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Amplification of *pfmdr1*, *pfcrt*, *pvmdr1*, and K13 Propeller Polymorphisms Associated with *Plasmodium falciparum* and *Plasmodium vivax* Isolates from the China-Myanmar Border

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Malaria in the China-Myanmar border region is still severe; local transmission of both falciparum and vivax malaria persists, and there is a risk of geographically expanding antimalarial resistance. In this research, the *pfmdr1*, *pfcrt*, *pvmdr1*, and K13-propeller genotypes were determined in 26 *Plasmodium falciparum* and 64 *Plasmodium vivax* isolates from Yingjiang county of Yunnan province. The *pfmdr1* (11.5%), *pfcrt* (34.6%), and *pvmdr1* (3.1%) mutations were prevalent at the China-Myanmar border. The indigenous samples exhibited prevalences of 14.3%, 28.6%, and 14.3% for *pfmdr1* N86Y, *pfcrt* K76T, and *pfcrt* M74I, respectively, whereas the samples from Myanmar showed prevalences of 10.5%, 21.1%, and 5.3%, respectively. The most prevalent genotypes of *pfmdr1* and *pfcrt* were $Y_{86}Y_{184}$ and $M_{74}N_{75}T_{76}$, respectively. No *pvmdr1* mutation occurred in the indigenous samples but was observed in two cases coming from Myanmar. In addition, we are the first to report on 10 patients (38.5%) with five different K13 point mutations. The F446I allele is predominant (19.2%), and its prevalence was 28.6% in the indigenous samples of Yingjiang county and 15.8% in samples from Myanmar. The present data might be helpful for enrichment of the molecular surveillance of antimalarial resistance and useful for developing and updating guidance for the use of antimalarials in this region.

Malaria is a serious public health problem in the Greater Mekong Subregion (GMS), which includes Cambodia, Laos, Thailand, Vietnam, Myanmar, and Yunnan province of China (1). Within this region, malaria transmission is particularly intense in international border areas. The malaria prevalence along the China-Myanmar border is particularly high, and malaria outbreaks have occurred frequently (2). In 2010, China initiated the National Malaria Elimination Action Plan, which aims to eliminate local malaria transmission nationwide by 2015 with the exception of the border region in Yunnan province and to completely eliminate malaria from China by 2020 (3). Despite a great reduction in the number of local malaria cases recently, both *Plasmodium falciparum* and *Plasmodium vivax* infections persist in the counties at the China-Myanmar border.

Effective chemotherapy is essential for malaria control, but the emergence and spread of multidrug-resistant (MDR) *P. falciparum* strains have led to the adoption of artemisinin-based combination therapies (ACTs) as the first-line treatment for uncomplicated falciparum malaria in the GMS. However, the confirmed emergence of artemisinin resistance in western Cambodia is a major threat for malaria control and elimination (4). Artemisinin resistance has since spread or emerged independently or both in other areas of mainland Southeast Asia (5). Because this area has been the origin of both chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) resistance (6, 7), the consequences of a similar spread of artemisinin resistance will be catastrophic. Therefore, the World Health Organization (WHO) is coordinating a large-scale elimination campaign in this region aiming to contain artemisinin resistance (8).

The national drug policy of China was updated in 2006; since then the first-line drugs used to treat uncomplicated falciparum malaria have been ACTs, including dihydroartemisinin-piperaquine (DHA-PPQ), artesunate-amodiaquine (AS-AQ), artimisinin-naphthoquine phosphate (ART-NQ), and artemisinin-piperaquine (ART-PPQ) (9). However, the China-Myanmar border area has the longest history of artemisinin monotherapy; it has been used for more than 3 decades, and *in vitro* studies have detected the reduced susceptibility of *P. falciparum* to artemisinins (10). In the city of Laiza, Myanmar, near the border, *P. falciparum* strains have a certain level of resistance *in vitro* to artesunate and dihydroartemisinin, and the resistance is gradually increasing (11). Moreover, the increasing border migration is a great challenge for the elimination of malaria in this region, and the mobile population remains a risk factor for the spread of ACT resistance (12). Therefore, close surveillance of artemisinin resistance in this area is necessary to detect and deter *P. falciparum* resistance development.

CQ was adopted as the first-line drug for treatment of the blood-stage infection of *P. vivax* in China more than 50 years ago. Although high-level resistance of *P. vivax* to CQ and SP was reported more than a decade ago in the north of Myanmar, the

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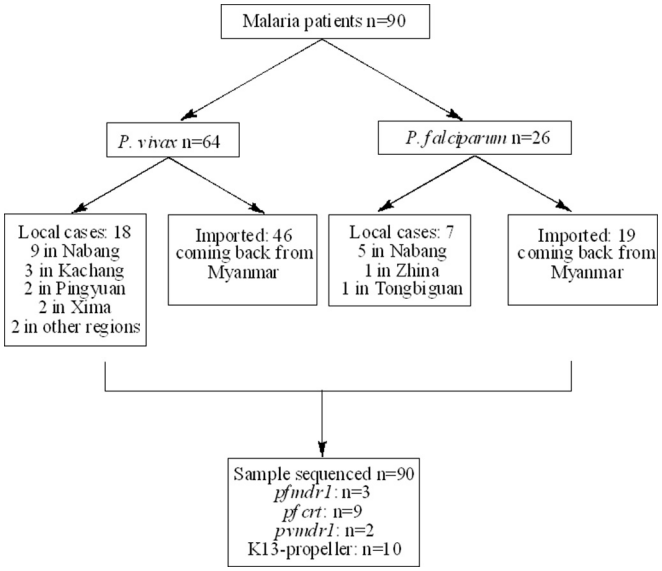

FIG 1 Screening, enrollment, and follow-up of subject patients.

resistance in the border counties of China remains unknown. Therefore, there is an urgent need to monitor drug resistance of vivax malaria in this area.

Several genetic polymorphisms can provide reliable data about the prevalence of drug resistance related *P. falciparum* and *P. vivax*. The *P. falciparum* chloroquine resistance transporter gene (*pfcrt*) T76 mutation and multidrug resistance 1 gene (*pfmdr1*) Y86 mutation have been linked to chloroquine and amodiaquine resistance (13, 14). Similarly, the *P. vivax* multidrug resistance 1 gene (*pvmdr1*) F976 mutation, which was shown to be associated with reduced susceptibility to chloroquine, was selected to evaluate the resistance of *P. vivax* (15). As for artemisinin resistance, the slowly clearing infections were strongly associated with single point mutations in the "propeller" region of the *P. falciparum* kelch protein gene on chromosome 13 (*kelch13*) (16). Recently, the WHO stated that artemisinin resistance should be suspected when ≥5% of patients carry K13 resistance-associated mutations (17).

Here we report an assessment of antimalarial resistance marker polymorphisms including *pfmdr1*, *pfcrt*, *pvmdr1*, and K13 propeller in samples collected from the China-Myanmar border region. The results might provide basic evidence for further molecular surveillance of drug-resistant *P. falciparum* and *P. vivax* strains in this region.

MATERIALS AND METHODS

Study site. The study was conducted in Yingjiang county, one of the 18 counties at the China-Myanmar border, in western Yunnan province. It has a long borderline of 214.6 km with the Kachin state of Myanmar, which is a tier II area according to the Global Plan for Artemisinin Resistance Containment (GPARC) by the WHO (17). The population of Yingjiang county is 307,960, and cross-border trade, logging, quarry, and plantation activities are frequent. In 2013, there were 72 malaria cases, including 18 indigenous cases in this county, which accounted for 21.2% of the total indigenous cases in China.

Sample collection. The blood samples at enrollment from confirmed malaria cases were collected from January 2013 to July 2014 in Yingjiang county. Approximately 200 μl of finger-prick blood was spotted on a piece of Whatman filter paper (3MM) and air dried. The samples were labeled with study numbers, names, and dates and stored at −20°C until DNA extraction.

Preparation of DNA template from blood samples. Parasite genomic DNA from all blood spot samples collected in microcentrifuge tubes was extracted by use of a QIAamp DNA blood kit (Qiagen, Valencia, CA), following the dried blood spot protocol provided in the kit. The known polymorphisms *pfmdr1* and *pfcrt* were assessed. Also, we investigated the mutation of the *PF3D7_1343700* kelch propeller domain (PF13_0238, also called K13 propeller), a molecular marker of artemisinin resistance. Sequences were evaluated using nested PCR followed by restriction fragment length polymorphism (RFLP) analysis, as described previously (14, 18). The *pvmdr1* single nucleotide polymorphisms (SNPs) at 976 were detected using a DNA template mismatch primer method (19). Polymorphisms were analyzed by Shanghai DNA BioTechnologies Co., Ltd. (Shanghai, China). Sequences were analyzed by the BLAST program (http://blast.ncbi.nlm.nih.gov/). Multiple nucleotide sequence alignments and analysis were performed using the BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Data analysis. Data were analyzed using Microsoft Excel 2003 and SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). The map was created by using ArcGIS 10.1 (Environmental Systems Research Institute, Inc.). The Fisher exact test was used to assess the differences in the gene polymorphisms between indigenous cases and cases from Myanmar. The *P* values were calculated, and results were considered statistically significant when $P < 0.05$.

Ethical considerations. The study was reviewed and approved by the ethical committee of the Chinese Centre for Disease Control and Prevention (China CDC).

RESULTS

Study samples. A total of 90 malaria cases were included in this study: 64 *P. vivax* and 26 *P. falciparum*. The *P. vivax* cases were composed of 18 indigenous cases and 46 cases from Myanmar, while the *P. falciparum* cases were composed of 7 indigenous cases and 19 cases from Myanmar (Fig. 1).

***pfmdr1*.** The *pfmdr1* gene was sequenced successfully in 3 isolates of all *P. falciparum* samples (11.5%, 3/26) that covered codons 86 and 184. Among all of the mutational types, no N1042D, S1034C, and D1246Y mutations were found. Mutations at codon N86Y (11.5%, 3/26) were common; $Y_{86}Y_{184}$ was the most prevalent (66.7%, 2/3) of all haplotypes (Tables 1 and 2). Further, one patient harboring the N86Y mutation was found in Nabang, China; the other two patients with this mutation were from Myanmar ($P = 1.0000$) (Table 1).

***pfcrt*.** Sequencing of the *pfcrt* gene was successful in 9 isolates (34.6%, 9/26) that covered codons 74, 75, and 76. Of all three mutated codons, K76T was the most prevalent (23.1%, 6/26) (Table 1). Two patients with the mutated genotype K76T were ob-

TABLE 1 Selection of *P. falciparum* and *P. vivax* polymorphisms

SNP	In China			From Myanmar			
	No.	Total no.	%	No.	Total no.	%	*P*
pfmdr1 N86Y ($n = 3$)	1	7	14.3	2	19	10.5	1.0000
pfmdr1 Y184F ($n = 1$)	0	7	0	1	19	5.3	NS[a]
pfcrt M74I ($n = 2$)	1	7	14.3	1	19	5.3	0.4738
pfcrt N75E ($n = 2$)	0	7	0	2	19	10.5	NS
pfcrt K76T ($n = 6$)	2	7	28.6	4	19	21.1	1.0000
pvmdr1 Y976F ($n = 2$)	0	18	0	2	46	4.3	NS

[a] NS, not significant.

TABLE 2 Prevalence of genotypes of candidate genes *pfmdr1*, *pfcrt*, and *pvmdr1*

Candidate gene	Prevalence (% [no.])	In China		From Myanmar	
		Genotype[b]	Proportion (%)	Genotype[b]	Proportion (%)
pfmdr1 ($n = 3$)	11.5 (3/26)	$\mathbf{Y}_{86}Y_{184}$	33.3	$\mathbf{Y}_{86}\mathbf{F}_{184}$	33.3
				$\mathbf{Y}_{86}Y_{184}$	33.3
pfcrt ($n = 9$)	34.6 (9/26)	$\mathbf{I}_{74}N_{75}K_{76}$	11.1	$\mathbf{I}_{74}N_{75}K_{76}$	11.1
		$M_{74}N_{75}\mathbf{T}_{76}$	22.2	$M_{74}N_{75}\mathbf{T}_{76}$	33.4
				$M_{74}\mathbf{E}_{75}K_{76}$	11.1
				$M_{74}\mathbf{E}_{75}\mathbf{T}_{76}$	11.1
pvmdr1 ($n = 2$)	3.1 (2/64)	ND[a]	ND	$\mathbf{Y}_{976}\mathbf{F}$	100.0

[a] ND, no mutated genotype detected in *pvmdr1* in the local samples.
[b] Mutations are in bold type.

served in Nabang, China; the other four patients with K76T were from Myanmar ($P = 1.0000$). Four different *pfcrt* genotypes were found, among which $M_{74}N_{75}T_{76}$ was the most common (55.6%, 5/9) (Table 2). One patient harboring $I_{74}N_{75}K_{76}$ was detected in Tongbiguan, China, and the other one was returning from Myanmar ($P = 0.4738$). Another two haplotypes, including $M_{74}E_{75}K_{76}$ and $M_{74}E_{75}T_{76}$, were all in patients from Myanmar.

***pvmdr1*.** Regarding *pvmdr1*, 64 *P. vivax* samples were assayed. Only 2 patients (3.1%, 2/64) harbored the Y976F allele, and the patients were returning from Myanmar (Table 2).

K13 propeller. To investigate the K13 propeller polymorphism, all 26 *P. falciparum* samples were assayed and sequenced. Ten patients (38.5%, 10/26) with five different point mutations, including two reported mutation sites and three unreported mutation sites, were observed (Table 3). Of all five nonsynonymous mutations, F446I was more frequent (19.2%, 5/26) than the others, and its prevalence was 28.6% (2/7) in the indigenous samples in Yingjiang county and 15.8% (3/19) in samples from Myanmar. Two out of five patients harboring the mutated condon F446I were found in Nabang, China, and one of these was found as a double-mutated allele of *pfmdr1* N86Y and K13 propeller F446I. The other three patients with mutated F446I were returning from Myanmar ($P = 0.5875$). In addition, one patient with the mutated condon A676D was observed in Zhina, China. The other four patients with other mutated condons were all returning from Myanmar.

DISCUSSION

Yunnan province is located in southern China, and malaria is one of the most important public health problems (20). The incidence of malaria transmission is more severe in the China-Myanmar border counties. The total number of malaria cases in Yunnan province according to the annual reported data was 576 in 2013, including 460 *P. vivax* cases, 106 *P. falciparum* cases, and 10 cases of other species. Most of them ($n = 463$, 80.4%) were observed in the 18 China-Myanmar border counties. The situation in Yingjiang county was the most severe, with an incidence rate of 2.3 per 10,000 people, and 18 local transmission cases were reported, representing 21.2% of the total local cases in China, with 54 other cases imported from Myanmar. Based on these facts, it was selected as the study site.

Resistance to antimalarial drugs has been a long-standing problem in the GMS. MDR *P. falciparum* strains that have emerged in the Thai-Cambodian border region, as well as the emerging resistance to chloroquine (CQ), to sulfadoxine-pyrimethamine (SP), and then to mefloquine (MQ), are gradually spreading in the tropical world (21). Malaria in the China-Myanmar border region is a topic of regional and national public health concern. The development and spread of MDR *P. falciparum* have led to the adoption of artemisinin-based combination therapy (ACT) as the first-line treatment for uncomplicated *P. falciparum* malaria in this region to improve treatment outcomes. However, widespread artemisinin resistance has been observed in the GMS (4, 22); this poses a great threat of resistant parasites being brought into China with the migrant population, immediately affecting therapy efficacy. In the China-Myanmar border area, artemisinins have been used for more than 30 years, mostly as monotherapies prior to 2005. Earlier *in vitro* assays had already detected a trend of declining sensitivity to artemisinins in the border area of Yunnan province (23, 24). Therefore, an understanding of whether artemisinin-resistant parasites have spread to the neighboring regions or emerged elsewhere in this area is essential for coordinating containment efforts.

In addition, *P. vivax* malaria persists in areas of Yunnan province along the China-Myanmar border (25), and CQ was adopted more than 50 years ago in China as the first-line drug used for treatment of the blood-stage *P. vivax* infection. Although high-level resistance of *P. vivax* to CQ and SP was reported more than a decade ago in the north of Myanmar (26, 27), the resistance in the border counties of China remains unknown.

The malaria parasite encodes many transporters, and some of them such as *pfmdr1* and *pfcrt* have been strongly connected with antimalarial drug resistance (28). The *pfmdr1* genotype is correlated with resistance of *P. falciparum* to CQ, MQ, and artemisinins, whereas knockdown of *pfmdr1* expression leads to increased susceptibility (29). The N86Y *pfmdr1* mutation that confers CQ resistance is also associated with decreased sensitivity to artemis-

TABLE 3 Polymorphisms observed in the K13 propeller in *P. falciparum* isolates

Codon position	Amino acid reference	Nucleotide reference	Amino acid mutation	Nucleotide mutation[a]	Prevalence (% [no.])	Location(s)
446 ($n = 5$)[b]	F	Ttt	I	**A**tt	19.2 (5/26)	2 in China, 3 from Myanmar
511 ($n = 2$)[b]	I	Ata	M	at**G**	7.7 (2/26)	Myanmar
537 ($n = 1$)	N	Aat	I	a**T**t	3.8 (1/26)	Myanmar
574 ($n = 1$)	P	Cct	L	c**T**t	3.8 (1/26)	Myanmar
676 ($n = 1$)[b]	A	Gcc	D	gAc	3.8 (1/26)	China

[a] Mutations are in bold type.
[b] A mutated site which was not reported before.

inins (18). In Thailand and Cambodia, the *pfmdr1* gene has become increasingly prevalent in field parasite populations and was responsible for the declining efficacy of ACTs (30, 31). In our assay, two mutated *pfmdr1* alleles, N86Y and Y184F, were observed, and one patient with a $Y_{86}Y_{184}$ amplification was found in the port city of Nabang, China. These findings are contradictory to those of Wang et al., who reported that *pfmdr1* N86Y had not been observed in this region (32). The possibility that patients take antimalarials with insufficient compliance cannot be excluded, although MQ was not frequently used in this region (33). Moreover, we have found one patient with the double mutation of *pfmdr1* N86Y and K13 propeller F446I, which may further indicate significance of *pfmdr1* mutations in artemisinin resistance. This uncommon *pfmdr1* polymorphism from this region offers opportunities for investigations of the mechanisms of artemisinins.

The *pfcrt* K76T mutation has been widely used as a reliable marker for CQ resistance, and it was found to have a high prevalence in China (34). Furthermore, it also influences *P. falciparum* susceptibility to MQ, halofantrine, and artemisinin (18). This is consistent with our study showing that *pfcrt* K76T played a predominant role in the *pfcrt* genotypes. Molecular assays have shown high prevalence of the $M_{74}N_{75}T_{76}$ (55.6%) genotype, which was similar to the findings of Huang et al. (35). No significant difference was observed between the local samples from Yingjiang county and those from Myanmar, since migration occurs in both directions. Despite the fact that China has not used CQ to treat *P. falciparum* infections for more than 30 years, the stable and high prevalence of this mutation may be a result of the continued use of CQ as a first-line drug for *P. vivax* infection over several decades. Another factor that may contribute to the high prevalence of *pfcrt* K76T is the use of CQ as a first-line drug for *P. vivax* infection for several decades in Myanmar, especially in the Myanmar-Thailand border area, where a high prevalence of *pfcrt* K76T was found, thus suggesting that the natural selection against CQ pressure for the maintenance of the *pfcrt* mutation in *P. falciparum* is still retained in the region (36, 37). However, we also found three other haplotypes ($I_{74}N_{75}K_{76}$, $M_{74}E_{75}K_{76}$, and $M_{74}E_{75}T_{76}$) in our study, suggesting that selection of other *pfcrt* haplotypes was still needed.

Resistance to common antimalarial drugs has been reported for *P. vivax* in the GMS, including Myanmar and Vietnam, and also in Indonesia (38–40). A trend for a gradual decline in the *in vitro* sensitivity of this parasite to CQ had also been reported during 2005 to 2008 around the China-Myanmar border and in central China (41, 42). Our findings revealed that the presence of the *pvmdr1* Y976F mutation in Yingjiang county in China is consistent with previous reports of declining sensitivity to CQ; this was also observed in Myanmar and Xishuangbanna (Yunnan province), which exhibit high frequencies of the *pvmdr1* Y976F allele (43, 44). The long history of CQ use and the frequent population movement across the borders may contribute to the CQ-resistant *P. vivax* strains detected in Yingjiang county. Close surveillance at sentinel sites in this region should continue so that the emergence and spread of *P. vivax* resistance can be carefully monitored.

Artemisinin and its derivatives have been used for falciparum malaria treatment in China since the late 1970s (45). *In vitro* assays showed that the susceptibility of *P. falciparum* to artemisinins was declining in China, but no evidence of the artemisinin resistance has been detected (46). In our study, five nonsynonymous mutations were found, and three of them were not reported previously. Furthermore, our study showed that F446I was the predominant allele (19.2%, 5/26), and two cases with this mutation were found in the port city of Nabang, China. Another mutation, K13 propeller A676D, was also observed in Zhina, China (Fig. 2). However, the C580Y allele, which was widely found in Cambodia (16), was not found in this study. Nevertheless, our results indicate that the mutated K13 propeller gene alleles exist in the China-Myanmar border area, and their presence should raise concerns regarding the risks of emerging artemisinin resistance in the GMS. We recommend further clinical trials associated with K13 propeller mutations, which might be useful for identifying additional genetic loci involved in monitoring the threat of artemisinin resistance.

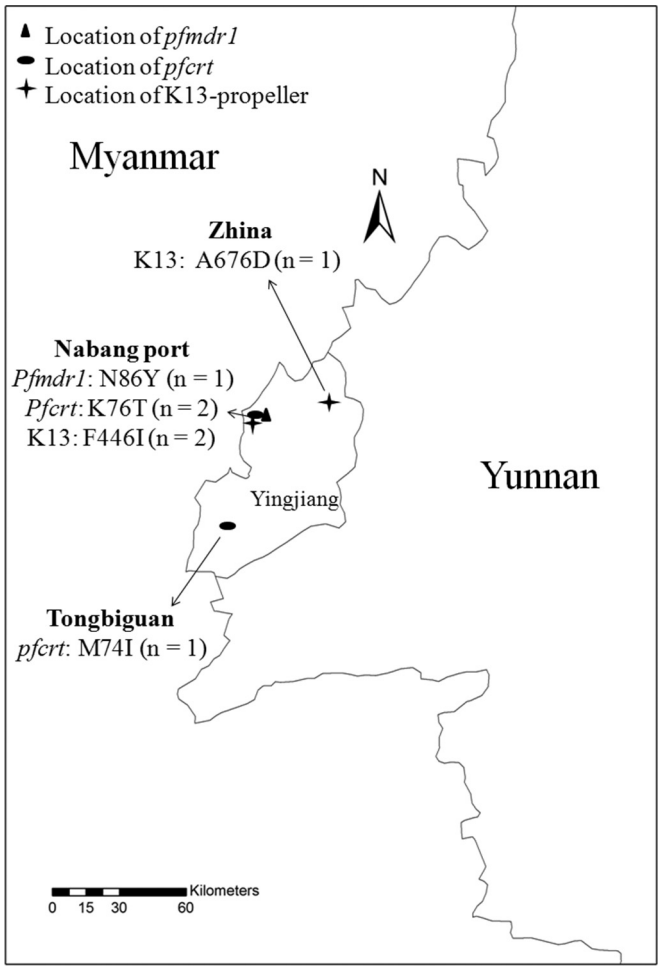

FIG 2 Location of antimalarial resistance marker polymorphisms in Yingjiang county. The numbers of mutated samples are shown in parentheses. The map was created by using ArcGIS 10.1 (Environmental Systems Research Institute, Inc.).

The prevalence of the K13 propeller polymorphism detected in Yingjiang county indicates that ACTs should be used in the China-Myanmar border area, and rational use of antimalarials against *P. falciparum* strains imported from Southeast Asia should be adopted. In addition, routine monitoring and surveillance, as recommended by the WHO Global Plan for Artemisinin Resistance Containment, should continuously be strengthened. Additional clinical investigations to complement sentinel surveillance, in-

cluding either analysis of the drug markers or risk factors or new approaches to monitor resistance, are required.

In conclusion, the present data might be helpful for enrichment of molecular surveillance of antimalarial resistance and for developing and updating guidance for the use of antimalarials in the region.

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