

## Original Article

# Molecular Pattern of Anti-malarial Drug Resistance of *Plasmodium falciparum* in Bangladeshi Troops Working in Endemic Areas of Bangladesh and Africa

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Members of Bangladesh Armed Forces work in two different malaria endemic area, Chittagong Hill Tracts (CHT) in Bangladesh and Sub-Saharan countries in Africa. This under-recognized group remained unexplored for long in respect to drug resistant falciparum malaria they usually suffer from. In this study, a total of 252 'dried blood samples on filter paper' were collected between November 2014 and February 2016, from *Plasmodium falciparum* positive Bangladeshi troops working in Chittagong Hill Tracts (CHT), Bangladesh and five Sub Saharan African Countries namely, Central African Republic (CAR), Democratic Republic of Congo (DRC), Liberia, Mali and Ivory Coast. After DNA extraction from all these samples (94 from Bangladesh and 138 from African countries), plasmodium species was confirmed by a nested PCR following standard protocol with minor modifications. Thereafter, a multiplex nested PCR followed by restriction fragment length polymorphism (RFLP) method was employed to investigate the presence of chloroquine resistance marker 'K76T mutation' in *P. falciparum chloroquine resistance transporters (pfert)* gene and lumefantrine and mefloquine resistance marker 'N86Y mutation' in *P. falciparum multidrug resistance1 (pfmdr1)* gene. The *P. falciparum* DNA was confirmed in 35 (37.23%) Bangladeshi and 45 (28.48%) African samples. The '*pfert* (K76T) mutation' that confers resistance to chloroquine, was detected in 93.10% Bangladeshi and 29.27% African samples. The '*pfmdr1* (N86Y) mutation' that confers resistance to lumefantrine and mefloquine, was detected in 20.69% Bangladeshi and only 2.44% African samples. The higher prevalence of chloroquine resistance of *P. falciparum* in Bangladesh than in African countries revealed that possible withdrawal of chloroquine from endemic areas and also periodic molecular survey to monitor *pf* resistance to chloroquine, mefloquine, lumefantrine and artemisinin among these troops working in both endemic areas.

**Key words:** *Plasmodium falciparum*, drug resistance, Chloroquine resistance

## Introduction

Despite achieving a trend of reduction in global morbidity and mortality from malaria since beginning of 21<sup>st</sup> century the parasitic disease continues to have a devastating impact on people's health and livelihoods in Asia and Africa. According to the World Malaria Report 2019 published by World Health Organization (WHO), an estimated 228 million cases of malaria occurred worldwide in 2018, most of them are in African Region (213 million or 93%) followed by South-East Asia Region with 3.4% of the cases<sup>1</sup>. Among 5 parasite species that cause malaria, *Plasmodium falciparum* is responsible for most malaria-related deaths globally.

*P. falciparum* has a long tradition of acquiring resistance against anti-malarial drugs. Antimalarial drug resistance of this falciparum species became a global problem for the first time in the 1960s when the parasite developed resistance to chloroquine (CQ), the then widely used antimalarial drug. Resistance first emerged in the Greater Mekong subregion spread to South-East Asia and then to Africa, triggering a dramatic increase in malaria-related illness and death<sup>2</sup>.

The problem of antimalarial drug resistance is compounded by cross resistance, in which resistance to one drug confers resistance to other drugs that belong to the same chemical group or which have similar modes of action. During the past decades, several highly efficacious antimalarial drugs had to be removed from markets after the development of parasite resistance to them<sup>3</sup>.

Malaria remains a major cause of military casualties in Southeast Asia and Africa. Members of Bangladesh Armed Forces working in the endemic areas of the country constitute one of the most vulnerable group to suffer from malaria casualty since long. The problem was amplified and complicated when these forces started operating in malaria endemic areas of Africa in peace keeping missions under United Nations exposing themselves to other probable resistant strains of *P. falciparum*.

Therefore, this study has attempted to examine the molecular pattern of *P. falciparum* resistance to antimalarial drugs in a population that remained under-represented but exposed to exceptional malarial situations at home and abroad. In this study we examined *P. falciparum* resistance to mefloquine which is

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consumed by Bangladeshi troops deployed in missions in Africa as a routine antimalarial prophylaxis and lumefantrine which in combination with artemisinin formulates one of the most potent antimalarial drug of present day. We also examined the parasite's resistance to chloroquine the most popular antimalarial of past.

### Materials and methods

The study was carried between 2014 and 2016 among Bangladeshi troops working in Chittagong Hill Tracts (CHT) and Cox's Bazar in Bangladesh and five Sub-Saharan African countries namely Democratic Republic of Congo (DRC), Central African Republic (CAR), Mali, Ivory Coast and Liberia. Ethical clearance was obtained from The Ethical Committee of Armed Forces Medical Services of Bangladesh (4614/35/T/DGMS/Ethi). Informed consent was obtained from each of the participant before collecting sample.

#### Sample collection

Three spots of finger prick blood were collected on a filter paper (Whatman UK) from *P. falciparum* malaria cases among the troops confirmed by microscopy or Rapid Diagnostic Test (RDT), before anti-malarial drug was started. The blood sample was then dried in the air for about half an hour in room temperature and preserved in a zip locked polybag with silica pack inside before final transportation to the laboratory of Department of Microbiology, University of Dhaka. In the laboratory samples were stored at 4°C refrigerator.

#### Extraction of DNA

*P. falciparum* DNA extraction was done using QIAamp® DNA Mini Kit (Qiagen GmbH, Germany 2015), following manufacturer's protocol of DNA Purification from Dried Blood Spots.

#### Species identification of *P. falciparum*

*P. falciparum* species was confirmed by a nested PCR following a standard protocol<sup>4</sup> with minor modifications. This nested based PCR targeted the 18 small subunit ribosomal RNA (ssr-RNA) gene amplifying a 1.2kb fragment of *pf* DNA. Two genus-specific primers rPLU5 (CCTGTTGTTGCCTTAAACTTC) and rPLU6 (TTAAAATTGTTGCAGTTAAAACG), were used for the first

cycle of amplification. An aliquot of the product thus obtained, was used as template for a second amplification cycle, in which parasite species was confirmed using species specific primers rFAL1 (TTAAACTGGTTTGGGAAAACCAAATATATT) and rFAL2 (ACACAATGAACTCAATCATGACTACCCGTC). The expected final band size was 205bp. Primers were constituted at Bioneer Corporation, Republic of Korea.

Genotyping of *pfcr* and *pfmdr1* genes of *P. falciparum* for drug resistance status

Antimalarial drug resistance has been associated with the presence of single nucleotide polymorphisms (SNPs) in particular gene of *P. falciparum*<sup>5</sup>. These include:

- The *pfcr* (*P. falciparum* chloroquine resistance transporters) K76T amino acid change is correlated to chloroquine and amodiaquine resistance.
- pfmdr1* (*P. falciparum* multidrug resistance1) N86Y alternation is associated with resistance to mefloquine and lumefantrine.

The presence of the *pfcr* K76T and *pfmdr1* N86Y mutations were assessed in the isolates using Multiplex PCR-RFLP method, following the protocol of Veiga *et al*<sup>5</sup>. with minor modifications. A set of first and internal (nest) primers were used to amplify DNA fragments containing *pfmdr1* N86Y and *pfcr* K76T (Table 1). After 1st round of amplification, amplicon from the 1st step was used as template for 2nd round amplification. The expected final band size of *pfmdr1* and *pfcr* were 418 bp and 145 bp respectively.

#### Restriction Fragment Length Polymorphism (RFLP) analysis

PCR products were used without any purification procedure for digestions with restriction enzyme. Restriction enzyme XpaI (ApoI) of Thermo Fisher Scientific, USA was used in this RFLP. It cleaves the wild types of *pfcr* allele in 76K position and *pfmdr1* in 86N position.

The products of multiplex nested PCR, after digestion with restriction enzyme were analyzed by electrophoresis on a 2% agarose gel. The cleaved fragments of *pfcr* and *pfmdr1* were categorized by their molecular weight.

**Table 1.** Sequence of the primers used for amplification of *pfcr* and *pfmdr1* genes by multiplex nested PCR

Steps	Alleles	Primers	Sequence (5' - 3')	Amplicon size
Step-1	<i>pfmdr1</i>	M1 FW	AAGAGGTTGAAAAAGAGTTGAAC	447 bp
		M1 REV	CCGTTAATAATAAATACACGCAG	
	<i>pfcr</i>	C1 FW	ATTCGTACCAATTCCTGAACT	538 bp
		C1 REV	CGGATGTTACAAAATATAGTTACC	
Step-2(nested)	<i>pfmdr1</i>	M2 FW	AGAGTACCGCTGAATTATTTAG	418 bp
		M2 REV	CCTGAACTCACTTGTTCTAAAT	
	<i>pfcr</i>	C2 FW	TGTGCTCATGTCTTTAAACTT	145 bp
		C2 REV	CAAACTATAGTTACCAATTTTG	

## Results

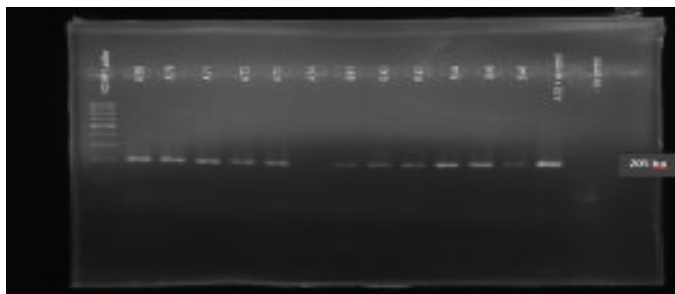
A total of 252 samples were collected, 94 from endemic areas of Bangladesh and 158 from five Sub-Saharan African countries (Table 2).

**Table 2.** Geographic distribution of blood samples

Origin of sample	Area/country	No of samples	Total
Bangladesh	CHT	94	94
Africa	D R Congo	74	158
	Ivory Coast	44	
	Liberia	12	
	Mali	13	
	C A R	15	

### Species identification of *P. falciparum*

*P. falciparum* species was confirmed in 35 out of 94 blood samples from Bangladesh and 45 out of 158 samples from Africa through a nested based PCR which targeted the 18 ssr-RNA gene amplifying a 1.2kb fragment of *pfDNA*. The expected final band size was 205 base pair. The presence of amplification product was detected by simple ethidium bromide staining following agarose gel electrophoresis (Figure 1).



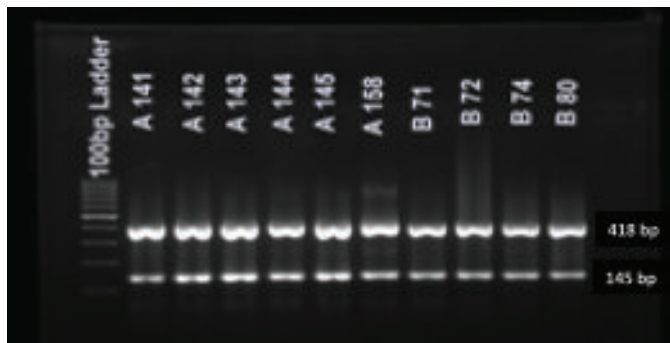
**Figure 1.** Bangladeshi and African samples were coded with the alphabets B and A respectively. Sample A74 and the negative control did not show any band, rest of the samples including the positive control (A63) showed band of 205 bp using a 100 bp DNA ladder.

We tried to compare the proportion of molecular confirmation of field diagnosis of the malaria patients by two methods namely, microscopy and ICT/RDT. We found that, 43% microscopically diagnosed patient were confirmed to have *P. falciparum* by PCR, whereas only 28% of ICT/RDT diagnosed cases were confirmed by PCR.

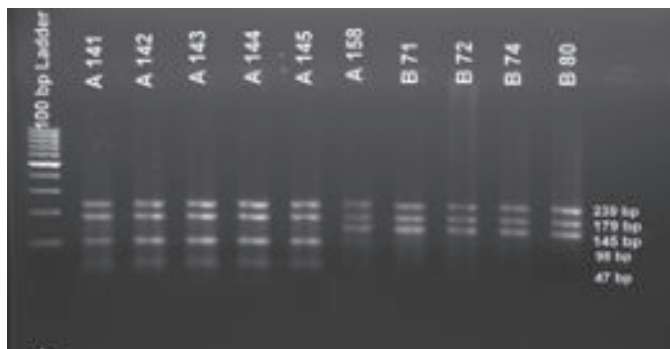
### Genotyping of *pfmdr1* and *pfprt* genes of *P. falciparum* for drug resistance status

A multiplex nested PCR followed by RFLP, successfully genotyped *pfprt* and *pfmdr1* genes among the samples. Two

nested primers pairs simultaneously, amplified two fragments, one fragment of *pfmdr1* and the other a *pfprt* amplicon. The amplicons resulted in sizes well distinguished in 2% agarose gel, 418 and 145 bp, respectively. Digestion with ApoI of the '86N harboring *pfmdr1* specific PCR product' gives rise into a 239 and 179 bp fragments. The restriction of the 76K carrying *pfprt* PCR amplicon resulted in a 98 and 47 bp fragment (Figure 2 and Figure 3).



**Figure 2.** *pfmdr1* gene and *pfprt* gene fragments of 6 African (A141, A142, A143, A144, A145 and A158) and 4 Bangladeshi (B71, B72, B74 and B80) isolates are seen at 418 bp and 145 bp respectively using a 100 bp DNA ladder.



**Figure 3.** Amplicons of *pfmdr1* (carrying 86N), cleaved into 239 bp and 179 bp fragment after digestion with restriction enzyme, denote wild type allele. Likewise, amplicons of *pfprt* (carrying 76K), cleaved into 98 bp and 47 bp fragments, denote wild type allele. Whereas, amplicons of both the genes, not cleaved at all, denote mutant type allele. The positive and negative controls are not shown in this gels as they were run in another gel with few other samples.

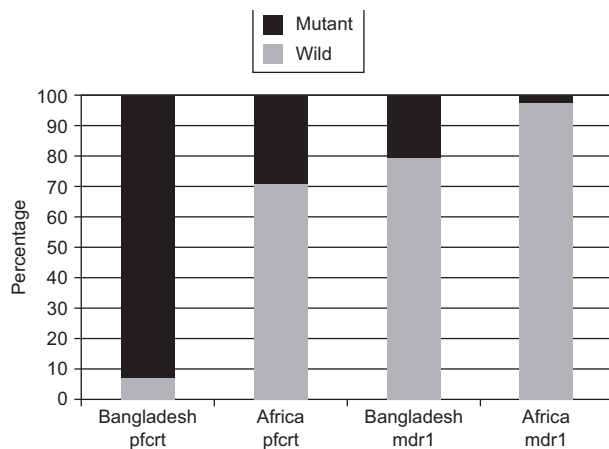
Almost 21% *pfmdr1* allele from Bangladesh were mutant, while in Africa, only 2.44% *pfmdr1* allele were found to be mutant denoting resistance to mefloquine and lumefantrine. In case of *pfprt* gene, 93% Bangladeshi allele and 29% African allele were mutant showing resistance to chloroquine (Table 3).

**Table 3.** Summary of genotyping of *pfmdr1* gene by PCR-RFLP method

Origin of isolates	Molecular confirmation of <i>Pf</i> species	Genotyping of <i>pfmdr1</i> by PCR-RFLP method		
		<i>pfmdr1</i> amplified	Wild	Mutant(N86Y)
Bangladesh	35	29	23(79.31%)	06(20.69%)
Africa	45	41	40(97.56%)	01(02.44%)
Total	80	70	63(90.00%)	07(10.00%)

Country wise comparison of mutation in *pfmdr1* and *pfert* genes.

Country wise data showed that, *pfert* K76T mutation was present in 93%, 24% and 44% isolates from Bangladesh Congo and Mali respectively. *pfmdr1* N86Y mutation was present in 21% and 44% isolates from Bangladesh and Mali respectively None of the isolates from Congo had *pfmdr1* N86Y mutation and none of the isolates from Ivory Coast had either of the mutations. However, a cumulative comparison of presence of mutations between samples from Bangladesh and Africa shows distinct upsurge in proportion of mutation in both the gene in Bangladeshi samples than those of Africa (Figure 4).

**Figure 4.** Comparison of presence of mutation in samples from Bangladesh and Africa

## Discussion

The global incidence rate of malaria was in a phase of decline (18% decline between 2010 and 2017). An increasing number of countries were progressing towards elimination<sup>6</sup>. The situation was almost comparable to the great eradication program of last century, which finally failed to the wide-spread resistance of malaria parasite to chloroquine, along with few other reasons. The most important challenge to present day elimination program was also drug resistance. Drug resistance of *P. falciparum* to multiple anti-malarial drugs is now threatening the achievements of our long fight against malaria.

The present study undertook the endeavor to investigate molecular pattern of anti-malarial drug resistance of *P. falciparum* for the first time in members of Armed Forces of Bangladesh working in endemic areas at home and Africa having frequent travel instances between these two endemic areas of the world.

In this study, 252 samples collected from Bangladesh and 5 African countries and proceed for molecular detection of *P. falciparum*. Of them, 37.23% of Bangladeshi samples and 28.48% of African samples (total 31.74%) were confirmed for the presence of the parasite. Molecular detection rate of *P. falciparum* among the microscopy/RDT confirmed blood samples in this study was higher than that of a study conducted by Ogbolu *et al.* in Nigeria<sup>7</sup> but lower than that of few other studies<sup>8,9,10</sup>. A lower rate of molecular detection of *P. falciparum* species among microscopy /RDT confirmed dried blood samples in this study might be due to few reasons. Microscopy/RDT were done before sample collection, samples were then preserved usually for months in the field condition before transported long way from CHT/Africa to the laboratory of University of Dhaka. The conflict ravaged field condition and long way transportation through different means might play some role in degradation of DNA in some of the samples. Lack of skills of field staffs in microscopy and probable confusion in distinguishing *P. falciparum* from *P. vivax* and other species while doing RDT might also play some role. However, the rate of detection of *pf* DNA in the samples from CHT, Bangladesh was higher than that from African countries. Results of this study also consistent with the findings of Ojurongbe *et al.*, where sensitivity of microscopy was higher than that of RDT in detecting *P. falciparum*<sup>11</sup>, while in some other studies RDT yielded higher sensitivity over microscopy<sup>12,13</sup>. Owing reverse result in a study, researchers felt it necessary to reinforce training in microscopy<sup>13</sup>.

Molecular evidence of *P. falciparum* resistance to chloroquine and partner drugs happened to be observed much before its removal from National Treatment Guideline as the first-line treatment of uncomplicated *P. falciparum* malaria in Bangladesh in 2004. Ingrid *et al.* conducted a study in Khagrachari Hill District of Bangladesh in the year 2002, which revealed that, 94% of parasites carried the K76T mutation at *pfert*, while 70% carried the N86Y mutation at *pfmdr1*<sup>14</sup>. In 2004, Kawai *et al.* detected *pfert* (K76T) mutation in 38 (84%) of 45 uncomplicated falciparum malaria patients' blood samples from Rangamati Hill District<sup>15</sup>. In an analysis of 33 paired recrudescence infections after the treatment in the period 2004 to 2008 in the Chittagong Hill Tracts, Akter *et al.* found prevalence of the *pfert* K76T chloroquine resistant allele in approximately 82% of the samples, while the resistant *pfmdr1* (N86Y) allele was present in 33% of the samples<sup>16</sup>.

The prevalence of chloroquine resistant *P. falciparum* strains continues to remain high in Bangladesh till present time. While carrying out a molecular analysis of 130 *P. falciparum* isolates collected between August 2014 and January 2015 from Bandarban, Alam *et al.* detected *pfprt* K76T and *pfmdr1* N86Y mutations in 81.5% and 13.9% isolates respectively<sup>17</sup>. Our present study reveals even higher rates of prevalence of these two mutations (93.10% and 29.27% respectively) in blood samples from CHT Bangladesh. Fact is that, in Bangladesh, chloroquine continues to be available in drug stores for self-treatment of malaria and for the treatment of *P. vivax*, that may have maintained chloroquine pressure on the local *P. falciparum* population, explaining the high prevalence of *pfprt* K76T mutants in this study.

Studies in neighboring countries of Bangladesh reported similar prevalence of mutant genotypes of *P. falciparum* in last couple of years. Pickard *et al.* reported polymorphism *pfprt* Thr76 in 97% of isolates from Thailand, Myanmar, Vietnam, and Bangladesh<sup>18</sup>. Goswami *et al.* recorded *pfprt* K76T and *pfmdr1* N86Y mutations in 100% and 52.6% treatment failure cases in Indo-Bangladesh border areas<sup>19</sup>. High prevalence of CQ and MQ-resistant *P. falciparum* isolates was also observed along the areas of Thai-Cambodian and Thai-Myanmar borders between 2006 and 2009<sup>20</sup>. In a study from Chhattisgarh, India between 2013 and 2015, mutation was detected in 78% and 59% of *pfprt* and *pfmdr1* gene respectively<sup>21</sup>.

Back in 2010, in D R Congo Koukoukila-Koussounda *et al.* detected mutations in *pfprt* gene in 92% of the samples under study<sup>22</sup>. In 2014 Mvumbi *et al.* detected mutations in *pfprt* gene among 63.9% of their samples from the same country<sup>23</sup>. These findings followed by the finding of our study indicate a gradual reduction in the prevalence of mutation in *pfprt* gene in D R Congo. This may be a consequence of cessation of malaria treatment with chloroquine and introduction of ACTs as 1st line of treatment in 2005.

Similar results were observed in many other African countries. In Malawi (the 1st African country to replace CQ with sulfadoxine pyrimethamin in 1993), Kublin *et al.* found that chloroquine-resistant *pfprt* genotype decreased from 85% in 1992 to 13% in 2000<sup>24</sup>. In Niger, 6 years after withdrawal of CQ and introduction of artemisinin-based combinations Salissou *et al.* observed low prevalence of *pfprt* resistance alleles among patients with uncomplicated falciparum malaria<sup>25</sup>. In Cameroon Moyeh *et al.* observed a significant reduction of *Pfprt* 76T and *Pfmdr1* 86Y genotypes i.e. 97.0% to 66.9% and 83.6% to 45.2% between 2003 and 2013 respectively<sup>26</sup>.

Likewise, recent studies in many African countries reported marked reduction in the prevalence of chloroquine resistant strains of *P. falciparum* indicating gradual return of the genotypes sensitive to chloroquine and other 4-aminoquinoline derivatives<sup>27,28</sup>. Chloroquine sensitive strains are becoming the predominant strains of *P. falciparum* in locations where

chloroquine has been almost completely removed from the market, including the private sector for 6–10 years<sup>17</sup>.

Prevalence of *pfmdr1* N86Y mutations in Bangladesh in this study (20.69%) was in moderate range which is in congruence with reports published in past few years<sup>16,17</sup>. This rate for five Sub-Saharan African countries in this study is even lower (2.44%) which is also lower than the finding of few reports published in the recent past<sup>26,29</sup>.

The higher prevalence of chloroquine resistance of *P. falciparum* in Bangladesh than in African countries revealed in this study suggests possible withdrawal of CQ from endemic areas of Bangladesh. *P. falciparum* resistance to mefloquine, lumefantrine and of course artemisinin requires continuous monitoring in both Bangladesh and Africa along with other part of endemic world as these are the medicines we have now in our hand for prophylaxis and first line curative treatment of falciparum malaria. We also recommend appropriate measures to be in place so that transmission of resistant strains of the parasite cannot take place between endemic areas by travelers.

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