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High prevalence of Sulfadoxine/pyrimethamine resistance alleles in *Plasmodium falciparum* parasites from Bangladesh

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A list of abbreviations

CQ; chloroquine, SP; sulfadoxine/pyrimethamine, PCR; polymerase chain reaction

Abstract

In Bangladesh, despite the official introduction of artemisinin combination therapy in 2004, chloroquine + sulfadoxine/pyrimethamine has been used for the treatment of uncomplicated malaria. To assess the distribution of *pfcrt*, *pfmdr1*, *dhfr*, and *dhps* genotypes in *Plasmodium falciparum*, we conducted hospital- and community-based surveys in Bandarban, Bangladesh (near the border with Myanmar) in 2007 and 2008. Using nested PCR followed by digestion, 139 *P. falciparum* isolates were genotyped. We found fixation of a mutation at position 76 in *pfcrt* and low prevalence of a mutation at position 86 in *pfmdr1*. In *dhfr*, a quadruple mutant which is the highest pyrimethamine-resistant genotype was found in 19% of isolates, which is a significantly higher prevalence than reported in a previous study in Khagrachari (1%) in 2002. Microsatellite haplotypes flanking *dhfr* of the quadruple mutants in Bangladesh were identical or very similar to those found in Thailand and Cambodia, indicating a common origin for the mutant in these countries. These observations suggest that the higher prevalence of the *dhfr* quadruple mutant in Bandarban is because of parasite migration from Myanmar. However, continuous use of sulfadoxine/pyrimethamine would have also played a role through selection for the *dhfr* quadruple mutant. These results indicate an urgent need to collect molecular epidemiological information regarding *dhfr* and *dhps* genes, and a review of current sulfadoxine/pyrimethamine usage with the aim of avoiding the widespread distribution of high levels of resistant parasites in Bangladesh.

Keywords: *Plasmodium falciparum*, sulfadoxine/pyrimethamine, *dhfr*, *dhps*, Bangladesh, microsatellite

Introduction

The spread of drug resistance is one of the major challenges of combating malaria in endemic regions. Until recently, many countries used sulfadoxine/pyrimethamine (SP) either as a monotherapy or in combination with other antimalarials because of the increase of chloroquine (CQ) resistance. However, *Plasmodium falciparum* has already exhibited resistance to SP in most Asian, South American, and African countries where it has been intensively used [1].

A genetic cross-analysis has shown that CQ resistance is associated with a point mutation from lysine to threonine at position 76 (K76T) in *pfcrt* [2]. A substitution from asparagine to tyrosine at position 86 (N86Y) in *pfmdr1* has been also reported to play some role in chloroquine resistance [3, 4]. Resistance to SP is associated with mutations in the dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes. In *dhfr*, point mutations at positions 51, 59, 108, and 164 are associated with pyrimethamine resistance [5-7]. The first mutation exclusively occurs at position 108, while additional mutations at other positions are associated with a stepwise increase of pyrimethamine resistance. Importantly, isolates harboring mutations at these four positions (IRNL at positions 51, 59, 108, and 164 with mutations underlined) show the highest pyrimethamine resistance [7]. Similarly, in *dhps*, mutations at positions 436, 437, 540, 581, and 613 are linked with sulfadoxine resistance in a stepwise manner [8, 9].

In Bangladesh, 84,690 confirmed malaria cases were reported in 2008, more than 80% of which were reported from the greater Chittagong Hill Tracts and neighboring Cox's Bazar districts [10]. The predominant species is *P. falciparum* at 75% of cases [10]. A combination of CQ and primaquine had been used as a first-line antimalarial for the treatment of uncomplicated malaria. SP was prescribed as a second-line treatment in combination with

quinine. However, the level of adequate clinical response to CQ was reported as only 34% in 1996-7 [11]. Similarly, failure of SP + quinine treatment also reached unacceptable levels (17%) by 2002 [12]. In 2004, artemisinin combination therapy (ACT) was officially introduced as the first-line treatment for confirmed *P. falciparum* cases. In the later half of 2007, it has been implemented in the health facilities in rural areas. However, despite the high prevalence of resistance, CQ + SP has been adopted in the national guidelines for treating presumptive malaria cases.

To date, only one molecular epidemiological study has been reported in Bangladesh, and it found that the highest pyrimethamine-resistant genotype, the *dhfr* quadruple mutant, was of considerably low prevalence (1%) in Khagrachari in 2002 [12]. Herein, we report the distribution of *dhfr, dhps, pfcrt*, and *pfmdr1* genotypes in *P. falciparum* isolates from Bandarban, where SP and CQ have been used in the rural area. Bandarban is located about 200 km south from the previous study site, Khagrachari. Khagrachari shares a border with India, whereas Bandarban borders Myanmar, where antimalarial-resistant parasites are widely distributed. In these geographical settings, we also considered how migration has affected the distribution of *dhfr*, *dhps*, *pfcrt*, and *pfmdr1* genotypes.

Materials and Methods

2.1 Study site

A hospital-based survey was conducted at Bandarban district hospital from October to December 2007 (Figure 1). We also carried out a community-based survey at six malaria-endemic villages in Bandarban in March 2008; namely, Sultanpur, Chemidalupara, Kyaching ghata natun para, Saingya daneshpara, Faruqpara, and Empupara. Bandarban is a hilly district located in the eastern part of the country bordering Myanmar. The majority Bengalis and an additional 11 different indigenous ethnic minorities live in this district. A majority of the inhabitants are farmers, forest workers, daily workers in development projects, and rubber plantation workers. In Bandarban, malaria is moderately endemic with perennial but seasonally intense transmission during pre- and post-monsoon periods (May-July and September-October). *P. falciparum* (75%) and *P. vivax* (25%) are the two most prevalent parasites reported from this district [10]. *P. malariae* has also been occasionally found. The principal vectors are *Anopheles dirus*, *A. minimus*, and *A. phillipinensis.*

2.2 Patients

In both hospital- and community-based surveys, patients were screened by a rapid diagnostic test using paracheque F (Orchid Biomedical System, Verna, Goa, India) and samples were also microscopically examined by two expert microscopists. Seventy-five microliters of blood were collected from all enrolled study subjects by heparinized capillary tube, and blotted onto 31ETCHR filter paper (Whatman International Ltd., Kent, UK). Blood on the filter papers was dried at room temperature and then transported to the Tokyo

Women's Medical University for molecular analysis. In the hospital-based survey, patients were mainly treated with Artemether + Lumefantrine or quinine for 7 days. In cases of severe malaria, intravenous administration of quinine was used. In the community-based survey, *P. falciparum* cases were treated with quinine for 7 days, while CQ + primaquine was used for *P. vivax* cases.

Before enrollment, written informed consent was obtained from all study subjects. In the case of children, consent was obtained from a legal guardian. This study was approved by the Bangladesh Medical Research Council and the local health regulatory body in Bandarban, Bangladesh.

2.3 Species-specific PCR and genotyping of dhfr, dhps, pfcrt, and pfmdr1

Parasite DNA was extracted from a quarter of the blood blotted on the filter paper using a DNA extraction kit (QIAGEN QIAmp DNA Mini Kit Catalog 51304, Germany) according to the manufacturer's instructions. To confirm the species identification, species-specific PCR was carried out on all positive and suspected cases as previously described [13]. Polymorphisms in *dhfr* at positions 51, 59, 108, and 164, and in *dhps* at positions 436, 437, 540, 581, and 613 were assessed by nested PCR followed by restriction digestion as described by Duraisingh et al. [14]. In *pfcrt*, the K76T polymorphism was assessed as described by Djimdé et al. [15]. In *pfmdr1*, polymorphisms at positions 86, 184, 1034, 1042, and 1246 were assessed by nested PCR, followed by restriction digestion as described by Duraisingh et al. [4].

2.4 Microsatellite haplotypes flanking dhfr

In isolates harboring the quadruple mutant in *dhfr*, nucleotide length variations (determined by the number of TA repeats) of microsatellite markers were determined to infer the origin of the mutants. We measured three microsatellite markers located at 0.1 kb and 3.87 kb upstream and 1.48 kb downstream of the *dhfr* locus as described by Nair et al. [16]. In brief, semi-nested PCR was performed using fluorescent end-labeled primers. Size variations of the amplified products were determined by electrophoresis on an ABI 377 and analyzed with Genescan software (Applied Biosystems, CA, USA). In the event of two or more polymorphisms being detected, we considered these isolates as mixed infections. The microsatellite haplotypes of samples from the present study were compared to those we previously reported in isolates harboring a *dhfr* quadruple mutant in Thailand and Cambodia [17].

2.5 Statistical analysis

Chi-square test, Fisher's exact test, and Student's t-test were carried out using JMP Ver 8 (SAS Institute Inc., NC, USA). Probability (P) values of less than 0.05 were considered to be statistically significant.

Results

3.1 Patients' background

In the community-based survey at six villages, malaria infection was found in 2.9% (30/1043) of individuals. These positive cases were confirmed by species-specific PCR: *P. falciparum* (n = 11), *P. vivax* (n = 16), and *P. falciparum* + *P. vivax* (n = 3). In the hospital-based survey, agreement to participate in this study was secured from 132 malaria-infected patients. Infection in these cases was also confirmed by species-specific PCR: *P. falciparum* (n = 128), *P. vivax* (n = 2), and *P. falciparum* + *P. vivax* (n = 2). Thus, a total of 139 *P. falciparum* blood samples were used for the molecular analysis (Table 1).

3.2 Mutations in dhfr, dhps, pfcrt, and pfmdr1

We did not find any significant differences in genotype prevalence in *dhfr, dhps, pfcrt,* and *pfmdr1* between the hospital- and community-based surveys (Supplementary Table 1), and thus the two groups were combined for subsequent analysis. A mixture of wild-type and mutant alleles was observed within some infections at positions 51 (n = 6) and 164 (n = 15) in *dhfr*, position 76 in *pfcrt* (n = 10), and positions 86 (n = 15) and 184 (n =5) in *pfmdr1*. These were excluded from subsequent analysis. In *dhfr*, a mutation at position 108 (S108N), which is believed to be the initial mutation for pyrimethamine resistance, was observed in all studied isolates (Table 2). I164L, the mutation that confers the highest pyrimethamine resistance, was detected in 21.8% of studied isolates. In *dhps*, 83.4% of the study isolates carried a mutation at position 437, which has been reported as the initial mutation for sulfadoxine resistance in many endemic regions. In *pfcrt*, all isolates harbored the K76T mutation. In *pfmdr1*, a

mutation at position 86 (N86Y) only was detected in 23.4% of isolates. No mutation was detected at positions 1034, 1042, or 1246.

3.3 Genotypes of dhfr, dhps, and pfmdr1

In *dhfr*, we detected six mutant genotypes: NCNI, NRNI, ICNI, NRNL, IRNI, and IRNL (amino acids at positions 51, 59, 108, and 164, mutations underlined) (Table 3). Nearly 50% of isolates were double mutants and most of these were NRNI. The other double mutant, ICNI, was found in only two isolates. Among the two types of triple mutants, IRNI was the major genotype (26.9%). The other triple mutant (NRNL) was found at a prevalence of 3.4%. The quadruple mutant (IRNL) that confers the highest level of pyrimethamine resistance was found at a prevalence of 19.3%. Only one isolate was a single mutant (NCNI). None of the samples harbored the wild genotype. In *dhps*, we detected nine distinct genotypes: three forms of triple mutant (AGEAA, SGEGA, and AGKGA (amino acids at positions 436, 437, 540, 581, and 613, mutations underlined), three forms of double mutant (AGKAA, SGEAA, and SGKGA), two forms of single mutant (AAKAA and SGKAA), and the wild-type SAKAA (Table 3). Nearly 50% of the isolates were triple mutants. The most prevalent triple mutant was AGEAA (37.4%), followed by SGEGA (8.6%). Among the three forms of double mutant, the two major genotypes were SGEAA (15.9%) and SGKGA (10.8%). The wild-type was detected in 15.9% of the study isolates. In *pfmdr1*, we detected three genotypes: YYSNN, NFSNN, and NYSNN (amino acids at positions 86, 184, 1034, 1042, and 1246, mutations underlined). Wild-type NYSNN was the most prevalent (63.7%).

3.4 Microsatellite haplotypes flanking dhfr in quadruple mutants

We successfully determined microsatellite haplotypes flanking *dhfr* in 21 isolates harboring *dhfr* quadruple mutants in Bangladesh (Table 4). Only two haplotypes were observed: 194/176/106 (n = 20) and 194/176/108 (n = 1). These were compared to our previous results in Cambodia and Thailand [17]. In all three countries, all parasites harboring the *dhfr* quadruple mutant shared identical or very similar microsatellite haplotypes, suggesting a common origin of the *dhfr* quadruple mutant.

Discussion

In the present study, in Bandarban, Bangladesh, the highest pyrimethamine-resistant genotype of *dhfr*, the quadruple mutant, was found in 19% of isolates. This is significantly higher than reported in a previous study in Khagrachari in 2002 (1%) [12]. It is possible that the high prevalence of the *dhfr* quadruple mutant in Bandarban is due to selection for this mutant because of continuing SP usage. Although ACT was officially introduced as the first-line treatment for confirmed *P. falciparum* cases in Bangladesh in 2004, it did not become widely available in Bandarban until the later half of 2007, just before the present survey. In addition, an antimalarial combination of CQ and SP is still widely used for treatment of presumptive *P. falciparum* cases. These two antimalarials are also easily available at many retail stores without a prescription. Thus, continuous SP use in Bandarban might have played a role in the high prevalence of the quadruple mutant.

In addition to selection due to SP use, parasite migration from Myanmar to Bandarban may also have contributed the high prevalence of the *dhfr* quadruple mutant in Bandarban. It has recently been indicated that *dhfr* quadruple mutants have had a single origin in Southeast Asia, putatively at the Thai/Cambodia border, and then spread to other Southeast Asian endemic regions [16, 17]. We found identical or very similar patterns of microsatellite haplotypes flanking *dhfr* among isolates from Bandarban, Thailand, and Cambodia, strongly suggesting a common origin of the quadruple mutants in these regions. At present, the prevalence of *dhfr* quadruple mutants is highest in Thailand (80%) followed by Cambodia (40%) [16, 17]. In Myothugy, on the western border of Myanmar, 29% of isolates harbored this genotype [16]. Although most of the malaria-endemic districts in Bangladesh, including Khagrachari, border with India, Bandarban borders Myanmar. Recently, mass human migration has occurred from Myanmar to Bangladesh; i.e., Rohingya refugees fleeing to

Bandarban and Cox's Bazar. Thus, these findings support the hypothesis of gene flow in the parasites from Thai/Cambodia to Bandarban, putatively through Myanmar.

The prevalence of mutant *dhps* alleles in the present study (84%) is similar to that in Myanmar in 2000 (>75%) [18], but higher than that in India (36% in 2003-4) [19]. This might also be explained by gene flow of resistant parasites from Myanmar to Bangladesh, and then to India. However, in Bandarban, high sulfadoxine-resistant triple mutant genotypes of *dhps* were found in 47% of isolates, which is lower than in Thailand (nearly 90%), Cambodia (70%), and Myanmar (55-60%) [18]. If SP is widely and continuously used in Bandarban, the prevalence of sulfadoxine-resistant genotypes will eventually be similar to that observed in the high SP-resistant countries.

The K76T mutation in *pfcrt* has already been fixed in Bandarban, and the prevalence is similar to that found in a previous study in Khagrachari (94%) [12]. This is consistent with the high prevalence of both *in vitro* (84%) and *in vivo* (66%) resistance to CQ previously reported from Bangladesh [11, 20]. A similar fixation of the *pfcrt* K76T mutation is also observed in neighboring countries like India, Myanmar, and Thailand [18, 21].

In Bandarban, the prevalence of the *pfmdr1* N86Y mutation was 24%, which is lower than in Khagrachari in 2002 (70%) [12], and lower than in Assam in India, which is about 400 km north of the present study site (66% in 2000-1 and 2003-4 [22] and 69% in 2006 [23]). However, these studies cannot be directly compared because of differences in time and location. The N86Y mutation plays a role in CQ resistance to some extent [24, 25]. In addition, it has been reported that complete withdrawal of CQ usage in the community can lead to decreased prevalence of the N86Y mutation [26]. However, in Bandarban, CQ was still widely used in the community at the time of the present study. In contrast to the relationship with CQ, the N86Y mutation is linked to increased sensitivity to artemisinin [4, 27]. In Zanzibar, after treatment with Artemether + Lumefantrine, the prevalence of the N86Y

mutation significantly decreased from 83% in pre-treatment patients to 56% in recurrent patients [27]. However, at the time of the present study, ACT was not widely-distributed in Bandarban, and thus ACT could not have strongly influenced the observed low prevalence of the N86Y mutation. Increased susceptibility to mefloquine has also been associated with the N86Y mutation [4, 28, 29]. In Bangladesh, a high prevalence (61%) of *in vitro* resistance to mefloquine was reported from the neighboring district of Cox's Bazar in 1999 [20]. Thus, the high frequency of mefloquine resistance might be associated with the observed low prevalence of the N86Y mutation in the present study. However, in Bangladesh, mefloquine has never been officially registered and has rarely been used for the treatment of CQ resistance cases. Mefloquine resistance has already been observed in Southeast Asian countries where, similar to the present result, the N86Y mutation is less frequently (<30%) observed [18, 30]. Taken together, these findings suggest that mefloquine-resistant parasites have migrated from these regions to Bandarban, Bangladesh. However, the increase in the *pfmdr1* copy number is also a key determinant for mefloquine resistance [31, 32], and thus, determination of the copy number in *pfmdr1* in the present isolates is planned.

In conclusion, we found a high prevalence of the quadruple mutant of *dhfr* in Bandarban. We also detected a low prevalence of the N86Y mutation in *pfmdr1*. Migration of SP- and mefloquine-resistant parasites to Bandarban explains the observed findings. In Bangladesh, SP is still used for uncomplicated presumptive malaria cases. Continued use of SP would accelerate the current progression of drug resistance [33]. Thus, there is an urgent need to collect molecular epidemiological information regarding drug resistance-related genes. The current usage of SP for treatment of presumptive malaria cases also needs to be reviewed.

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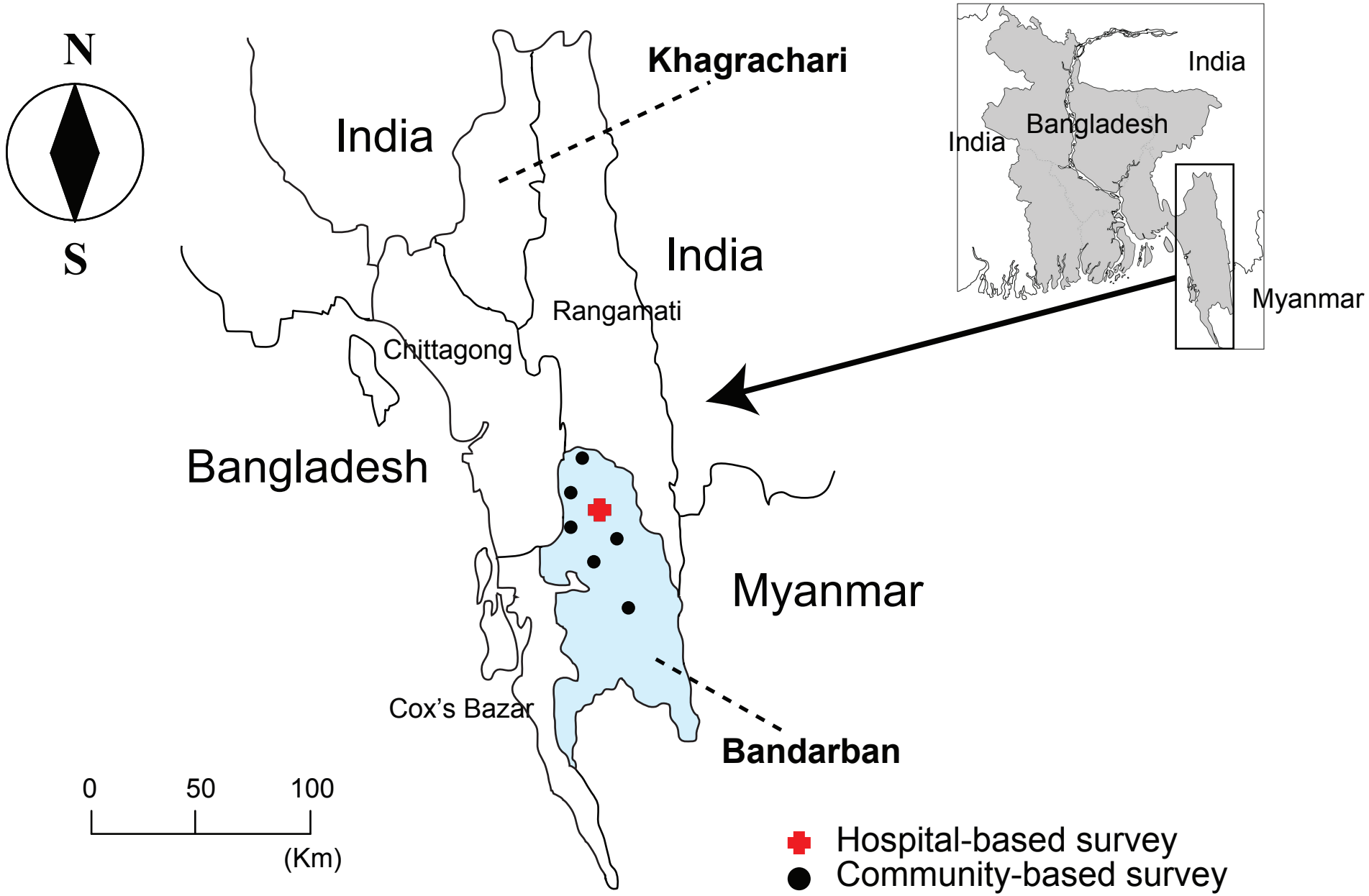
Figure legends

Figure 1. Map of study sites in Bandarban, Bangladesh.

The map shows the greater Chittagong Hill Tracts (Bandarban, Khagrachari, and Rangamati) and Cox's Bazar districts. Bandarban borders Myanmar in the south and southeast. All neighboring districts are malaria-endemic.

Figure 1


N
S
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Rangamati
Chittagong
Bangladesh
Myanmar
Cox's Bazar
Bandarban
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India
Myanmar
0
50
100
(Km)
Hospital-based survey
Community-based survey

Table 1. Charactersistics of *Plasmodium falciparum*- infected patients from hospital-based (n = 128) and community-based (n = 11) studies in Bandarban, Bangladesh

	Hospital (n = 128)	Community (n = 11)
Gender		
Male	86	6
Female	42	5
Age (years)		
<5 years	10	2
5-14 years	33	6
≥15 years	85	3
Splenomegaly (%)	27.2	18.2
Median parasite density (μL^{-1})	27159	6020
(25 percentile, 75 percentile)	(12383, 85970)	(815, 11465)

Table 2. Prevalence of *dhfr* , *dhps* , *pfcrt,* and *pfmdr1* mutations in 139 *P. falciparum* isolates from Bandarban, Bangladesh

Gene	n[a]	Amino Acid (Wild-type/Mutant)	Wild-type (%)	Mutant (%)
dhfr				
51	133	N/I	69 (51.9)	64 (48.1)
59	139	C/R	2 (1.4)	137 (98.6)
108	139	S/N	0 (0.0)	139 (100)
164	124	I/L	97 (78.2)	27 (21.8)
dhps				
436	139	S/A	80 (57.6)	59 (42.4)
437	139	A/G	23 (16.6)	116 (83.4)
540	139	K/E	53 (38.1)	86 (61.9)
581	139	A/G	111 (79.9)	28 (20.1)
613	139	A/S	139 (100)	0 (0.0)
pfcrt				
76	129	K/T	0 (0.0)	129 (100)
pfmdr1				
86	124	N/Y	95 (76.6)	29 (23.4)
184	134	Y/F	118 (88.1)	16 (11.9)
1034	139	S/C	139 (100)	0 (0.0)
1042	139	N/D	139 (100)	0 (0.0)
1246	139	D/Y	139 (100)	0 (0.0)

a; Mixed infections were excluded.

Table 3. Prevalence of *dhfr* , *dhps,* and *pfmdr1* genotypes in *P. falciparum* isolates from Bandarban, Bangladesh

Genes[a]	Genotypes	n (%)
dhfr (n=119)	IRNL[b]	23 (19.3)
	IRNI	32 (26.9)
	NRNL	4 (3.4)
	ICNI	2 (1.7)
	NRNI	57 (47.9)
	NCNI	1 (0.8)
dhps (n=139)	AGEAA[c]	52 (37.4)
	SGEGA	12 (8.6)
	AGKGA	1 (0.7)
	AGKAA	5 (3.6)
	SGEAA	22 (15.8)
	SGKGA	15 (10.8)
	AAKAA	1 (0.8)
	SGKAA	9 (6.5)
	SAKAA	22 (15.8)
pfmdr1 (n=121)	YYSNN[d]	29 (23.9)
	NFSNN	15 (12.4)
	NYSNN	77 (63.7)

a; In *pfcrt* , only position 76 was determined.

b; Amino acids at positions 51, 59, 108, and 164 with mutations underlined

c; Amino acids at positions 436, 437, 540, 581, and 613 with mutations underlined

d; Amino acids at positions 86, 184, 1034, 1042, and 1246 with mutations underlined

Table 4. Microsatellite haplotypes around *dhfr* in *P. falciparum* isolates harboring the *dhfr* quartet mutation from Bangladesh (n = 21), Cambodia (n = 14), and Thailand (n = 44)

Country	Microsatellite haplotype[a]		
	194/176/106	194/176/108	194/178/106
Bangladesh	20	1	0
Cambodia[b]	14	0	0
Thailand[b]	41	0	3

a; size (bp) of microsatellite markers (-0.1 kb/-3.87 kb/+1.48 kb flanking *dhfr*)

b; Data from ref [17].

Suplementary Table 1. *dhfr* , *dhps* and *pfmdr1* genotypes in *Plasmodium falciparum* isolates in community-based (n = 11) and hospital-based (n = 128) survey in Bandarban, Bangladesh

Gene[a]	Genotypes	Hospital	Community
dhfr	IRNL[b]	21	2
(n=119)	IRNI	29	3
	NRNL	4	0
	NRNI	54	3
	ICNI	2	0
	NCNI	1	0
dhps	AGEAA[c]	45	7
(n=139)	SGEGA	12	0
	AGKGA	0	1
	AGKAA	5	0
	SGEAA	21	1
	SGKGA	15	0
	AAKAA	1	0
	SGKAA	8	1
	SAKAA	21	1
pfmdr1	YYSNN[d]	26	3
(n=121)	NFSNN	15	0
	NYSNN	70	7

a; Mixed infections were excluded. In *pfcrt* , position 76 alone was determined.

b; Amino acid at positions 51, 59, 108 and 164 with mutations underlined.

c; Amino acid at positions 436, 437, 540, 581 and 613 with mutations underlined.

d; Amino acid at positions 86, 184, 1034, 1042 and 1246 with mutations underlined.