INTRODUCTION

The widespread occurrence of chloroquine (CQ)–resistant malaria due to *Plasmodium falciparum* is a major health problem, especially in sub-Saharan Africa. Southeast Asia is also another major focus, although the mortality rates are lower than in Africa. The *P. falciparum* multidrug resistance 1 (*Pfmdr1*) homolog that encodes the P glycoprotein was initially proposed as determinant of the CQ-resistant phenotype, but a direct association of mutations in this gene with the resistant phenotype has not been substantiated. More recently, the locus for CQ resistance has been mapped to a 36-kb segment on chromosome 7 of *P. falciparum* and specifically linked to the polymorphic gene *Pfcr*. Among other mutations, a lysine to threonine mutation at amino acid residue 76 (K76T) is present in all documented clinical CQ failures and laboratory-adapted field isolates of CQ-resistant strains, although this change is observed with different amino acid haplotypes (*Pfcrt* haplotypes) corresponding to amino acid residues 72-76. In particular, CQ-resistant strains from Africa/Southeast have the haplotype CVIET, whereas most South American strains studied have been observed to have the SVMNT haplotype, and CVIET *Pfcrt* haplotypes. These two geographic regions are considered to be the two major foci for the origin of CQ resistance. Field studies in Mali with uncomplicated malaria have indicated that the *Pfcr* K76T mutation is strongly associated with the development of CQ resistance and can be used as a marker for surveillance of the resistant phenotype. In addition, these investigators have shown that the *Pfmdr1* N86Y mutation is also selected, since it was detected in 86% of the patients with CQ-resistant parasite infections, although its co-selection is not indicative of a direct causal role of the *Pfmdr1* gene in resistance.

In a recent study from India, the *Pfcr* K76T mutation has been shown to be rampant (96–100%) in blood samples collected from patients infected with *P. falciparum*, especially in the northern and northeastern parts of India. These studies were based on results obtained by amplification with a refractory mutation-specific polymerase chain reaction (PCR). However, the DNA sequence information needed to identify the *Pfcr* haplotype or the *Pfmdr1* mutation is not available. In the present study, archived and fresh blood samples collected from *P. falciparum*-infected patients from different parts of India were analyzed for the *Pfcr* haplotypes (amino acids 72-76) and the *Pfmdr1* N86Y mutation by PCR amplification, followed by restriction enzyme digestion and DNA sequencing of the primary PCR product.

MATERIALS AND METHODS

**Study samples.** A genotype study was performed on the blood samples from *P. falciparum*-infected patients essentially as described by Djimde and others, with slight modifications. DNA was isolated from blood samples obtained in different regions of India. These included samples collected over heparin and stored in liquid nitrogen, as well as those spotted on filter papers discs. The samples were collected between 1996 and 2002, except for two samples collected in 1988. The samples from northern and northeastern India were from the States of Delhi (Delhi city), Uttar Pradesh (Ghaziabad, Shankargarh town), Madhya Pradesh (Jagadalpur, and Assam (Kamrup), those from eastern India were from Orissa (Keonjhar), those from western India were from Maharashtra (Gondi), and those from western India were from Karnataka (Raichur). The names given in parenthesis are the districts except where indicated. These areas represent a range from high-endemicity malaria to sporadic occurrence. The archived samples were from both sexes and the age group of the patients ranged from 6 to 45 years, except for one sample that was obtained from a 95-year-old patient. The different institutions involved collected blood samples after informed consent was obtained and with the approval of the respective institutional ethical committees.

**Chloroquine resistance in vitro.** The samples (northern and northeastern) were used for assessing CQ resistance in vitro before storing in liquid nitrogen. This was carried out in the field using predosed plates supplied by the World Health Organization (WHO) according to a standard microtest protocol using smear analysis to assess the effect on schizont maturation. The stored samples were later revived for cultivation and CQ sensitivity was retested after synchronizing the culture.

Isolates showing schizont maturation in wells containing ≥ 8 pM CQ were considered resistant. Since the cut-off value
reported earlier by the WHO for in vitro CQ resistance was \( \geq 5.7 \) pM, isolates showing sensitivity in the range 6–7 pM were classified as borderline cases.

**Isolation of DNA.** DNA was isolated from whole blood or samples blotted onto filter paper discs. For DNA isolation from whole blood, the kit supplied by Life Technologies, Pvt. Ltd. (New Delhi, India) was used as per the manufacturer’s protocol, which was based on digestion of the sample with proteinase K in the presence of sodium dodecyl sulfate, precipitation of DNA with isopropanol, and absorption and elution from a silica gel membrane column. In the case of filter paper discs, they were stored in 100°C water for 10 minutes, air-dried, and incubated at 95–100°C in 50 μL of water for 10 minutes and vortexing the sample three times. An aliquot was used for as the DNA source.

**Polymerase chain reaction amplification, restriction enzyme digestion, and DNA sequencing.** DNA from whole blood samples or those blotted onto filter paper discs was amplified by PCR using the primer pair 5'-GGCTCACGTTAGTTGGA-3' and 5'-TGAATTTCTTTTTATTTCCAAA-3' to give a 264-basepair product corresponding to amino acid residues 32 to 119 of the Pfcrt gene product. Digestion of this PCR product with Apo I results in two fragments of 128 and 136 basepairs if the CQ-sensitive haplotype (CVMNK) is present. The K76T mutation renders the fragment resistant to digestion with Apo I. For the Pfmdr1 gene, the primer pair 5'-ATGGGTAAGAGCGAAAGA-3' and 5'-AACGCAAGTAGTACATAAAAGTCG-3' was used to give a 603-basepair product. Digestion of this PCR product with Afl III results in two fragments of 253 and 350 basepairs if an A to T mutation is present, which results in the N86Y mutation. The wild-type PCR product is resistant to digestion with Afl III. After screening the DNAs for PCR amplification and restriction enzyme digestion, the primary PCR products were all subjected to DNA sequencing by the dideoxy termination enzymatic method using the single capillary ABI prism 310 sequencer (Applied Biosystems, Foster City, CA).

**RESULTS**

All DNA samples (PCR products) were digested with Apo I and Afl III and sequenced. The enzyme digestion patterns correlated with the DNA sequence and some examples are shown in Figure 1. Apo I did not digest any of the DNA samples except 6 and 18. As expected, DNA sequence data showed that the DNA samples resistant to Apo I carried the CQ-resistant haplotypes. In the case of DNA samples 6 and 18, although Apo I digested most of the DNA, a small amount was left undigested even after overnight digestion. The DNA sequence of the PCR product indicated a CQ-sensitive (CVMNK) haplotype. However, careful analysis of the individual bands indicated that a small proportion of the DNA might contain the CQ-resistant haplotype (SVMNT). To confirm this result, the DNA that remained undigested on the gel was eluted and sequenced. This clearly showed the presence of the CQ-resistant haplotype. Thus, samples 6 and 18 were classified as mixed. Sample 6 was a field collection, while sample 18 was an old culture available in the laboratory. In the case of digestion with Afl III, all the PCR DNA samples digested were found to carry the N86Y mutation.

The haplotype analysis based on DNA sequence information of the PCR products is shown in Table 1. The results indicate that of the 20 P. falciparum-infected blood samples analyzed from northern and northeastern India, 18 had the

![Figure 1](pfcrt-gene-haplotype-in-india-257.png)

**TABLE 1**

<table>
<thead>
<tr>
<th>Region and period of collection</th>
<th>Pfcrt haplotype</th>
<th>Pfmdr1 haplotype</th>
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<tr>
<td>North and northeast, 1988–1996</td>
<td>CVMNK (2)</td>
<td>N86 (16)</td>
</tr>
<tr>
<td></td>
<td>SVMNT (18)</td>
<td>Y86 (4)</td>
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<tr>
<td>(Sporadic cases)</td>
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</tr>
<tr>
<td>South, 1999</td>
<td>CVMNK (2)</td>
<td>N86 (16)</td>
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<tr>
<td></td>
<td>SVMNT (18)</td>
<td>Y86 (4)</td>
</tr>
<tr>
<td>(Early treatment failures)</td>
<td>SVMNT (11)</td>
<td>SVMNT (12)</td>
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<tr>
<td>(Hospitalized tribal patients)</td>
<td>SVMNT (12)</td>
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\* Pfcrt = *P. falciparum* chloroquine resistance transporter; Pfmdr1 = *P. falciparum* multidrug resistance 1.
† All four blood samples with the Pfcrt (CVIET) haplotype had the Y86 Pfmdr1 mutation. However, of the 11 blood samples with the Pfcrt (SVMNT) haplotype, only one sample had the Y86 Pfmdr1 mutation.
‡ Mixed infection.
CQ-resistant haplotype (SVMNT) and only two had the CQ-sensitive haplotype (CVMNK). Data on in vitro CQ resistance is available on these samples and 15 of the 18 carrying the CQ-resistant haplotype are clearly resistant and three show borderline resistance. The two samples with the CQ-sensitive haplotype are highly sensitive, responding to 1 PM CQ. All 15 blood samples from southern India analyzed had the CQ-resistant haplotype. Interestingly, four of these had the African/Southeast Asian CQ-resistant haplotype (CVIET) and the remaining 11 had the SVMNT haplotype. In the field, these 15 patients were classified as early treatment failures and were resistant to CQ, sulfadoxine, and pyrimethamine therapy. The blood samples from the western India were from the Gondia District of Maharasthra, an endemic region with a high incidence of P. falciparum malaria. The clinicians were not clear as to the efficacy of CQ in this region. All but one of the samples had the CQ-resistant haplotype (SVMNT). The one exception was a mixed infection with CQ-sensitive (predominant) and CQ-resistant P. falciparum. The blood samples from eastern India were collected from an endemic tribal population and all of them had the CQ-resistant haplotype (SVMNT). Thus, 70 of the 73 archived samples collected from different regions of India had the CQ-resistant Pfcr haplotypes.

Analysis of the Pfmdr1 gene showed that 20 of the 69 DNA samples had the N86Y mutation. The regional proportion of the N86Y mutation ranged from 11% to 33%, except for four samples from southern India that had the Pfcr CQ-resistant haplotype CVIET and the Pfmdr1 N86Y mutation.

**DISCUSSION**

The study had three interesting findings. The first was that the predominant Pfcr CQ-resistant haplotype in India seems to be SVMNT rather than CVIET. It is interesting to note that isolates from Papua New Guinea and India share this haplotype with the South American isolates. However, studies based on an analysis of P. falciparum strains using microsatellite haplotypes showed a strong clustering of Papua New Guinea isolates with Southeast Asian (Thailand)/Africa isolates, which is indicative of a greater evolutionary relatedness and distinction from South American isolates. It has been suggested that the CQ-resistant Pfcr allele (SVMNT) in Papua New Guinea and South America (Brazil) has evolved independently due to CQ-selective sweeps (changes), since the Pfcr-flanking microsatellite haplotypes are notably different. While the CQ-resistant haplotype in Papua New Guinea is exclusively SVMNT, Indian isolates appear to be unique in carrying a mixed haplotype, although the Southeast Asian/African haplotype was found in only four of the 73 samples analyzed. The predominant SVMNT haplotypes seen in the blood samples from India raise the question of whether independent sweeps due to CQ pressure with similar results are possible in geographically isolated regions of the world. If so, multiple geographic foci for the origin of CQ resistance mutations need to be considered, in addition to the foci already identified as originating from the Old World (Southeast Asia/Africa) and the New World (South America). Analysis of a large number of samples across a region can shed light on the proportion and preponderance of the SVMNT haplotypes in the Indian subcontinent.

The second interesting finding was the high frequency (70 of 73) of the Pfcr K76T mutation seen in archived and fresh blood samples from different parts of India. Although there is a strong correlation between the Pfcr K76T mutation and CQ resistance in vitro, the in vivo response to CQ therapy is also influenced by host factors, including acquisition of partial immunity in endemic areas with age and the potential for drug metabolism. Field studies in Africa and Laos have led to the conclusion that this mutation is necessary, but not sufficient, to predict the response to CQ in vivo. Nevertheless, a high preponderance of the CQ-resistant haplotype, even in specific geographic regions, needs to be viewed seriously. This indicates that the parasite with a mutation for CQ resistance is already widespread in such areas, and this can have serious implications for children and malaria-naive individuals who are infected for the first time with P. falciparum carrying this mutation, if this is the primary mechanism for drug resistance. Therefore, it is necessary to conduct large-scale correlative studies on the prevalence of CQ resistance Pfcr haplotypes, including the other mutations in the field, and the response to CQ therapy. The practice of large-scale CQ therapy for P. falciparum malaria in India needs to be re-evaluated, since this drug constitutes the frontline therapy in this country. However, there is a trend towards increasing CQ-resistant P. falciparum cases in India.

The third interesting finding was that co-selection of the CQ-resistant Pfcr haplotype and the Pfmdr1 N86Y mutation in the Indian samples was infrequent, unlike the results observed in Papua New Guinea and Mali. However, it is interesting to point out that all four samples with the African/Southeast Asian haplotype (CVIET) observed in the present study had the Pfmdr1 N86Y mutation. Nevertheless, a larger sample size would be needed to establish such a correlation. The suggestion that the Pfmdr1 N86Y mutation may represent a compensatory mutation conferring fitness or some other advantage for coping with CQ pressure may not be valid in all geographic areas.

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