INTRODUCTION

In Sri Lanka, sulfadoxine-pyrimethamine (SP) is currently used as the second-line drug for treatment of chloroquine-resistant, uncomplicated Plasmodium falciparum infections. Resistance of P. falciparum to chloroquine has increased since it was first reported in 1984 and currently accounts for more than 50% of treatment failures in certain parts of the country. Evidence of clinical failures with SP was first published in 1994, but only a few such cases have been found in the country to date.

Resistance of P. falciparum to SP is due to point mutations in the dihydrofolate reductase (Pf-dhfr) and dihydropteroate synthase (Pf-dhps) genes. In Pf-dhfr, point mutations changing Asn51 to Ile (N51I), Cys59 to Arg (C59R), Ser108 to Asn (S108N), and Ile164 to Leu (I164L) have been shown to confer resistance to pyrimethamine. Sulfadoxine resistance also depends on mutations in its target, the DHPS domain of the bifunctional protein dihydro-hydroxymethylpyrophosphokinate-DHPS, at codons 436 (S436A/F), 437 (A437G), 540 (K540E), 581 (A581G), and 613 (A613S/T).

An increase in the proportion of mutant alleles of Pf-dhfr and Pf-dhps and a increasing parasitologic resistance at mild and moderate levels has usually preceded the decrease in clinical effectiveness of SP. Initial clinical failures to SP usually become evident when an isolate carries a triple mutant (N51I + C59R + S108N) in the Pf-dhfr gene with or without additional mutations in Pf-dhps. As such, determining the prevalence of mutant alleles of Pf-dhfr and Pf-dhps and comparing it with in vivo and in vitro outcome of parasite isolates can be extremely valuable in making predictions about the likely efficacy of antifolate drugs in a particular region. The longitudinal monitoring of in vitro response of parasite isolates to an antifolate drug in parallel with molecular analyses and in vivo assays can provide valuable information to adopt a predictive method of molecular surveillance for antifolate resistance, especially in areas where therapeutic failure is uncommon, by the time the clinical failure begins to establish.

MATERIALS AND METHODS

Study site and population. Sample collection for this study was carried out from January to June 2002 in an operational area in the Mannar district of the Northern Province of Sri Lanka approximately 28 km east of Mannar town on the main supply route from Mannar to Vavuniya. The study area was exclusively inhabited by approximately 800 Sri Lankan armed forces and police personnel who had been deployed to protect...
the main supply route from rebel attacks. The screening was carried out in camps scattered within a 10-km stretch along this route. A temporary field station, staffed by a medical officer, a technical officer and a technical assistant, was established for the purposes of this study in one of the camps within this study area.

**Study participants and protocol.** Patients with any symptoms suggestive of acute malaria were screened by examining a thick blood smear stained with 2% Giemsa. At least 100 thick film fields were examined before a patient was confirmed as negative. All positive patients were initially treated with chloroquine as recommended by the national Anti Malaria Campaign (AMC), Sri Lanka; 600 mg on day 1, 600 mg on day 2, and 300 mg on day 3. Primaquine was also given as recommended for *P. vivax* and *P. falciparum*, i.e., 7.5 mg twice a day for 14 days and a 45-mg single dose, respectively. Those who received treatment were subjected to the World Health Organization (WHO) extended field assay to determine the *in vivo* response to chloroquine, according to the guidelines given by WHO. During this assay, enrolled patients were reviewed clinically as well as parasitologically on days 1, 2, 7, 14, 21, and 28 as described elsewhere. All patients who had chloroquine-resistant *P. falciparum* infections were enrolled in the *in vivo* assay for SP provided that they fulfilled the following criteria: 1) an elevated axillary temperature ≥ 37.5°C (99.5°F) at recrudescence or a history of fever in the previous 24 hours; 2) mono-infection with *P. falciparum* regardless of parasite density at recrudescence; 3) absence of severe malaria; 4) absence of a history of allergy to either SP or primaquine and absence of a history of glucose-6-phosphate dehydrogenase deficiency; and 5) willingness to provide informed consent to participate in the study. All patients with uncomplicated *P. falciparum* recrudescences were treated with a single dose of SP as recommended by the AMC, Sri Lanka: 1,500 mg of sulfadoxine and 75 mg of pyrimethamine. Those who had sexual stages at the recrudescence were treated with a single dose of 45 mg of primaquine. On enrollment, each patient was examined and/or evaluated by a medical officer for symptoms, duration of the illness, anti-malarial therapy during the last four weeks, previous malaria episodes, and axillary temperature. Information was recorded in a pre-tested questionnaire. The severity of each infection was determined using a previously validated clinical scoring system. Before commencing the treatment (day 0), finger prick blood samples were collected from each enrolled patient using sterile disposable lancets, and blotted onto filter paper strips of 0.5 × 3 cm (3 MM; Whatman, Florham Park, NJ). They were stored at −20°C until further analysis. Parasite densities were calculated by counting the number of asexual parasites per 200 white blood cells in a thick film assuming an average white blood cell count of 8,000/µL of blood. All drugs were administered under supervision and re-administered if a patient vomited within 30 minutes of administration.

**Follow-up of patients.** After treatment with SP, each enrolled patient was followed-up for 42 days to determine the clinical and parasitologic responses to SP. During the follow-up period, each patient was reviewed daily until the fever subsided, as determined by measuring the axillary temperature. The severity of each infection was monitored based on the total clinical scores measured using the clinical scoring system. A thick blood film was repeated in each patient 48–72 hours after treatment to ascertain the clearance of asexual stages. If any patient seemed to be deteriorating clinically within first 48 hours after the treatment with SP, a thick blood smear was repeated immediately to determine whether the parasite density was increasing. Subsequently, each patient was reviewed clinically as well as parasitologically on days 14, 28, and 42. In addition, since we were visiting these camps daily for screening of new patients and for reviewing of patients enrolled in the *in vivo* assay for chloroquine, all patients enrolled in the *in vivo* assay for SP and the medical staff of each camp were advised to report to us immediately if anyone developed fever or any other symptoms suggestive of malaria on days other than their routine follow-up days. If so, they were assessed clinically as well as parasitologically in addition to their routine review.

On each review, the axillary temperature and the total clinical scores of each patient were recorded. Thick and thin smears were prepared using a finger prick blood sample and examined for the reappearance of asexual stages of *P. falciparum*. At the same time, a blood sample from each patient was collected onto filter paper strips. The parasite densities of positive samples were calculated as described earlier in this report. The treatment failures with SP were treated with quinine as recommended by the AMC, Sri Lanka. The parasitologic responses were classified into RI, RII, and RIII grades, and clinical response was classified into adequate clinical response, early treatment failure, and late treatment failure according to modified WHO criteria for areas with low-to-moderate transmission.

**PCR-restriction fragment length polymorphism (PCR-RFLP) assay.** Parasite DNA was extracted from filter paper strips using a modified method previously published. The *Pf-dhfr* was amplified by PCR using a nested protocol and was subjected to RFLP analysis as previously described. The oligonucleotides used in the first round were M1 5′-TTATGATGGAACAAAGTCTGC-3′ and M2 5′-AGTATATACATCGCTAACAGA-3′. In the nested reactions, the following primers were used: F1 5′-AAATGTAACTCCATTATGATGAAATTT-3′ and M1 5′-TTAATTTCCAGAGAAAACATTAGAGGTAGTC-3′; F1 5′-AAATGTAACTCCATTATGATGAAATTT-3′ and M2 5′-TTATGATGGAAACAAAGTCTGCAG-3′. Mutations at codon positions 51 (51I), 59 (59R), 108 (S108N), and 164 (164L) were detected by digesting the PCR products with the restriction enzymes *Tsp* 509I, *Xmn* I, *Bsr* I/*Alu* I, and *Dra* I (New England Biolabs, Beverly, MA), respectively. Digestions were carried out overnight according to the manufacturer’s instructions. The digested products were subjected to electrophoresis on 6% denaturing polyacrylamide gels and visualized by silver staining. Digested products of the field isolates were compared with those of reference isolates K1, (59R/108N), FCR3 (S108N), W2 (S108N/108N), and V1/S (S108N/108N/164L) that were known to have wild type and mutant alleles at the codon positions listed.

Similarly, the *Pf-dhps* gene was amplified by PCR using oligonucleotides R2 5′-AACCTAAACGTGTGCTTCA-3′ and R′ 5′-AATTGTTGATTTTGTCACAA-3′ in the first round followed by a second round of amplification with K 5′-TGCTAGTTATGATGATATGAGGAGA TC-3′ and K′ 5′CTAAACGAGGTAGTcATTTAATGCAAGA-3′, L 5′-ATAGGATACTATTTTGATATTTGGACCA AGGATTcG-3′, and L′ 5′-TATTAACACATTTTGATCAT-
TGGCAAccGG-3' using a protocol described elsewhere.30 In this study, mutations at codons 436 (S436A/F), 437 (A437G), 540 (K540E), 581 (A581G), and 613 (A613S/T) were detected by digesting nested PCR products with the restriction enzymes Mnl I/Msp AI, Mwo I/Ava II, Fok I, Bst UI/Bol I, and Mwo I/Bsa WI/Age I (New England Biolabs), respectively. The digestions, visualization of digested products, and identification of alleles were carried out as described elsewhere.

Cloning and sequencing of Pf-dhfr. Pf-dhfr was first amplified by PCR as described previously.31 The primer sequences for the amplification were 5'-TCTCCTTTATGATGGAAAACAAGTCCTGCAGCTTTTCG-3' and 5'-TCATATGACATGTATCTTTGTTCATATTCTTTAAAAGGC-3'. The 5' end of each primer is complementary to the sequence of the yeast shuttle vector plasmid GR7, a derivative of the pRS314 yeast shuttle vector.32 Yeast transformations were performed using a lithium acetate–mediated method.33 They were then grown on yeast extract, peptone, and dextrose broth (YEPD) plates for 3–5 days without deoxythymidine monophosphate to select the successful transformants that express a functional Pf-dhfr gene.

Plasmids carrying Pf-dhfr were isolated by transforming total yeast DNA into the chemically competent Escherichia coli strain DH5α. The transformations were grown overnight on Luria-Bertani (LB) plus ampicillin plates at 37°C. Single colonies were grown in liquid LB media for 12–16 hours at 37°C, after which plasmid DNA was isolated using Qiagen miniprep kits (Qiagen, Valencia, CA). The following primers were used for PCR amplification of plasmid DNA in sequencing reactions: 5'-TGAAAAGCACAAGCAGCTACG-3' and 5'-GGCATATCTATATTTTTTTCTTTCTTTTAC-3'. The PCR protocol was as follows: initial denaturation at 96°C for 10 seconds, 24 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, and extension at 60°C for 4 minutes. The reactions were terminated at 4°C. Sequencing was conducted using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Perkin Elmer Applied Biosystems Division, Foster City, CA). Sequencing was performed using the manufacturer's protocols, and sequences were analyzed using Sequencher® software (Gene Codes Corporation, Ann Arbor, MI).

IC50 assay for in vitro susceptibility to pyrimethamine. In the absence of treatment failures to SP during the in vivo assay, we determined the in vitro response of those field isolates to SP. This was achieved by measuring IC50 values of field isolates to pyrimethamine in a yeast system. The IC50 assays were performed according to a standard protocol described elsewhere.31 In this study, the IC50 was defined as the concentration of drug at which the growth of the yeast culture was inhibited by 50% relative to that of the same cells grown with the solvent dimethylsulfoxide (DMSO) alone. Yeast cells that were transformed with Pf-dhfr alleles were grown to mid-log phase. For each strain, a final concentration of 5 × 10^4 yeast cells and pyrimethamine (Sigma Aldrich, St. Louis, MO) at concentrations between 10^-4 and 10^-8 M were used in each well. Cell and drug dilution mixtures were grown in liquid YEPD medium in 96-well plates for 23–24 hours at 30°C, and the absorbance was measured at 650 nm. YEPD medium alone was used to measure background absorbance. Yeast dependent on the Pf-dhfr alleles of the pyrimethamine-sensitive wild type, the triple mutant 51I/59R/108N, and quadruple mutant 51I/59R/108N/164F were used as reference strains. The IC50 assays were performed in duplicate for each strain to ensure reproducibility.

Inhibition curves were obtained using Kaleidagraph version 3.51 (Synergy Software, Reading, PA) by plotting the percentage of growth of each strain, relative to the no drug (DMSO) control, against the drug concentrations used in the assay. The IC50 values were determined using this plot.

Ethical clearance. Ethical clearance for this study was obtained from the Ethics Committee of the Faculty of Medicine, University of Kelaniya, Sri Lanka.

RESULTS

In vivo assay. Of 38 patients who received SP, 30 (78.9%) completed the 42-day follow-up period. None of the infections showed either clinical or parasitologic evidence of resistance to SP during the 42-day follow-up period.

PCR-RFLP assay. The samples analyzed were taken before treatment with SP from 30 patients who completed the in vivo assay for SP resistance. Thus, they represent a reasonably unbiased sample with respect to the SP alleles found in the P. falciparum samples.

Our first goal was to identify isolates that carried mutations at the four codons most frequently observed in the Pf-dhfr gene that confer resistance to pyrimethamine: 51, 59, 108, and 164. These data are summarized in Table 1. Three of the 30 samples analyzed by PCR-RFLP assay showed mixed allelic infections. Of the 27 pure infections, 23 (85.1%) field isolates showed mutant alleles at codon 108 (S108N), as well as at codon 59 (C59R) of Pf-dhfr, and four carried only the wild type allele. None of the isolates had single mutant alleles and we did not identify allelics with mutations at codons 51 or 164.

In the Pf-dhps gene, two (6.7%) isolates had mixed alleles at codon 436 (wild type and S436A), one isolate (3.3%) showed mutant alleles at codon 437 (A437G), and three isolates showed mixed alleles at codon 437 (wild type and A437G). Moreover, four (13.3%) isolates showed mutant alleles at codon 581 (A581G) and four isolates had mixed alleles (wild type and A581G) at the same codon position. All isolates were of wild type at codons 540 and 613 of the same gene (Table 1).

Sequence analysis. Since there was no prior information about Pf-dhfr alleles from this region, we also sequenced the coding region of the dhfr domain in each isolate. Of 30 isolates sequenced, 25 showed S108N + C59R mutations in Pf-dhfr. This included two alleles isolated from patients who carried mixed infections identified in the PCR-RFLP assay (Table 2). All isolates were wild type at codons 16, 51 and 164, and no new mutations were detected in the dhfr sequence. Sequence analysis confirmed the results of PCR-RFLP analysis by not detecting any alleles with single mutations at either codons 108 or 59.

IC50 assay. Under the field conditions in which these isolates were collected, it was not possible to measure the in vitro response of the parasites to pyrimethamine. As a surrogate
measure, we transferred these Pf-dhfr alleles to yeast that lacked endogenous DHFR activity, and measured the sensitivity of these yeast strains to the drug. Each strain was grown in 0–10^{-4} M pyrimethamine. Growth was measured after 24 hours and IC_{50} values were determined. Graphic analysis of these IC_{50} values showed two patterns of in vitro response to pyrimethamine (Figure 1). The response curves of the wild type isolates (mean IC_{50} values ranged from 6.6 \times 10^{-9} to 1.0 \times 10^{-8} M, summarized in Table 2) were in the same range as that of the wild type reference strain (IC_{50} value = 1.7 \times 10^{-8} M). In contrast, 10 field isolates had IC_{50} values ranging from 4.0 \times 10^{-6} to 1.6 \times 10^{-5} M, which were slightly lower than yeast dependent upon the triple mutant reference strain (1.9 \times 10^{-5} M). When matched with sequencing and PCR-RFLP data, it was evident that these strains all carried the double mutant allele of Pf-dhfr and were approximately 200-fold more resistant to pyrimethamine than the wild type isolates.

**DISCUSSION**

Based on few cases of clinical failures with SP reported since 1994,4–6 it is believed that in vivo resistance of P. falciparum to SP is not yet common in Sri Lanka. However, proper data are not available to confirm this situation because of the lack of large epidemiologic studies with regard to SP resistance in this country. To the best of our knowledge, this study was the first attempt to determine the prevalence of SP clinical failures at the community level in Sri Lanka. Although only a moderate number of samples were analyzed, our results suggest clinical failure of SP treatment of P. falciparum is not common in an area with a high level of chloroquine resistance in Sri Lanka.5 However, double mutant alleles that confer increased resistance to pyrimethamine are already well established in this area. In our survey of 30 P. falciparum isolates, only two types of alleles of Pf-dhfr: wild type and double mutants (S108N + C59R). However, 85% of the isolates were double mutants in Pf-dhfr, with in vitro drug sensitivity patterns in yeast slightly lower than that of a triple mutant reference strain. Several in vitro studies have previously shown that P. falciparum parasites that carry double mutant alleles of dhfr are moderately resistant to pyrimethamine,13,37 but the patients who carry parasites with this genotype have an adequate clinical response to SP.14,20,21 Extrapolating from work done in countries where SP resistance is more extensive,21,38–44 it is likely that continued use of SP will result in selection for strains that carry the triple mutant allele of dhfr that is associated with clinical SP resistance.

**TABLE 1**

Distribution of alleles in Plasmodium falciparum dihydrofolate reductase (dhfr) and dihydropteroate synthase (dhps) genes of Sri Lankan field isolates*

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Codon in dhfr</th>
<th>Codon in dhps</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE4</td>
<td>Asn Arg Asn Ile</td>
<td>Ser Ala Lys Ala Alal</td>
</tr>
<tr>
<td>NE6</td>
<td>Asn Arg Asn Ile</td>
<td>Ser Gly Lys Gly Alal</td>
</tr>
<tr>
<td>NE8</td>
<td>Asn Arg Asn Ile</td>
<td>Ser Ala Lys Ala Alal</td>
</tr>
<tr>
<td>NE10</td>
<td>Asn Arg Asn Ile</td>
<td>Ser Ala Lys Ala Alal</td>
</tr>
<tr>
<td>NE15</td>
<td>Asn Arg Asn Ile</td>
<td>Ser Ala Lys Ala Alal</td>
</tr>
<tr>
<td>NE22</td>
<td>Asn Cys Ser Ile</td>
<td>Ser Ala Lys Ala Alal</td>
</tr>
<tr>
<td>NE24</td>
<td>Asn Mix Mix Ile</td>
<td>Ser Ala Lys Mix Alal</td>
</tr>
<tr>
<td>NE30</td>
<td>Asn Cys Ser Ile</td>
<td>Mix Mix Ala Lys Ala Alal</td>
</tr>
<tr>
<td>NE35</td>
<td>Asn Arg Asn Ile</td>
<td>Ser Mix Lys Gly Alal</td>
</tr>
<tr>
<td>NE39</td>
<td>Asn Arg Asn Ile</td>
<td>Ser Ala Lys Mix Alal</td>
</tr>
<tr>
<td>NE42</td>
<td>Asn Arg Asn Ile</td>
<td>Ser Mix Lys Gly Alal</td>
</tr>
<tr>
<td>NE51</td>
<td>Asn Cys Ser Ile</td>
<td>Mix Mix Ala Lys Ala Alal</td>
</tr>
<tr>
<td>NE56</td>
<td>Asn Arg Asn Ile</td>
<td>Ser Ala Lys Ala Alal</td>
</tr>
<tr>
<td>NE58</td>
<td>Asn Arg Asn Ile</td>
<td>Ser Ala Lys Ala Alal</td>
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<tr>
<td>NE67</td>
<td>Asn Cys Ser Ile</td>
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<td>NE72</td>
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</tr>
<tr>
<td>NE74</td>
<td>Asn Arg Asn Ile</td>
<td>Ser Ala Lys Mix Alal</td>
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<td>NE75</td>
<td>Asn Arg Asn Ile</td>
<td>Ser Ala Lys Ala Alal</td>
</tr>
<tr>
<td>NE78</td>
<td>Asn Mix Mix Ile</td>
<td>Ser Ala Lys Ala Alal</td>
</tr>
<tr>
<td>NE79</td>
<td>Asn Arg Asn Ile</td>
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</tr>
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<td>Ser Ala Lys Ala Alal</td>
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<td>NE83</td>
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<td>Ser Ala Lys Ala Alal</td>
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<tr>
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<td>NE91</td>
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<td>NE92</td>
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<td>Ser Ala Lys Mix Alal</td>
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<tr>
<td>NE93</td>
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<td>Ser Ala Lys Ala Alal</td>
</tr>
<tr>
<td>NE95</td>
<td>Asn Arg Asn Ile</td>
<td>Ser Ala Lys Ala Alal</td>
</tr>
</tbody>
</table>

* All codons were determined by an allele-specific polymerase chain reaction-restriction fragment length polymorphism methods. White boxes = wild types; black boxes = mutants; gray boxes = mixed genotypes.

**TABLE 2**

Comparison of molecular and in vitro analyses of the dihydrofolate reductase gene of Plasmodium falciparum field isolates*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of isolates</th>
<th>IC_{50} (M) range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>4</td>
<td>6.6 \times 10^{-9}–1.03 \times 10^{-8}</td>
</tr>
<tr>
<td>C59R + S108N</td>
<td>23</td>
<td>3.96 \times 10^{-6}–1.62 \times 10^{-5}</td>
</tr>
<tr>
<td>Mixed</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

* Genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism methods and confirmed by complete sequencing. Each allele was transferred into yeast and the 50% inhibitory concentration (IC_{50}) was determined.
are observed principally in populations in which the Pf-dhfr allele distribution at codon 59 of Pf-dhfr, along with additional mutations in Pf-dhps, can be used as a good predictive marker for SP clinical failure. However, we found a high frequency of mutant alleles at codon 59 of Pf-dhfr in the absence of clinical failures to SP in our population. Our results, as well as those of recent studies in India,18,47,50 have shown that most of the double mutant alleles have the 59R/108N genotype, rather than the 51I/108N allele that is most often seen in east Africa.20,22,26,38,46 Thus, it appears that allele distribution at amino acid residue 51 of Pf-dhfr rather than at codon 59 would be a good marker for the triple mutant and impending clinical failure of SP on the Indian subcontinent. This conclusion stresses that the mutation acquisition pattern in Pf-dhfr of P. falciparum is one of the important determinants of molecular markers for the detection and/or prediction of SP resistance in different geographic settings.

It has always been assumed that additional point mutations and the subsequent selection of parasites that carry triple and quadruple mutant alleles of Pf-dhfr and mutant alleles of Pf-dhps would follow in each location where SP was introduced. However, several recent studies have suggested that single and double mutant alleles do arise independently on many different genetic backgrounds, but that the triple mutant Pf-dhfr alleles observed in widely separated populations shared a common ancestor.33,41,48,49 Roper and others further suggest that there does not appear to be a strong selection for single mutants, so that they may not persist long enough to accumulate further mutations.41 If so, this could also explain the predominance of double mutants in the absence of pure single mutants in our study population. As such, further studies are necessary to test these models and to understand the sequence of molecular events in the process of SP resistance.

Molecular surveillance methods should allow one to monitor the prevalence of alleles that are correlated with drug resistance and to relate those data to the prediction of therapeutic efficacy in that population. Based on recent work in Malawi46 and Uganda,20,22 it has been suggested that the allele distribution at codon 59 of Pf-dhfr, along with additional mutations in Pf-dhps, can be used as a good predictive marker for SP clinical failure. However, we found a high frequency of mutant alleles at codon 59 of Pf-dhfr in the absence of clinical failures to SP in our population. Our results, as well as those of recent studies in India,18,47,50 have shown that most of the double mutant alleles have the 59R/108N genotype, rather than the 51I/108N allele that is most often seen in east Africa. Thus, it appears that allele distribution at amino acid residue 51 of Pf-dhfr rather than at codon 59 would be a good marker for the triple mutant and impending clinical failure of SP on the Indian subcontinent. This conclusion stresses that the mutation acquisition pattern in Pf-dhfr of P. falciparum is one of the important determinants of molecular markers for the detection and/or prediction of SP resistance in different geographic settings.

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Authors’ addresses: Hapuarachchige C. Hapuarachchi, Meegoda Y. D. Dayanath, Kandeyaye Bandaralage A. T. Bandara, Sudusinge Abeyesundara, Wimaladhama Abeywickreme and Nilanthi R de Silva, Department of Parasitology, Faculty of Medicine, University of Kelaniya, P.O. Box 6, Thalagolla Road, Ragama, Sri Lanka, Telephone: 94-11-295-3412, Fax: 94-11-295-3412. Sonia Y. Hunt and Carol Sibley, Department of Genome Sciences, Box 357730, University of Washington, Seattle, WA 98195-7730, Telephone: 206-685-9578, Fax: 206-543-0754, E-mail: sibley@gs.washington.edu.

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