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Persistence of Markers of Chloroquine Resistance in *Plasmodium falciparum* in Bangladesh

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Abstract. The control of malaria, in terms of drug resistance, remains a significant global challenge, with Bangladesh, a malaria-endemic country, being no exception. The aim of this study was to explore antimalarial resistance in Bangladesh by molecular analysis of *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) and *P. falciparum* multidrug resistance transporter 1 (*pfmdr1*) genetic markers of *P. falciparum*. Samples were obtained from uncomplicated malaria patients between 2009 and 2014 from six malaria-endemic districts. Based on parasite transmission intensity, the endemic districts were divided into high-transmission (Chittagong Hill Tracts [CHT]) and low-transmission (non-CHT) regions. Falciparum malaria-positive isolates were genotyped for K76T of the *pfcrt* gene, and N86Y and Y184F of the *pfmdr1* gene: in total, 262 *P. falciparum* clinical isolates were analyzed. In CHT areas, the prevalence of polymorphisms was 70.6% for 76T, 14.4% for 86Y, and 7.8% for 184F. In non-CHT areas, 76T and 86Y mutations were found in 78.0% and 19.5% of the samples, respectively, whereas no 184F mutations were observed. We compared our data with previous similar molecular observations, which shows a significant decrease in *pfcrt* 76T mutation prevalence. No *pfmdr1* amplification was observed in any of the samples suggesting an unaltered susceptibility to amino alcohol drugs such as mefloquine and lumefantrine. This study provides an updated assessment of the current status of *pfcrt* and *pfmdr1* gene mutations in Bangladesh, and suggests there is persistent high prevalence of markers of resistance to aminoquinoline drugs.

INTRODUCTION

In 2018, *Plasmodium falciparum* contributed to 50% of all reported malaria cases in Southeast Asia (SEA).[1] Southeast Asia has always been an important area for the observation of antimalarial drug resistance, as this region contains the hotspot zone (Greater Mekong Sub-region)[2] for the onset and spread of drug resistance against chloroquine ([CQ] a 4-aminoquinoline antimalarial) monotherapy, as well as to different multidrug combination therapies used in malaria treatment.[3,4] *Plasmodium falciparum* has demonstrated resistance to nearly every antimalarial drug, although in most regions, artemisinin (ART)-based combination therapies (ACTs) offer effective therapy[5]: in the absence of a vaccine, this resistance threatens the WHO Roll Back Malaria action plan in areas of high transmission.[6]

Bangladesh, a malaria-endemic country in SEA, shares international borders with other malaria-endemic countries, such as India and Myanmar. In 2016, it had an annual parasitic index of 2.08/1,000 population from 13 endemic districts.[7] According to the national malaria elimination program (NMEP), there were a total of 107,226 malaria cases between 2015 and 2018 in Bangladesh, 88% of which were associated with *P. falciparum* alone, or in combination with *Plasmodium vivax*.[8] More than 90% of these cases occurred in the high-transmission areas of Bandarban, Khagrachhari, and Rangamati (regionally known as Chittagong Hill Tracts [CHT]),[8–10] and other cases have been reported from non-CHT (low-transmission)[8] areas.

In malaria-endemic countries, the WHO recommends ACTs as the frontline treatment for uncomplicated malaria.[11] The ACT, artemether–lumefantrine (AL), has excellent efficacy in the SEA region[12] and has been recommended as the firstline treatment in Bangladesh since 2004.[13,14] The fast-acting component of ACTs, ART, or its derivatives dihydroartemisinin (DHA), artesunate, and artemether, works in combination with various partner drugs, such as amino alcohols (lumefantrine [LUM] and mefloquine [MQ]), 4-aminoquinolines (amodiaquine, piperaquine, and pyronaridine), and a sulfa-antifolate combination (sulfadoxine–pyrimethamine).[15] The first documentation of ART resistance was reported in western Cambodia[16]; this was followed by the discovery of multiple *kelch*13 gene mutations,[17] which eventually spread as well as emerged independently to the rest of mainland SEA.[18] Failure of ART treatment is defined by delayed parasite clearance.[19] ART treatment failure also selects for ACT partner drug resistance.[20] In Bangladesh, clinical trials of ACTs, for example, AL, artesunate–mefloquine, artesunate–amodiaquine, and artesunate–azithromycin, have shown excellent efficacy, with rapid parasite clearance and high rates of treatment success.[21–25] Although AL is the mandated treatment for uncomplicated malaria, CQ remains available as an over-the-counter medication for self-treatment of malaria, as well as for vivax malaria treatment in some parts of the country.[26,27]

Several drug resistance mechanisms have been reported in *P. falciparum*; CQ resistance (CQR) has been linked to the mutation in the *P. falciparum* chloroquine resistance transporter gene (*pfcrt*) at codon 76.[28] A single nucleotide polymorphism in the *P. falciparum* multidrug resistance transporter 1 gene (*pfmdr1*) at codon 86 is associated with hypersensitivity to LUM, MQ, and also to DHA, an active artemisinin metabolite.[29] Mutation at codon 86 is also associated with decreased susceptibility to aminoquinolines.[29] In addition, some studies also reported the association of mutation at codon 184 with parasite susceptibility to various antimalarials.[29,30] A copy number increase in this gene is also associated with decreased susceptibility to LUM.[31–33]

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Recent studies found no *kelch*13 gene mutations in *P. falciparum* from Bangladesh.[34,26] A handful of studies have also investigated CQR-associated mutations from specific endemic districts in Bangladesh.[26,35–39] However, there has not been a large-scale study of the currently used antimalarials, CQ and AL, resistance-associated molecular markers, in Bangladesh. Hence, in this study, we examined the prevalence of *pfcrt* and *pfmdr1* markers for resistance to various antimalarials in clinical samples across the malaria-endemic districts of Bangladesh.

METHODS

Study sites. Samples were collected from 11 subdistricts (Upazilas) in three CHT areas: Bandarban (subdistricts: Bandarban Sadar, Naikhongchhari, and Lama), Khagrachhari (subdistrict: Matiranga), and Rangamati (subdistrict: Rajasthali); and three non-CHT areas: Cox's Bazar (subdistricts: Ramu and Ukhiya), Moulvibazar (subdistricts: Kamalganj and Sreemangal), and Netrokona (subdistricts: Kalmakanda and Durgapur) (Figure 1). All the sites share an international border with malaria-endemic countries, either India or Myanmar.

Sample collection. Blood samples were collected directly into an EDTA tube, from adults (5 mL) and children (3 mL) with clinical symptoms of malaria, and stored at –20°C. A total of 296 microscopy-positive falciparum malaria samples were collected. DNA extraction was performed using the QIAamp Blood Mini Kit (Qiagen Inc., Hilden, Germany) following the manufacturer's instructions for collected whole blood. To determine mono-infection of *P. falciparum*, PCR[40] was performed for all the samples, along with real-time quantitative PCR (qPCR) confirmation, following the methods in Alam et al.[41] A total of 262 samples (CHT: 180; non-CHT: 82) were confirmed as *P. falciparum* mono-infection. These samples were selected for drug resistance marker analysis. The institutional Ethics Review Committee of the International Centre for Diarrhoeal Disease Research, Bangladesh, approved the original study. All study participants provided their informed consent for future use of the sample.

Genotyping of *pfcrt* and *pfmdr1*. All DNA samples were analyzed for *pfcrt* and *pfmdr1* gene mutations using PCR and restriction fragment length polymorphism. Polymerase, and all the restriction enzymes were obtained from New England Biolabs Inc. (Ipswich, MA). Analysis of the K76T mutation in the *pfcrt* gene was conducted by the method described by Veiga et al.[42] In brief, the *pfcrt* gene containing codon 76 was amplified by nested PCR, resulting in a 145-bp amplicon. This amplicon was then restriction digested with the *Apo*I enzyme. In the absence of 76T mutation (145 bp mutant allele), the PCR amplicon results in 98 bp and 47 bp fragments.

To analyze mutations of codons 86 and 184 in the *pfmdr1* gene, the protocol described by Duraisingh et al.[43] was followed. In brief, the *pfmdr1* gene fragment containing codons 86 and 184 was amplified by nested PCR, resulting in a 560-bp amplicon. This amplicon was subsequently digested with restriction enzymes, *Apo*I and *Dra*I. In the absence of the 86Y

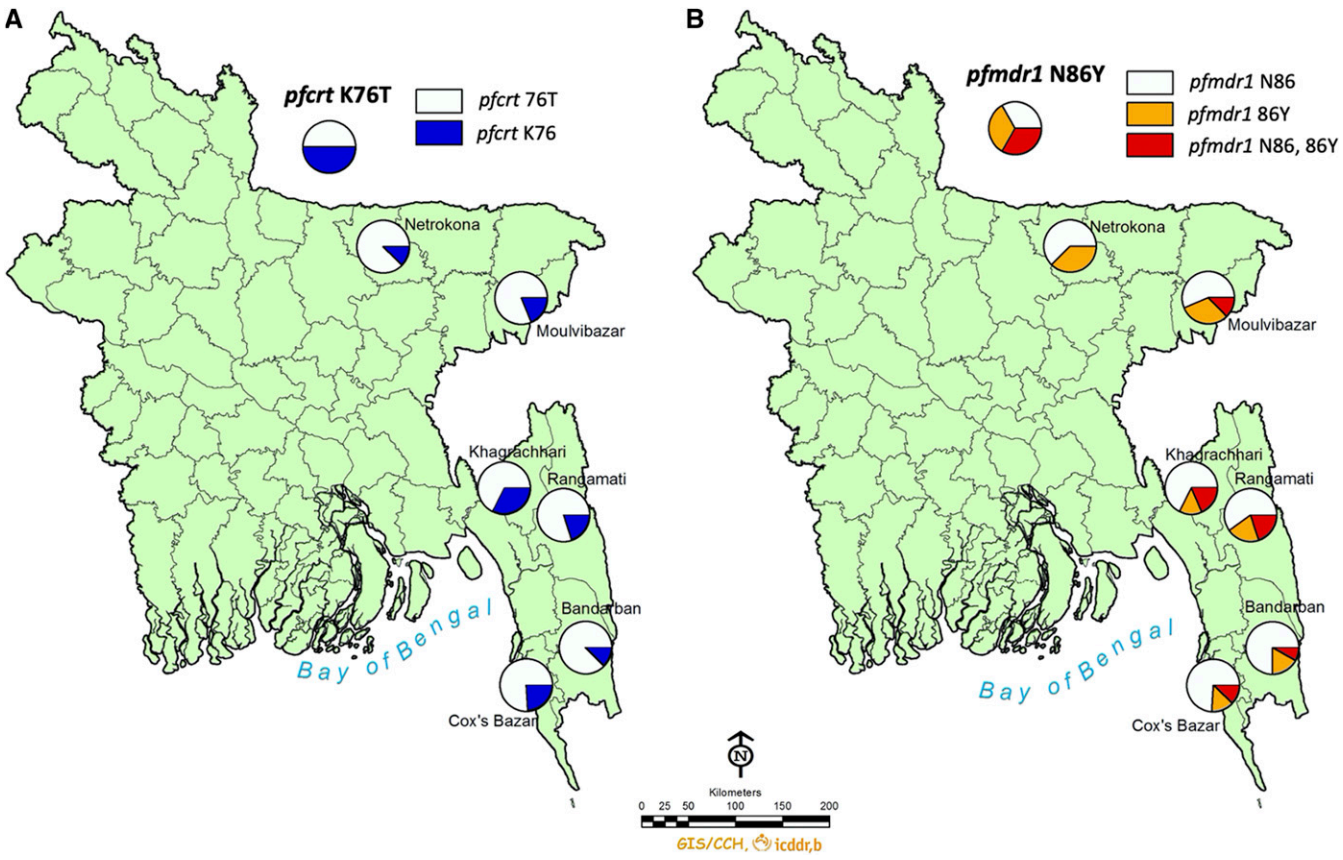

FIGURE 1. Study areas showing the prevalence of both mutant and wild-type alleles at each site. (**A**) *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) K76T; (**B**) *Plasmodium falciparum* multidrug resistance transporter 1 (*pfmdr1*) N86Y. This figure appears in color at www.ajtmh.org.

mutant allele, the *Apo*I restriction digestion generates 249 bp and 256 bp fragments, whereas *Dra*I digestion in the presence of the 184F mutant allele results in 242 bp, 173 bp, and 114 bp fragments. For both *pfcrt* and *pfmdr1*, a sample can show heterozygosity, that is, can contain both wild-type and mutant alleles, which suggests infection by at least two genotypically different parasites. All the PCR and restriction digestion products were resolved on 2–2.5% agarose gels containing 0.1 μg/mL ethidium bromide (Invitrogen™ Life Technologies, Carlsbad, CA).

***Plasmodium falciparum* multidrug resistance transporter 1 copy number analysis.** The *pfmdr1* copy number was estimated using qPCR, following the method described by Ferreira et al.[44] In brief, in a final 10 μL PCR reaction volume, 5 μL iQ™ SYBR® Green Supermix (BioRad Laboratories Inc., Hercules, CA), 300 nM of each forward and reverse oligonucleotide (primer) (Integrated DNA Technologies Inc., Coralville, IA), and 2 μL of template DNA were added. In each run of qPCR, DNA from *P. falciparum* clone 3D7 (which has a copy number of 1) was used as a calibrator, and *P. falciparum* clone Dd2 DNA (which has a copy number of ~4) was used as an amplification control for multiple copies of *pfmdr1*. The *P. falciparum* clones 3D7 and Dd2 DNA were from MR4 (BEI resources, Manassas, VA). We used the *P. falciparum* β-actin gene as the reference gene for this experiment. All samples were run in duplicate. The ΔΔCt method was used for the analysis of qPCR results, where ΔΔCt = (Ct of *pfmdr1* gene - Ct of reference gene) of the sample - (Ct of *pfmdr1* gene - Ct of reference gene) of the 3D7 calibrator. The copy number of *pfmdr1* was calculated as $2^{-\Delta\Delta Ct}$; a copy number of 1.5 was set as a threshold to define multiple copies of *pfmdr1*.[45]

Statistical analysis. The prevalence of *pfcrt* and *pfmdr1* alleles within CHT and non-CHT areas was computed in Microsoft Excel (Microsoft Corporation, Redmond, WA). A proportion comparison test was performed using Stata (version 15.1, Stata Corp., College Station, TX) to observe any changes between the resistance status of these areas. The statistical significance level was defined as $P < 0.05$.

RESULTS

***Plasmodium falciparum* chloroquine resistance transporter and *pfmdr1* polymorphisms.** We identified *pfcrt* 76T mutations in 191 of 262 samples (72.9%), from all the study areas (Table 1). From the 180 CHT samples, the 76T mutation was observed in 127 samples (70.6%), whereas 64 of the 82 non-CHT samples (78.0%) had the 76T mutation. No mixed alleles were recorded in any of the isolates for the *pfcrt* 76 position. The proportion comparison test showed there was no significant difference ($P > 0.05$) in the prevalence of the *pfcrt* 76T mutation between CHT and non-CHT regions.

TABLE 1
Prevalence of polymorphisms in *pfcrt* and *pfmdr1* genes.

	pfcrt, *n* (%)	*pfmdr1*, *n* (%)		
Polymorphism	76T	86Y	184F	N86, 86Y
Total (*n* = 262)	191 (72.9)	42 (16.0)	14 (5.3)	40 (15.3)
CHT region (*n* = 180)	127 (70.6)	26 (14.4)	14 (7.8)	31 (17.2)
Bandarban (*n* = 24)	21 (87.5)	4 (16.7)	0 (0.0)	2 (8.3)
Khagrachhari (*n* = 151)	102 (67.5)	21 (13.9)	14 (9.3)	28 (18.5)
Rangamati (*n* = 5)	4 (80.0)	1 (20.0)	0 (0.0)	1 (20.0)
Non-CHT region (*n* = 82)	64 (78.0)	16 (19.5)	0 (0.0)	9 (11.0)
Cox's Bazar (*n* = 58)	44 (75.9)	8 (13.8)	0 (0.0)	7 (12.1)
Moulvibazar (*n* = 16)	13 (81.3)	5 (31.3)	0 (0.0)	2 (12.5)
Netrokona (*n* = 8)	7 (87.5)	3 (37.5)	0 (0.0)	0 (0.0)

CHT = Chittagong Hill Tracts; *pfcrt* = *Plasmodium falciparum* chloroquine resistance transporter; *pfmdr1* = *Plasmodium falciparum* multidrug resistance transporter 1.

For *pfmdr1*, we evaluated polymorphisms in the N86 and Y184 positions in all the samples (262) and found the 86Y mutant allele in 42 (16.0%) and the 184F mutant allele in 14 (5.3%) (Table 1). Most of the 86Y mutations and all the 184F mutations were from the CHT area (Khagrachhari district). We also found N86 and 86Y mixed alleles in 40 samples (15.3%), which suggests a mixed infection in these samples. As for the mixed allele, 31 (17.2%) of the mixed alleles were from the CHT region. A proportion comparison of both 86Y and mixed mutations between the CHT and non-CHT regions did not show any significant difference ($P > 0.05$) in the prevalence of these mutations between the regions.

For the analysis of the *pfmdr1* haplotype for polymorphic positions 86 and 184 (Table 2), we did not include the samples with the mixed allele for position 86. We found $N^{86}Y^{184}$ was the most prevalent in both the CHT (82.6%) and non-CHT (78.1%) regions, whereas $Y^{86}Y^{184}$ was more prevalent (22.0%) in the non-CHT region. $Y^{86}F^{184}$ haplotypes were only found in CHT regions (3.4%).

Changes in resistance markers over time. Over the last two decades, several molecular studies have been conducted using *pfcrt* K76T and *pfmdr1* N86Y markers to investigate malaria drug resistance in the malaria-endemic districts of Rangamati,[35] Bandarban,[26,36] Khagrachhari,[38] and Cox's Bazar.[37] We used a proportion comparison test to compare our data with these published studies to observe the changes, if any, in the prevalence of antimalarial resistance–associated molecular markers (Figure 2). This analysis revealed a persistently high prevalence of the *pfcrt* 76T allele and a low prevalence of the *pfmdr1* 86Y allele. We observed a decreasing trend in *pfcrt* 76T mutation in Khagrachhari, Bandarban, and Cox's Bazar districts over the last two decades (Figure 2A). Likewise, the *pfmdr1* 86Y mutation in the Khagrachhari district declined (Figure 2B). As for *pfmdr1* 184F mutations, there were no previous studies in Khagrachhari; therefore, we could not do a proportion comparison test for this mutation.

Mixed alleles of polymorphic *pfmdr1* N86 have been reported previously,[26,35–38] although these studies have either excluded or apportioned the mixed population of *pfmdr1* N86, 86Y for their analysis. In this study, we compared only the mixed *pfmdr1* N86, 86Y population with mixed populations from previous studies, using a proportion comparison test for each district (Figure 3). We found that the prevalence of mixed alleles in the Khagrachhari district has significantly increased.

***Plasmodium falciparum* multidrug resistance transporter 1 copy number.** Amplification of the *pfmdr1* gene to investigate copy number increase was successful in 256 of the 262 isolates (97.7% [CHT: 176; non-CHT: 80]). The control DNA gave reproducible results, as 3D7 and Dd2 had a mean copy number of 1.0 and 3.0, respectively.[45] Using a copy number threshold of 1.5 to define multiple copies,[45] when rounded to the nearest integer, all the isolates were found to contain a single gene copy with a mean of 0.8 (SD = ±0.3).

DISCUSSION

In 2004, the NMEP introduced AL as an alternative to CQ, to treat uncomplicated falciparum malaria in Bangladesh; however, it took 3 years to implement AL treatment in all 13

TABLE 2

Plasmodium falciparum multidrug resistance transporter 1 haplotypes for position 86 and 184.

pfmdr1 haplotype		Total (n = 262)	CHT region (n = 149)	Non-CHT region (n = 73)
86	184	Frequency of isolates	Frequency of isolates	Frequency of isolates
N	Y	180	123 (82.6%)	57 (78.1%)
N	F	ND	ND	ND
Y	Y	37	21 (14.1%)	16 (22.0%)
Y	F	5	5 (3.4%)	ND

CHT = Chittagong Hill Tracts; ND = not detected; *pfmdr1* = *Plasmodium falciparum* multidrug resistance transporter 1. Samples with mixed infection at position 86 (CHT: 31; non-CHT: 9) were excluded for this analysis.

malaria-endemic districts.[14] This study and previous studies demonstrate that the CQR allele is dominant in Bangladesh (Figure 2A). Among the study districts, Khagrachhari, Bandarban, and Cox's Bazar showed a significant decrease in CQR over time (Figure 2A). Some African countries have shown a reversion to CQ sensitivity in *P. falciparum* isolates after the complete cessation of CQ use.[46–50] A limitation of this study is that complete *pfcrt* haplotype data are not available in Bangladesh; therefore, the possibility of a reversion to CQ sensitivity cannot be addressed. Cox's Bazar (on the Myanmar border) has more than 95% CVIET haplotype frequency, which is similar to Myanmar,[37] whereas in India (which borders other study districts), SVMNK prevails over CVIET, in terms of haplotype frequency.[51] Thus, the different endemic districts of Bangladesh, which are surrounded by endemic countries, may display different *pfcrt* haplotypes. However, despite the fact that CQ has not been used for falciparum malaria treatment for more than a decade, it is still used for the treatment of mono-infection of *P. vivax*.[27] As a result, CQ remains accessible via local drug store, reducing the likelihood of the CQR allele reverting to the sensitive allele in Bangladesh.

In this study, we found that the *pfmdr1* 86Y mutation occurred in 14.4% of samples from the CHT region (Table 1); another recent study in a CHT subdistrict found 13.9% occurrence.[26] The presence of the 86Y mutant allele can be attributed to the circulating CQ pressure; this mutation leads to decreased susceptibility to CQ.[29] However, there was a significant decrease in *pfmdr1* 86Y prevalence in the Khagrachhari district (Figure 2B), which could potentially facilitate the reversion of CQ sensitivity in this district.

The *pfmdr1* 86Y mutation has been linked to hypersensitivity to LUM, MQ, and DHA.[29,32,33] The stable prevalence of *pfmdr1* 86Y mutation over the last two decades in Bangladesh suggests susceptibility to LUM (the partner drug for ACT in Bangladesh) is unaffected. However, the significant increase in the prevalence of the wild-type *pfmdr1* N86 allele in Khagrachhari (Figure 2B) is of significant concern, as it has been reported that parasites with the N86 allele have reduced susceptibility to LUM, compared with parasites with the 86Y allele.[52,53] The *pfmdr1* N86 mutation cannot be used as a predictive marker for LUM resistance on its own[54]; several studies have reported the presence of *pfmdr1* 184F

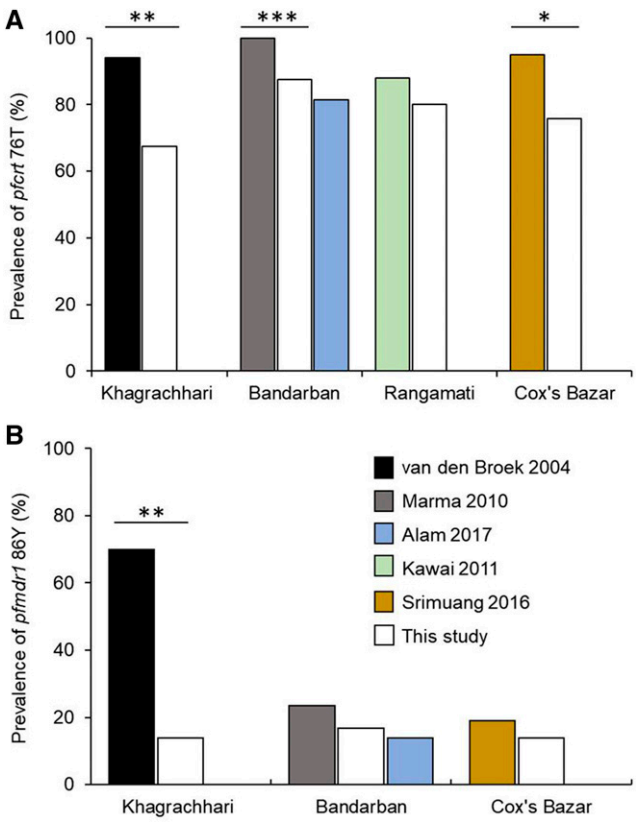

FIGURE 2. Proportion comparison of the prevalence of (**A**) *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) 76T and (**B**) *Plasmodium falciparum* multidrug resistance transporter 1 (*pfmdr1*) 86Y in the present study with previous published studies[26,35–38] from Bangladesh. Samples from non-CHT (Chittagong Hill Tracts) districts (Netrokona, n = 8 and Moulvibazar, n = 16) were not included in this analysis. *P < 0.05, **P < 0.01, ***P < 0.001; proportion comparison test. This figure appears in color at www.ajtmh.org.

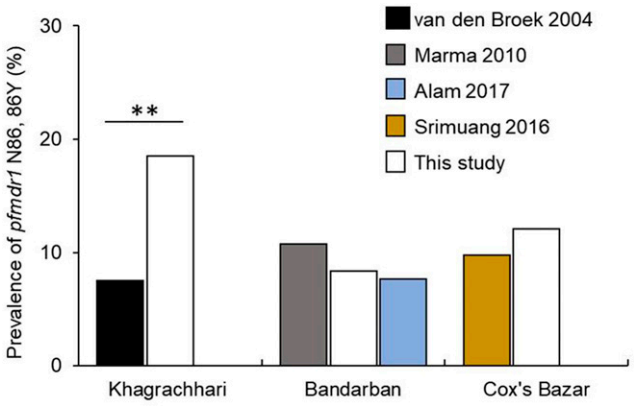

FIGURE 3. Proportion comparison of the prevalence of *Plasmodium falciparum* multidrug resistance transporter 1 (*pfmdr1*) N86, 86Y in the present study with previous published studies[26,36–38] from Bangladesh. Samples from non-CHT (Chittagong Hill Tracts) districts (Moulvibazar, n = 2) were not included in this analysis. **P < 0.01; proportion comparison test. This figure appears in color at www.ajtmh.org.

and D1246 markers, along with N86 after prolonged AL treatment.[30,55–57] In our study, we observed a low prevalence of the *pfmdr1* 184F mutation, which is consistent with other studies.[37,39] This mutation appears to have a weaker relationship with drug susceptibility[29,30,58] and may not by itself cause the failure of drug treatment. However, a clinical tolerance is observed to aminoquinolines when 184F is present with N86 mutation in parasites with predominant Asian/African variant of *pfcrt*.[29] We did not analyze our sample for the *pfmdr1* D1246Y polymorphism; future deep sequencing or targeted sequencing analyses might help identify new or known polymorphisms in the *pfmdr1* gene of the circulating parasite population of Bangladesh.

In parasites from SEA, the circulating *pfmdr1* haplotype was either $N^{86}F^{184}$ or $N^{86}Y^{184}$.[29] In this study, the $N^{86}Y^{184}$ haplotype has been observed at a high frequency in both the CHT and non-CHT regions (Table 2), which is similar to the findings from a previous study in the Cox's Bazar and Bandarban regions.[39]

A copy number increase of *pfmdr1* is linked to resistance to LUM[32] and MQ.[31–33] Although MQ is not the ACT partner drug in Bangladesh, Noedl et al.[59] showed a high prevalence of MQ resistance in Bangladesh, in vitro. They concluded this might be due to circulating parasite populations of inherent MQ resistance, or resistant parasite strains, from neighboring Myanmar. In our study, we did not observe an increase in the copy number of the *pfmdr1* gene. This finding is consistent with a previous study in this region[37] and provides reassurance that the efficacy of LUM has not been affected.

In the past two decades, before and after the introduction of ACTs in Bangladesh, only a handful of molecular studies on drug-resistant markers of antimalarials have been conducted, focusing on either one or two endemic regions. In this study, we analyzed clinical samples from a wider geographical setting, covering both high- and low-transmission areas of the country. Regardless of the transmission area, there was no statistically significant difference between the prevalence of *pfcrt* and *pfmdr1* markers of drug resistance.

A previous study from Bangladesh that investigated molecular markers for ART resistance, with this same sample set, did not report the presence of any established resistance markers, except for a *kelch*13 A578S mutation, which appears to not mediate resistance, in two isolates.[34,60] The monitoring of ART-resistant markers is important as AL is the frontline antimalarial treatment. It is also important to monitor partner drug (LUM) efficacy of this combination therapy in this region. Therefore, the real-time monitoring of *pfmdr1* and *pfcrt* markers enables tracking the emergence of drug resistance in advance and allows the regional malaria control programs to make informed decisions to prevent rise of widespread treatment failure and/or resistance.

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