Detection of antimalarial drug resistance markers in *Plasmodium falciparum* samples from CARAGA, Philippines: a retrospective study

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The eradication of malaria globally is hindered by the emergence and spread of antimalarial drug resistance. Therapeutic efficacy surveillance (TES) of antimalarial drugs is done to monitor the susceptibility of malaria parasites to the treatment regimen being implemented. This retrospective study investigated the prevalence of molecular markers associated with resistance to the antimalarial drugs chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) in samples collected from CARAGA (then composed of Agusan del Sur, Agusan del Norte, Surigao del Sur and Surigao del Norte provinces), Mindanao, Philippines from 2005 to 2006 (see Espino et al 2006). DNA from malarial parasites were extracted from 38 dried blood spot samples, and parasite identification in the blood samples by species-specific nested PCR confirmed the cases as *Plasmodium falciparum*. Single nucleotide polymorphisms in the *P. falciparum* chloroquine resistance transporter (*pfcrt* C72S, M74I, N75E/D, and K76T), dihydrofolate reductase (*pfdhfr* A16V, C50R, N51I, C59R, S108N, and I164L) and dihydropteroate synthetase (*pfdhps* S436A/F, A437G, K540E, A581G, and A613S/T) genes were determined by nested PCR and sequencing. Genotyping of the *msp1, msp2* and *glurp* polymorphic loci for two samples which had late treatment failure (LTF) identified them as recrudescence. All samples had the *pfcrt* K76T mutation and harbored the SVMNT mutant haplotype. Moreover, 25% of the isolates were quadruple *pfdhps/pfdhfr* mutants, with detected polymorphisms at codons 436 and 437 in the *pfdhps* gene, and codons 59 and 108 in the *pfdhfr* gene, while 75% were quintuple mutants, including the LTF samples, the additional mutation being codon 540 in the *pfdhps* gene. No significant difference in the mutation profile was found between recrudescent isolates and those that responded to treatment. These results suggest that mutations that can confer resistance to the administered antimalarial drugs had alarmingly high prevalence in the region. Although previous studies determined the presence of these mutations to be indicative of resistant phenotype, this was not the case for the CARAGA isolates, as samples which harbored mutations still had adequate clinical response. It should be considered that aside from the presence of markers, phenotypic resistance depends on many factors such as host immunity and initial parasite biomass. Nonetheless, the presence of these multiple mutations even sensitive phenotypes could be associated with increased risk of CQ+SP resistance in the CARAGA region thereby providing molecular evidence for the need to shift the national drug policy. The national treatment guidelines were revised in 2009, nevertheless, based on the *in vivo* results. Further studies can look into the use of these molecular techniques to analyze and compare past and present molecular profiles of antimalarial drug resistance in order to determine possible reversion of resistance. Thus, *in vivo* studies can be complemented, and a solid basis for

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formation, execution and validation of public health policies for malaria control and elimination can be provided.

KEYWORDS

antimalarial drug resistance, *Plasmodium falciparum*, chloroquine, sulfadoxine-pyrimethamine, molecular markers, *pfcrt*, *pfdhps*, *pfdhfr*

INTRODUCTION

Malaria is caused by the parasite *Plasmodium* spp. transmitted by a blood meal of infected female *Anopheles* mosquitoes. *Plasmodium falciparum* accounts for more than 80% of the malaria cases in the Philippines (World Health Organization 2018). Although there has been a remarkable decrease in malaria morbidity and mortality in the country since the 1990s because of effective malaria control, this is threatened by the emergence of multidrug resistance of the parasite (Iwagami et al. 2009; World Health Organization 2010). A mainstay in the control and eventual elimination of malaria in the Philippines and in other endemic countries is highly effective antimalarial drugs. Parasite susceptibility to the administered drug regimen is important information for the control of the spread of drug resistance in pathogens. In 1993, the World Health Organization (WHO) introduced the therapeutic efficacy surveillance (TES) of antimalarial drugs in the wake of reported resistant phenotypes. Antimalarial drug resistance associated genes have been identified for the 4-aminoquinoline chloroquine phosphate (CQ), and antifolate and sulfonamide (i.e., sulfadoxine-pyrimethamine) combination (SP), wherein point mutations in target regions of these genes that affect parasite susceptibility to drugs are detected and analyzed (Bray et al. 2005; Picot et al. 2009). The complementary *in vitro* assays, such as molecular assays for drug-resistant markers, provide added information for the National Malaria Program's decision-making process in updating treatment guidelines and a sound antimalarial drug policy.

The TES consists of monitoring for recurrence of fever and parasitaemia at specified intervals over a period of 28 days after treatment for a patient's malaria episode. The protocols used for TES in the Philippines follow the regularly updated standard WHO protocols, with the evaluation of antimalarial drug efficacy based on a classification of therapeutic responses. An adequate clinical and parasitological response (APCR) is the absence of fever and parasitaemia during the entire observation period following the administration of the drug under investigation. A person's response to treatment is characterized as early treatment failure (ETF) when there is persistence of parasitaemia until Day 3, and late treatment failure (LTF) when parasitaemia is observed on any day from Day 4 to Day 28 without meeting ETF criteria. LTF is classified further into late clinical failure, wherein fever (or severe signs of malaria) and parasitaemia are observed anytime from Day 4 to Day 28 after the start of treatment, and late parasitological failure when, in the absence of fever after Day 4 to Day 28, a person is observed to have parasitaemia (WHO 2003; WHO 2009; WHO 2010). A treatment failure rate of 15% was arbitrarily set as the level at which a shift in drug policy is recommended.

CQ was the first-line drug of choice from the 1950s to 2002 (Department of Health - Philippines 2009). However, within decades after its introduction against *Plasmodium* spp., the presence of chloroquine-resistant phenotypes were reported (Sibley and Price 2012). Within the same period, rapid spread of resistance to SP, introduced in the 1980s as a second-line antimalarial drug of choice in the Philippines, was also observed (Wongsrichanalai et al. 2002). Fortunately, the Philippines National Malaria Program TES was well underway. Based on observations, the combination of CQ+SP became the first-line treatment regimen in the country (DOH 2002). TES observations were pivotal in amending the antimalarial treatment regimen for falciparum malaria and by 2009, artemisinin combination therapies (ACTs) artemether-lumefantrine, and primaquine (AL+PQ) were the first-line drugs of choice for uncomplicated falciparum malaria (DOH 2009).

There have been a few studies on the prevalence of molecular markers in drug resistance associated genes in *P. falciparum* isolates. These include those by Chen *et al* (2003, 2005), who studied *pfcrt* mutations in isolates from Morong, Bataan and other provinces in the Philippines, and by Hatabu *et al* (2009) who related *in vitro* CQ resistance to *crt* and *mdr1* molecular markers in isolates from three provinces – Kalinga, Palawan and Davao. There are also unpublished reports from RITM Malaria Study Group, which studied the genes *pfcrt, dhps* and *dhfr* in Philippine isolates collected through TES studies in Kalinga, Isabela provinces, Apayao, Davao del Norte, Compostela Valley, Palawan, Davao del Sur, Sultan Kudarat and Zamboanga City.

The TES of CQ+SP for uncomplicated falciparum malaria in the CARAGA region in Mindanao from 2005 to 2006 revealed an 8.1% treatment failure rate for the drug combination. In 2014, molecular analysis for *pfcrt, dhps* and *dhfr* markers involved in the mechanism of multidrug resistance of *P. falciparum* (Hyde 2005), were carried out on the archived samples. Mutations of these markers were correlated with *in vivo* observations and are reported here.

MATERIALS AND METHODS

Samples

Archived samples were obtained from a TES study of CQ+SP combination for uncomplicated falciparum malaria. The study was conducted from June 2005 to July 2006 in four municipalities from three provinces in the CARAGA region, namely (a) Esperanza, Agusan del Sur, (b) Buenavista and (c) Las Nieves in Agusan del Norte, and (d) San Miguel, Surigao del Sur. Patients were screened based on specific inclusion and exclusion criteria, as shown in Table 1.

After patients were recruited and written informed consents were obtained, CQ+SP antimalarial drugs were administered. The blood samples were collected for parasitological assessment by microscopy before treatment (Day 0), and after (Days 2, 3, 7, 14, 21 and 28). Dried blood spots on filter paper were also collected on these days and stored in 4°C at the RITM-Molecular Biology Laboratory.

DNA Extraction

The CARAGA TES samples consisted of 74 samples of *P. falciparum* and *P. vivax*, only 38 of which were identified as *P. falciparum* (Pf). Parasite DNA was extracted from 38 dried blood strips in filter paper samples using QIAamp DNA Mini Kit (QIAgen), following the manufacturer's instructions, modified by adding 100 uL Buffer EB instead of 150 uL to elute extracted DNA.

Confirmation of Parasite Species

To detect the presence and confirm the identification of parasites by blood film microscopy, a 2-step nested polymerase chain reaction (PCR) assay was carried out. For each PCR step, the PCR mixture was prepared and run on a thermal cycler, and products were visualized using agarose gel electrophoresis loaded together with a molecular weight marker (100 bp ladder, Invitrogen) and controls, as described below. For the first

Table 1: Inclusion and exclusion criteria for selecting TES enrolled patients.

Inclusion criteria	Exclusion criteria
• > 6 months old • Mono-infection with *P.falciparum* (>1000 and <100,000 asexual parasites per mm^3) • Fever on consultation or history of fever 24 hours before consultation • Able to return for/accessible to follow-ups • Ability and willingness to participate based on patient information sheet • Signed informed consent to participate in the study	• One or more danger signs of severe malaria • Clinical condition requiring hospitalization • Mixed malaria infection • Pregnant women • Acute febrile illness other than malaria • Severe malnutrition or any severe, chronic medical condition; skin conditions • History of hypersensitivity reactions to drugs in the study

Table 2: Distribution of the uncomplicated falciparum malaria patients among the 4 municipalities.

Municipality	Male	Female	Total
Buenavista, Agusan del Norte	3	5	8
Esperanza, Agusan del Sur	4	2	6
Las Nieves, Agusan del Norte	11	10	21
San Miguel, Surigao del Sur	3	0	3
		TOTAL	38

amplification (Nest 1), the genus-specific primers for *Plasmodium* spp. were used. These are rPLU1 + rPLU5 ('N1 1 + 5') which amplifies a ~1600bp of the small subunit ribosomal RNA (SSU rRNA) gene.

A second PCR step (Nest 2) was performed to identify the species of the detected parasite. Each sample was subjected to 5 species-specific primer pairs in separate reactions, corresponding to the five *Plasmodium* species that can infect humans. DNA samples of the five species were used as positive controls. For Pf, the positive control used was MRA-151 *Plasmodium falciparum* 3D7A genomic DNA, while the rest of the positive controls used were extracted DNA from clinical samples confirmed to be *P. vivax* (Pv), *P. malariae* (Pm), *P. ovale* (Po) or *P. knowlesi* (Pk) by the same nested PCR assay protocol. Water was added instead of a DNA template for the negative control. The primers used were from Snounou *et. al.* (1993) for Pf, Pv, Pm and Po, and Singh *et. al* (2004) for Pk. The PCR profile follows the modified method of Snounou & Singh (2002). The samples were run on an agarose gel (Invitrogen) for visualization. For better resolution of the amplified products, a 2% gel was used for the detection of PCR products for the Pf, Pm and Pk protocols, while 1.5% gel was used for Po and 3% for Pv.

Analysis of *pfcrt*, *pfdhps* and *pfdhfr* genes

Drug resistance analysis was performed by detecting the presence or absence of point mutations or single nucleotide polymorphisms (SNPs) in drug resistance associated genes. Nested PCR was done, which targeted different regions in the genes of interest. Specifically, for chloroquine resistance, *pfcrt* was the gene of interest, with codons 72-76 as the target region, following the methodology of Djimdé *et al* (2001). For *pfdhfr*, primary amplification made use of M1F and M5R primers, and the target region containing the codons 16, 50, 51, 108 and 164 was amplified using M3 and F/ primers, while F and M4 were used to detect mutations at codons 59 and 108. For *pfdhps*, the primers for primary amplification were R2F and R/R, and there were two secondary amplifications using K-K/ and L-L/ primer pairs which targeted regions encompassing codons 436, 437 and 540, and codons 581 and 613, respectively. The primer sequences and cycling conditions used were from the study by Das *et al* (2013), and were first described in the study by Duraisingh *et al* (1998).

The PCR products of the secondary amplification for the 5 gene fragments were purified using QIAquick PCR Purification Kit (Qiagen) and samples that had multiple bands after PCR were purified using PureLink® Quick Gel Extraction Kit by excising the desired DNA fragment from the agarose gel. The samples were quantified and assessed for purity using NanoDrop spectrophotometer (Thermo Scientific), which read the absorbances at wavelengths 260 nm and 280 nm, and were later sent for sequencing to 1st BASE in Singapore. Resulting DNA sequences were aligned and single-nucleotide polymorphisms (SNPs) were detected using Mega 6 software (Tamura et al. 2013). Both the forward and reverse reads of the sequences were analyzed, and the published gene sequences used as reference were as follows: GenBank Accession No. NC_004328 (*pfcrt* cds) for *pfcrt*, GenBank Accession Nos. Z30659 and U07706 for *pfdhps*, and GenBank Accession Nos. M22159 and J04643 for *pfdhfr*.

Recrudescence and Reinfection Analysis

To distinguish whether samples with treatment failures were either recrudescence or reinfection, PCR correction through genotyping of polymorphic genes encoding the merozoite surface proteins-1 and -2 (*msp1* and *msp2*) and the glutamate-rich protein (*glurp*) was done (WHO 2007). Two of the 38 samples had late treatment failures. Band patterns of amplifications for their Day 0 (pre-treatment) and Day 28 (post-treatment) samples were compared. A multiplex primary PCR for *msp1* and *msp2* was followed by a nested PCR for the amplification of 3D7-type and FC27-type *msp2* alleles. The primary and nested PCR for *glurp* was also done, and if recrudescence was observed for both, *msp1* nested PCR was also done. The positive control used was MRA-151 *Plasmodium falciparum* 3D7A genomic DNA.

Data Analysis

Results were interpreted based on the previous TES CARAGA 2005-06 technical report. Confirmation of the infecting *Plasmodium* species was compared to microscopy results. Recrudescence analysis followed the WHO guidelines: (a) recrudescence is considered when at least one common band or allele was observed in the Days 0 and 28 samples for the three markers; and (b) reinfection when a different band pattern was observed. Prevalence of mutations was determined for each drug-resistance associated gene, calculated as allele proportions, or the number carrying a certain allele (wild-type or mutant) divided by the number of samples which had positive PCR amplification. Moreover, presence or absence of drug resistance markers was interpreted in relation to the *in vivo* response to treatment (*i.e.* had adequate response or treatment failure) as previously reported. Statistical analysis was performed using XLStatistics Version 13.04.14 in Microsoft Office Excel

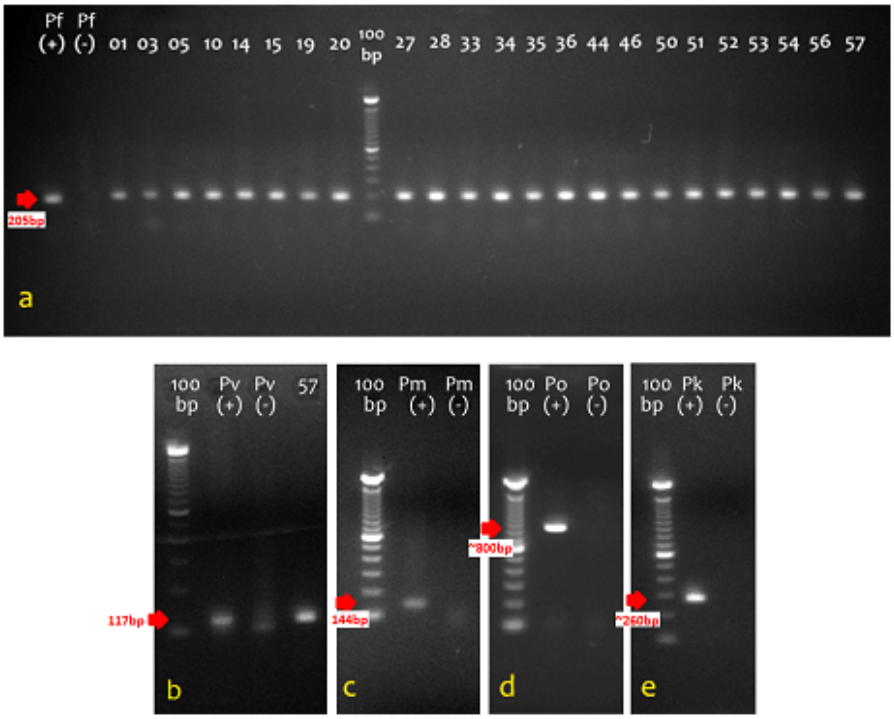

Figure 1: Sample gel electrophoresis results for the identification of malaria parasites by nested PCR. (a) Samples 01 – 57 (lanes labeled with last 2 numbers of patient ID) were loaded together with a positive and negative PCR control in a 2% agarose gel. Amplification of a 205bp product indicates presence of *P. falciparum*; (b,c,d,e) Amplification of 117 bp, 144 bp, ~800 bp and ~260 bp products indicate presence of *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*, respectively. Agarose gel concentrations used: 3% for Pv, 1.5% for Po, and 2% for Pf. Pm and Pk.

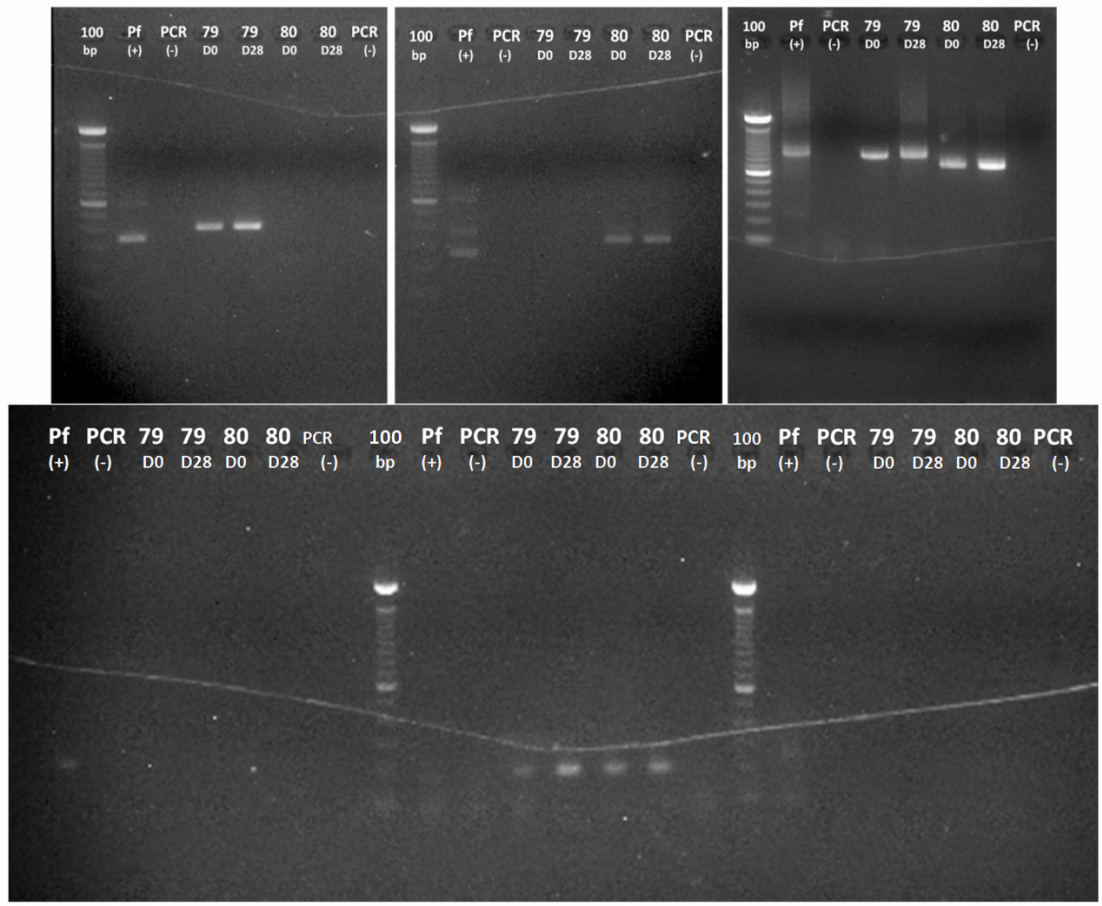

Figure 2: Gel electrophoresis results of *msp2*, *glurp* and *msp1* genotyping. Samples in each gel were loaded as follows: 100bp ladder (Invitrogen), MRA-151 *P. falciparum* 3D7A (positive control), negative control, Sample 79 Day 0, Sample 79 Day 28, Sample 80 Day 0, Sample 80 Day 28, negative control. (A) *msp2* marker amplifying FC27-type *msp2* alleles; (B) *msp2* marker amplifying 3D7-type *msp2* alleles; (C) *glurp* marker; (D) *msp1* marker amplifying K1, MAD20 and RO33 allelic families.

Table 3: Prevalence of *pfcrt*, *pfdhps*, and *pfdhfr* mutations in *P. falciparum* isolates from CARAGA, Mindanao, Philippines

Gene and encoded amino acids (wild-type/mutant)	Wild-type (%)	Mutant (%)
pfcrt		
C72S	0 (00)	38 (100)
K76T	0 (00)	38 (100)
pfdhps[a]		
S436F	0 (00)	36 (100)
A437G	0 (00)	36 (100)
K540E	9 (25)	27 (75)
A581G	34 (100)	0 (00)
A613S/T	34 (100)	0 (00)
pfdhfr		
A16V	38 (100)	0 (00)
C50R	38 (100)	0 (00)
N51I	38 (100)	0 (00)
C59R	0 (00)	38 (100)
S108N	0 (00)	38 (100)
I164L	38 (100)	0 (00)

[a] Two samples, namely 0205034 and 0205046, failed to amplify *pfdhps* gene regions, while samples 0203005 and 0205020 did not amplify *pfdhps* for the region encompassing codons 581 and 613. Hence, sample size is n=36 for S436F, A437G and K540E, and n=34 for A581G and A613S/T.

(Carr 2013). Prevalence of mutations and its correlation with clinical outcome and municipality was analyzed using Fisher's exact test. A *p*-value of less than 0.05 was considered to be statistically significant.

Ethical considerations

The human rights in research for the subjects, as indicated in the Declaration of Helsinki (2000 version), was observed in this study for concerns such as informed consent, patient safety and welfare, and subject confidentiality. The protocols for the TES and genotyping studies were also reviewed and approved by the institutional and ethical review boards of RITM. Informed consent was obtained for all participants, including the use of the samples for genetic analyses. Molecular assays were approved at the time of TES study approval but were only conducted in 2013 when funding was available.

RESULTS AND DISCUSSION

Identification by nPCR assay and Recrudescence Analysis

The parasites in the blood samples were identified by microscopy as *P. falciparum*, except for one that had a mixed infection of *P. falciparum* and *P. vivax*. To confirm the microscopy diagnosis, species-specific nested PCR was carried out for all samples. DNA was successfully extracted from all 38 samples, and results indicated 100% concordance between the identification results for microscopy and nested PCR. Since all samples had *P. falciparum,* the 38 isolates were then used for subsequent molecular analyses.

For recrudescence and reinfection analysis, only 2 of the 38 samples had a late treatment failure (LTF) as clinical outcome, with the rest having adequate clinical and parasitological response (ACPR). The samples with LTF were subjected to genotyping of three mentioned polymorphic genes – *msp1*, *msp2* and *glurp,* to determine whether the treatment failure was due to recrudescence or reinfection.

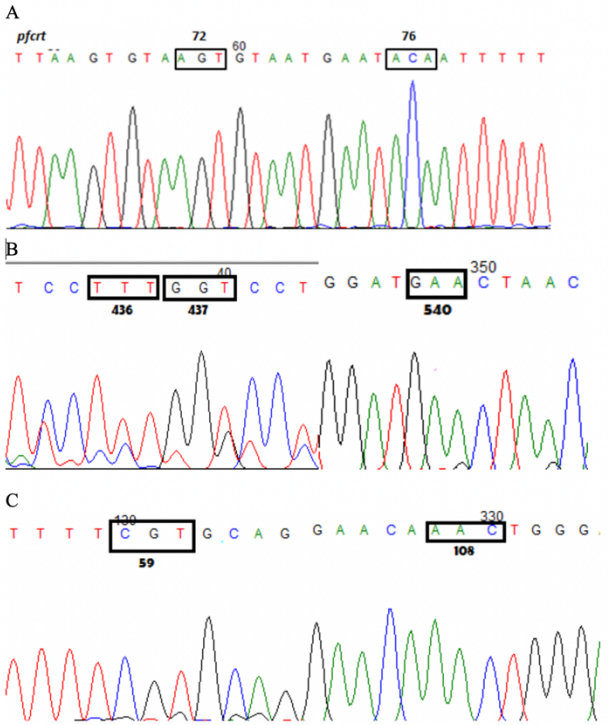

Figure 3: Sample chromatograms of each gene fragment showing point mutations at different gene loci of interest. (A) *pfcrt* codons 72 and 76 having polymorphisms. (B) Sites 436, 437 and 540 in *pfdhps* also have mutations. (C) Sites 59 and 108 for *pfdhfr* gene.

Figures 1 and 2 show the gel electrophoresis results for the species-specific nested PCR and recrudescence analysis. Analysis confirmed that the Day 0 and Day 28 samples for the two LTF samples had the same banding patterns, thus they are considered recrudescent infections. The genotyping done also determined that one of the samples carried a 3D7-type *msp2* allele, while the other sample carried a FC27-type *msp2* allele, and both had different alleles for the *glurp* marker. Both samples carried a MAD20 *msp1* allele.

Analysis of *pfcrt, pfdhps* and *pfdhfr* polymorphisms

Table 3 shows the point mutations or single nucleotide polymorphisms (SNPs) at different codons of the drug resistance associated genes *pfcrt, pfdhps* and *pfdhfr.* Chromatogram results for codons with mutations are illustrated in Figure 3.

From 1970s until 2002, CQ was the drug of choice for malaria in the Philippines; SP (also known as Fansidar) was reserved for second-line treatment. In 2000, TES disclosed CQ treatment failure rates of 45 to 70% in the CARAGA region and southern Mindanao. Further, treatment failure rates of SP ranged from 9 to 43% in Kalinga-Apayao, Palawan and Agusan del Sur (DOH 2002). This led to the decision of the combination of chloroquine and sulfadoxine-pyrimethamine (CQ+SP) as the first-line drug for the treatment of uncomplicated falciparum malaria. TES treatment failure rate was observed to be 8.1% in CARAGA. The surveillance conducted in Sultan Kudarat and Zamboanga (catchment for Basilan, Sulu, Tawi-Tawi) in 2006-07 ranged from 7 to 23%. These findings eventually led to the decision in 2009 to shift from CQ+SP as first-line drug-of-choice for uncomplicated falciparum malaria to AL. Given the *in vivo* observations, the presence of mutations in the drug resistance associated genes in the region is not surprising.

Table 4: Prevalence of single nucleotide polymorphisms (SNPs) in *pfcrt*, *pfdhps* and *pfdhfr* genes in the 4 municipalities

Municipality	*pfcrt*[a]		*pfdhps*[b]			*pfdhfr*[c]	
	CVMNK	**S**VMN**T**	SAKAA	**FG**K(AA)	**FGE**(AA)	ACNCSI	ACN**RN**I
Prevalence	**0.00**	**100.00**	**0.00**	**25.00**	**75.00**	**0.00**	**100.00**
Buenavista, Agusan del Norte	0	8	0	5	3	0	8
Esperanza, Agusan del Sur	0	6	0	0	6	0	6
Las Nieves, Agusan del Norte	0	21	0	3	14	0	21
San Miguel, Surigao del Sur	0	3	0	1	2	0	3
Total	0	38	0	9	25	0	38

In this study, no wild-type allele was observed for positions 72 and 76, as all samples harbored the K76T mutation, along with the C72S, resulting in the SVMNT haplotype for *pfcrt* codons 72 to 76. Molecular epidemiological studies have identified at least six different areas of origin of CQR although these still share common phenotypes (Awasthi and Das 2013). Later on, the *pfcrt* gene has been identified as a CQR determinant, and mutations in this gene are found to be strongly associated with *in vivo* and *in vitro* drug resistance; moreover, the mutation of codon 76 from lysine (K_{AAA}) to threonine (T_{ACA}) played a critical role (Djimdé et al. 2001; Wellems and Plowe 2001).

The results of the current study are consistent with previous studies which used TES samples (Segubre 2005), wherein all isolates from different regions in the Philippines, namely Kalinga, Apayao, Davao del Norte, Compostela Valley and Palawan, carried this haplotype, suggesting that the isolates from CARAGA, Philippines are more closely related to isolates from Papua New Guinea, Bougainville, East Timor and Lombok, Indonesia (Mehlotra et al. 2001; Huaman et al. 2004). This is also supported by the study of Takahashi *et al* (2012), which showed that one of the major CQ resistant lineages that occurs in the Philippines is distinct from other lineages. The study of Chen *et al*. (2003) of CQR isolates from Morong, Bataan, Philippines observed that all samples also had the K76T mutation. Moreover, Hatabu *et al* (2009) observed that either majority or all of the isolates from Kalinga (100%), Palawan (80%), and Mindanao (87%) likewise had the mutation.

There is varied association between the mutation and *in vitro* and *in vivo* CQ resistance, as seen in previous reports from other countries (Chen et al. 2001; Lakshmanan et al. 2005; Rungsihirunrat et al. 2009). Some found strong association and gave evidence that the single mutation caused CQR, while other reports showed a weak association. It is suggested that for areas with a high prevalence of the mutations, there is the fixation of the K76T mutation in the *P. falciparum* populations because of continuous drug pressure (Wellems and Plowe 2001; Afsharpad et al. 2012). It was also suggested that for isolates observed in other countries in Southeast Asia, Africa and South America where K76T is highly prevalent, additional mutations such as A220S, Q271E, N326S and R371I may be required to confer CQR to parasite populations (Daily et al. 2003). The mechanism of how the *pfcrt* mutation confers resistance is not known, but it has been observed that compared to the wild-type, mutant parasites accumulate less chloroquine in its digestive vacuole. The most plausible explanation given is that the mutation allows for the resistant parasites to actively transport chloroquine (Petersen et al. 2011).

For the molecular analysis of SP resistance, two genes were targeted, encoding the enzymes dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*). The gene *pfdhps* is linked with sulfadoxine resistance, while *pfdhfr* is linked with pyrimethamine resistance, and both are said to acquire higher levels of resistance in a stepwise fashion (Plowe et al. 1997; Duraisingh et al. 1998; Triglia et al. 1998; Lozovsky et al. 2009). All isolates had mutations in different positions of these genes. For *pfdhps*, the codons of interest were 436, 437, 540, 581 and 613, all of which were reported to have SNPs conferring drug resistance (Plowe et al. 1997; Alifrangis et al. 2003; Das et al. 2013). Out of the 36 samples that successfully amplified the gene fragments, 9 (25%) had a double mutation at codons 436 (S_{TCT}→F_{TTT}) and 437 (A_{GCT}→G_{GGT}), while 27 (75%) had a triple mutation, the additional mutation being the change of lysine (K_{AAA}) at position 540 to glutamine (E_{GAA}). The two samples that did not amplify the region containing codons 581 and 613 also had a triple mutation (FGE). The mutation at codon 437 is considered to be the most closely linked polymorphism to SP resistance, and it was observed in all samples, along with mutations at codon 436 in the isolates. The most common mutation for codon 436 (S_{TCT}→A_{GCT}) was not observed, and it was instead a change from serine (S_{TCT}) to phenylalanine (F_{TTT}), which is reportedly prevalent in Africa and observed in Thailand (Sridaran et al. 2010; Alam et al. 2011). The mutation at 540 was the only mutation which differentiated the isolates, since the other mutations, if present, were present in all samples. The wild-type (K540) constituted only 25% of the isolates, indicating that there is a high prevalence for this mutation, and all other mutations observed. Double mutations of 437 and 540 are said to be predictive of therapeutic failure, perhaps since the combination therapy CQ+SP was used and not a monotherapy, majority of the isolates were cleared by the treatment.

Pyrimethamine resistance is linked with the single mutation at codon 108, and higher-level resistance has been reported for isolates with additional mutations at codons 51 and/or 59 (Plowe et al. 1997). Out of the 6 codons analyzed in *pfdhfr*, polymorphisms were observed only for codons 59 (C_{TGT}→R_{CGT}), and 108 (S_{AGC}→N_{AAC}), with all isolates having this double mutation. Thus 9 (25%) of the isolates had *pfdhps/pfdhfr* quadruple mutations and 27 (75%) had quintuple mutations. This suggests that there is selection pressure for a high frequency of antifolate drug resistance in the region. However, clinical outcome of the isolates showed that most samples responded to CQ+SP treatment despite this observed genotype. Moreover, statistical analysis found no significant difference in the prevalence of these mutations among the four municipalities (p>0.05).

The quintuple mutations at *pfdhps* 437/540 and *pfdhfr* 51/59/108 is strongly associated with treatment failure of SP in African isolates (Happi et al. 2005). A double mutation at codons 108 and 51 already resulted in resistant phenotype in India (Das et al. 2013). No mutation at codon 51 was observed for all samples, and this could have contributed to the observed clinical outcome wherein only two had treatment failure. The small sample size (n=38) could have also affected the low variability observed in

the genotypes. The presence of a minimum of quadruple mutations for such a small population, however, cannot be disregarded. The use of these molecular markers as predictors of drug resistance will need to be further evaluated in other areas of the country.

Relating Occurrence of SNPs and Clinical Outcome

In *pfcrt*, the mutations at codons 72 and 76 were observed in all 38 isolates, resulting in the SVMNT mutant haplotype. For *pfdhps,* which is linked with sulfadoxine resistance, three out of the five codons analyzed had mutations, namely at codons 436, 437 and 540. All isolates had the wildtype allele for codons 581 and 613. For *pfdhfr* which is linked with pyrimethamine resistance, only two codons out of the six analyzed were observed to have mutations. The mutations at codons 59 and 108 were seen in all samples, and wild-type alleles were observed for codons 16, 50, 51 and 164. Table 3 shows the prevalence of multiple mutations for each gene, while Table 4 shows the distribution in each municipality. All samples were either quadruple or quintuple *pfdhps/pfdhfr* mutants. No significant difference was found in the prevalence of mutations between the clinical outcomes (ACPR or LTF) and also among the four municipalities.

There were only two cases of treatment failure from the isolates, and these were confirmed to have recrudesced. Both had quintuple mutations and the SVMNT haplotype. However, all isolates had polymorphisms at the studied markers for both CQ and SP resistance. Expectedly, statistical analysis found no significant difference between the prevalence of mutations (whether it was SVMNT+quintuple or SVMNT+quadruple mutations) and the clinical outcome of either ACPR or LTF. Based on previous studies, it was expected that the presence of these mutations would lead to the drug resistance phenotype. However, data from this study indicates that these mutations did not lead to the expected resistant phenotypes.

Treatment failure of drugs is due to the complex interplay of host, parasite, and drug, such that it has been difficult to identify markers that are predictive of resistance (Méndez et al. 2002). Clinical outcomes could vary because of individual differences in factors, including age, level of host immunity and initial parasite biomass, and also drug absorption, such that immunity and not necessarily the drug administered was able to clear the parasites harboring these mutations (Sakihama et al. 2007; Afsharpad et al. 2012). It is also suggested that the presence of these mutations may have arisen due to the extensive use of the drug in that country (Alifrangis et al. 2003). CQ had been used for the treatment of malaria for almost five decaseds when the 2005-06 Philippine TES was CQ+SP was conducted. The continuous drug pressure may have greatly contributed to the selection of mutant genotypes.

In a study by Bustos *et al.* (1999), which compared the efficacy of CQ+SP to atovaquone-proguanil for treatment of multidrug-resistant malaria, CQ+SP had 87.5% cure rate in Palawan isolates, while the rate for atovaquone-proguanil treatment was 100%. The reported 23-39% rate of chloroquine resistance in Palawan indicates that the mutations in *pfcrt* as well as *pfdhps* and *pfdhfr* were already present in these isolates. Moreover, Sakihama *et al* (2007) reported the co-existence of CQ-resistant and CQ-sensitive *pfcrt* alleles in Palawan in the mid-1990s and Chen *et al* (2005) also reported the presence of resistant phenotypes in the Palawan isolates. That the combination therapy of CQ+SP still had a high cure rate could mean that its synergistic activity as a combination treatment may have been able to overcome the possible presence of these mutations resulting in successful treatment, even if these mutations already exhibited a resistant phenotype in other countries (Happi et al. 2005; Ogouyèmi-Hounto et al. 2013). Méndez *et al* (2002) also observed that although mutations were present in the *dhfr* gene at codons 51 and 108, SP still had 96.7% efficacy in 120 isolates from Colombia. However, the mutations were shown to be associated with longer parasite clearance time and presence of gametocytes, since the parasites were able to survive longer and differentiated into gametocytes. This could then be transmitted by vectors and may promote the spread of drug resistance. This supposition is supported by observations in Mindanao where three patients of the 36 in the 2005-06 TES had gametocytemia until Day 28 while this was observed in only one patient in a 2001 TES of the same drug combination (Espino et al. 2006).

Studying the spread of SP resistance is needed since SP can be used as a partner drug in artemisinin combination therapies (ACTs) and it is still the WHO recommended drug for intermittent preventive therapy for pregnant women and infants (Ako et al. 2012; Venkatesan et al. 2013). Emerging resistance to the current first-line drug artemether-lumefantrine is being monitored, so the drug policy may need to be altered. Reversion to chloroquine (or CQ+SP) as a treatment is a possibility, especially when it has been observed that years of discontinued use of a drug increases the frequency of sensitive phenotypes in the population, as observed in Zambia and Cameroon (Ekland and Fidock 2007; Mwanza et al. 2016; Moyeh et al. 2018). It has also been suggested that artemisinin has an opposing selective force to CQ, and *pfcrt* can be used as predictor of its resistance. Knowing that these mutations can bring about a change in the susceptibility or sensitivity not just to chloroquine but also to other drugs can help predict the possible clinical outcomes of treatment regimens in a particular area (Summers et al. 2012).

CONCLUSION

Malaria parasites from CARAGA, Mindanao, Philippines (n=38) that were confirmed by nested PCR to be *P. falciparum* were screened for polymorphisms in antimalarial drug resistance associated genes, namely *pfcrt, pfdhps* and *pfdhfr*. Consistent with previous studies in the Philippines, all isolates had the critical K76T *pfcrt* mutation, and the SVMNT haplotype for codons 72 to 76. Moreover, a high prevalence of quadruple (25%) and quintuple (75%) *pfdhps/pfdhfr* mutants was observed, although the *in vivo* clinical outcome resulted in mostly successful treatment. The findings of this study suggest that by the year 2005 to 2006, when the CARAGA samples were collected, the risk of treatment failure was already present in the region because the malaria parasites harbored multiple mutations that could have conferred CQ and SP resistance. However, any resistance that did develop had not yet reached the stage where CQ+SP treatment became largely unsuccessful. This retrospective study has produced evidence that can be used to evaluate a reversal to susceptibility of parasite strains to chloroquine plus sulfadoxine-pyrimethamine combination in the remaining endemic areas in the country. This can be through a comparison of the molecular profiles of TES isolates that were collected after 2009 when AL replaced CQ+SP as first-line antimalarial drug. Determining the utility of these molecular markers in predicting the reversion of parasites to sensitive phenotype for the antimalarial drugs can be explored to determine the potential of these antimalarial drugs to return as a 'new' alternative to current artemisinin-based therapies.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS' CONTRIBUTIONS

MLM Macalinao performed the experiments and data analysis, and assisted in study design. FEJ Espino and E Segubre - Mercado conceptualized, designed and supervised the study. RJ Calugay participated in the coordination and supervision of experiments. MLM Macalinao, FEJ Espino, E Segubre-Mercado and RJ Calugay revised the manuscript. SA Galit and JHL Acuna provided technical support for data interpretation and analysis. All authors participated in the interpretation of the results, read and approved the final manuscript.

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