In response to the emergence of chloroquine-resistant *Plasmodium falciparum* malaria in Africa, drugs targeting parasite folate biosynthesis (antifolates) were deployed as the first-line treatment for uncomplicated malaria. The most commonly used antifolate is pyrimethamine-sulfadoxine (PYR-SDX). PYR targets *P. falciparum* dihydrofolate reductase (PfDHFR), a key enzyme involved in the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate.

The greatest disadvantage of the use of PYR-SDX has been the rapid evolution of drug-resistant parasites. This is mediated by the sequential acquisition of point mutations in the Pf*dhfr* and Pf*dhps* genes (8, 25). With respect to PYR, the single Pf*dhfr* mutation S108N is responsible for low-level resistance and is followed by an N51I or C59R mutation (resulting in a double mutant). A combination of all three mutations gives rise to the highly PYR resistant triple mutant. A fourth point mutation in Pf*dhfr*, I164L, is found extensively in Southeast Asia and South America, but there is debate over its existence in Africa (5, 11, 12, 17, 29). The presence of the I164L quadruple mutant confers high-level resistance to PYR, and in Africa this mutation would severely compromise the continued use of PYR-SDX. This mutation would also compromise the use of Lapdap, a combination of chlorproguanil (CPG) and dapsone (DDS) (30), and CPG-DDS-artesunate in Africa. The development and use of these compounds have now been halted due to toxicity in children with glucose-6-phosphate dehydrogenase deficiency.

Many studies have looked for the presence of the I164L mutant allele in Africa; Malawi was the first African country to switch from chloroquine to PYR-SDX in 1993. Using conventional PCR, most studies have not detected this mutation (2, 3, 7, 15, 18, 19, 22–24, 26, 27), but it has been reported at low prevalences in five different African countries.

Of particular interest is the study by Alker et al. (1), who used real-time PCR with fluorescent probes specific for the mutation and reported a 4.7% prevalence in parasites collected from human immunodeficiency virus-positive pregnant women in Malawi between 2001 and 2003, a finding that they validated more recently using a heteroduplex tracking assay (9). However, on biological grounds, if the quadruple-mutant alleles were present in reasonable proportions, it is hard to imagine that they would not have been selected to high levels by this time. This is the highest prevalence reported, and it is a high priority for the public health that this finding be further evaluated.

The aims of this study were to confirm and validate the sensitivity, specificity, and reproducibility of the assay reported by Alker et al. (1) and to confirm the presence of the mutant I64 allele in parasites collected from the same location in Malawi. We also tested the hypothesis that sustained antifolate use would have resulted in an increased prevalence of the I164L mutant in subsequent years. Finally, we wanted to determine if treatment failure after treatment with PYR-SDX resulted in the selection of this mutation. The prevalence of the I164L allele was also investigated in clinical isolates from Zambia, a neighboring country with a shorter history of PYR-
SDX deployment, and in clinical isolates from the Thailand-Myanmar border in Southeast Asia, an area known to have a high prevalence of the 1164L mutation (5, 10).

MATERIALS AND METHODS

Samples. Five Plasmodium falciparum reference strains (K1, 3D7, HB3, Dd2, and V1/S) were selected for optimization and validation of the Pf
dhfr 1164L mutation-specific assays. K1, 3D7, HB3, and Dd2 are wild type at position 164 (isoleucine), while V1/S is mutant (leucine). The genotypic and phenotypic statuses of the strains were established through PCR–restriction fragment length polymorphism, real-time PCR, and in vitro determination of 50% inhibitory concentrations of PYR, as previously described (13). Further confirmation was performed by DNA sequencing of the region of Pf
dhfr encompassing the 1164L allele.

Field isolates were obtained from studies conducted in Malawi between 2003 and 2005 (n = 210), in Zambia in 2005 (n = 55), and around the Thailand-Myanmar border in 2005 (n = 50). The Malawian samples were from children less than 5 years old presenting with uncomplicated malaria and were treated as part of a study with PYR-SDX alone or PYR-SDX plus either chloroquine, artemether, or amodiaquine. The study took place at a government health center 10 km outside of Blantyre, Malawi, where malaria transmission occurs all year round. Parasite isolates were collected prior to treatment and from children who had recurrent parasitemia after treatment. For 57 children, both pre- and post-treatment isolates were available. Genotypic analysis of the msps2 gene showed that approximately one-third of these recurrent parasitemias were due to reinfection and the remainder were due to recrudescence. The pretreatment prevalences of Pf
dhfr triple-mutant parasites was 96%, compared to a prevalence of 80% in the parasites described by Alker et al. (1). Details of the Malawian study, which includes Pf
dhfr 164 genotype typing using a less-sensitive methodology (PCR and allele-specific restriction analysis) have been published elsewhere (4).

The Zambian samples were collected from adults with uncomplicated malaria before treatment as part of a randomized clinical trial with either PYR-SDX or artesunate-lumefantrine. The samples were a fair representation of the population, and inclusion/exclusion criteria have been published previously (14, 28).

Isolation and extraction of total DNA. Total genomic DNAs (host and parasite) were extracted either from EDTA-treated whole blood or from blood-spotted filter papers with the QIAamp DNA blood minikit (Qiagen) according to the manufacturer’s instructions.

Whole-genome amplification. A high degree of variability in parasitemia and a low recovery rate of parasite genomic DNA from filter paper were observed. Therefore, when the total-parasite DNA concentration was below 10,000 copies per µL, whole-genome amplification by improved primer extension PCR was conducted. This procedure was performed as previously described (6) in order to increase the quantity of DNA and maximize the number of experiments that could be conducted on each sample.

Real-time PCR-based discrimination of Pf
dhfr 1164L alleles by the method of Alker. In order to assess the sensitivity of the assay, real-time PCR was conducted using the methodology of Alker et al. (1) using a PTC-200 Peltier thermal cycler with a Chromo 4 continuous fluorescence detector (Bio-Rad). This PCR was conducted on reference strains as well as on samples obtained from Malawi that had not undergone whole-genome amplification. The primer and probe sequences are identical to those published previously (1) and were sourced from Applied Biosystems, Cheshire, United Kingdom.

Normalization of Plasmodium DNA by quantification of EF1-α. Owing to the high degree of variability in parasite DNA content, it was necessary to normalize the samples according to the parasite DNA copy number. This was achieved by quantification of elongation factor 1 alpha (EF1-α) as a marker for the parasite genome copy number. Primers and fluorescent probes specific for the EF1-α gene were designed so as to avoid introns (Table 1).

In order to construct a standard curve, DNAs from five reference strains (K1, Dd2, HB3, 3D7, and V1/S) were normalized to 20 ng/µL in a 25-µL final reaction volume with 1× Absolute QPCR mix (ABgene, Surrey, United Kingdom), 0.9 µM EF1-α forward and reverse primers, and 0.25 µM EF1-α probe. The samples were amplified in a PTC-200 Peltier thermal cycler with a Chromo 4 continuous fluorescence detector. The program consisted of an initial activation at 95°C for 10 min, followed by 45 cycles of denaturation at 92°C for 14 s and annealing/extension at 60°C for 60 s.

Amplons were checked for the presence of nonspecific products by electrophoresis on a 1% agarose gel. Each amplon was gel purified by using the Promega Gel Wizard prep kit according to the manufacturer’s instructions. The copy number of amplons was then quantified as described previously (20) and diluted to 1, 10, 100, 1,000, 10,000, and 100,000 copies per µL. EF1-α was then quantified alongside clinical samples as described above. Following amplification, the cycle threshold (Ct) was determined for each sample and standard, the data plotted, and the equation of the linear regression used to determine the numbers of copies of parasite DNA in the clinical samples. Parasite DNA was then normalized to 100, 250, 500, 1,000, and 10,000 copies per µL for validation of the assay.

Validation of real-time PCR-based discrimination of Pf
dhfr 1164L alleles. For quantification of the Pf
dhfr 1164L DNA, mutant (V1/S) and wild-type (K1) genomes were combined in fixed ratios of 100:0, 99:1, 95:5, 90:10, 75:25, 50:50, and 0:100. These standards were then amplified, and the Ct values for the mutant probe were divided by the corresponding Ct values for the wild-type probe. These values were then plotted against log-transformed percentages as a standard curve.

All reactions were carried out in duplicate in a total volume of 25 µL. Each reaction mixture contained 1× Absolute QPCR mix (ABgene), 200 nM each probe, 288 nM forward primer, and 490 nM reverse primer (Table 1). The program consisted of an initial activation at 95°C for 10 min, followed by 45 cycles of denaturation at 92°C for 14 s and annealing/extension at 60°C for 60 s.

Initial experiments were conducted with 100, 250, 500, 1,000, and 10,000 copies per µL of parasite DNA in order to determine the optimum concentration. The intrarun precision of the analyses, expressed as a percentage, was calculated as 100 – [(standard deviation/mean) × 100]. The intrarun precision, also expressed as a percentage, was calculated as (calculated log copy number)/(nominal log copy number added) × 100. Determinations were performed using the same amplicon stock solutions. Intrarun precision and accuracy were assessed on six replicates of standards containing 10% and 75% concentrations of the mutant genome. Similarly, intrarun precision and accuracy were assessed on six separate runs of standards containing 10% and 75% concentrations of the mutant genome. The limit of detection was defined as the percentage at which the mutant allele could be reliably differentiated from the wild-type allele, and the limit of quantification was defined as values between 90 and 110% for both intra- and interrun accuracy and precision.

For quantification of the percentage of an individual’s total parasite population containing the mutant allele, normalized DNAs were amplified as described above. A standard curve was constructed on each plate, and both standards and samples were assessed at least in duplicate.

RESULTS

Limitations of the original methodology. In transferring the methodology to our laboratory, a number of problems were
encountered. First, a large intersample variability in amplification was observed (Fig. 1A). Second, the wild-type probe was found to bind nonspecifically to the mutant sequence (Fig. 1B), and vice versa (Fig. 1C). This phenomenon could lead to isolates being interpreted as mixed alleles, particularly in Africa, where most infections are polyclonal. The combination of intersample variability and the suboptimal specificity of the probes led us to conclude that the assay was likely to call samples with high parasite DNA concentrations mutant, even if they were actually wild type. A strategy was therefore developed to normalize parasite DNA concentrations (irrespective of total DNA) and to subsequently validate a method for detection of the PfDHFR I164L mutant by capitalizing on the higher specificity of the mutant probe for the mutant sequence and of the wild-type probe for the wild-type sequence. In order to achieve this, an optimized standard curve was included within every run.

**Standardization of parasite DNA copy number.** The amount of parasite DNA within the total DNA of an experimental sample (containing parasite and host DNAs) was quantified by real-time PCR amplification of EF1-α (Fig. 2A). A standard curve generated from purified, quantified prerun amplicons was constructed alongside samples (Fig. 2B). Using this methodology, the median copy number per μl of parasite DNA isolated from Malawian samples was 27,776 (range, 4 to 948,664,062). The median level of parasitemia in the Malawian patients was 66,585 parasites per μl (range, 39 to 644,840 parasites per μl). For log-transformed data, a significant correlation was observed between parasitemia and the copy number of isolated parasite DNA ($R^2 = 0.13; P < 0.0001$).

**Validation of real-time PCR genotyping methodology.** The assay was initially tested at parasite DNA copy numbers of 100, 250, 500, 1,000, and 10,000. At 10,000 copies, efficient discrimination of mutant and wild-type alleles was possible at concentrations between 5 and 100% (Fig. 3A and B). Insufficient amplification occurred at parasite DNA concentrations lower than this (data not shown). For the standard curve of mixed mutant (V1S) and wild-type (K1) DNAs (where wild-type and mutant DNAs were mixed so as to contain 0% to 100% mutant DNA), the CT values obtained for the mutant probe (labeled with 6-carboxyfluorescein [FAM]) were then divided by those for the wild-type probe (labeled with VIC) and plotted against the log-transformed mutant DNA concentration (i.e., the percentage of total DNA that was mutant) (Fig. 3C).

The limits of detection and of quantification were shown to be 5% and 10%, respectively. The intrarun accuracy and interrun accuracy were 98.4% and 106.7% at high mutant concentrations (75%) and 89.7% and 96.9% at low mutant concentrations (10%), respectively. The intrarun precision and interrun precision were 92.1% and 95.1% at high mutant concentrations (75%) and 92.2% and 96.8% at low mutant concentrations (10%).

**Assessment of PfDHFR I164L frequency in Malawian, Zambian, and Thai cohorts.** In samples where the total-parasite DNA concentration was below 10,000 copies per μl, whole-genome PCR was conducted prior to genotypic analysis. Subsequently, sufficient DNA was obtained from 158 of the 210 Malawian isolates (94 pretreatment isolates and 64 isolates from recurrent parasitemia after treatment), 42 of 55 Zambian isolates, and 38 of 50 Thai isolates. Using the optimized real-time PCR methodology, the frequency of the I164L mutation in these isolates was monitored (Table 2). For the Thai isolates, the I164L mutation was present in 36 out of 38 samples tested. The two remaining samples showed indications that if the mutation was present, then it was below the 5% confidence.
level of the assay. Conversely, the I164L mutation was below the confidence level in all Malawian and Zambian isolates tested. Furthermore, there was no evidence of the selection of this I164L mutation in any of the 64 Malawian isolates appearing within 42 days of treatment with PYR-SDX (Table 3).

**DISCUSSION**

We observed a large variability in the degree of amplification from field isolates. This was partly explained by variability in parasitemia, but the correlation coefficient was low (0.13), indicating that other factors contribute to this variability (e.g., DNA extraction efficiency). Furthermore, only partial selectivity of the mutant probe for the mutant sequence and of the wild-type probe for the wild-type sequence was observed.
TABLE 2. Frequency of the Pf dhfr I164L alleles in isolates from Thailand, Zambia, and Malawi

<table>
<thead>
<tr>
<th>Genotype (%)</th>
<th>No. of isolates with I164L alleles/total isolates (%) from:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Thailand</td>
</tr>
<tr>
<td>Pure mutant (100)</td>
<td>26/38 (68.42)</td>
</tr>
<tr>
<td>Partial mutant (5–99) within patients</td>
<td>10/38 (26.32) (80–97%)</td>
</tr>
<tr>
<td>Wild type (&lt;5%)</td>
<td>2/38 (5.26)</td>
</tr>
</tbody>
</table>

TABLE 3. Frequency of different Pf dhfr I164L alleles in Malawian isolates from patients naïve to therapy and from patients having received therapy

<table>
<thead>
<tr>
<th>Isolate collection</th>
<th>No. (%) of isolates with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;5% Ile164</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>94 (100)</td>
</tr>
<tr>
<td>Posttreatment</td>
<td>64 (100)</td>
</tr>
<tr>
<td>Matched pre- and posttreatment</td>
<td>57 (100)</td>
</tr>
</tbody>
</table>

5% of the mutant allele, and it would not have been detected. Nonetheless, the rarity of this allele in Africa despite more than a decade of use of PYR-SDX as frontline antimalarial therapy is an intriguing phenomenon, particularly given its rapid selection in Southeast Asia under similar circumstances (5, 16) and the fact that it is easily selected for in vitro studies (21).

Using a fully validated methodology, we could not identify I164L mutants within clinical isolates from Malawi and Zambia, even in posttreatment failure parasites from Malawi. These data are reassuring, because even though CPG-DDS and CPG-DDS-artesunate have now been withdrawn, PYR-SDX is still used extensively for treatment in Africa and plays a major role in intermittent presumptive therapy programs in pregnancy. Our results are in agreement with the majority of previous reports, and coupled with the need for specialized equipment and the cost associated with real-time PCR, there appears to be no urgent need for field application of this method. The failure of antifolate chemotherapy to select I164L mutant parasites in Africa compared to Southeast Asia is an important phenomenon that clearly requires further investigation of the underlying mechanisms.

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S.A.W. and A.O. designed the study. E.O., D.J.J., and A.O. analyzed the data. E.O., D.J.B., D.J.J., P.A.W., P.G.B., and S.A.W. contributed to the writing of the paper. U.D. and M.M. conducted the trial in Zambia, and S.M. conducted the trial in Thailand. E.O. extracted DNA from all samples used in the study and performed the real-time PCR.

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