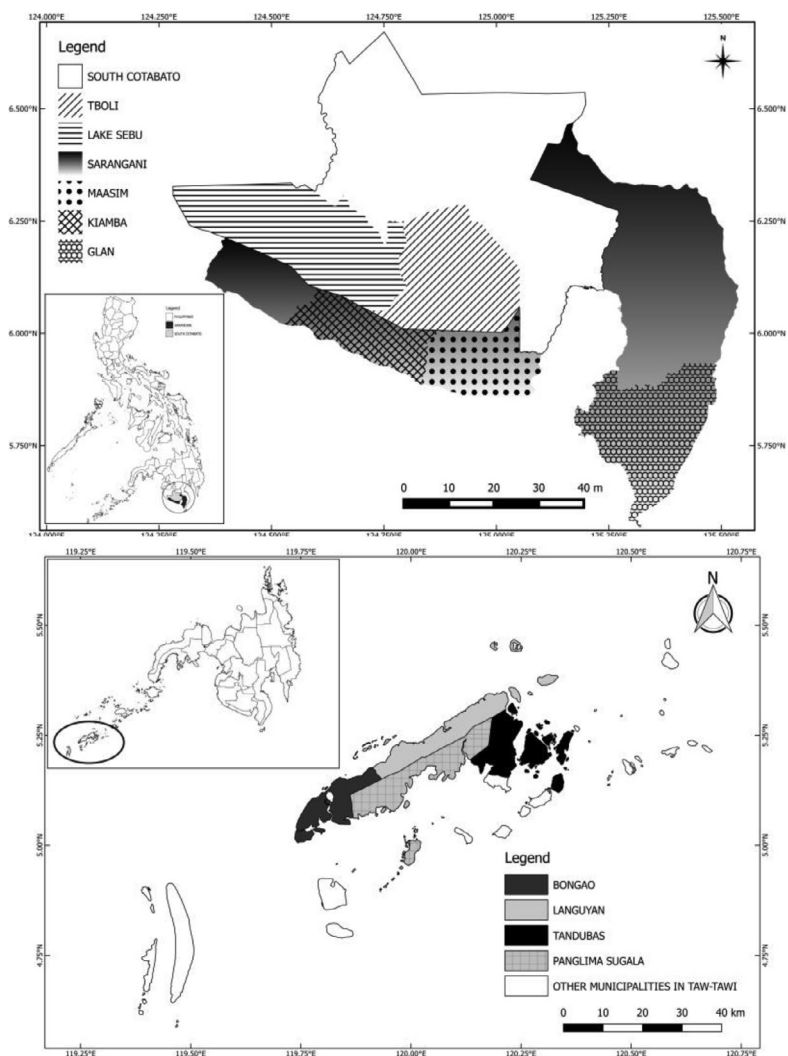


Figure 1. Study site locations. (A) The coastal province of Sarangani and the inland province of South Cotabato where the cross-sectional surveys were conducted in selected municipalities per province in 2010 and 2013, respectively (inset: map of the Philippines). (B) Map showing the island municipalities of Tawi-Tawi Province where the cross-sectional surveys were conducted in 2012 (inset: map of Mindanao showing the location of Tawi-Tawi).

participants' blood films for the presence of *Plasmodium* following national guidelines. Any person diagnosed with malaria based on parasite detection by the respective POC tests was referred to the Provincial Malaria Control Program for appropriate treatment following national guidelines. Finger-prick blood samples as a 6-mm spot on 3MM Whatman chromatography paper were collected from all participants for post hoc molecular detection of malaria parasites.

2.4. Parasite detection by PCR

Genomic DNA was extracted from an aliquot of each participant's original diagnostic blood sample collected on the filter paper, using the Chelex method as described previously (Plowe et al., 1995). Parasite DNA was detected by nested PCR amplification of the 18S ribosomal RNA gene. Primers rPLU6 and rPLU5new were used in the genus-determining PCR, while the following primers were used in the species-determining PCR: rFAL 1 and rFAL 2 for *P. falciparum* detection, rVIV 1 and rVIV 2 for *P. vivax* detection, rMAL 1 and rMAL 2 for *P. malariae* detection (Snounou, 1996, Fançonou et al., 2012), PadPo and rOVA2v for *P. ovale* spp. detection (Padley et al., 2003, Calderaro et al., 2007), and Pmk8 and Pmk9

for *P. knowlesi* detection (Singh et al., 2004). PCR diagnosis was repeated twice for samples that were PCR-positive for any *Plasmodium* species.

2.5. Genotyping *pfprt* and *pfmdr1* genes of Mindanao isolates

Genomic DNA of samples previously confirmed as PCR-positive for *P. falciparum* was extracted from additional 6-mm blood spots using the Qiagen DNA mini kit (Qiagen, Germany). These samples were used for *pfprt* and *pfmdr1* genotyping. The primers used to amplify selected *pfprt* polymorphic codons were designed from the *P. falciparum* 3D7 *pfprt* gene (NCBI accession number NC_004328.3, Gene ID 2655199) (Table 1). *P. falciparum* 3D7, Dd2, and 7G8 were reference isolates (Ponnudurai et al., 1981, Foote et al., 1990, Burkot et al., 1984). The *pfprt* amino acid haplotype CVMNKHALLAQN (codons 72–76, 97, 144, 148, 160, 220, 271, and 326) was taken as the wild type (Gadalla et al., 2015). Polymorphisms in the *pfmdr1* gene were also examined as described previously, with the *pfmdr1* amino acid haplotype NYSND at codons 86, 184, 1034, 1042, and 1246 as the reference wild type in this study (Humphreys et al., 2007, Beshir et al., 2010).

Table 1
PCR primers, annealing temperature, and expected PCR product size for direct sequencing of polymorphisms in the *pfcr* gene

Primer ^a	Primer sequence (5'–3')	Annealing temp. (°C)	Expected product size (bp)
1. <i>pfcr</i> codons 72–76, 97			
Primary amplification			
CRT1F1	GGCTCACGTTTAGGTGGAGG	55	325
CRT1R1	GGTAGGTGGAATAGATTCTC		
Hemi-nested amplification ^b			
CRT1F2	GTGGAGGTTCTGTCTTGGA	55	312
2. <i>pfcr</i> codons 144 and 160			
Primary amplification			
CRT2F1	GACCTTTTAGGAACGACACC	56	167
CRT2R1	AAAGCAGAAGAACATATTAATAGG		
Hemi-nested amplification ^b			
CRT2F2	AGGAACGACACCGAAGCTTA	56	158
3. <i>pfcr</i> codon 220			
Primary amplification			
CRT3F1	CACTTATACAATTATCTCGGAGC	55	318
CRT3R1	AACTATTTCCCTTGTATGTTTG		
Hemi-nested amplification ^b			
CRT3F2	TCTCGGAGCAGTTATTATTGTTG	55	304
4. <i>pfcr</i> codons 326			
Primary amplification			
CRT4F1	GTCTTGGTATGGCTAAGTTATGTG	56	285
CRT4R1	TATTCCTCTGTATGTATCAACG		
Hemi-nested amplification ^b			
CRT4R2	GATTGTGACGGAGCATGGGTAA	56	259

^a Forward primer F, reverse primer R.

^b In each case, the reverse primer in the primary PCR is used in the hemi-nested PCR.

Table 2
PCR primers and expected PCR product size for genotyping the *pvmdr1* gene

Primer	Primer sequence (5'–3')	Annealing temp. (°C)	Expected product size (bp)
1. <i>pvmdr1</i> codon 91			
Primary PCR			
P91_For1	CCGTCAAGTCATAGGAAGCTGTT	62	195
P91_Rev1	GAAGCTCGAAATGAAGGACAGAAT		
Hemi-nested PCR ^a			
P91_For2	TAGGAAGCTGTGGGGGTGT	62	184
2. <i>pvmdr1</i> codon 976			
Primary PCT			
P976_For1	GACCAGGATAGTCATGCCCCA	60	256
P976_Rev1	TGACTCGTCTTCTCTACATCC		
Hemi-nested PCR ^a			
P976F2	ATGCCCCAGGATTGCTGTCAG	60	243
3. <i>pvmdr1</i> codon 1076			
Primary PCR			
P1076N1	ACGGGCTGGAGGATTACTTCTG	62	241
P1076R1	TTCCCGCGTAGCTTCCCG		
Hemi-nested PCR ^a			
P1076N2	GGAGGATTACTTCTGCCACTGAT	62	234

^a In each case, the reverse primer in the primary PCR is used in the hemi-nested PCR.

2.6. Genotyping polymorphisms in the *pvmdr1* gene

P. vivax isolates from Mindanao were genotyped for polymorphisms in the *pvmdr1* codons 91, 976, and 1076 by direct sequencing of PCR products. These were compared to reference *P. vivax* clinical isolates from French Guyana (MRL 14/450), India (MRL 12/985), Indonesia (MRL 12/1103), and the Solomon Islands (MRL 12/594) provided by the LSHTM Malaria Reference Laboratory. The primers given in Table 2 were designed for this study, based on the *pvmdr1* gene of the chloroquine-sensitive *P. vivax* Sal-1 strain (NCBI accession number AY68622; Gene ID 5473000).

2.7. Viewing and sequencing of PCR amplicons

Amplification products were viewed by agarose gel electrophoresis. Direct sequencing in forward and reverse directions

of *pfmdr1*, *pfcr*, and *pvmdr1* fragments was performed with the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems), fractionated in the ABI 3730 sequencer (Applied Biosystems). Sequence data were analysed using Geneious version 7.0 (Biomatters, New Zealand) and Chromas version 1.61 (Technelysium Pty Ltd., Australia), and aligned with appropriate sequences in publicly available databases.

2.8. Data analyses

Data were entered into Microsoft Excel (Microsoft Corp., USA) and checked for errors before importing into Stata version 12 (Stata Corp LP, College Station, TX, USA) for all statistical analyses. Proportions were compared using the Chi-square test, or Fisher's exact test when an expected value in the 2 × 2 tables was less than 5.

Table 3
Characteristics of participants from Mindanao, Philippines

Demographics ^a	Sarangani	South Cotabato	Tawi-Tawi
Participants (n)	950	601	1088
Age (years), median (IQR) ^b	19 (6–38)	12 (7–30)	23 (10–35)
Participants <5 years old	17.7% (166/939)	8.3% (41/492)	2.5% (23/1084)
Female participants	64.1% (602/939)	67.7% (348/514)	51.7% (560/1083)
Bed net use	98.1% (893/910)	87.2% (422/484)	87.0% (931/1070)
Member of indigenous tribe	89.4% (816/913)	100% (519/519)	83.9% (877/1045)
Occupation of adults			
Farming	21.9% (206/410)	5.2% (85/165)	16.2% (116/714)
Housekeeping	32.9% (135/410)	30.3% (50/165)	30.7% (219/714)
Fishing	2.8% (26/410)	0.6% (1/165)	9.2% (66/714)
Others	10.5% (43/410)	17.6% (29/165)	43.8% (313/714)
<i>P. falciparum</i> prevalence ^c	1.1% (10/919)	0.2% (1/582)	0.0% (0/1088)
<i>P. vivax</i> prevalence ^c	0.1% (1/919)	4.0% (23/582)	0.0% (0/1088)
Mixed infection (<i>Pf</i> and <i>Pv</i>) ^c	0.0% (0/919)	0.3% (2/582)	0.0% (0/1088)

IQR, interquartile range (25th–75th percentile).

^a Values are given as the percentage (number of positives/total number of respondents) unless indicated otherwise.

^b Age data missing for 11, 109, and 4 persons, respectively, in the three provinces.

^c Parasite prevalence estimated using the RDT Falcivax in Sarangani and South Cotabato, and microscopy in Tawi-Tawi.

2.9. Ethical approval

The London School of Hygiene and Tropical Medicine Ethics Committee (Reference No. 5712) and the Philippines National Ethics Committee approved the conduct of this study. Adult participants provided prior written informed consent. Participants who could not read or write provided a thumbprint in the presence of an impartial literate adult witness. A parent or a guardian gave consent for children less than 18 years of age. Children who were 7 to less than 18 years of age also provided consent in addition to parental consent.

3. Results

Cross-sectional surveys were conducted in the three provinces where 2639 participants were enrolled (Table 3). Of these, a POC diagnostic test result was provided for 919 in Sarangani (RDT), 582 in South Cotabato (RDT), and 1088 in Tawi-Tawi (microscopy). The majority (84%) of these participants were indigenous people or 'Lumads' of Mindanao. Malaria prevalence by RDT was 1.2% in Sarangani and 4.5% in South Cotabato, where *P. vivax* predominated. None of the participants in Tawi-Tawi was diagnosed with malaria by microscopy (Table 3). Only one person was diagnosed with symptomatic *P. falciparum* malaria by RDT in Sarangani Province. This individual complained of feeling unwell, while the other nine participants with positive malaria RDT remained healthy during the survey and were considered asymptomatic. All were treated with standard antimalarial chemotherapy according to Government and World Health Organization guidelines.

Parasite prevalence by PCR was as follows in the three provinces: 3.8% (95% confidence interval (CI): 2.7–5.2%) in Sarangani Province, 10% (95% CI: 7.7–12.7%) in South Cotabato Province, and 4.2% (95% CI: 3.2–5.6%) in Tawi-Tawi Province. By PCR detection, *P. falciparum* and *P. vivax* infections were both present in all three provinces surveyed (Table 4). No *P. knowlesi* or *P. ovale* spp infections were identified. One symptomatic participant from South Cotabato Province was diagnosed with *P. malariae* by microscopy and this was later confirmed by PCR. This person had visited Sultan Kudarat, an adjacent malaria-endemic province,

weeks before clinical signs of malaria were observed. Since *P. malariae* is uncommon in the Philippines and may be a misdiagnosed *P. knowlesi* infection, the participant's blood film was confirmed and the result validated in the provincial diagnostic laboratory in South Cotabato.

There was no significant difference ($P = 0.306$, Fisher's exact test) in PCR-determined prevalence between *P. falciparum* and *P. vivax* infections in Sarangani Province. Similarly, there was no significant difference in the prevalence rates of *P. falciparum* and *P. vivax* infections in Tawi-Tawi Province ($P = 1.0$, Fisher's exact test). Meanwhile, the PCR-detected prevalence of *P. vivax* was significantly higher than that of *P. falciparum* ($P = 0.047$, Chi-square = 3.94) in South Cotabato (Table 4).

3.1. Diagnostic performance of RDT and microscopy

RDT and microscopy POC test results for diagnosing *P. falciparum* and *P. vivax* infections were compared with post hoc PCR results as the reference standard (Table 5). Inferior sensitivity of the RDT for the diagnosis of asymptomatic *P. falciparum* and *P. vivax* infections was observed in Sarangani Province and South Cotabato (Table 6). In both provinces, the specificity of the RDT for *P. falciparum* and *P. vivax* was acceptable. In Tawi-Tawi, microscopy failed to detect 26 samples that were PCR-positive for *P. falciparum* and 20 samples that were PCR-positive for *P. vivax*. The positive predictive value and positive likelihood ratio could not be calculated when comparing microscopy to PCR in diagnosing *P. falciparum* and *P. vivax* malaria in Tawi-Tawi, because no microscopy-positive individuals were identified (Table 6).

3.2. Characterization of *pfmdr1* alleles and haplotypes

Sixty-seven out of the 71 *P. falciparum* isolates were sequenced for polymorphisms in the *pfmdr1* gene. Two uncommon *pfmdr1* alleles 86F (TTT) and 86C (TGT) with two nucleotide differences from the wild type base sequence (AAT) at *pfmdr1* codon 86 were found in isolates from South Cotabato Province. Only the former has been described previously to our knowledge (Beshir et al., 2010). Seven haplotypes at *pfmdr1* codons 86, 184, 1034, 1042, and 1246 were constructed from 33 *P. falciparum* isolates with complete or near complete data. Examples of these haplotypes are shown in Table 7. The *pfmdr1* codons 1042 and 1246 were invariant in the genotyped *P. falciparum* but these were included in the haplotype analysis for clarity. The frequencies of the *pfmdr1* haplotypes were as follows: NYSND (18.2%), NFSND (21.2%), YFSND (24.2%), YYSND (24.2%), FYSND (6.1%), NYSNY (3%), and YYSNY (3%).

3.3. Characterization of *pfcr1* alleles and haplotypes

The *pfcr1* haplotype for codons 72–76 was constructed for 62 out of 67 *P. falciparum* isolates with complete data. The *pfcr1* wild type CVMNK was found in 27.4% (95% CI: 87.0–99.5%). The mutant haplotypes CVIET and S[_{ACT}]VMNT were found in 59.7% (95% CI: 46.4–71.9%) and 9.7% (95% CI: 7.9–13.2%) of *P. falciparum* isolates, respectively. Two samples harboured a mixture of CVMNK and CVIET haplotypes. The SVMNT haplotype was absent among *P. falciparum* isolates from South Cotabato, while the CVMNK and CVIET haplotypes were present in the three provinces surveyed. None of the *P. falciparum* isolates in this study that carried the *pfcr1* K76T allele also encoded the *pfcr1* A144T and L160Y variants. Haplotypes for *pfcr1* codons 72–76, 144, 160, 220, and 326 in seven *P. falciparum* isolates from Mindanao with complete data are presented in Table 7.

Table 4
Parasite detection by PCR in three provinces of Mindanao, Philippines

Province	Species by PCR, % (n)				
	<i>P. falciparum</i>	<i>P. vivax</i>	<i>P. malariae</i>	Mixed <i>falciparum</i> + <i>vivax</i>	Combined infections per province
Sarangani (n = 950)	2.0 (19)	1.7 (16)	0	0.1 (1)	3.8 (36)
South Cotabato (n = 601)	3.2 (19)	5.5 (33)	0.2 (1)	1.2 (7)	10.0 (60)
Tawi-Tawi (n = 1088)	2.4 (26)	1.8 (20)	0	0	4.2 (46)

Figures in parentheses represent the actual observed frequencies.

Table 5
Frequency of *P. falciparum* and *P. vivax* diagnosed by RDT and microscopy compared to PCR^a

Province	RDT	PCR				Total
		Negative	<i>Pf</i>	<i>Pv</i>	<i>Pf</i> and <i>Pv</i>	
Sarangani	Negative	877	16	14	1	908
	<i>Pf</i>	7	3	0	0	10
	<i>Pv</i>	0	0	1	0	1
	Total	884	19	15	1	919
South Cotabato	Negative	512	12	29	3	556
	<i>Pf</i>	1	0	0	0	1
	<i>Pv</i>	11	6	2	4	23
	<i>Pf</i> and <i>Pv</i>	1	1	0	0	2
Total	525	19	31	7	582	
Tawi-Tawi	Microscopy					
	Negative	1042	26	20	0	1088
	<i>Pf</i>	0	0	0	0	0
	<i>Pv</i>	0	0	0	0	0
	Total	1042	26	20	0	1088

Pf, *Plasmodium falciparum*; *Pv*, *Plasmodium vivax*; RDT, rapid immunochromatographic diagnostic test.

^a Values are actual counts of samples screened.

3.4. Polymorphisms in the *pvmdr1* gene

The *pvmdr1* 91N wild type allele was found in all 59 *P. vivax* isolates from Mindanao that were successfully genotyped out of 71 samples diagnosed by PCR. Nine *P. vivax* isolates were successfully genotyped at *pvmdr1* codon 976. Four of these encoded the wild type Y allele, while five encoded the Y976F mutant allele. Three *P. vivax* isolates successfully genotyped at *pvmdr1* codon 1076 all carried the F1076L mutant allele. The *pvmdr1* haplotypes at codons 91, 976, and 1076 were constructed for three *P. vivax* isolates from

Mindanao with complete data and four geographically diverse isolates from UK travellers that were sequenced in parallel (Table 8).

4. Discussion

There is general optimism that the Philippines can attain malaria elimination by 2030 as more provinces report a sustained reduction in malaria cases (The Global Health Group 2013). The provinces of Sarangani, South Cotabato, and Tawi-Tawi in Southern Mindanao were selected for this study because of insufficient data from these provinces to inform progress towards elimination. Against expectations, almost all participants diagnosed with *P. falciparum* and *P. vivax* infections either by RDT, microscopy, or PCR were asymptomatic during the survey; only two participants presented with fever. One participant in Sarangani Province was diagnosed with *P. falciparum* by RDT and PCR. The second, from South Cotabato, was diagnosed with *P. malariae* by microscopy and PCR. Those who carried malaria infections in these two provinces were members of indigenous tribes in geographically remote communities with poor access to health care. Thus, foci of malaria are still present in Sarangani and South Cotabato, with partial immunity in chronically infected individuals suppressing parasite density. Hence, an infectious reservoir is maintained in communities in the apparent absence of significant disease (Harris et al., 2010, Belizario et al., 1997).

There were disagreements between RDT and microscopy results and PCR. The low *P. falciparum* and *P. vivax* density in the sampled blood might have influenced the poor performance of the RDT in Sarangani and South Cotabato provinces. Participants who were RDT-positive but PCR-negative for *P. falciparum* might have carried persistent *P. falciparum* HRP2 antigen in their blood after detectable parasitaemia had been cleared (Mayxay et al., 2001). In

Table 6
Diagnostic performance of RDT in Sarangani Province and South Cotabato Province and microscopy in Tawi-Tawi Province compared to PCR

	Sarangani ProvinceRDT		South Cotabato ProvinceRDT		Tawi-Tawi ProvinceMicroscopy	
	<i>P. falciparum</i> ^a	<i>P. vivax</i> ^a	<i>P. falciparum</i>	<i>P. vivax</i>	<i>P. falciparum</i>	<i>P. vivax</i>
Sensitivity	15	6.2	6.2	15.4	0	0
% (95% CI)	(3.2–37.9)	(0.2–30.2)	(0.2–30.2)	(5.9–30.5)	(0–13.2)	(0–16.8)
Specificity	99.2	100	99.6	96.5	100	100
% (95% CI)	(98.4–99.7)	(99.6–100)	(98.7–100)	(94.6–97.9)	(99.7–100)	(99.7–100)
PPV	30	100	33.3	24	-	-
% (95% CI)	(6.7–65.2)	(2.5–100)	(0.8–90.6)	(9.4–45.1)	-	-
NPV	98.1	98.3	97.3	94.1	97.6	98.2
% (95% CI)	(97.0–98.9)	(97.3–99.1)	(95.6–98.5)	(91.8–95.9)	(96.5–98.4)	(97.2–98.9)
LR (+)	19.24	-	16.97	4.39	-	-
% (95% CI)	(5.36–69.06)	-	(1.62–177.6)	(1.86–10.35)	-	-
LR (-)	0.86	0.94	0.94	0.88	1.0	1.0
% (95% CI)	(0.71–1.03)	(0.83–1.06)	(0.83–1.07)	(0.77–1.00)	(1.0–1.0)	(1.0–1.0)

RDT, rapid immunochromatographic diagnostic test; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; LR, likelihood ratio.

^a The diagnostic accuracy of point-of-care tests for *P. falciparum* was estimated after excluding those positive for *P. vivax* and vice versa.

Table 7

Amino acid variants encoded by the *pfmdr1* and *pfprt* genes in seven isolates from Mindanao, Philippines, compared to previously published haplotypes

Source	Isolates	PFMDR1					PFCRT								Origin
		86	184	1034	1042	1246	72	74	75	76	144	160	220	326	
Previous papers	3D7	N	Y	S	N	D	C	M	N	K	A	L	A	N	NL (Padley et al., 2003)
	P2a ^a	Y	Y	S	N	D	C	N	N	T	T	Y	A	D	PH (Chen et al. 2003)
	P2b ^a	Y	Y	S	N	D	S	M	N	T	T	Y	A	D	PH Chen et al. 2003
	E1a ^b	N	F	C	D	D	C	I	E	T	A	L	S	S	PH Chen et al. 2003
This study	TGP 075	N	Y	S	N	Y	C	M	N	K	A	L	A	N	Sar
	TT 489	N	F	-	-	D	C	M	N	K	A	L	A	S	TT
	TGP 084	Y	Y	S	N	D	C	I	E	T	A	L	A	N	Sar
	TMJ 068	Y	F	S	N	-	C	I	E	T	A	L	A	S	Sar
	TT 290	N	Y	S	N	D	C	I	E	T	A	L	S	S	TT
	TMJ 080	Y	Y	S	N	D	S	M	N	T	A	L	S	D	Sar
	TMK 050	N	F	S	N	D	S	M	N	T	A	L	A	N	Sar

Amino acids different from the reference sequence (3D7) are shaded in grey. NL, Netherlands; PH, Philippines; Sar, Sarangani; TT, Tawi-Tawi.

^a Isolates with novel *pfprt* alleles: Thr-144 and Tyr-160.

^b Southeast Asian allelic type described in the Philippines.

Table 8

PvMDR1 haplotypes from Mindanao, Philippines compared to haplotypes of *P. vivax* clinical isolates from other geographic origins

Isolate	Origin	Alleles ^a		
		91	976	1076
<i>P. vivax</i> Sal-1 (Gene ID AY618622)	El Salvador, Central America	N	Y	F
UK Traveller 01 ^b	French Guyana	N	Y	F
UK Traveller 02	India	N	Y	F
UK Traveller 03	Indonesia	N	Y	L
UK Traveller 04	Solomon Islands	N	F	L
TKE022	Sarangani Province, Philippines	N	F	L
TKE065	Sarangani Province, Philippines	N	F	L
TT531	Tawi-Tawi Province, Philippines	N	F	L

^a Alleles different from the wild type are shaded grey.

^b DNA from four *P. vivax* isolates taken in 2012 from UK travellers was provided by the Malaria Reference Laboratory (MRL) at LSHTM for *pvmr1* sequencing.

Tawi-Tawi Province, none of the 46 participants who were PCR-positive for malaria were detected by microscopy. Since two expert microscopists independently examined the blood films, this suggested that either the blood films prepared in the field were not well preserved during shipment to the Municipality of Bongao in Tawi-Tawi for examination, or parasite density was below the level of detection of microscopy in all cases (Harris et al., 2010, Mosha et al., 2013).

Based on national estimates, *P. falciparum* is more prevalent than *P. vivax* in Sarangani Province and Tawi-Tawi Province. However, there was no significant difference in the proportions of *P. falciparum* and *P. vivax* infections in our survey. South Cotabato has not reported a malaria case since 2010 and yet PCR identified 60 *Plasmodium* spp. infections among the 601 participants surveyed in 2013. This previously unknown burden includes a significantly higher proportion of *P. vivax* infections by PCR compared to *P. falciparum*. These findings suggest that subpatent infections of this species are prevalent in the provinces surveyed and so measures designed to control *P. falciparum* transmission may not be effective in reducing *P. vivax* (Kaneko et al., 2014).

This study is novel in characterizing the *pfmdr1* and *pfprt* genes of *P. falciparum* isolates from Mindanao for polymorphisms that might influence the effectiveness of AL (Sisowath et al., 2009, Venkatesan et al., 2014), which is currently used to treat *P. falciparum* in the Philippines. This was only partially successful, as attempts were limited by sample size, low parasite density, and the difficulty of amplification across introns in the *pfprt* gene. Never-

theless, seven *P. falciparum* isolates from Mindanao were identified with the *pfmdr1* NFSND haplotype, which has repeatedly been associated with prolonged parasite survival following AL treatment in Africa and Asia (Humphreys et al., 2007, Henriques et al., 2014, Lubis et al., 2020). This haplotype was found in asymptomatic infections in Mindanao and further studies are needed to evaluate its effect on AL treatment in clinical *P. falciparum* malaria in the Philippines.

The *pfprt* CVIET haplotype was dominant in all three provinces, suggestive of continuing pressure from CQ. This was widely used in the Philippines from the 1940s until AL replaced it for the treatment of *P. falciparum* infections in 2009, and remains recommended for *P. vivax* infections (Department of Health Philippines 2010). In the Philippines *P. falciparum* isolates with the *pfprt* 76T allele were described previously as having compensatory A144T and L160Y mutations (Chen et al., 2003), but these were not found in the present study. This suggests that (1) there might be other mutations in the *pfprt* gene of isolates from Mindanao to compensate for the *pfprt* 76T polymorphism, and/or (2) *P. falciparum* isolates from Sarangani, South Cotabato, and Tawi-Tawi experienced a different history of CQ pressure than did provinces where the Philippine-specific *pfprt* A144T and L160Y mutations were reported. This is likely to have included widespread use of amodiaquine in the past, which selects for the SVMNT haplotype at codons 72–76 (Beshir et al., 2010), but further studies will be required to explore this. Future studies should also examine genotype variation at the *pfk13* locus, which had not yet emerged as a candidate marker for artemisinin susceptibility at the time of the laboratory work described here.

There was limited success in characterizing the *pvmr1* gene among *P. vivax* isolates from Mindanao, mainly due to low *P. vivax* peripheral blood density among asymptomatic infected participants. The few results obtained showed that there were *P. vivax* isolates with the *pvmr1* Y976F mutation, suggested to be an important marker of *P. vivax* resistance to CQ elsewhere (Suwanarusk et al., 2007). In addition, the *pvmr1* NFL haplotype identified here in three *P. vivax* isolates from Mindanao and one from a UK traveller to the Solomon Islands, has previously been reported in *P. vivax* from settings where CQ-resistant *P. vivax* is present, including the Solomon Islands (Suwanarusk et al., 2007, Tjitra et al., 2008, Brega et al., 2005).

This work shows that *P. falciparum* and *P. vivax* isolates from Mindanao harbour gene variants that could influence the effectiveness of AL for *P. falciparum* and CQ for *P. vivax* malaria. Further studies are needed to explore the implications for the treatment of *P. vivax* malaria in the Philippines.

Declaration of Competing Interest

None.

Author contributions

MGD designed the study, supervised the field data collection, analysed the data, and wrote the manuscript. CD contributed to the study design, data collection, and analyses. JCD, JAB, FA, VM, DKB, SA, FY, WN, EB, and JB conducted the field surveys. MO was involved in the genotyping analysis. CJS and RH supervised the overall study design, laboratory procedures, data collection and analyses, and writing of the manuscript.

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