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Population-based validation of dihydrofolate reductase gene mutations for the prediction of sulfadoxine–pyrimethamine resistance in Uganda

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Abstract

Mutations in the dihydrofolate reductase gene (*dhfr*) of *Plasmodium falciparum* have been proposed as molecular markers for the surveillance of sulfadoxine–pyrimethamine (SP)-resistant malaria, but such proposals have not been validated. At 7 Ugandan sites in 1999, we determined the population-based prevalence of infections with mutations and the mutant allele frequency of *dhfr* codons 108, 51, and 59 using a random sample of infected individuals aged 1–45 years. Sulfadoxine–pyrimethamine treatment failure was independently estimated by *in vivo* tests in 327 children aged 6–59 months with clinical malaria. The prevalence of infections with the single point mutations and the *dhfr* codons 108 and 51 mutant allele frequency were not correlated to SP treatment failure. However, the *dhfr* codon 59 mutant allele frequency was positively correlated to SP treatment failure ($r = 0.72$, $P = 0.06$). The ratio of the infections with the mutant to wild genotype (M/W) and that of the mutant to wild allele (MA/WA) had the same values. Both *dhfr* codon 59 M/W and MA/WA ratio were significantly and positively correlated to SP treatment failure ($r = 0.73$, $P = 0.05$). Moreover, the prevalence of infections with only 2 mutations (Asn-108 plus Ile-51) was significantly and inversely correlated to the prevalence of infections with 3 mutations (Asn-108 plus Ile-51 plus Arg-59) ($r = 0.92$, $P = 0.004$), suggesting the stepwise accumulation of the *dhfr* mutations is Asn-108 Ile-51 Arg-59 and further supporting the idea of using the *dhfr* codon 59 M/W ratio as a molecular index for the prediction of SP treatment failure. At the population level, the *dhfr* codon 59 M/W ratio is a simple and stable index for the estimation of SP treatment failure.

Keywords: malaria, *Plasmodium falciparum*, chemotherapy, drug resistance, sulfadoxine–pyrimethamine, *dhfr* gene mutations, Uganda

Introduction

The point mutations at codons 108, 51, 59, and 164 in the parasite gene encoding for the enzyme dihydrofolate reductase (DHFR) have been linked to pyrimethamine resistance (Reeder *et al.*, 1996; Plowe *et al.*, 1997; Wang *et al.*, 1997; Nzila-Mounda *et al.*, 1998; Doumbo *et al.*, 2000). A mutation to asparagine at codon 108 (S108N) is the key mutation for conferring pyrimethamine resistance *in vitro* (De Pécoulas *et al.*, 1996). An asparagine to isoleucine change at codon 51 (N51I) and a cysteine to arginine change at codon 59 (C59R) appear to modulate higher levels of pyrimethamine resistance *in vitro* when they occur with the asparagine-108 mutation. An isoleucine to leucine mutation at codon 164 (I164L) in combination with the other 3 mutations has been found in *Plasmodium falciparum* parasites highly resistant to both pyrimethamine and cycloguanil in Asia (Basco *et al.*, 1995). It has been suggested that the *dhfr* triple mutant genotype determines resistance to sulfadoxine–pyrimethamine (SP) (Watkins *et al.*, 1997) and that different rates of SP resistance *in vivo* are probably due to the progressive stepwise accumulation of the *dhfr* mutations (Peterson *et al.*, 1990; Sirawaraporn *et al.*, 1997). If mutations occur in a stepwise fashion, then the prevalence of the mutant allele for the last single point mutation could predict the occurrence of the triple mutant genotype and consequently estimate the prevalence of SP resistance. In Malawi, the presence of 3 *dhfr* mutations in pre-treatment host infections was associated with a very high sensitivity (95%) and a relatively low specificity (63%) in predicting SP treatment failure (Kublin *et al.*, 2002). In all studies such a relationship has been determined mainly in patients with clinical malaria attending health facilities and the clinical outcome has been analysed in relation to the presence of a particular mutation or set of mutations, sometimes with conflicting results.

The prevalence of mutations in host infections, i.e. the number of infected individuals carrying the mutations over the total number of infected individuals, has often been used although it might differ from the frequency of the mutant allele in the parasite population, possibly a more appropriate measure to estimate the amount of circulating mutant parasites. A major problem when estimating the mutant allele frequency is the occurrence of polyclonal infections. This is even more pronounced if resistance is based on mutations occurring in multiple loci and in more than 1 gene.

Considering that 2 recent studies (Kublin *et al.*, 2002; Mutabingwa *et al.*, 2001) reported the link between *dhfr* mutations and SP resistance for individual patient outcomes, we investigated the relationship between the population-based prevalence of *dhfr* mutations in individuals aged 1–45 years and treatment failure *in vivo*, independently determined from a sample of children aged 6–59 months from the same population and with clinical malaria. Our aim was to identify a valid, simple and easy to use molecular index for estimating SP drug resistance at population level.

Materials and Methods

Study setting

The Uganda National Malaria Control Programme (UNMCP) and the East Africa Network for Monitoring Antimalarial Treatment (EANMAT) jointly chose 8 sentinel sites for the surveillance of antimalarial drug efficacy and we conducted the study in 7 of these sites. The study sites were selected according to the following criteria: transmission intensity (high, medium, low), moderate/high population density, easy accessibility/practicability, inclusion of at least 1 country border area, geographical representation, health unit with a defined catchment population (hospitals excluded), and the presence of a stable population with limited migration in and out. The sites lie between 945 m and 1219 m above sea level. The main occupation of the

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population in the sentinel sites is subsistence agriculture. Population-based cross-sectional surveys and efficacy studies *in vivo* were conducted at all the 7 sites. The Uganda National Council for Science and Technology (UNCST) and the research ethics committee at the Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium reviewed and approved the study.

Determination of sulfadoxine–pyrimethamine treatment failure

Efficacy tests at all sites used the EANMAT field manual, a simplified version of the World Health Organization protocol for areas of intense transmission (WHO, 1996). Details of the methodology of the efficacy tests *in vivo* and test outcomes are reported elsewhere (Talisuna *et al.*, 2002). Briefly, children aged 6–59 months with fever (axillary temperature ⩾ 37.5 °C) or history of fever in the past 24 h, with a *P. falciparum* monoinfection and a parasite density of 2000–100 000/μL were recruited, treated with SP and followed-up to day 14 after treatment. After enrolment, the study team saw patients on days 1, 2, 3, 7, 14 and on any other day if they developed symptoms. Quality-assured SP (500 mg sulfadoxine and 25 mg pyrimethamine) was administered orally under supervision and according to bodyweight. Clinical response was classified in 3 groups: early treatment failure (ETF), late treatment failure (LTF), and adequate clinical response (ACR) as defined previously (WHO, 1996). Sulfadoxine–pyrimethamine treatment failure is the sum of ETF and LTF.

Determination of the population-based prevalence of host infections with mutant genotypes and the mutant allele frequency

A population-based cross-sectional survey was conducted independently of the drug efficacy test. Prior to the survey, a census of the population around each site was carried out. Each enumerated individual was given a 9-digit identification code to represent the site, village (smallest administrative unit), household and position in the household. For each site a sample of 250 people aged 1–45 years was selected randomly from the census database. During the survey, conducted during the second peak transmission season, from September to December 1999, duplicate thick and thin blood films for parasitological examination were collected. A blood sample for polymerase chain reaction (PCR) analysis was collected from each individual on 3MM Whatman filter-paper and stored at room temperature in an individual zip-lock dry polythene bag. For the analysis of *dhfr* mutations we randomly selected 50 microscopy-positive samples per site or all positive samples if there were fewer than 50. Identification of *dhfr* genotypes at codons 108, 51, and 59 was done by nested mutation-specific PCR performed according to the techniques already described (Plowe *et al.*, 1995, 1997).

Statistical analysis

The prevalence of infections with wild or mutant genotypes at *dhfr* codons 108, 51, and 59 was computed as the number of infections with a specific genotype divided by all the infections successfully assayed. As many infected individuals carried *P. falciparum* polyclonal infections, the sum of the prevalence of infections with *dhfr* mutant and wild genotypes was more than 100%. We therefore used the mutant to wild genotype prevalence ratios (M/W) as stable indices. We also computed the mutant and wild-type allele frequencies for *dhfr* codons 108, 51, and 59. The allele frequency at each codon was derived as the number of a given allele detected divided by the total number of alleles (mutant + wild) observed. For each sentinel site the ratio between the frequency of the mutant and wild alleles (MA/WA) was also computed. The relationship between SP-treatment failure and prevalence of the *dhfr* mutant genotypes among infections, the allele frequency, the genotype and allele ratios (M/W and MA/WA) were examined using linear regression. Correlation coefficients were derived based on ordinary least squares (OLS) and P-values ⩽ 0.05 were considered significant.

Results

A total of 352 children were recruited and followed-up for the *in vivo* test and of these, 7% (24) were lost to follow-up. We were able to establish a clinical outcome for 93% (327) of the children. SP treatment failure (ETF + LTF) varied significantly between sites ($P = 0.04$). The lowest SP treatment failure was 0% and the highest 14.8% (median 10%) (Table 1).

We were able to genotype a total of 254, 247, and 252 samples for *dhfr* codons 108, 51, and 59, respectively. The prevalence of host infections with mutant genotypes was very high for *dhfr* Asn-108 (median 100%, range 85.1–100%) and for *dhfr* Ile-51 (median 100%, range 87.5–100%) (Table 1). The median prevalence of infections with the *dhfr* Arg-59 genotype was

Table 1. Prevalence of sulfadoxine–pyrimethamine (SP) treatment failure, prevalence of host infections with the wild and mutant genotypes and ratio of mutant to wild genotypes at *dhfr* codons 108, 51 and 59, Uganda, September–December 1999

Study site	SP treatment failure	Wild genotype Codon 108	Wild genotype Codon 51	Wild genotype Codon 59	Mutant genotype Codon 108	Mutant genotype Codon 51	Mutant genotype Codon 59	Mutant to wild genotype ratio Codon 108	Mutant to wild genotype ratio Codon 51	Mutant to wild genotype ratio Codon 59
	% (no. of infections with wild or mutant genotypes/total no. of infections)									
Jinja	2.4 (1/42)	33.3 (4/12)	33.3 (3/9)	100 (11/11)	100 (12/12)	100 (9/9)	72.7 (8/11)	3.00	3.00	0.73
Rukungiri	0 (0/55)	8.6 (3/35)	28.6 (10/35)	82.8 (29/35)	100 (35/35)	100 (35/35)	74.3 (26/35)	11.6	3.49	0.90
Tororo	14.8 (9/61)	28.9 (13/45)	69.6 (32/46)	91.3 (42/46)	100 (45/45)	97.8 (45/46)	93.5 (43/46)	3.46	1.40	1.02
Mubende	11.6 (5/43)	10.5 (2/19)	38.5 (5/13)	66.7 (10/15)	100 (19/19)	100 (13/13)	60.0 (9/15)	9.52	2.60	0.90
Kyenjojo	12.5 (2/16)	29.8 (14/47)	28.3 (13/46)	83.0 (39/47)	97.9 (46/47)	100 (46/46)	89.4 (42/47)	3.29	3.53	1.08
Arua	9.8 (5/51)	74.5 (35/47)	72.9 (35/48)	81.2 (39/48)	85.1 (40/47)	87.5 (42/48)	79.2 (38/48)	1.14	1.20	0.97
Apac	10.2 (6/59)	73.5 (36/49)	88.0 (44/50)	98.0 (49/50)	100 (49/49)	100 (50/50)	96.0 (48/50)	1.36	1.14	0.98

80.9% (range 60–96.0%). At each site the prevalence of infections with the *dhfr* codon 59 mutant genotype was consistently lower than that of the *dhfr* codon 51 mutant genotype, which was lower than, or just equal to, that of the *dhfr* 108 mutant genotype. The prevalence of infections with the single point *dhfr* mutants was not correlated to SP treatment failure for codons 108 ($r = 0.14$, $P = 0.7$), 51 ($r = 0.08$, $P = 0.86$), and 59 ($r = 0.31$, $P = 0.46$). Similarly the prevalence of infections with 2 mutations ($r = 0.54$, $P = 0.22$) and 3 mutations ($r = 0.28$, $P = 0.54$) was not correlated to SP treatment failure. The highest *dhfr* mutant allele frequencies were observed for *dhfr* codon 108 (median 76.7%, range 53.3–92.1%) and the lowest for *dhfr* codon 59 (median 49.4%, range 42.1–51.9%) (Table 2). The mutant allele frequencies of *dhfr* codon 108 ($r = 0.26$, $P = 0.6$) and 51 ($r = 0.48$, $P = 0.3$) were not correlated to SP treatment failure. However, the *dhfr* codon 59 mutant allele frequency ($r = 0.72$, $P = 0.06$) was positively correlated to SP treatment failure. The ratio of infections with the M/W genotype and the MA/WA allele ratio had the same values. No correlation between SP treatment failure and the *dhfr* codon 51 ($r = 0.61$, $P = 0.14$) or *dhfr* codon 108 ($r = 0.42$, $P = 0.33$) M/W or MA/WA ratios was found. The *dhfr* M/W ratios were > 1 at all sites for *dhfr* codon 108 (median 3.29, range 1.17–11.6) and for *dhfr* codon 51 (median 2.6, range 1.14–3.53). However, the *dhfr* codon 59 M/W ratio (median 0.97, range 0.73–1.08) was > 1 in only 2 of the 7 sites (Tables 1 and 2) and was directly and significantly correlated to SP treatment failure ($r = 0.73$, $P = 0.05$) (Fig. 1). A *dhfr* codon 59 M/W ratio < 1 was observed in sites with SP treatment failure < 10% while sites with SP treatment failure of 10–15 % had a M/W ratio of 1–1.5.

We also analysed the data for the occurrence of 2 or 3 mutations in the same infection. Only 1 sample had the *dhfr* codon 59 mutant genotype without the *dhfr* Ile-51 mutation being present. The prevalence of infections with only 2 mutations (*dhfr* Asn-108 plus Ile-51 only) significantly decreased as the prevalence of infections with 3 mutations (*dhfr* Asn-108 plus Ile-51 plus Arg-59) increased ($r = 0.92$, $P = 0.004$) (Fig. 2).

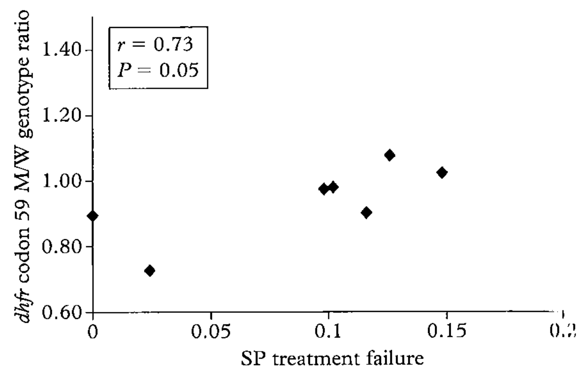

Fig. 1. Relationship between the *dhfr* codon 59 mutant to wild (M/W) genotype ratio and sulfadoxine–pyrimethamine (SP) treatment failure in Uganda, September–December 1999. Correlation coefficients are based on ordinary least squares for simple regression.

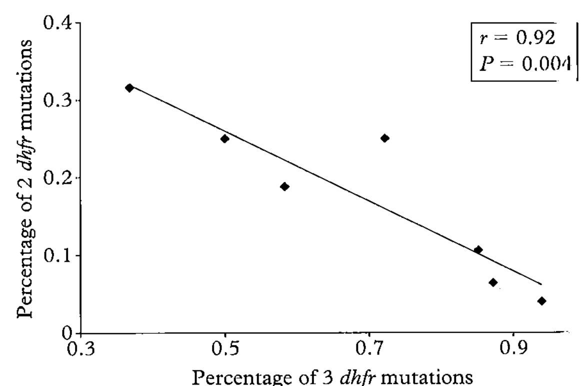

Fig. 2. Inverse correlation and corresponding regression line between the prevalence of isolates with 2 *dhfr* mutations (Asn-108 and Ile-51 only) and the prevalence of isolates with 3 mutations (Asn-108, Ile-51, and Arg-59).

Discussion

The *dhfr* 59 M/W genotype and the MA/WA allele ratios but not the other molecular markers such as the prevalence or the allele frequency of the *dhfr* single and double mutation or the *dhfr* codons 108 and 51 M/W genotype ratios, were correlated to SP treatment failure *in vivo*. In a clinical trial carried out in Malawi, infections with 3 *dhfr* mutations were found to be associated with SP treatment failure. However, the presence of 5 mutations (*dhfr* Asn-108/Ile-51/Arg-59 plus *dhps*

Table 2. Frequency of the mutant and wild alleles (MA and WA) and ratio of MA/WA *dhfr* codons 108, 51 and 59, Uganda, September–December 1999

Study site	Wild allele			Mutant allele			Mutant to wild allele ratio		
	Codon 108	Codon 51	Codon 59	Codon 108	Codon 51	Codon 59	Codon 108	Codon 51	Codon 59
	% (no. of wild or mutant alleles/total no. of both alleles)								
Jinja	25.0 (4/16)	25.0 (3/12)	57.9 (11/19)	75.0 (12/16)	75.0 (9/12)	42.1 (8/19)	3.00	3.00	0.73
Rukungiri	7.9 (3/38)	22.2 (10/45)	52.7 (29/55)	92.1 (35/38)	77.8 (35/45)	47.3 (26/55)	11.7	3.54	0.90
Tororo	22.4 (13/58)	41.5 (32/77)	49.4 (42/85)	77.6 (45/58)	58.4 (45/77)	50.6 (43/85)	3.46	1.40	1.02
Mubende	9.5 (2/21)	27.8 (5/18)	52.6 (10/19)	90.5 (19/21)	72.2 (13/18)	47.4 (9/19)	9.52	2.60	0.90
Kyenjojo	23.3 (14/60)	22.0 (13/59)	48.1 (39/81)	76.7 (46/60)	78.0 (46/59)	51.8 (42/81)	3.29	3.53	1.08
Arua	46.7 (35/75)	45.4 (35/77)	50.6 (39/77)	53.3 (40/75)	54.5 (42/77)	49.3 (38/77)	1.14	1.20	0.97
Apac	42.3 (36/85)	46.8 (44/94)	50.5 (49/97)	57.6 (49/85)	53.2 (50/94)	49.5 (48/97)	1.36	1.14	0.98

Glu-540/Gly-437) was more strongly associated with SP treatment failure and has been proposed as a molecular marker for the surveillance of SP resistance. Moreover, the *dhfr* Arg-59 and the *dhps* Glu-540 mutant genotypes could predict the presence of quintuple mutations (Kublin *et al.*, 2002). However, the link between these 2 mutant genotypes and SP treatment failure was established for the treatment outcomes of patients attending the local health clinic and has not been validated at community level, possibly a more valid approach for the estimation of mutation frequencies. Malaria patients attending health facilities may have already taken antimalarial treatment and selected mutant parasites. We assessed the value of the *dhfr* mutations for the prediction of SP treatment failure at population level and we observed a high prevalence of infections with the *dhfr* point mutations with a relatively low prevalence of SP resistance. For example, in Kyenjojo, with 12.5% SP treatment failure, the prevalence of infections with the *dhfr* codon 59 mutation was 89%; in Arua, with about 10% SP treatment failure, the prevalence of the *dhfr* codon 59 mutation was 79%. Such high prevalence of mutant genotypes is probably a consequence of the large number of polyclonal infections. Therefore, a molecular index that takes into account the prevalence of both the wild and mutant genotypes might partly solve this problem. Intuitively, the prevalence of the wild genotype at any *dhfr* codon should decrease as parasite resistance evolves and the M/W genotype ratio should increase. Consequently, such a ratio should be a more robust index to measure the progressive spread of parasites with the *dhfr* mutations and hence SP treatment failure than the prevalence of infections with the mutant genotype alone or the mutant allele frequency. While the mutant allele frequency varies from 0% to 100%, the M/W ratio varies from zero to infinity and does not require adjustment for the number of clones that is necessary when considering the allele frequency or the prevalence of infections with the mutant genotype. Our data confirm that the *dhfr* codon 59 M/W genotype ratio is associated with the prevalence of SP treatment failure in Ugandan sentinel sites with different rates of malaria transmission. An M/W genotype ratio for the *dhfr* codon 59 of < 1 would suggest SP treatment failure of $< 10\%$ while an M/W ratio of 1–1.5 would indicate a higher SP treatment failure of 10–15%. Such a prediction could be more precise if the *dhps* M/W genotype ratios were considered and, more specifically, the *dhps* codon 540 M/W ratio. However, in view of the complexities involved in deriving the frequency of the multiple mutant genotypes at more than 1 locus, we believe that the prediction based on only the *dhfr* codon 59 M/W ratio is fairly precise and its reliability should be tested in other epidemiological settings. It is important to notice that a major limitation of this study is the small sample size for the estimation of SP resistance. A consequence is that such an estimation might vary quite substantially if repeated. For future studies, it would be important to recruit a larger number of patients for the *in vivo* tests. We are currently examining the *dhps* loci and investigating the statistical models that could be used to derive valid allele frequencies for 2 or more loci/genes.

An inverse relationship between the prevalence of infections with 2 and those with 3 *dhfr* mutations was observed. In previous studies, the prevalence of the *dhfr* codon 59 mutation has been found to be lower than that of the other 2 *dhfr* mutations (Asn-108 and Ile-51) (Sirawaraporn *et al.*, 1990; Peterson *et al.*, 1997; Plowe *et al.*, 1997). Our observation is consistent with the current thinking that the stepwise accumulation of the *dhfr* mutations predominantly follows the order Asn-108 → Ile-51 → Arg-59 and further supports the idea of using the *dhfr* codon 59 M/W ratio as a molecular index for prediction of SP treatment failure.

Molecular markers are promising tools but need to be validated in various epidemiological settings with different rates of malaria transmission. The correlation of parasite genotypes with drug efficacy should be considered as a population phenomenon (Sibley *et al.*, 2001). However, as far as we are aware, there are no population-based studies that have investigated the role of molecular markers for estimating the magnitude of drug resistance. Most studies have investigated the association between mutant genotypes and individual treatment success and obtained contradictory results as in several cases the molecular markers could not predict treatment outcome in the individual patient. A limitation of molecular techniques that needs to be urgently addressed is the standardization of the methods for the identification of mutant genotypes and the interpretation of the observed mutant prevalence as they presently vary from study to study. Molecular markers should be further validated for the surveillance of antimalarial drug efficacy at population level rather than for the diagnosis of treatment failure in the individual patient. Because of the ease of sample collection, validated molecular markers could become a fundamental tool for early warning that could give policy makers some lead time to prepare for policy change. As a complementary tool and not as a replacement for *in vivo* tests, they could be used for large-scale mapping and estimation of parasite resistance. *In vivo* tests could then be conducted on the basis of molecular data to map out the rate of clinical failure relevant for the antimalaria treatment policy evaluation and re-evaluation. The *dhfr* codon 59 M/W genotype ratio, estimated through cross-sectional surveys involving a random sample from the general population, is a simple and robust molecular marker that could be used for early detection of low SP treatment failure. Existing networks such as EANMAT and the Multilateral Initiative for Malaria (MIM) drug resistance network should collect population-based samples with the aim of testing the reliability of this marker in a wide range of epidemiological settings in sub-Saharan Africa.

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ROYAL SOCIETY OF TROPICAL MEDICINE AND HYGIENE
Garnham Fellowships

Professor Cyril Garnham was one of the UK's leading parasitologists in the 20th century and his work was characterized by outstanding achievement as both laboratory scientist and field worker in the tropics. The special place that Garnham occupies among his colleagues is recognized by the Fund set up in his memory to establish research fellowships for young scientists.

The aim of the Garnham Fellowship is to encourage young scientists to carry out short-term field projects. Suitable applicants are invited to apply to the Fund, which is administered by the Royal Society of Tropical Medicine and Hygiene.

There are no restrictions by nationality or age, and fellowship of the Royal Society of Tropical Medicine and Hygiene is not a requirement. Applications from non-Fellows should be supported by a Fellow who can attest to the value of the project and to the competence of the applicant to carry out the work.

- One Garnham Fellowship of up to £2000 will be awarded annually
- The Garnham Fellowship is to be used for short-term field projects of up to 2 years' duration
- Preference will be given to topics in parasitology or medical entomology and to applicants with less than 5 years' postdoctoral experience
- Applicants are required to submit a detailed project, with costing of the work proposed, and a supporting statement from their head of department or supervisor, at least 6 months before the date of commencement
- A short report should be submitted within 3 months of completion of the study

Application forms may be obtained from the Administrator, Royal Society of Tropical Medicine and Hygiene, 50 Bedford Square, London, WC1B 3DP, UK; fax +44 (0)20 7436 1389, e-mail mail@rstmh.org
The closing date for receipt of applications is 15 September annually.