FACTORS ASSOCIATED WITH REGIONAL BIAS OF *PFCRT* (*PLASMODIUM FALCIPARUM* CHLOROQUINE RESISTANCE TRANSPORTER) HAPLOTYPES IN NAPAL

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Abstract. Evidences of reappearance of chloroquine sensitive *Plasmodium falciparum* haplotypes after cessation of chloroquine in many countries provide a rationale for the search of chloroquine sensitive haplotypes in *P. falciparum* isolates in Nepal where the use of chloroquine for falciparum malaria treatment has been ceased since 1988. *P. falciparum* chloroquine resistant transporter gene (*pfcrt*) haplotypes were determined and the factors associated with *pfcrt* haplotypes in the Eastern and Central regions of Nepal were identified. Blood samples from 106 microscopy-positive falciparum malaria patients (62 from the Eastern and 44 from the Central region) were collected on filter paper. *Pfcrt* region covering codons 72-76 was amplified by PCR and sequenced. SVMNT haplotype was predominant in the Central region, whereas CVIET haplotype significantly more common in the Eastern region. In multivariable analysis of factors associated with CVIET haplotype, the Eastern region and parasite isolates from patients visiting India within one month are significant at 5% level of significance. These findings suggest that antimalarial pressure is different between Eastern and Central regions of Nepal and there is a need of an effective malaria control program in the border areas between India and Nepal.

Keywords: *Plasmodium falciparum*, *pfcrt* haplotypes, Eastern and Central Nepal

INTRODUCTION

Malaria is a major public health problem in most of the tropical countries causing morbidity of 300-500 million people worldwide and mortality between 1 and 1.5 millions every year (WHO/UNICEF, 2005) with the major burden in Africa (Winstanley *et al*, 2004). Malaria remains a public health problem because of emergence of the drug resistant parasites and pesticide resistant mosquito vectors as well as non-availability of suitable and effective malaria vaccine (Sharma, 2005). Resistance to antimalarials especially by *Plasmodium falciparum* has spread throughout the world and is causing a

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serious obstacle to malaria control program (Wongsrichanalai *et al*, 2002). Resistance against chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) antimalarials has been reported in many countries and recently tolerance to artemisinin has also been reported by several studies (Jambou *et al*, 2005; Prugger *et al*, 2008; Andriantsoanirina *et al*, 2009; Dondorp *et al*, 2009). It has been suggested that molecular epidemiological data generated for each country would provide useful information for more effective usage of limited numbers of antimalarial drugs (Wongsrichanalai *et al*, 2002).

P. falciparum achieve CQ resistance by reducing CQ accumulation in their acid digestive vacuole (DV) where the drug does its damage. Point mutation in *P. falciparum* chloroquine resistant transporter gene (*pfcrt*) encoding PfCRT, a putative transporter, is involved in CQ efflux and confers CQ resistance (Fidock *et al*, 2000; Martin *et al*, 2009). K76T mutation in *pfcrt* is the key point mutation reported in all CQ resistant parasites (Hyde, 2005; Egan, 2006) and therefore has been proposed as molecular marker for monitoring CQ resistance. On the basis of mutation at codons 72-76 of *pfcrt*, *P. falciparum* has been classified into different haplotypes. Under CQ pressure, parasites with various genetic backgrounds have developed CQ resistance independently by mutating at different positions in the *pfcrt* gene (Chen *et al*, 2005). Their association with CQ resistance is different in different locations or countries.

In Nepal, SP has been the first-line drug for laboratory-confirmed falciparum malaria since 1988 and recently artemether/lumefantrine combination has been started (Department of Health Services, Nepal, 2008). Several studies revealed the recurrence of CQ sensitive parasite lines after few years of cessation of CQ use (Kublin *et al*, 2003; Mita *et al*, 2003; Wang *et al*, 2005). Besides, high dose of CQ than the normal dose was found efficacious in preventing the development of CQ resistance in Guinea-Bissau (Ursing *et al*, 2009). In neighboring country India, from which the drug resistance is considered to have been imported into Nepal, the first-line drug is still CQ in some parts of the country. In addition, CQ is the widely used successful drug for the treatment of *P. vivax* cases in Nepal. Therefore, the haplotypes of *P. falciparum* resistant to CQ may be different in Nepal. Moreover, different drug resistant patterns have been found in different geographic loci (Vathsala *et al*, 2004; Dittrich *et al*, 2005).

Limited information on antimalarial drug resistance and its relation with molecular markers of drug resistance is available in Nepal. Therefore, this study explored *pfcrt* haplotypes in the Eastern and Central region of Nepal and identified the factors associated with *pfcrt* haplotypes distribution in the Eastern and Central region.

MATERIALS AND METHODS

Study districts in Nepal

Among 12 falciparum malaria endemic districts, falciparum malaria in Jhapa accounts for almost 50% of the total malaria cases (Epidemiology and Disease Control Division, 2005). Although Nepal is a low transmission area for malaria, Jhapa district of Eastern Nepal is a relatively moderate endemic area of both falciparum and vivax malaria. More than 50% of national total falciparum malaria cases occur in this district. As Jhapa borders a high malaria endemic region of India, both imported and indigenous malaria cases are believed to be found in this

Fig 1–Map showing the location of study districts in Nepal.

district. Dhanusha District of Central Nepal is a low endemic area of malaria and malaria cases are considered to be indigenous to this district. Although these districts are only about 250 kilometers apart, they represent different geographical and malarial endemicity situation (Fig 1).

Sample collection

Blood samples were collected from 106 falciparum malaria positive patients attending healthcare facilities, 62 from Eastern and 44 from Central regions. Approximately 2 ml of venupuncture blood samples were collected and laboratory analysis (blood smear examination, hemoglobin measurement, and parasitemia count) were performed. Parasitemia was estimated by counting parasites against 200 WBC and converted to parasites per ml by assuming a standard count of 8,000 WBC/ml. A slide was considered negative if parasite was not found after examination of 100 fields. For those samples that were positive for *P. falciparum* by microscopy, blood samples initially collected from malaria-infected individuals were transferred onto filter papers (3MM Whatman) in the form of 3 spots of blood containing approximately 50 µl in each spot. After confirmatory diagnosis of falciparum malaria, the treatment was provided by the clinician according to national malaria treatment policy and existing hospital protocol. The healthcare facility followed up the patients for any inadequate treatment or treatment failure. The protocol was reviewed and approved by the Institutional Review Board (IRB) of the Institute of Medicine, Tribhuvan University, Kathmandu, Nepal as per the national research policy, Ethics Committee (EC) of Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand and Ethical Review Committee (ERC), World Health Organization, Geneva, Switzerland. Before interviewing and collecting blood samples, a written informed consent

was completed by each patient/caretaker, and assent form (if child) of malaria patients.

DNA extraction and detection of *pfcrt* haplotypes

DNA extraction from dried blood spots on filter papers was performed using QIAamp DNA Mini Kit and QIAamp DNA Blood Mini Kit protocol (QIAGEN, Hilden, Germany) following manufacturer's protocol and stored at -70ºC until use. PCR and direct DNA sequencing were used for *pfcrt* mutation and haplotype identification at codon 72-76. In brief, PCR primers CRT1 5'-CGTTTA GGTGGAGGTTCTTG-3' and CRT2 5'-CTTCGGTGTCGTTCCTAAAA-3' were used for amplification of 453 bp of the *pfcrt* covering codons 72-76. Each 50 µl PCR reaction contained 1X HiFi GC rich buffer, 2 mM Mg^{2+}, 0.3 mM dNTPs, 0.3 µM each primer, 0.5 U of KAPPA HiFi *Taq* polymerase and 5 µl of template DNA. Thermocycling conditions were as follows: denaturation at 95ºC for 5 minutes, 40 cycles of 98ºC for 30 seconds, 57ºC for 15 seconds, 72ºC for 30 seconds and a final heating at 72ºC for 1 minute. PCR amplicons were separated by 1.5% agarose gel-electrophoresis and visualized after staining with ethidium bromide.

Amplicons of *pfcrt* were purified using Nucleospin Extract II protocol (Macherey-Nagel-04/2008/Rev 07) and were sequenced using ABI 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA). The nucleotides sequences were converted into amino acid sequences using EXPASY DNA translate tool. The *pfcrt* haplotypes (amino acid sequences of codons 72-76) were identified using BioEdit (version 7.0.9) for alignment of amino acid sequences with the reference *pfcrt* amino acids sequence of *P. falciparum* 3D7 strain (Gen Bank accession number CAD508421).

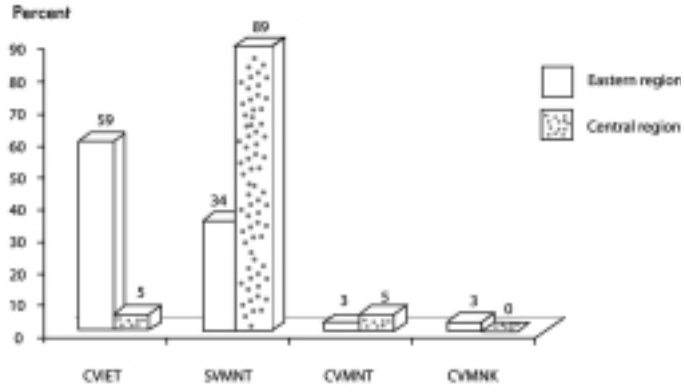

Fig 2–*Pfcrt* haplotypes (codons 72-76) in Eastern and Central regions of Nepal. Fragment of *pfcrt* covering codons 72-76 were PCR amplified and sequenced (n=106).

Data management and analysis

Data were analyzed using SPSS version 13.0. Logistic and regression analysis was conducted to identify factors associated with specific *pfcrt* haplotypes.

RESULTS

Sequences of *pfcrt* covering codons 72-76 were determined from 106 *P. falciparum* obtained in Eastern and Central Nepal. SVMNT was the predominant *pfcrt* haplotype in Nepal followed by CVIET, the latter haplotype being predominant in the Eastern region and the former haplotype predominant in the Central region ($p < 0.001$) (Fig 2). In bivariate analysis using logistic regression, the Eastern region and travel history of the patient within one month are highly significant factors associated with CVIET haplotype than SVMNT ($p < 0.001$). Similarly, water logging (stagnant water in ditches) near the

house and hemoglobin level are significant at 5% level of significance, whereas presence of gametocytes and *dhfr* I164L mutation (data not shown) are significant at 10% level of significance. In *pfcrt* SVMNT haplotype infection, parasitemia was high compared to low parasitemia in CVIET haplotype infection (Table 1).

Those variables, which are significant in bivariate analysis at 10% level of significance, then were used in multivariable analysis. The Eastern region and parasite isolates from patients visiting outside the region within one month were found to be associated with *pfcrt* CVIET haplotype at 5% level of significance (Table 2).

DISCUSSION

Studies conducted in many countries have reported the re-emergence of CQ and SP sensitive *P. falciparum* parasites after cessation of CQ monotherapy for the treatment of *P. falciparum* malaria (Kublin *et al*, 2003; Mita *et al*, 2003; Wang *et al*, 2005; Zhou *et al*, 2008; Bacon *et al*, 2009; Gama *et al*, 2009; Mwai *et al*, 2009). These data provide a rationale for the search of CQ sensitive haplotypes in *P. falciparum* isolates in Nepal where the use of CQ for falciparum malaria treatment has been ceased since 1988 (Epidemiology and Disease Control Division, 2005). In addition, this is the first study to document and compare the *pfcrt* haplotypes in the Eastern and Central regions of Nepal.

We detected more than 98% of falciparum parasite having K76T mutation, which indicates that there is still CQ resistance in *P. falciparum* in Nepal (Table 1). Persistence of CQ resistance mutant even after more than 20 years of official withdrawal suggests that local *P. falciparum* may still be inadvertently exposed to CQ, that mutant parasites are being imported into the country from Assam, India, and/or that low genetic diversity and low parasite transmission help to maintain mutant haplotypes in Nepal. In Kenya, the frequency of the *pfcrt*-76 mutant significantly decreases from around 94% to 63% over a period of 13 years and it is predicted that *P. falciparum* will revert back to CQ sensitive phenotype within 20 years after official removal of the drug (Mwai *et al*, 2009). However, in Malawi this level of sensitivity was reached within 9 years following CQ withdrawal (Kublin *et al*, 2003).

Our study found that SVMNT was the predominant *pfcrt* haplotype in Nepal and was present about double the frequency of CVIET. In the Eastern region, CVIET and SVMNT haplotypes were observed as major haplotypes, whereas SVMNT constituted 90% of the haplotypes in the Central region. Analysis of factors associated with CVIET compared to SVMNT haplotype suggests that CVIET haplotype may have been imported from Assam, India as this haplotype is reported as being predominant in Assam which borders to Eastern region of Nepal (Valecha *et al*, 2009). Thapa *et al* (2007) also reported limited diversity of falciparum parasites in Jhapa District of the Eastern region suggesting importation of the parasite from India or some other districts of Nepal and local transmission.

Based on analysis of *pfcrt* haplotypes, it suggests that Eastern region of Nepal represents at relatively high antimalarial pressure region and Central region a low antimalarial pressure region. It has been observed that CVIET haplotype is predominant in countries (Africa and Asia) with high prevalence of falciparum malaria and high antimalarial pressure and SVMNT haplotype predominant in countries with low prevalence of falciparum

Table 1
Factors associated with *pfcrt* CVIET and SVMNT haplotypes in Nepal.

Factor	CVIET (%)	SVMNT (%)	Odds ratio	95% CI	*p*-value
Region					
Central	2 (6)	33 (94)	1		
Eastern	19 (63)	11 (37)	28.5	5.70-142.42	<0.001
Median age (years)	32	32			0.384[a]
Gender					
Female	5 (56)	4 (44)	1		
Male	16 (29)	40 (71)	3.1	0.74-13.15	0.120
Body temperature (°C)	36.9	37.3			0.202[a]
Parasitemia per µl[b]	1,431	2,308			0.378[a]
Gametocytes					
No	19 (39)	30 (61)	1		
Yes	2 (12)	14 (88)	0.2	0.04-1.10	0.066
Hemoglobin (g/dl)	10.7	9.9			0.237
Travel history within 1 month					
No	7 (15)	41 (85)	1		
Yes	14 (82)	3 (18)	27.3	6.21-120.36	<0.001
History of malaria within 3 months					
No	18 (31)	40 (69)	1		
Yes	3 (43)	4 (57)	1.6	0.34-8.23	0.531
Taking antimalarial drug before confirmatory diagnosis					
No	14 (27)	37 (72)	1		
Yes	7 (50)	7 (50)	2.6	0.78-8.91	0.117
Forest near the house					
No	15 (31)	33 (69)	1		
Yes	6 (35)	11 (65)	1.2	0.37-3.86	0.759
Water logging near the house					
No	14 (26)	39 (74)	1		
Yes	7 (58)	5 (42)	3.9	1.06-14.31	0.040

[a]Mann-Whitney *U* test
[b]Geometric mean

Table 2
Multivariable analysis of factors associated with *pfcrt* CVIET compared to SVMNT haplotype.

Factor	Odds ratio	95% CI	*p*-value
Eastern region *vs* Central region	10.6	1.79-62.34	0.009
Gametocytes *vs* no gametocytes	0.3	0.05-2.34	0.275
Visited outside within one month *vs* no visit	8.2	1.41-47.34	0.019
Water logging *vs* no water logging near house	2.6	0.35-19.38	0.346

malaria and low antimalarial pressure (South America and Pacific regions) (Mita *et al*, 2009). SVMNT haplotype is predominant in some parts of India with low CQ pressure (Keen *et al*, 2007) and CVIET are predominant in high CQ pressure areas (Valecha *et al*, 2009).

In summary, based on *pfcrt* haplotypes, falciparum malaria in the Eastern region of Nepal was probably due to importation and local transmission, whereas in the Central region falciparum malaria is due to indigenous cases. Thus, there is a need of effective malaria control program in the border areas between India and Nepal.

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