

## VALIDATION OF A SIMPLIFIED METHOD FOR USING MOLECULAR MARKERS TO PREDICT SULFADOXINE-PYRIMETHAMINE TREATMENT FAILURE IN AFRICAN CHILDREN WITH FALCIPARUM MALARIA

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**Abstract.** Surveillance of molecular markers for key mutations in *Plasmodium falciparum* dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS) has been proposed as a means of predicting sulfadoxine/pyrimethamine (SP) treatment outcomes in Africa. This study assessed the association between DHFR and DHPS mutations and standardized clinical outcomes in children treated with SP for uncomplicated malaria in Kampala, Uganda. Two mutations (DHFR Asn-108 and Ile-51) were too common to be useful predictors. Three other mutations (DHFR Arg-59, DHPS Gly-437, and DHPS Glu-540) were associated with clinical treatment failure after 14 days, although associations were not significant. When follow-up was extended to 28 days and genotyping was used to distinguish recrudescence from new infections, associations were significantly strengthened. The presence of both the DHFR Arg-59 and DHPS Glu-540 mutations had the strongest association with clinical treatment failure (odds ratio = 10.7,  $P = 0.009$ ). These results support a previously proposed method of predicting clinical outcomes based on the prevalence of these two mutations.

### INTRODUCTION

Resistance to antimalarial therapy is a major concern in Africa.<sup>1</sup> Widespread chloroquine (CQ) resistance has led to increasing use of the antifolate combination sulfadoxine/pyrimethamine (SP) due to its efficacy, low cost, and simplicity of use. Several countries in eastern and southern Africa have adopted SP as first-line therapy for uncomplicated malaria,<sup>1</sup> and it is the second-line antimalarial drug after CQ in most other African countries. Resistance to SP has developed rapidly in other areas of the world following widespread use,<sup>1</sup> and a similar phenomenon may be occurring in parts of Africa.<sup>2–4</sup> The emergence of SP resistance in Africa is of great concern because few affordable alternatives to CQ are available. Monitoring the spread of SP resistance is therefore a major public health objective.

Clinical studies are currently the gold standard for monitoring antimalarial drug efficacy and establishing drug treatment policy in endemic areas of Africa.<sup>5</sup> However, such studies are costly and time-consuming, and are thus not ideal for large-scale epidemiologic surveys. *In vitro* drug sensitivity testing is impractical due to technical limitations and poor correlation with clinical outcomes.<sup>5</sup> Evaluation of molecular markers of drug resistance may offer a simple, low-cost means of drug efficacy surveillance.<sup>6</sup> Point mutations in genes encoding parasite dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) are associated with *in vitro* resistance to pyrimethamine and sulfadoxine, respectively.<sup>7–13</sup> Molecular assays have been developed to detect these mutations and have been adapted for field samples.<sup>14</sup>

Correlation between molecular markers of SP resistance and *in vivo* outcomes has been complex. Field surveys have led to conflicting reports on the relative importance of various mutations in mediating treatment failure.<sup>15–18</sup> Many previous studies have been limited by small sample size, low SP resistance levels, and parasitologic (but not clinical) measures of treatment outcome.<sup>15–17,19,20</sup> Many of these limitations were addressed in a recent study from Malawi that found that a DHFR/DHPS “quintuple” mutant (DHFR Asn-108, Ile-51, Arg-59 and DHPS Gly-437, Glu-540) was strongly associated

with seven-day parasitologic treatment failure, and that this genotype was best predicted by the presence of two mutations (DHFR Arg-59 and DHPS Glu-540).<sup>21</sup> However, it remains that few studies have tested the utility of molecular markers of SP resistance to predict clinical outcomes, the most meaningful parameter for formulating treatment policy. In the present study, we analyzed a large number of isolates from children treated with SP in Kampala, Uganda, with treatment outcomes based on standard clinical classifications and genotyping used to distinguish recrudescence from new infection. Our results offer validation that the analysis of only two mutations, DHFR Arg-59 and DHPS Glu-540, is an effective means of predicting SP treatment failure.

### METHODS

**Clinical study.** The clinical portion of this study took place between July 2000 and August 2001 in Kampala, Uganda. Full details of the clinical trial have been reported elsewhere.<sup>22</sup> Briefly, 316 healthy children between the ages of six months and five years were enrolled and randomly assigned to one of three treatment regimens, which were administered for all future episodes of uncomplicated malaria diagnosed during 12 months of follow-up. Samples for this study were restricted to the 105 patients assigned to the SP treatment arm. Cases were included in this study if they fulfilled the following criteria: 1) *Plasmodium falciparum* infection with  $\geq 2,000$  asexual parasites/ $\mu\text{L}$ , 2) tympanic temperature  $\geq 38.0^\circ\text{C}$  or history of fever in the previous 24 hours, and 3) absence of severe malaria or danger signs. All treatments were directly observed to ensure compliance. Treatment outcomes were classified according to standardized World Health Organization (WHO) 14-day clinical criteria with slight modifications and dichotomized into success (adequate clinical response) or failure (early or late treatment failure).<sup>22,23</sup> Given the longitudinal nature of the study design, all patients with an adequate clinical response after 14 days were followed until their next episode of malaria or the end of the study. For recurrent episodes, molecular genotyping based on merozoite surface protein-2 polymorphisms was used to distinguish re-

crudescent from new infections, as previously described.<sup>24</sup> Briefly, an outcome was defined as recrudescence if a subsequent episode contained identical alleles or a subset of the alleles present in the previous episode and reinfection if a subsequent episode contained only new alleles. If a subsequent episode contained both alleles present in the previous episode and new alleles (10% of episodes), the outcome was considered a reinfection if half or more of the bands were new, and recrudescence if more than half of the bands were present in the previous episode. With follow-up extended to 28 days, treatment failure was defined as fulfillment of the WHO criteria for early or late treatment failure in the presence of recrudescence parasites. This study was reviewed and approved by the institutional review boards of Makerere University and the University of California, San Francisco.

**Mutation analysis.** Polymorphisms of interest in the *P. falciparum* DHFR and DHPS genes were assessed using a nested polymerase chain reaction (PCR) amplification followed by mutation-specific restriction enzyme digestion. Blood was collected on filter paper each time a patient was diagnosed with malaria or classified as a treatment failure. Parasite DNA was isolated from filter paper using the Chelex extraction method.<sup>25</sup> Primers, enzymes, and amplification conditions for PCR assays to detect DHFR Asn-108, Ile-51, and Arg-59 and DHPS mutations Gly-437 and Glu-540 were as described in a prior report<sup>14</sup> except that 1) DHFR first-round primers were modified (sense, 5'-TTTATGATGGAACAAGTCTGCGACGTTTTTC-3' and antisense, 5'-AATTTGATACTCATTTTCATTTATTCTGG-3') to yield a 622-basepair fragment, 2) nested primers for codons 51 and 108 were modified (sense, 5'-GTCTGCGACGTTTTCGATATTTATGC-3' and antisense, 5'-AAATTCTTGATAAACAACGGAACCTTTTA-3') to yield a 506-basepair fragment, 3) Vent polymerase (New England Biolabs, Beverly, MA) was used for the DHFR first-round reaction, and 4) annealing temperatures were changed to 54°C for five cycles followed by 56°C for 35 cycles for the first round DHFR reaction and to 50°C for five cycles followed by 54°C for 35 cycles for both nested DHFR reactions.

The presence of mutations at each codon was determined using restriction enzyme digestion of nested PCR products using published methods.<sup>14</sup> Digestion products were visualized by electrophoresis and results classified as wild type, pure mutant, or mixed (both wild type and mutant genotype present in the same infection). Investigators were blinded to clinical outcomes at the time of molecular analysis.

**Statistical analysis.** Associations between individual mutations were assessed using the chi-square test. Associations between DHFR and DHPS mutations (either alone or in combination) and clinical treatment failure were estimated using generalized estimating equations with exchangeable correlation and robust standard errors controlling for pre-treatment log-transformed parasite density and repeated measures in the same patients.<sup>26</sup> We controlled for pre-treatment (day 0) parasite density, as higher parasite densities were an independent risk factor for treatment failure.<sup>22</sup> All data were entered and verified using Epi-Info version 6.04 (Centers for Disease Control and Prevention, Atlanta, GA) and SPSS (SPSS, Inc., Chicago, IL) software. Analysis was performed using STATA (State Corp., College Station, TX) statistical software. All confidence levels were set at 95%.

## RESULTS

**Sample selection and clinical treatment outcomes.** Over the one-year follow-up period, 192 episodes of uncomplicated falciparum malaria treated with SP fulfilled our selection criteria, of which 183 (95%) had successful genotyping results and were included in the analysis. These 183 episodes occurred in 57 different children (median number of episodes = 3, range = 1–10). Based on the WHO 14-day clinical classification system, 36 (20%) of 183 episodes were classified as treatment failures. With follow-up extended to 28 days and genotyping used to distinguish recrudescence from new infections, 60 (33%) of 183 episodes were classified as treatment failures.

**Prevalence of DHFR and DHPS mutations alone and in combination.** We performed molecular assays for five DHFR and DHPS mutations known to occur commonly in Africa (DHFR Asn-108, Ile-51, and Arg-59, and DHPS Gly-437 and Glu-540) on all pretreatment samples. Mutation analysis was successful for at least 96% of samples for all individual mutations of interest and 90% of samples had successful results for all five mutations. All five evaluated mutations were common (Figure 1). A breakdown of the prevalence of each unique combination of mutations is shown in Table 1. In our study population, the prevalence of the DHFR Asn-108 (98%) and Ile-51 (95%) mutations (including mixed genotypes) was very high. Among samples with both the DHFR Asn-108 and DHFR Ile-51 mutations, 71% had the DHFR Arg-59 mutation, while among those with the DHFR Arg-59 mutation, 96% had both the DHFR Asn-108 and DHFR Ile-51 mutations. This observation is consistent with previous conclusions that DHFR mutations are acquired in a stepwise fashion, with DHFR Asn-108 and DHFR Ile-51 acquired first, followed by DHFR Arg-59.<sup>27</sup> Due to their high prevalence and concordance with the DHFR Arg-59 mutation, the DHFR Asn-108 and DHFR Ile-51 mutations were not further considered as independent markers. However, it should be reiterated that when we discuss the predictive value of the DHFR 59 mutation, virtually all of the samples that contained this mutation were actually what is commonly referred to as the DHFR “triple mutant” (DHFR 108 + 51 + 59).

The DHFR Arg-59 and DHPS Gly-437 mutations had a 67% concordance (results for both codons were the same) and were not statistically associated ( $P = 0.29$ ). The DHFR Arg-59 and DHPS Glu-540 mutations had a 69% concordance and were statistically associated ( $P = 0.02$ ). The DHPS

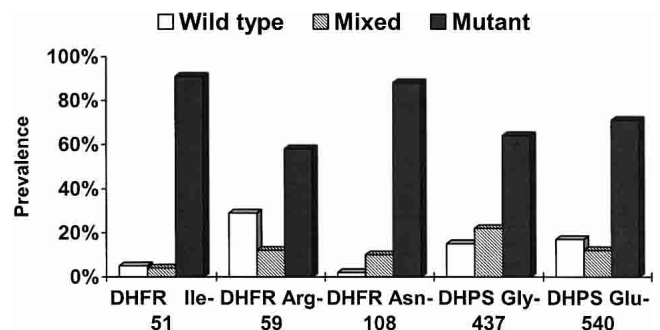


FIGURE 1. Prevalence of key dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS) mutations.

TABLE 1  
Combinations of mutations for samples with successful polymerase chain reaction results for all five mutations (n = 164)

Number of mutations	Total	Number of failures		Subgroups (codon numbers with mutant genotype)	Total	Number of failures	
		14-day	28-day			14-day	28-day
0 or 1	0	0	0	—	—	—	—
2	10	1	1	108 + 51	9	1	1
3	19	3	4	108 + 59	1	0	0
				108 + 51 + 59	10	3	3
				108 + 51 + 437	4	0	0
				51 + 437 + 540	1	0	1
				108 + 59 + 540	1	0	0
4	45	8	13	108 + 437 + 540	3	0	0
				108 + 51 + 437 + 540	31	5	7
				108 + 59 + 437 + 540	3	2	2
				51 + 59 + 437 + 540	3	0	1
				108 + 51 + 59 + 540	4	1	2
				108 + 51 + 59 + 437	4	0	1
5	90	18	35	108 + 51 + 59 + 437 + 540	90	18	35

Gly-437 and DHPS Glu-540 mutations had a 93% concordance and were strongly associated ( $P < 0.001$ ). Unlike the DHFR mutations, there was no clear step-wise accumulation of DHPS mutations. In the 13 (7%) samples that were discordant (one wild type and the other mutant) for the two DHPS mutations, five were wild type for the 540 mutation and eight were wild type for the 437 mutation.

**Associations between DHFR and DHPS mutations and clinical treatment failure.** We investigated associations between the DHFR Arg-59, DHPS Gly-437, and DHPS Glu-540 mutations alone and in combination and clinical treatment failure. In all of our analyses we controlled for parasite density, which itself was an independent risk factor for treatment failure. Age was not an independent risk factor for treatment failure, likely due to the restricted age range of our patient population (six months to five years). After 14 days the risk of clinical treatment failure for mixed genotypes was similar to that of pure mutants (Table 2). To simplify the interpretation of results and improve statistical power, mixed genotypes and pure mutants were combined in all subsequent analyses.

Associations between mutations of interest and clinical treatment failure are summarized in Table 3. Individual mutations were associated with clinical treatment failure based on the WHO 14-day classification system, but none of these

comparisons reached statistical significance. When follow-up was extended to 28 days and genotyping used to distinguish recrudescence from new infections, these associations were strengthened. Considering combinations of mutations, there was generally a “dose response” relationship, with increasing numbers of mutations resulting in stronger associations with treatment failure. The strongest association was found when considering only the DHFR Arg-59 and DHPS Glu-540 mutations (odds ratio = 10.7, 95% confidence interval = 1.79–64.4,  $P = 0.009$ ). These results suggest that a combination of these two mutations alone provides the most useful marker of clinical outcomes in our patient population. Considering overall genotypes rather than efficient predictive markers, 90% of samples with both the DHFR Arg-59 and DHPS Glu-540 mutations also contained the other three mutations commonly seen in Africa (DHFR Asn-108, DHFR Ile-51, and DHPS Gly-437).

**Early treatment failures and the prevalence of DHFR and DHPS mutations.** Considering all five mutations, the one treatment failure with only two mutations (DHFR 108 + 51) and three of the four treatment failures with three mutations (DHFR 108 + 51 + 59) occurred within four days of the onset of therapy. There was no association between the prevalence of mutations and these early treatment failures, while there was a clear association between the prevalence of mutations and treatment failures that occurred later (Table 4). These data suggest that factors other than the mutations evaluated in this study may be of greater importance in determining early treatment failures in our patient population. When treatment failures that occurred within four days were removed from the analysis, all of the associations between our molecular markers and treatment failure were strengthened.

## DISCUSSION

This study demonstrates an association between molecular markers of SP resistance and clinically relevant treatment outcomes. When outcome classification was limited to 14 days, associations between the studied mutations (alone or in combination) and treatment failure did not reach statistical significance. However, when follow-up was extended to 28 days and genotyping used to distinguish treatment failure

TABLE 2

Results of mutation analysis and risk of 14-day clinical treatment failure\*

Codon	Clinical treatment failure	Clinical treatment success	Risk of treatment failure
<b>DHFR 59</b>			
Wild type	2	50	3.8%
Mixed	3	30	9.1%
Mutant	10	93	9.7%
<b>DHPS 437</b>			
Wild type	0	24	0%
Mixed	6	47	11.3%
Mutant	10	98	9.3%
<b>DHFR 540</b>			
Wild type	1	28	3.6%
Mixed	2	25	7.4%
Mutant	14	119	10.5%

\* Based on standardized World Health Organization criteria. DHFR = dihydrofolate reductase; DHPS = dihydropteroate synthetase.

TABLE 3  
Associations between various combinations of molecular markers and clinical treatment failure

Mutation(s)*	Comparison groups (n)	14-day follow-up† OR‡ (95% CI)	28-day follow-up† OR‡ (95% CI)
Individual mutations			
59	59 present (126) 59 not present (52)	2.32 (0.98–5.49) ( <i>P</i> = 0.06)	3.13 (1.31–7.51) ( <i>P</i> = 0.01)
437	437 present (150) 437 not present (26)	1.14 (0.41–3.23) ( <i>P</i> = 0.80)	1.75 (0.73–4.18) ( <i>P</i> = 0.21)
540	540 present (150) 540 not present (30)	2.09 (0.71–6.22) ( <i>P</i> = 0.18)	3.11 (1.09–8.93) ( <i>P</i> = 0.03)
Combinations of two mutations			
59 + 437	Neither 59 or 437 present (10)	1.0 (reference)	1.0 (reference)
	Only 59 or 437 present (57)	1.73 (0.24–12.2) ( <i>P</i> = 0.59)	2.82 (0.53–14.9) ( <i>P</i> = 0.22)
	Both 59 and 437 present (105)	2.76 (0.44–17.4) ( <i>P</i> = 0.28)	6.99 (1.19–40.9) ( <i>P</i> = 0.03)
59 + 540	Neither 59 or 540 present (14)	1.0 (reference)	1.0 (reference)
	Only 59 or 540 present (54)	2.20 (0.34–14.3) ( <i>P</i> = 0.40)	4.27 (0.78–23.3) ( <i>P</i> = 0.09)
	Both 59 and 540 present (108)	4.25 (0.76–23.7) ( <i>P</i> = 0.10)	10.7 (1.79–64.4) ( <i>P</i> = 0.009)
All three mutations combined			
59 + 437 + 540	None of the 3 mutations present (10)	1.0 (reference)	1.0 (reference)
	Only one mutation present (15) (59 or 437 or 540)	2.22 (0.23–21.7) ( <i>P</i> = 0.49)	2.52 (0.29–22.1) ( <i>P</i> = 0.40)
	Only two mutations present (46) (59 + 437 or 59 + 540 or 437 + 540)	1.37 (0.21–8.92) ( <i>P</i> = 0.75)	2.87 (0.57–14.5) ( <i>P</i> = 0.20)
	All three mutations present (100)	2.94 (0.48–18.1) ( <i>P</i> = 0.24)	7.16 (1.25–41.1) ( <i>P</i> = 0.03)

\* Ignoring the presence or absence of other mutations.

† 14-day follow-up based on World Health Organization (WHO) clinical classification system. 28-day follow-up based on WHO criteria with treatment failures confirmed by genotyping.

‡ Controlling for day 0 parasite density and repeated measures. OR = odds ratio; CI = confidence interval.

from new infections, many cases initially classified as adequate clinical responses were reclassified as treatment failures. When we used this improved method of outcome classification, associations between molecular markers and treatment outcomes achieved statistical significance. The strongest associations were found when the analysis considered the combination of two mutations, DHFR Arg-59 and DHPS Glu-540.

Multiple studies have identified common DHFR and DHPS mutations in *P. falciparum* parasites circulating in Africa<sup>17–19,21</sup> and key mutations have been linked to *in vitro* resistance to pyrimethamine and sulfadoxine.<sup>7–13</sup> However, clinical evaluations of molecular markers of SP resistance have been limited by cross-sectional study design, small sample size, low levels of SP resistance, and short-term and/or non-clinical outcome measures.<sup>17–19,21</sup> Currently, drug treatment policy is primarily influenced by *in vivo* studies using standardized criteria based on clinical outcomes.<sup>23,28</sup> For molecular markers to be useful surrogates of *in vivo* studies, they

should be assessed in the context of the clinical outcomes that are relevant for policy decision making.

The role of various DHFR and DHPS mutations in mediating clinical treatment failure to SP in Africa is uncertain. For DHFR, associations with treatment failure appear only to occur with multiple mutations, generally Asn-108 with Ile-51 and/or Arg-59.<sup>27</sup> The relative importance of DHPS mutations in mediating SP treatment failure has been debated. Recent reports suggest that the Gly-437 and Glu-540 mutations increase the risk of treatment failure only with coexisting DHFR Asn-108, Ile-51, and Arg-59 mutations.<sup>27</sup> In Cameroon, the presence of the DHFR Asn-108, Ile-51, and Arg-59 “triple” mutant was associated with 14-day clinical treatment failure, but DHPS mutations had no influence on the clinical outcome (although no samples contained the Glu-540 mutation).<sup>16</sup> In Kenya, the DHFR triple mutant was associated with seven-day parasitologic treatment failure, and this association was strengthened by the additional presence of the DHPS Gly-437 and Glu-540 “double” mutant.<sup>17</sup> A recent study in Malawi found a strong association between the DHFR Asn-108, Ile-51, Arg-59 and DHPS Gly-437, Glu-540 “quintuple” mutant and parasitologic failure, and that the presence of both the DHFR Arg-59 and DHPS Glu-540 mutations accurately predicted the presence of this “quintuple” mutant.<sup>21</sup>

We attempted to build upon previous work by using a large sample size, focusing on clinically relevant outcomes, and providing a more accurate assessment of drug efficacy by extending follow-up to 28 days with genotyping used to distinguish recrudescence (true treatment failure) from new infections. This improvement was important, since we recently showed that a majority of SP treatment failures at our study site were

TABLE 4

Number of dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS) mutations and risk of treatment failure stratified by the day of failure

Number of mutations*	Percentage with failures occurring on days 1–4†	Percentage with failures occurring on days 4–28†
2	10	0
3	16	5
4	11	18
5	9	30

\* Considering DHFR Asn-108, Ile-51, Arg-59 and DHPS Gly-437, Glu-540 mutations.

† Proportion of samples with clinical treatment failure at the indicated times after therapy.

identified only after more than 14 days of follow-up.<sup>22</sup> In addition, recent recommendations from the WHO point out the need for a longer period of follow-up (accompanied by genotyping) when assessing the efficacy of SP in areas of intense transmission.<sup>28</sup>

Our primary goal was to optimize a practical method of using molecular markers to predict SP treatment failure. We first concluded that samples with mixed and pure mutant genotypes could be combined, which greatly simplified our analysis. Next we assessed which combinations of the five mutations commonly reported in Africa had the strongest association with treatment failure. The DHFR Asn-108 and Ile-51 mutations were very common, and their inclusion in analyses did not add to consideration of the DHFR Arg-59 mutation alone. Considering the remaining three mutations, the strongest association between molecular markers and clinical outcomes was achieved with consideration of the DHFR Arg-59 and DHPS Glu-540 mutations in combination. It is encouraging that our conclusions agree with those reached in Malawi, where the DHFR Arg-59 and DHPS Glu-540 mutations in combination were found to be the most useful predictor of treatment outcomes.<sup>21</sup> One potential limitation recognized in our study is the lack of association between molecular markers and treatment failures occurring within four days. A similar finding was reported in Malawi, where such treatment failures were excluded from their analysis.<sup>21</sup> In contrast, in a study from Cameroon, where all treatment failures occurred within three days, the DHFR "triple mutant" (Asn-108, Ile-51, and Arg-59) was associated with these early treatment failures.<sup>16</sup> The reasons for our lack of association between molecular markers and early treatment failure and for differences between studies are unexplained.

A long-standing goal has been to use molecular markers as a rapid and inexpensive means of surveillance to help guide antimalarial treatment policy decisions. This study confirms the key role of *P. falciparum* DHFR and DHPS mutations in mediating clinical failure after treatment with SP in Africa. A logical next step is to use mutation prevalence data to predict the risk of treatment failure. Indeed, unless molecular markers can accurately predict the risk of clinical treatment failure, they will likely be of little value to policy makers. It is well recognized that treatment of patients infected with parasites containing resistance mediating mutations may result in an adequate clinical response, since host immunity and possibly other factors play a role in the clearance of parasites and resolution of symptoms.<sup>21</sup> One approach that has been proposed to account for differences between the prevalence of genetic markers and the risk of clinical treatment failure is the use of a genotyping failure index (GFI).<sup>21,29</sup> This is a correction factor derived by dividing the prevalence of molecular markers of interest by the known risk of clinical treatment failure. If this index is consistent across different study sites (if necessary controlling for relevant factors, such as age) it could be used to routinely predict the risk of clinical treatment failure based on the prevalence of molecular markers in a representative sample of patients with uncomplicated malaria. In the study from Malawi, considering the prevalence of the DHFR Arg-59 and DHPS Glu-540 combination (65%) and using a seven-day parasitologic definition of treatment failure (30%), the GFI was equal to 2.2. In this study, considering the prevalence of the same combination of mutations (61%) and

our 28-day clinical definition of treatment failure (33%), the GFI was equal to 1.9. Despite differences in methodology, the similarity in calculated indices between these two studies is noteworthy.

In conclusion, we found that a simplified method based on the prevalence of only two mutations (DHFR Arg-59 and DHPS Glu-540) provided the best means of predicting clinical treatment outcomes in our patient population of children from an endemic area of Africa. Such a focused approach when using molecular markers for surveillance of antifolate drug resistance could save valuable time and money. Implementation of this research tool in the arena of public health practice will require additional studies to test specific molecular markers and validate whether the prevalence of these markers can be used to accurately estimate clinically relevant outcomes across widely differing epidemiologic settings. As has been recommended previously, this can best be accomplished by collaborative efforts using comparable study designs and analytical techniques.

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