Timing of Treatment Failure and Mutations in *Plasmodium falciparum* Dihydrofolate Reductase In Uganda

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Summary

The spread of drug resistant strains of malaria parasites has made prophylaxis and treatment of the disease increasingly difficult. The resistance of *Plasmodium falciparum* to chloroquine and sulfadoxine-pyrimethamine (Fansidar) has been reported in various parts of Uganda.

A study was carried out in Tororo, Uganda, in order to investigate by polymerase chain reaction/dot-blot hybridisation the association of mutations in the *DHFR* gene in *Plasmodium falciparum* parasite isolates with clinical and parasitological treatment failure following sulfadoxine-pyrimethamine or a combination of sulfadoxine-pyrimethamine and chloroquine treatment in Uganda. The study was also done to determine if the number of mutations in *DHFR* is correlated with the time to recrudescence following sulfadoxine-pyrimethamine and sulfadoxine-pyrimethamine plus chloroquine treatment.

In collaboration with the Ministry of Health, blood samples were collected on filter paper on the first day of treatment-Day 0 (D0) and on any other day symptoms developed after administering Fansidar alone and a combination of chloroquine and Fansidar to children with uncomplicated malaria aged between 6 months and 5 years old who were followed up on days 1, 2, 3, 7, 14, 21 and 28. Forty seven paired chloroquine plus Fansidar D0 and R (day of recrudescence) patient DNA samples in the RI-RIII group, were genotyped at position 76 of the *Pfcrt* locus. Following genotyping at the *MSP1* and *MSP2* loci, 22 of these patient samples showing evidence of recrudescence were genotyped at the 3 *DHFR* codons: 108, 51 and 59. For *Pfcrt* codon 76, all (100%) of the pre- and post-treatment isolates possessed the *Pfcrt* T76 allele. Only 6 (13%) pre- and 3 (7%) post-treatment isolates possessed both *Pfcrt* K76 and T76 alleles simultaneously. No isolate possessed only the *Pfcrt* K76 allele alone. For codon 108, of the 22 (pre-treatment) patient DNA samples successfully typed, all (100%) had only the *DHFR* Asn108 allele. The same trend was observed in the post-treatment isolates. No isolate pre- /or post-treatment possessed the *DHFR* Ser108 allele alone. No isolate pre- /or post-treatment possessed both *DHFR* Ser108 and Asn 108 alleles simultaneously. At codon 51, there was a high

prevalence (96%) of the 51Ile allele alone in the pre-treatment isolates and of the 51Ile allele alone in the post-treatment isolates (100%). No isolate both pre- and post-treatment possessed the *DHFR* 51Asn allele alone. Only one pre-treatment isolate possessed both *DHFR* 51Asn and 51Ile alleles simultaneously. At codon 59 there was a low prevalence (14%) of the 59Arg allele alone in the pre-treatment isolates and 38% of the isolates had the 59Arg allele alone in the post-treatment isolates. Seventeen (77%) patient samples possessed both *DHFR* 59Cys and 59Arg alleles present simultaneously in their pre-treatment isolates. This dropped to 38% in the post-treatment isolates. Only 2 (9%) patient samples pre-treatment had only the 59Cys allele while this increased to 24% in the post-treatment isolates.

Thirty three out of 48 paired Fansidar D0 and R (day of recrudescence) patient DNA samples in the RI-RIII group, showing evidence of recrudescence were genotyped for the 3 *DHFR* codons 108, 51 and 59. For codon 108, of the 33 (pre-treatment) patient DNA samples successfully typed, all (100%) had only the *DHFR* Asn108 allele. Thirty samples, representing 100% of the successfully typed post-treatment isolates, had only the *DHFR* Asn108 allele. No isolate pre- or post-treatment possessed the *DHFR* Ser108 allele alone. No patient pre- or post-treatment possessed both *DHFR* Ser108 and Asn 108 alleles simultaneously. For codon 51, 100% of samples had only the *DHFR* 51Ile allele in the pre-treatment isolates and 85% had only the *DHFR* 51Ile allele in the post-treatment isolates. No patient pre-treatment possessed the *DHFR* 51Asn allele alone. Only one patient isolate post-treatment possessed the *DHFR* 51Asn allele alone. One patient pre- and post-treatment possessed both *DHFR* 51Asn and 51Ile alleles present simultaneously. At codon 59, there was a low prevalence (18%) of the 59Arg allele alone in the pre-treatment isolates and a high prevalence (77%) for 59Arg allele alone in the post-treatment isolates. Nineteen (58%) patient samples possessed both the *DHFR* 59Cys and 59Arg alleles present simultaneously in their pre-treatment isolates. This dropped to 17% in the post-treatment isolates. Only 8 (24%) patient samples pre-treatment had only the 59Cys allele while this reduced to 7% in the post-treatment isolates.

In conclusion, the findings showed there was no correlation between the presence of *Pfcrt* alleles encoding T76 and time to recrudescence following treatment with a combination of chloroquine and Fansidar. There was no correlation between the *DHFR* alleles encoding Asn108 and/ or Ile51 and time to recrudescence following treatment with chloroquine plus Fansidar as a combination and also with Fansidar treatment alone. There was however a correlation between the presence of the *DHFR* double mutant allele Asn 108/ Arg 59, and a stronger correlation between the triple mutant allele Asn 108/ Ile 51/ Arg 59, and time to recrudescence following Fansidar treatment in Tororo, Uganda. There was no correlation of theses alleles in the chloroquine plus Fansidar treatment group.

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List of abbreviations

ACR	Adequate Clinical Response
Arg 59	Amino acid arginine at position 59
Asn 51	Amino acid asparagine at position 51
Asn 108	Amino acid asparagine at position 108
cg1	Candidate gene 1
cg2	Candidate gene 2
Cys 59	Amino acid cysteine at position 59
CQ	Chloroquine
dATP	Deoxyadenine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanine triphosphate
dTTP	Deoxythymine triphosphate
DHFR	Dihydrofolate reductase protein
DHFR	Dihydrofolate reductase gene

DHFR-TS	Dihydrofolate reductase-thymidylate synthase enzyme
DHPS	Dihydropteroate synthase protein
DHPS	Dihydropteroate synthase gene
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ETF	Early Treatment Failure
Ile 51	Amino acid isoleucine at position 51
K76T	Amino acid substitution from lysine to threonine at *Pfcrt* position 76
LTF	Late Treatment Failure
MSP1	Merozoite surface protein 1
MSP2	Merozoite surface protein 2
MS-PCR	Mutation specific-polymerase chain reaction
PCR	Polymerase chain reaction
PFCRT	*Plasmodium falciparum* chloroquine resistance transporter Protein
Pfcrt	*Plasmodium falciparum* chloroquine resistance transporter gene
pfmdr 1	*Plasmodium falciparum* multidrug resistant gene 1
RI	Resistance level I
RII	Resistance level II
RIII	Resistance level III
Ser 108	Amino acid serine at position 108
SP	Sulfadoxine-pyrimethamine or Fansidar
Thr 108	Amino acid threonine at position 108

TIMING OF TREATMENT FAILURE AND MUTATIONS IN *PLASMODIUM FALCIPARUM* DIHYDROFOLATE REDUCTASE IN UGANDA

AIM: The aims of this thesis were

(i) To investigate the association of mutations in dihydrofolate reductase (*DHFR*) with clinical and parasitological treatment failure following sulfadoxine-pyrimethamine (Fansidar-SP) or a combination of SP and chloroquine (CQ) treatment (SP/CQ) in Uganda.

(ii) To determine if the number of mutations in *DHFR* is correlated to the time to recrudescence following SP and the combination SP/CQ.

1. INTRODUCTION:

Malaria is caused by parasites of the genus *Plasmodium*, protozoan parasites of the blood. There are four species which affect man: *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. The virulence of *P. falciparum*, and its ability to develop drug resistance, marks it out from other species of human malaria parasite as one of the main threats to global public health. Worldwide there are over 300-500 million new cases of malaria per year which are responsible for 1.5 to 2.7 million deaths annually, mainly in children below 5 years of age and pregnant women in sub-Saharan Africa (WHO Report, 2001). In Uganda, malaria is the principle cause of death in children under 5 years and is responsible for 25% of all hospital admissions (Ministry of Health) [http://www.health.go.ug/malaria.htm]). However, in many areas the effectiveness of treatment and efforts to prevent *falciparum* malaria are being limited by the development of drug resistant strains (Peters, 1998). Although drug resistance is a major problem in other parts of the world, in Africa it is distinguished by two main factors: a) the enormous scale of the disease (Snow *et al.*, 1999) and of anti-malarial consumption for 'fever' and b) the small scale of the resources ranged against it.

1.1 Definition of Antimalarial drug resistance

Antimalarial drug resistance has been defined as the "ability of a parasite strain to survive and/ or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject" (WHO, 1973).

This definition was later changed in reference to the drug in question by indicating that the drug must "gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action" (Bruce-Chwatt, 1986). This was interpreted by a majority of other researchers to refer only to the persistence of parasites after antimalarial treatment other than prophylaxis failure. The continued presence of parasitaemia after antimalarial treatment is an indicator for early warning of the occurrence of drug resistance (Lobel *et al.*, 1986).

1.2 Malaria treatment failure

There are several factors that are able to contribute to malaria treatment failure including the following: incorrect dosing, non-compliance with duration of dosing regimen, poor drug quality, drug interactions, poor drug metabolism and misdiagnosis. These factors at the same time may contribute to the development and strength of true drug resistance by elevating the rate at which the parasites are exposed to sub-optimal drug levels. Therefore, drug resistance can cause treatment failure but not all treatment failure is a result of drug resistance (Bloland, 2001).

Chloroquine (CQ) and the combination drug sulfadoxine-pyrimethamine (SP) are the commonest antimalarial drugs used in Uganda. Due to its reduced efficacy, CQ is no longer used as the first-line antimalarial drug in Uganda and in some other parts of Africa. SP was one of the most important second-line drugs used in Uganda for the treatment of CQ-resistant *P. falciparum* (Bloland *et al.*, 1993). However, there are already anecdotal stories about SP resistance

in several Ugandan urban populations (Staedke *et al.*, 2001, Kamya *et al.*, 2002, Legros *et al.*, 2002, Talisuna *et al.*, 2002). Recently, the drug policy in Uganda was changed and now dictates that a combination of CQ and SP (SP/CQ) be administered for treatment of uncomplicated *P. falciparum* malaria (Communication from the Uganda Ministry of Health, Malaria Control Program, 2001). Combination therapy has been suggested to confront *P. falciparum* resistant malaria, but the suitable combination therapy for sub-Saharan Africa is not yet clear. Since drug treatment is the only efficacious control strategy used in Uganda, monitoring of levels of resistance is important. Delaying the spread of SP and CQ resistance is a major public health objective of the Ugandan Malaria Control Programme.

1.3 Classification of *in vivo* resistance

Clinical drug resistance is defined according to the World Health Organisation (WHO) classification of Early treatment failure (ETF), Late treatment failure (LTF) and Adequate clinical response (ACR). If monitoring of parasitaemia is performed, it is possible to classify resistance on three levels, known as RI, RII and RIII resistance (WHO, 1996; Bruce-Chwatt, 1986). The standard drug efficacy test is performed by the monitoring of patients for 14 days following treatment. In some circumstances (especially in lower transmission areas) this is extended to a 28-day test.

ETF is defined as a response to chemotherapy monitored from day 1 to day 3 post-treatment. During this period, a patient with ETF will develop signs and symptoms of severe malaria in the presence of parasitaemia, or will have a fever and a parasitaemia on day 2 which is greater than the day 0 count, or will have a fever on day 3 with a parasitaemia greater or equal to 25% of the day 0 count.

LTF is defined as a response following treatment which does not qualify for classification as ETF, where the patient shows signs and symptoms of severe malaria in the presence of parasitaemia, on any day from day 4 to day 14 (or to day 28 for the extended test) or has a fever in the presence of parasitaemia on

any day from day 4 to day 14 (or day 28) post-treatment.

ACR is defined as the absence of parasitaemia throughout the follow-up period, with or without fever, or where there is no fever irrespective of the presence of parasitaemia, where the patient response is not classified as ETF or LTF.

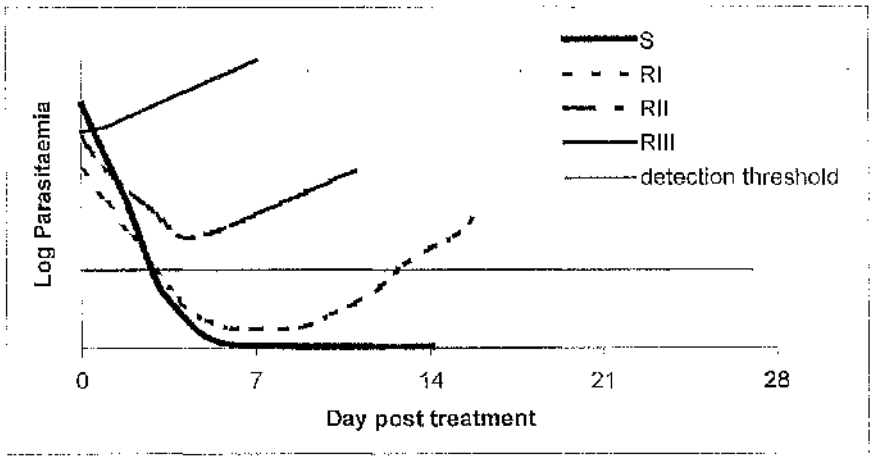
Parasites can also be defined as sensitive or resistant at various levels by monitoring parasitaemia during the follow-up period. Sensitive (S) parasites are those that are cleared by the drug within 7 days of treatment, and which do not reappear for the rest of the follow-up period. RI resistant parasites are initially cleared by drug treatment within 7 days, but parasites later reappear as a recrudescence before the end of the study follow-up period (day 14 or day 28). RII resistant parasites decrease initially but recrudescence occurs before day 7. RIII is a response with no decrease in parasitaemia; parasitaemia continues to increase following treatment.

In the study reported in this thesis, the treatment response pattern has been classified by both methods. Clinical response has been classified as ETF, LTF, or ACR, according to the standard WHO definitions. In addition, parasitaemia has been monitored, allowing the parasitological outcome of treatment to be assessed as S, RI or RII/RIII.

One explanation for the different types of resistant parasites observed is that RI parasites may be the result of a low proportion of resistant parasites in a mixed infection. Drug treatment would kill the sensitive parasites, thereby reducing the resistant parasite population to a parasitaemia below that detectable by microscopy and possibly below the threshold for symptomatic malaria. These resistant parasites would continue to grow in the presence of the drug and eventually reach microscopically detectable parasitaemias (RI) or the threshold for symptomatic malaria to return (LTF). An alternative explanation is that resistance is not an 'all-or-nothing' response. Some parasites could be highly resistant and their growth would be unaffected by the

treatment; these would be classified as RIII resistant. Other parasites could show slightly lower resistance, such that their growth would be initially inhibited by the drug, but as concentrations fall they would be able to grow again; these would be RII. Finally, RI parasites could be resistant to the drug at a lower level still, such that growth is inhibited until the drug concentration drops further, although this level would still be able to prevent growth of parasites sensitive to the drug. At present there are no studies supporting either hypothesis; this thesis has attempted to address the latter hypothesis.

Figure 1
Graph showing parasite resistance after treatment over a 28-day period



There is some correlation between clinical and parasitological response, but generally the rate of resistance/treatment failure is higher when measured by parasitaemia than by clinical response.

Some *in vivo* studies do not have a clear- cut definition of SP clinical resistance, which can complicate their interpretation and comparisons with other data.

For example, Basco and co-workers (1998) considered ETF as RII, LTF as early RI and late RI as recrudescence. Masimirembwa and colleagues (1999) associated treatment failure with the presence of parasites on day 2 greater than half of the parasitaemia on day 0, and the presence of parasitaemia on day

7 as recrudescence with the clearance of parasitaemia by day 7 defined as sensitive.

In conclusion, the evidence presented in the studies discussed indicates that the correlation between *in vitro* resistance and *in vivo* resistance of SP is still not clear, and further studies are necessary.

There is also a problem of parasite clearance by acquired immunity especially in more immune populations. This factor cannot be easily quantitated but can be hypothesised to play an important role. Individual patients may have different immunological capacities (independent of resistant phenotype of the parasites) to clear parasites. The level of acquired immunity is related to both age (older children and adults have higher levels of acquired immunity) and exposure (individuals living in areas of high endemicity have higher levels of acquired immunity than those of the same age living in areas of lower endemicity) (Djimde *et al.*, 2001, Omar *et al.*, 2001). The importance of acquired immunity in the clearance of drug resistant parasites is demonstrated by the results of a study carried out in Mali. Sixty-six percent (66%) of adults and older children treated with chloroquine were able to clear their parasitaemia despite having parasites with the mutated form of *Pfcrt76*, whereas only 32% of the younger children carrying the *Pfcrt 76* mutant form were able to clear their parasitaemia (Djimde *et al.*, 2001). In another study done in a holo-endemic area in Kenya where patients were treated with SP it was observed that treatment success increased with age (Omar *et al.*, 2001). The ability to clear drug-resistant parasites increased with age, implying that age-acquired immunity plays an important role in the clearance of chloroquine resistant parasites. Studies with the rodent model *P. chabaudi* showed that partially immune mice were able to clear drug resistant parasites more efficiently than naive mice (Cravo *et al.*, 2001).

1.4 Mechanisms of antimalarial drug resistance

The way in which parasites can acquire resistance depends on the mechanism

by which the drug kills them. This is an important factor in determining how fast parasite resistance can occur.

1.4.1 Chloroquine resistance

The mechanism of chloroquine action is not yet clear but the formation of a complex with haem is thought to be important (Mungithin *et al.*, 1998). The molecular basis of drug resistance to CQ is now partially understood, although there is still some debate regarding the contribution of mutations in different genes to resistance. Initial reports associated resistance with mutations in the gene *pfmdr1* (*P. falciparum* multidrug resistance gene 1) (Foote *et al.*, 1989). Associations between *pfmdr1* point mutations and *in vivo* or *in vitro* CQ resistance were inconsistent. An early study suggested that it was possible to predict whether an isolate was resistant or sensitive to CQ by the presence of defined mutations in *pfmdr*1 (Foote *et al.*, 1990). Studies that followed established an association between the presence of a mutation at codon-86 (causing an amino acid substitution from asparagine in the wild type to tyrosine in the mutant form) and *in vitro* CQ resistance, with sub- Saharan isolates (Basco *et al.*, 1995a, Adagu *et al.*, 1996). A similar association was not observed with culture- adapted Sudanese (Awad El Kariem *et al.*, 1992) and Thai isolates (Wilson *et al.*, 1993). Other studies using fresh clinical isolates showed that the mutant allele of *pfmdr1* encoding tyr-86 could be used to predict *in vivo* drug resistance to CQ (Duraisingh *et al.*, 1997). However, extensive field studies (Wellems *et al.*, 1990, Bhattacharya *et al.*, 1999, Adagu *et al.*, 1999, Price *et al.*, 1999, McCutchcon *et al.*, 1999, Duraisingh *et al.*, 2000, Pillai *et al.*, 2001) produced conflicting results, casting doubts on a direct causal association between polymorphisms in *pfmdr1* and CQ resistance. Analyses of a genetic cross between CQ-sensitive and CQ-resistant parasite clones demonstrated that *pfmdr1* mutations were not linked to the resistance phenotype (Wellems *et al.*, 1990). Transfection studies that followed showed that substitution of the mutant *pfmdr1* with the wild type sequence in resistant parasites decreased chloroquine resistance from high to

moderate levels (Reed *et al.*, 2000). Therefore, it appears that, although mutations in *pfmdr1* may not be absolutely required for chloroquine resistance, polymorphisms in this gene may play a role in modulating the resistance phenotype (Wellems *et al.*, 1991, Su *et al.*, 1997, Reed *et al.*, 2000, Dorsey *et al.*, 2001).

Linkage analyses of a genetic cross identified two loci, known as *cg2* (candidate gene 2) and *Pfcrt* (*P. falciparum* chloroquine resistance transporter), implicated in chloroquine resistance (Su *et al.*, 1997, Fidock *et al.*, 2000b, Djimde *et al.*, 2001). Inheritance of a 36 kb segment of chromosome 7 from the CQ resistant parent in the cross conferred resistance in the progeny clones. DNA sequencing and searches for open reading frames (ORF) encoding more than 100 amino acids within this segment identified nine ORFs. Of these, one ORF, denoted *cg2*, contained multiple and complex polymorphisms associated with CQ resistance both in the progeny clones of the genetic cross, and also in a group of isolates from Asia and Africa (Su *et al.*, 1997). However, larger scale field studies failed to demonstrate a clear association between the complex polymorphisms within *cg2* and CQ resistance (Adagu *et al.*, 1999, Price *et al.*, 1999, Durand *et al.*, 1999, Basco *et al.*, 1999, McCutcheon *et al.*, 1999, McCutcheon *et al.*, 2000, Pillai *et al.*, 2001). Transfection-based studies confirmed that the *cg2* gene was not the gene responsible for CQ resistance (Fidock *et al.*, 2000a). Extra analysis of the initial 36 kb locus comprising the *cg2* gene led to the discovery of a highly interrupted gene, denoted *Pfcrt*. Polymorphisms in this gene were absolutely associated with CQ resistance *in vitro* in forty known parasite lines (Fidock *et al.*, 2000b). A mutation in codon 76, causing an amino acid change from lysine (K) to threonine (T) was identified as the key mutation necessary for resistance. Transformation of a CQ-sensitive *P. falciparum* clone using plasmid constructs that expressed a *Pfcrt* sequence with the K76T codon change produced a highly CQ resistant line. However, the resistant parasites ended up losing the transfected DNA and instead acquired a novel mutation encoding K76I in the chromosomal *Pfcrt* gene. The change in the sequence

directly contributed to the change in CQ phenotype (Fidock *et al.*, 2000b). There is therefore strong evidence that an amino acid change at position 76 in *Pfcrt* is associated with CQ resistance in African and Asian *P. falciparum* isolates. This is supported by field studies showing a strong link between the presence of *Pfcrt76* mutation and clinical CQ resistance (Djimde *et al.*, 2001). Further *in vivo* studies on field isolates have shown that the *Pfcrt 76* mutation is often also present in CQ-sensitive isolates (Pillai *et al.*, 2001, Mayor *et al.*, 2001, Dorsey *et al.*, 2001, Kyosiimire *et al.*, 2002, Chen *et al.*, 2002). The *Pfcrt* mutant allele is always present in CQ treatment failure samples. This therefore suggests that the *Pfcrt* 76 mutation may be necessary but is not always sufficient for CQ resistance *in vivo*.

It has been suggested that CQ resistance is a multigenic phenomenon, involving changes to *Pfcrt* and other genes such as *pfmdr1* (Reed *et al.*, 2000). Support for this hypothesis is provided by field studies in which clinical treatment failure with CQ is significantly associated with parasites with the mutated forms of both *Pfcrt 76* and *pfmdr1* 86 (Babiker *et al.*, 2001, Dorsey *et al.*, 2001, Mockenhaupt *et al.*, 2001). In addition, the relatively long time taken for CQ resistance to arise and spread could indicate that multiple mutations are required.

1.4.2 Pyrimethamine resistance

Sulfadoxine-pyrimethamine (SP)-(Fansidar) is a synergistic combination of drugs that interact with two enzymes of the folate biosynthetic pathway. Dihydrofolate reductase (DHFR) is the biochemical target of pyrimethamine, and also of cycloguanil, the biologically active metabolite of proguanil. In *P. falciparum*, two genes involved in the pyrimidine biosynthetic pathway, *DHFR* and *TS* (thymidylate synthase) are contiguous and are expressed as a bifunctional enzyme DHFR-TS (Bzik *et al.*, 1987). Pyrimethamine binds to the parasite DHFR with a high affinity, inhibiting enzyme activity, resulting in the depletion of dTMP and the disruption of DNA synthesis (Hyde, 1990). The

selective activity of pyrimethamine has usually been attributed to higher affinity of the drug for *Plasmodium* DHFR-TS than for human DHFR (Hitchings, 1960; Ferone and Hitchings, 1969; Peterson *et al.*, 1990, Foote *et al.*, 1990). Recent work has shed light on the differential specificity of the drug for parasite and human DHFR. The DHFR-TS of both species binds its cognate messenger RNA (mRNA) and inhibits its own translation. Pyrimethamine treatment removes the translational repression of the human enzyme, and more DHFR is produced to partially overcome the drug barrier. However, in *Plasmodium* DHFR-TS, mRNA binding is not coupled to enzyme active sites. Therefore, antifolate treatment does not relieve translational inhibition and the parasites do not have the ability to make fresh enzyme (Zhang and Rathod, 2001).

Resistance to pyrimethamine is linked to a point mutation in *DHFR* leading to an amino acid substitution at codon 108 from serine to asparagine (Peterson *et al.*, 1988, Peterson *et al.*, 1990). Resistance to cycloguanil has been linked to paired mutations in codon 16 (alanine to valine) and 108 (serine to threonine) (Foote *et al.*, 1990; Peterson *et al.*, 1990). Other mutations at positions 51 (asparagine to isoleucine), 59 (cysteine to arginine) and 164 (isoleucine to leucine) are associated with higher levels of pyrimethamine and cycloguanil resistance (Foote *et al.*, 1990, Peterson *et al.*, 1990).

Following the introduction of pyrimethamine monotherapy, resistance was reported within a year (Maberti, 1960), most likely as a result of the simple resistance mechanism. Pyrimethamine is now used only in synergistic combinations with sulfadoxine or other antifolates (Chulay *et al.*, 1984), so it is not possible to correlate *in vivo* pyrimethamine treatment failure with *DHFR* genotype. However parasites taken from infected individuals can be tested for their pyrimethamine susceptibility *in vitro*. In these tests, resistance is denoted by an IC_{50} value of > 100 nM (de Pécoulas *et al.*, 1996). Such studies reveal a very strong link between mutations in *DHFR* and pyrimethamine and cycloguanil resistance. For pyrimethamine, *DHFR* Asn108 is almost completely associated with resistance to pyrimethamine, and the presence of

parasites encoding DHFR Ser108 alone is a good indicator of sensitivity *in vitro* to pyrimethamine (e.g. de Pécoulas *et al.*, 1996, Basco *et al.*, 1995b, Basco *et al.*, 1996, Foote *et al.*, 1990, Hyde *et al.*, 1990, Khan *et al.*, 1997, Peterson *et al*, 1990, Reeder *et al.*, 1996 and Zindrou *et al.*, 1996). Geometric mean 50% inhibitory concentration values (IC_{50}) were over 1000-fold higher for parasites with *DHFR* Asn108 from African patients compared to parasites with *DHFR* Ser108 (IC_{50} of 4050 nM for Asn 108 and 12.5 nM for Ser 108).

The central mutation implicated in pyrimethamine resistance is at position 108 (Cowman *et al.*, 1988, Peterson *et al.*, 1988, Zolg *et al.*, 1990), a finding that was confirmed by transfection studies (Wu *et al.*, 1996). It is believed that additional mutations in *DHFR* codons 51 and 59, combined with the Asn108 change, confer higher levels of resistance. Kinetic analysis of the *P. falciparum* DHFR-TS enzyme after expression in *Escherichia coli* confirmed the role of these mutations in the different levels of resistance. The inhibition constant (K_i) values for the double mutants Asn108/Arg59 and Asn108/Ile51 are 72 nM and 37 nM respectively, compared to 13 nM for the single mutated allele encoding Asn108 (Sirawaraporn *et al.*, 1997). The inhibition constant for parasites carrying the triple mutant Asn108/ Ile51/ Arg59 was 120 nM (Sirawaraporn *et al.*, 1997) indicating a much higher level of resistance as compared to the single and double mutated alleles.

Transfection experiments have also been performed using DHFR as a selectable marker that confers resistance to pyrimethamine (Wu *et al.*, 1996). Pyrimethamine-sensitive parasites transformed with the DHFR allele from parasite Dd2 (triple mutant form of *DHFR* Asn108/Ile51/Arg59) yielded drug-resistant parasites with an IC_{50} of 16 nM. Transformation with the *DHFR* gene from parasite HB3 (single mutant form Asn108) yielded parasites with an IC_{50} value of 1.0 nM. Investigating the effect of the additional mutation in *DHFR* at codon 51 was also done by transfection of *P. falciparum* DHFR alleles into yeast (Wooden *et al.*, 1997). This suggests that the double mutant form *DHFR* Asn108/Ile51 (*pf-dhfr*-Mikenga, a resistant construct) causes a further ten-fold

increase in IC_{50} compared to the single mutant form *DHFR* Asn108 (*pf-dhfr*-Honduras, a resistant construct), which itself produces a ten-fold increase in IC_{50} compared to the wild type form *DHFR* Ser108 (*pf-dhfr*-D6, a sensitive construct).

However it is difficult to predict what the effects of these mutations may be on *in vivo* treatment failure. *In vitro* studies usually describe IC_{50} values, which is the lowest inhibitory concentration of the drug which kills 50% of the parasites. This implies that 50% of the parasites remain viable. *In vivo* this is most likely to result in treatment failure.

1.4.3 Sulfadoxine resistance

Sulfadoxine, the other component of the combination drug SP, is also never used as monotherapy for malaria. The information on the resistance mechanisms to sulfadoxine alone has therefore been obtained from *in vitro* work. The molecular target of sulfadoxine is the enzyme DHPS, a part of a bifunctional protein with hydroxymethylpterin pyrophosphate. Sulfadoxine is a structural homologue of 4-amino benzoic acid (PABA) the natural substrate of DHPS (Brooks *et al.*, 1994, Triglia and Cowman, 1994) and is a competitive inhibitor of the enzyme. Mutations in the *DHPS* gene are found in natural populations, and have been correlated with resistance to SP *in vivo,* for example, mutations leading to amino acid changes at positions 437 (alanine to glycine), 436 (serine to phenylalanine), 613 (alanine to serine), and 581 (alanine to glycine) (Triglia *et al.*, 1997, Plowe *et al.*, 1997, Wang *et al.*, 1997a).

Some studies have attempted to correlate *in vitro* sulfadoxine resistance and *DHPS* mutations with isolates taken from the field and with laboratory lines, but these have not been conclusive (Table 1: a, b, d, and f). *In vitro* assays for sulfadoxine have been done under varying folate conditions (Wang *et al.*, 1997b), and some parasite isolates are known to use host folate to antagonise

sulfa drugs irrespective of their *DHPS* genotype (Milhous *et al.*, 1985, Watkins *et al.*, 1985). Sulfadoxine *in vitro*-drug testing is technically difficult but can be done using culture medium depleted of folate and PABA that antagonise sulfadoxine (Chulay *et al.*, 1984, Watkins *et al.*, 1985, Milhous *et al.*, 1985). Published data for IC_{50} (50% Inhibitory concentration) values show very large differences, varying from 10 nM (Milhous *et al.*, 1985) to 150 nM (Watkins *et al.*, 1987), or from 1000 nM (Chulay *et al.*, 1984) to as high as 3 X 10^6 nM (Schapira *et al.*, 1986) for sulfadoxine- sensitive strains. Relatively small differences in IC_{50} are observed between parasites classified as resistant or sensitive to sulfadoxine.

A direct correlation between *DHPS* point mutations and *in vitro* sulfadoxine resistance was demonstrated using the progeny of a cross between the sulfadoxine- sensitive parasite clone HB3 and the sulfadoxine-resistant clone Dd2 (Wang *et al.*, 1997b). Consistently reproducible results showing large differences between the most sensitive and most resistant progeny clones were obtained as a result of using an improved drug assay in the absence of folate. Differences in sulfadoxine responses of the individual progeny from the HB3-Dd2 cross were monitored. Parental clone HB3 with a 'wild type' (unmutated) allele of *DHPS* had the lowest IC_{50} value of 12.9 nM. Progeny clones inheriting the DHPS allele from this parent had IC_{50} values in the same range. The Dd2 parent clone carrying the three *DHPS* mutations 436 Phe/437 Gly/613 Ser showed the highest IC_{50} value of 12.8 X 10^3 nM, 1000-fold higher, as did progeny clones inheriting the Dd2 allele.

Site directed mutagenesis studies involving alterations in the amino acids in the DHPS enzyme yielded alleles with varying inhibition constants for sulfadoxine (Triglia *et al.*, 1997). Eight plasmid constructs were made that expressed the alleles of the functional *P. falciparum DHPS* in *Escherichia coli*. After purification, the inhibitory constant for sulfadoxine was measured for the eight DHPS enzymes by a competitive mechanism. The K_i for sulfadoxine for all the eight enzymes varied significantly by up to 811-fold (3

orders of magnitude). Enzyme D10-C (with a sequence identical to *P. falciparum* 3D7) present in sulfadoxine-sensitive isolates had the lowest K_i value of 1.4 X 10^2 nM and W2 mef-C, which is present in highly sulfadoxine-resistant isolates, had the highest K_i value of 11.2 X 10^4 nM.

Another mechanism associated with folate uptake and utilisation is thought to have an effect on the sulfadoxine resistance levels (Wang *et al.*, 1999). Low levels of folate in the culture medium can result in a reduction in the susceptibility of the parasites to sulfadoxine (known as the folate effect). Parasite clone Dd2 had markedly lower IC_{50} values under low folate conditions, whereas clone HB3 showed little or no change in the IC_{50} values observed compared with those obtained in normal medium. The difference between the two clones was suggested to be due to another gene that strongly influences resistance of a specific clone to sulfadoxine depending on the levels of exogenous folate.

1.4.4 SP resistance

The association of mutations in both *DHFR* and *DHPS* with resistance to SP in malaria parasites has been investigated (Hyde *et al.*, 1990, Brooks *et al.*, 1994). Previous studies have analysed the correlation between *in vitro* sulfadoxine resistance and *DHPS* genotype separately from *in vitro* pyrimethamine resistance and *DHFR* genotype. Generally, laboratory adapted strains of *P. falciparum* have been used, although field isolates have also been studied occasionally. It is not clear what role point mutations in the *DHFR* and *DHPS* genes play in *in vivo* pyrimethamine and sulfadoxine resistance, as these drugs are never used alone (Hyde, 1990, Wang *et al.*, 1997a). Although an *in vitro* SP susceptibility test has been described, the 'folate effect' described above can complicate their interpretation, and most studies have used samples from clinical SP treatment failure. The molecular criteria for *in vitro* antifolate resistance have not been confirmed or correlated with *in vivo* response of patients treated with SP (Rallón *et al.*, 1999). Furthermore, the

DHFR and *DHPS* allelic combinations that may be associated with SP therapeutic failure are not yet clearly known.

Controversies have arisen between different studies showing evidence for and against mutations in *DHFR* and *DHPS* being implicated in SP resistance of *P. falciparum* strains, and these will be described below. Studies dealing with the determination of genetic changes as the basis of clinical resistance have been carried out in West Africa (Plowe *et al.*, 1995; 1996). These studies have confirmed that the molecular diagnostic techniques used are appropriate for epidemiological studies of resistance where it is not possible using standard *in vitro* and *in vivo* methods for detecting resistance. They also show that surveillance studies carried out at intervals to determine resistance levels in different regions using these molecular assays may assist health officials in changing antimalarial drug treatment policies accordingly. Arguments have been made for limiting the distribution and the use of specific antimalarial drugs, but evidence that such an approach can slow down the spread of resistance has been lacking (Price and Nosten, 2001).

Evidence for or against the role of *DHFR* and *DHPS* mutations in SP resistance *in vivo* and *in vitro*

Several studies have been carried out to investigate the role of mutations in *DHFR* and *DHPS* in clinical SP resistance. Field tests carried out in Kenya both *in vivo* and *in vitro* have suggested that pyrimethamine resistance alone (associated with mutations in *DHFR*) is sufficient to cause SP failure (Nguyen-Dinh *et al.*, 1982). This was supported by a study done in Tanzania, which showed evidence of *in vivo* selection under SP drug pressure of parasites with alleles encoding the DHFR Asparagine 108 mutant (Curtis *et al.*, 1996). An *in vivo* study carried out in Mali showed that SP selected for *DHFR* mutants rapidly, with Arginine 59 (55.0%) and Asparagine 108 (55.0%) being more common in post-treatment isolates, supporting their role in SP failure (Diourte *et al.*, 1999). However, because there was a low level of

in vivo SP resistance in the study area, it was not possible to identify any group of *DHFR* or *DHPS* mutations that were predictive of clinical SP failure. A study carried out in Cameroon showed that the triple *DHFR* mutant encoding Asn 108/Arg 59/Ile 51 was strongly associated with *in vivo* resistance to SP (Basco *et al.*, 2000).

There is also evidence that mutations in both *DHFR* and *DHPS* are necessary for *in vivo* SP resistance. Studies carried out in West and East Africa, the Middle East and Vietnam (Wang *et al.*, 1997a, Schapira *et al.*, 1986) suggest that *DHFR* 108 and *DHPS* 437 changes are necessary for SP treatment failure. The study by Schapira also performed *in vitro* sulfadoxine tests on parasites from SP-resistant cases and found that the *in vitro* results reflected the *in vivo* treatment failure. A study carried out in Peru (Kublin *et al.*, 1998) showed that a collection of mutations in *DHFR* (Asn 108/Ile 51/Leu 164) and *DHPS* (Gly 437/Glu 540/Gly 581) was strongly associated with *in vivo* SP resistance. In Vietnam, a combination of multiple *DHFR* antifolate resistance mutations coupled with the 437 Glycine mutation in *DHPS* correlated best (72%) with *in vivo* resistance to SP (Masimirembwa *et al.*, 1999). In Kenya, a combination of the triple *DHFR* mutant (Asn 108/Ile 51/ Arg 59) coupled with the *DHPS* double mutant (Gly 437/Glu 540) was associated (55.2%) with *in vivo* resistance to SP (Nzila *et al.*, 2000).

In other studies, however, mutations in *DHFR* and *DHPS* were not informative for predicting SP resistance. A study done in Colombia showed that *DHFR* alleles encoding Asn 108 were detected in 75% of clinical samples with adequate clinical response (ACR) and in all the treatment failure samples, rendering it inadequate for predicting SP treatment failure (Rallón *et al.*, 1999). Studies carried out in Tanzania found no association between the presence of mutations at different loci of the *DHFR* gene before treatment and subsequent clinical SP resistance (Jelinek *et al.*, 1997).

It has also been argued that *DHPS* mutations do not play a role in SP failure

(Watkins *et al.*, 1997). A study carried out in Mali found no significant selection by SP for *DHPS* mutations, including no significant selection against the *DHPS* allele encoding Ala-436 (Diourte *et al.*, 1999). In Vietnam, only two out of seven samples resistant to SP *in vivo* were fully explained by the known *DHPS* mutations as investigated by mutation-specific PCR (Masimirembwa *et al.*, 1999). A study carried out in Cameroon to assess SP efficacy showed there was no effect on clinical outcome by the *DHPS* genotype (Basco *et al.*, 2000). From these studies and those listed in Tables 1 and 2, it is difficult to judge whether molecular changes in the *DHFR* and/or *DHPS* correlate with *in vivo* treatment failure with SP. Tables 1 and 2 summarise some of the studies performed to investigate the association between *DHFR/DHPS* mutations and SP resistance.

Table 1

DHFR/DHPS mutations and SP resistance *in vitro*
The listed references provide evidence of a correlation between mutations in *DHFR* and *DHPS* and resistance to pyrimethamine and sulfadoxine respectively

Evidence supporting the role of Asn-108 and other *DHFR* mutant codons in pyrimethamine *in-vitro* resistance	**Evidence supporting the role of Gly-437 and other *DHPS* mutant codons in sulfadoxine *in-vitro* resistance**
1. Basco *et al.*, 1996 Exp. Parasitol. 82: 97-103	a. Basco *et al.*, 1998a Am. J. Trop. Med. Hyg. 58: 374-378
2. Basco *et al.*, 1995 Mol. Biochem. Parasitol. 69: 135-138	b. Brooks *et al.*, 1994 Eur. J. Biochem. 2: 397-405
3. Foote *et al.*, 1990 Proc. Natl. Acad. Sci. USA. 87: 3014-3017	c. Reeder *et al.*, 1996 Am. J. Trop. Med. Hyg. 55: 209-213
4. Hyde *et al.*, 1990 Pharmacol. Ther. 48: 45-49	d. Triglia *et al.*, 1994 Proc. Natl. Acad. Sci. USA 91: 7149-7153
5. Khan *et al.*, 1997 Trans. R. Soc. Trop. Med. Hyg. 91: 456-460	e. Triglia *et al.*, 1997 Proc. Natl. Acad. Sci. USA. 94: 13944-13949
6. Nzila-Mounda *et al.*, 1998 Antimicrob. Agents Chemother. 42: 164-169	f. Wang *et al.*, 1995 Mol. Biochem. Parasitol. 71: 115-125
7. Peterson *et al.*, 1990 Proc. Natl. Acad. Sci. USA. 87: 3018-3022	g. Wang *et al.*, 1997b Mol. Microbiol. 23: 979-986
8. Reeder *et al.*, 1996 Am. J. Trop. Med. Hyg. 55: 209-213	h. Triglia *et al.*, 1998 EMBO J. 17: 3807-3815
9. Zindrou *et al.*, 1996 Exp. Parasitol. 84: 56-64	
10. Zolg *et al.*, 1989 Mol. Biochem. Parasitol. 36: 253-262	
11. Cowman *et al.*, 1998 Proc. Natl. Acad. Sci. USA. 85: 9109-9113	
12. Peterson *et al.*, 1998 Proc. Natl. Acad. Sci. USA. 85: 9114-9118	
13. Basco *et al.*, 1998b Antimicrob. Agents Chemother. 42: 1811-1814	
14. Wilson *et al.*, 1993 Mol. Biochem. Parasitol. 57: 151-160	
15. Awad-El-Kariem *et al.*, 1992 Trans. R. Soc. Trop. Med. Hyg. 52 (6): 565-568	

Table 2

DHFR/DHPS mutations implicated in SP *in vivo* resistance
The listed references which provide evidence of a correlation between mutations in *DHFR* and/or *DHPS* and resistance to SP respectively.

Paper and study region	**Correlation of SP resistance to *DHFR* mutations**	**Correlation of SP resistance to *DHPS* mutations**	**Recommended combinations for genotyping tests**
Curtis *et al.*, 1996 Trans.R. Soc.Trop.Med. Hyg. 90: 678-680 (Tanzania)	Asn 108	Gly 437	Asn 108 and Ile51/Arg 59 **AND** Gly 437 and/or Gly 437/Glu 540
Basco *et al.*, 1998b Antimicrob. Agents Chemother. 42: 1811-1814 (Cameroon)	Asn 108	Gly 437	Asn 108 and Ile 51/Arg59
Kublin *et al.*, 1998 Lancet 351: 1629-1630 (Peru)	Asn 108	Gly 437	Asn 108 and Ile 51/Leu 164 **AND** Gly 437 and/or Glu 540/Gly581
Masimirembwa *et al.*, 1999 Int. J. Antimicrob. Agents. 12: 203-211 (Vietnam)	Asn 108	Gly 437	Asn108/Ile 51/Arg 59/Leu 164 **AND** Gly 437 and/or Gly 581/ 613 Ser or Thr
Diourte *et al.*, 1999 Am. J. Trop. Med. Hyg. 60: 475-478 (Mali)	Asn 108 and Arg 59	No correlation observed with any mutation	Asn 108 and Arg 59
Basco *et al.*, 2000 J. Infect. Dis. 182: 624-628 (Cameroon)	Asn 108	Ala or Ser 436/Gly or Ala 437/Lys 540/Ala 581/ Ala 613	Asn 108/Arg 59/Ile 51 **AND** Ala 436/Ala 437/Lys 540/Ala 581/Ala 613 or Ser 436/Gly 437/Lys540/Ala 581/Ala 613

1.5 Hypothesis:

The hypothesis being tested is that early treatment failure is caused by parasites with several mutations in *DHFR*, i.e. a higher level of resistance, as indicated by more mutated codons in *DHFR*, is predicted in parasites causing early treatment failure than in parasites causing late treatment failure.

Molecular techniques to monitor drug resistance are dependent on polymerase chain reaction (PCR)-based methods to amplify genes associated with drug resistance (Plowe *et al.*, 1995). Mutation-specific PCR techniques are able to indicate the presence of parasites within a sample carrying alleles of the gene under investigation which are associated with resistance or sensitivity to the drug. However the sensitivity and specificity of these techniques may be compromised by the multiplicity of *P. falciparum* genotypes in natural infections (Ranford-Cartwright *et al.*, 2002). A PCR/dot-blot hybridisation technique for detecting point mutations at residues 108, 51 and 59 of the *P. falciparum DHFR* gene, which are associated with pyrimethamine resistance, has recently been developed (Abdel-Muhsin *et al.*, 2002). The technique combines PCR amplification and hybridisation of amplified products using radiolabelled allele-specific oligonucleotide probes. The dot-blot method was as sensitive as the mutation-specific PCR technique, and was more specific in a series of comparisons (Ranford-Cartwright *et al.*, 2002). Characterisation of parasite genes was possible from samples with a parasitaemia as low as 100 parasites/µl of blood. It was also possible to identify a minority parasite genotype down to 1% in a mixture. The dot-blot technique is more suited to high throughput studies, because each dot-blot can contain PCR products from over 90 samples.

In this study, mutation-specific PCR has been used to type alleles of *Pfcrt* codon 76, because the dot-blot method had not yet been developed for this locus. The PCR/dot-blot hybridisation technique was applied to detect alleles of *DHFR* associated with drug resistance in parasites with recrudescent

infections. The patient samples were collected during an *in vivo* drug efficacy study of SP and the combination of SP and CQ, carried out in Uganda.

The results from the typing were used to investigate if mutations in the *DHFR* gene in *P. falciparum* pre-treatment isolates are predictive of failure of SP/CQ and SP treatment during follow-up. In addition, we investigated if the number of mutations in *DHFR* was correlated with time to recrudescence, i.e. does the number of days to recrudescence correlate with more *DHFR* mutations in the resistant parasites at the time the drug was given?

2. MATERIALS AND METHODS:

2.1. Study area and population

The study was carried out during the period December 2001-March 2002 in Nagongera Health Centre in Tororo district, Eastern Uganda, one of the sentinel sites chosen by the East Africa Network for Monitoring Antimalarial Treatment (EANMAT) and the Uganda National Malaria Control Programme (UNMCP) for surveillance of antimalarial drug efficacy. A clinical drug efficacy trial was performed to compare the efficacy of SP and a combination of Chloroquine and SP in symptomatic children with uncomplicated *falciparum* malaria.

Tororo District lies at an altitude approximately between 1,097 m and 1,219 m above sea level and has a population of approximately 400,000. It borders the Republic of Kenya to the East, and has the Ugandan districts Mbale to the North East, Iganga to the West, Busia to the South and Pallisa to the North. Tororo District has a bimodal rainfall pattern with peak rainfall during the months of March to May and August to September and high temperatures most of the year. The area is holo-endemic for malaria, registering an average of 4500 malaria cases monthly (Epidemiological Surveillance Division, Ugandan Ministry of Health). The parasite prevalence in children aged 2-9 years is 88% (Talisuna *et al.*, 2002). The main occupation of the population (the Japadhola, the Banyole and the Iteso) in this rural region is subsistence agriculture.

Children aged between 6 months and 5 years old were enrolled into a drug efficacy trial according to the EANMAT field manual and the standard World Health Organisation (WHO) protocol for areas of intense transmission (WHO 1996), but adapting the trial to a 28-day test and not the standard 14-day test. Inclusion criteria were: uncomplicated malaria, with *P. falciparum* monoinfection, axillary temperature $\geq 37.5^{\circ}C$ or history of fever in the past 24 hours, and an asexual parasitaemia between 2000 parasites per µl blood and

100,000 parasites per μl of blood. The children were recruited with informed consent from their parents or guardians, treated and followed up to day 28 after treatment. Active recrudescent case detection was initiated at the point of enrolment into the study, when parents or guardians were asked to return the children after treatment on appointment i.e. day 3, 7, 14, 21 and 28, irrespective of their clinical status at that time. Passive recrudescent case detection was done at any time during follow-up. Any child who felt ill within the 28 days after initial treatment was requested to return to the clinic for resampling and retreatment with an alternative drug where appropriate. Children who presented with or subsequently developed severe malaria symptoms or other causes of fever, those with severe malnutrition and those with a history of allergic reactions to sulfa drugs were not recruited to the trial. Children who did not complete the follow up, those who took antimalarial drugs from other sources, and those who developed complications were not considered in the analysis as per the protocol.

Ethical approval for the study was obtained from the Uganda National Council of Science and Technology (UNCST) and the Institutional Review Board (IRB) of the University of Glasgow, UK.

2.2. Sample collection, treatment and follow-up

One hundred eighty one (181) children were recruited to the trial. A finger-prick blood sample was taken at admission to the trial. Thin and thick smears were prepared and stained with Giemsa's stain for the assessment of parasitaemia on the first day of treatment (known as D0) and on each day of subsequent visits. A finger-prick sample of blood from each study subject was also taken onto filter paper (3MM Whatman) for later molecular studies (polymerase chain reaction (PCR) analysis) at each sampling point. The filter papers were air dried at room temperature and placed into individual self-sealing plastic bags and stored at room temperature.

The recruited children were followed up on days 1, 2, 3, 7, 14, 21 and 28, and/or on any day that they developed symptoms. On entry to the trial, children were treated with either a combination of CQ (150mg base) and SP (500mg S + 25mg P), or with SP alone (500mg S + 25mg P), administered orally under the direct supervision of a study nurse. The two trials were done simultaneously. The first recruited child was assigned to the SP/CQ treatment group and the second child to the SP treatment group. That same order was followed for all the other recruited children. For CQ, the drug was given over 3 days. The total CQ given was calculated according to body weight at 25mg CQ per kg body weight of the child. This was divided into three doses of 10 mg/kg on day 0 and day 1, and 5 mg/kg on day 2). The drugs were all quality assured. Beginning on day 3, follow up was stopped after confirming the presence of malaria parasites in the blood (≥25% of D0 parasite count) of a child. This was termed as a recrudescent sample to await confirmation by PCR. At this point the child was treated with an alternative drug as advised by the treating clinician, and took no further part in the study beyond the treatment failure sampling at that time.

2.3. Definition of outcomes

Clinical outcome was defined as described earlier according to the WHO classification of adequate clinical response (ACR), early treatment failure (ETF), and late treatment failure (LTF) (WHO, 1996). Parasitological response was defined using the standard sensitive (S), resistance level I (RI), resistance level II (RII), and resistance level III (RIII) classification system (Bruce-Chwatt, 1986).

2.4. Slide examination

Thin films were fixed with methanol and both thin and thick films were stained with 10% Giemsa's stain for 30 minutes. The number of asexual

P. falciparum parasites per 200 white blood cells (WBC) was counted under 100 times (high power) magnification oil immersion fields. The number of parasites was multiplied by 40 to calculate the parasite density per microlitre of blood. This assumes a WBC count of 8000/μl (WHO, 1991). Where less than 10 parasites per 200 WBC were found, the count was continued to 500 WBC and the parasite count determined correspondingly. A sample was declared negative after examining 200 thick film fields without observing any asexual parasites.

2.5. DNA extraction, PCR amplification and product analysis

Extraction and purification of parasite DNA from blood samples on filter paper was performed using the Chelex-100 method as described (Plowe *et al.*, 1995). All of the blood from a single spot was used and the final volume of DNA solution obtained was approximately 200μl. Extracted DNA was used immediately or stored at -80°C for later PCR amplification of DNA.

2.5.1. Genotyping at the* Pfcrt *locus

Mutations in *P. falciparum* chloroquine resistance transporter gene (*Pfcrt*), specifically those causing an amino acid substitution from lysine to threonine at position 76 (K76T), were detected by mutation-specific polymerase chain reaction (MS-PCR) (Djimde *et al.*, 2001). In the first round PCR, oligonucleotide primer pairs TCRP1 (5'-CCG TTA ATA ATA AAT ACA CGC AG-3') and TCRP2 (5'-CGG ATG TTA CAA AAC TAT AGT TAC C-3') were included in a single reaction. Five microlitres of genomic DNA was used as template in the primary reaction. The reaction took place in a total volume of 25 μl containing 20 mM Tris-Cl pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM of each dNTP (dGTP, dATP, dTTP, dCTP), primers at a final concentration of 0.1 μM each and 0.625 units of Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany). The amplification program for *Pfcrt* was as follows: denaturation for 30 seconds at 94°C, annealing for 30 seconds at

56°C, extension for 1 minute at 60°C. Forty-five cycles were performed for the primary reaction. The last extension was carried out for 3 minutes at 60°C. Ten microlitres of primary PCR product were mixed with 1-2 µl of 10X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 25% ficoll (type 400) in water) and analysed by agarose gel electrophoresis on a 1.5% agarose gel stained with ethidium bromide (0.8 µg /ml) in Tris-acetate-EDTA buffer.

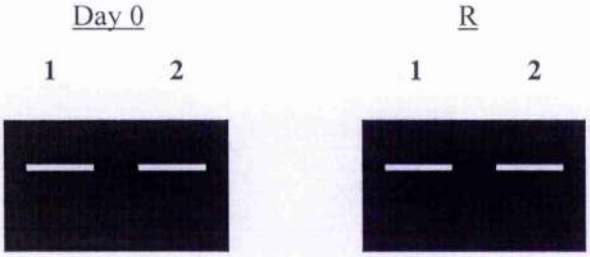
A primary PCR product present as a strong DNA band on the agarose gel was diluted 1:100 with DNase and RNase-free water (ICN Biomedicals, Inc., Irvine, CA, USA) before use as template in the nested reaction. A primary PCR product present as a faint band following electrophoresis was diluted 1:10 before use. A PCR product with no band visible on the gel was used undiluted in the nested PCR.

The PCR product generated from the primary reaction was used as template in the nested PCR. Two separate reaction mixes were prepared, one containing primers specific to the wild type allele encoding Pfcrt Lys76, and the other to the mutant form encoding Pfcrt Thr76. The primers used were TCRP4w (5'-GTT CTT TTA GCA AAA ATC T-3') for the wild type allele or TCRP4m (5'-GTT CTT TTA GCA AAA ATT G-3') for the mutant allele. Each specific primer was paired with the common primer TCRP3 (5'-TGA CGA GCG TTA TAG AG-3'). One microlitre of the neat or 1µl of the diluted primary PCR product was used as template in the nested PCR reaction. The reaction was performed in a total volume of 25µl containing 20mM Tris-Cl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP (dGTP, dATP, dTTP, dCTP), primers at a final concentration of 1 µM each and 0.625 units of Taq DNA polymerase. The amplification program for *Pfcrt*76 was as follows: denaturation for 30 seconds at 94°C, annealing for 30 seconds at 47°C, extension for 1 minute at 64°C. Fifteen cycles were performed for the nested reaction. Twenty microlitres of the nested PCR product were mixed with 1-2 µl of 10X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 25% ficoll (type 400) in water) and analysed by agarose gel electrophoresis on a

1.8% agarose gel stained with ethidium bromide (0.8 μg/ml) in Tris-acetate-EDTA buffer. For each day 0 (D0) sample, the two amplification reactions (specific amplification of the wild type and mutant alleles of *Pfcrt*) were run side-by-side on the agarose gel. The same was done for the day of recrudescence (R) PCR products as shown in Figure 2.

Figure 2:

A schematic to indicate loading of gels for *Pfcrt* analysis



Lane 1 shows PCR product band after amplification of DNA with *Pfcrt* wild type primer
Lane 2 shows PCR product band after amplification of DNA with *Pfcrt* mutant primer

DNA was visualised by ultraviolet transillumination and the band sizes determined by comparison with a standard size 100-base pair DNA ladder (Roche Diagnostics, Mannheim, Germany). *P. falciparum* DNA clones 3D7 and FCB-1 were included in each run as controls for the wild type and mutant alleles, respectively.

2.5.2. Distinguishing recrudescent from new P. falciparum *infections*

To distinguish genuine recrudescence of resistant parasites from the appearance of parasites arising from reinfection during the follow-up period, day 0 (pre-treatment) and the day of recrudescence–R (post-treatment) isolates were genotyped at two highly polymorphic gene loci, merozoite surface protein-1 (*MSP1)* and merozoite surface protein-2 (*MSP2*), as described (Ranford-Cartwright *et al.*, 1993).

2.5.2.1. Genotyping at the **MSP1** *locus*

In a primary PCR, oligonucleotide primer pairs 01 (5'-CAC ATG AAA GTT ATC AAG AAC TTG TC-3') and 02 (5'-GTA CGT CTA ATT CAT TTG CAC G-3'), corresponding to the conserved sequence on either side of the repetitive region of *MSP1* (block 2), were included in a single reaction. Using the PCR product generated from the primary reaction as template, the polymorphic repetitive region of block 2 of *MSP1* was amplified in a nested PCR. Specific primers N1 (5'-GCA GTA TTG ACA GGT TAT GG-3') and N2 (5'-GAT TGA AAG GTA TTT GAC-3') were used.

Amplifications were performed using a PTC-100™ PCR System (MJ Research, Inc., Waltham, MA, USA). In the primary and nested *MSP1* PCR amplification, the reaction took place in total volume of 25 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 75 µM of each dNTP (dGTP, dATP, dTTP, dCTP), primers at a final concentration of 0.1 µM each and 0.5 units of Taq DNA Polymerase (Roche Diagnostics, Mannheim, Germany). Five microlitres of parasite DNA was used as template in the primary reaction, and 2 µl of the primary PCR product was used as template in the nested PCR reaction. The amplification program for *MSP1* was identical for both reactions: denaturation for 25 seconds at 94°C, annealing for 35 seconds at 50°C, extension for 2 minutes 30 seconds at 68°C. Thirty cycles were performed for both primary and nested reactions. The final extension was carried out for 8 minutes at 68°C. Ten microlitres (or 15 µl for nested PCR product) of primary PCR product were mixed with a 1-2 µl of 10X loading dye and analysed by agarose gel electrophoresis on a 1.5% (or 2.0% for nested PCR products) agarose gel stained with ethidium bromide (0.8 µg/ml) in Tris-acetate-EDTA buffer. Each day 0 (D0) isolate was run alongside its day of recrudescence (R) isolate. DNA was visualised by ultraviolet transillumination and band sizes determined by comparison with a standard 100-base pair DNA ladder.

2.5.2.2. Genotyping at the MSP2 *locus*

Oligonucleotide primer pairs S2 (5'-GAG GGA TGT TGC TGC TCC ACA G-3') and S3 (5'-GAA GGT AAT TAA AAC ATT GTC-3') corresponding to the conserved sequence surrounding the central repetitive and polymorphic regions of *MSP2* were included in a single reaction. Using the PCR product generated from the primary reaction as template, the polymorphic repetitive regions of block 2 and 3 of *MSP2* were amplified by a nested PCR. In this case, specific primers S1 (5'-GAG TAT AAG GAG AAG TAT G-3') and S4 (5'-CTA GAA CCA TGC ATA TGT CC-3') were used. Amplifications were performed using a PTC-100^{TM} PCR System. In the primary and nested *MSP2* PCR amplification, the reaction took place in a total volume of 25 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 75 µM of each dNTP (dGTP, dATP, dTTP, dCTP), primers at a final concentration of 0.1 µM each and 0.5 units of Taq DNA Polymerase. Five microlitres of parasite DNA was used as template in the primary reaction, and 2 µl of the primary PCR product was used as template in the nested PCR reaction. The amplification program for *MSP2* was as follows: denaturation for 25 seconds at 94°C, annealing for 60 seconds at 42°C (or 60 seconds at 50°C with nested reaction), extension for 2 minutes at 65°C (or 60 seconds at 70°C with nested reaction). Thirty cycles were performed for both primary and nested reactions. The final extension was carried out for 8 minutes at 65°C (or 8 minutes at 70°C in the nested reaction). Ten microlitres (or 15 µl for nested PCR product) of primary PCR product were mixed with a 1-2 µl of 10X loading dye and analysed by agarose gel electrophoresis on a 1.5% (or 2.0% for nested PCR products) agarose gel stained with ethidium bromide in Tris-acetate-EDTA buffer. Each day 0 (D0) isolate was run alongside its day of recrudescence (R) isolate. DNA was visualised by ultraviolet transillumination and band sizes determined by comparison with a standard 100-base pair DNA ladder. Three categories of recrudescent infections were identified: (i) genuine recrudescent

infections, (ii) infections with evidence of recrudescent and also of new infections and (iii) entirely new infections.

2.6. Polymerase chain reaction and dot-blot at the *DHFR* locus

Oligonucleotide primer pairs AMP1 (5'-TTT ATA TTT TCT CCT TTT TA-3') and AMP2 (5'-CAT TTT ATT ATT CGT TTT CT-3') were included in a single outer PCR reaction. Using the PCR product generated from the primary reaction as template, a nested PCR was then carried out using primers based on conserved sequences, so that the PCR product would include codons 108, 51 and 59 in *DHFR*. SP1 (5'-ATG ATG GAA CAA GTC TGC GAC-3') and SP2 (5'-ACA TTT TAT TAT TCG TTT TC-3') were the nested primers used (Plowe *et al.*, 1995). Control amplifications, using DNA from *P. falciparum* parasite clones known to contain the possible different alleles at positions 108, 51 and 59 of *DHFR*, were used to ensure specificity and sensitivity of the technique (Table 3).

2.6.1. AMP1/AMP2 PCR amplification

Amplifications were performed using a PTC-200™ PCR System (MJ Research, Inc., Waltham, MA, USA). In the primary PCR amplification, the reaction took place in a total volume of 20 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP (dGTP, dATP, dTTP, dCTP), primers at a final concentration of 0.1 µM each and 1 unit of Taq DNA Polymerase. Five microlitres of parasite DNA was used as template in the primary reaction. Two microlitres of DNA extracted from *in vitro* cultures of *P.falciparum* clones 3D7 and Dd2, which have *DHFR* encoding Asn and Ile respectively at position 51, Cys and Arg respectively at position 59, and Ser and Asn respectively at position 108, were used as the major positive controls (Table 3). *P. falciparum* clones HB3 and V1/S were used as alternative controls. *P. falciparum* clone T9-94 which has *DHFR* encoding Thr at position 108 was not used because the mutant form encoding DHFR Thr108 is rare in

Africa (Plowe *et al.*, 1997, Nzila-Mounda *et al.*, 1998).

Table 3

Positive and negative control parasite clones for the *DHFR* locus

Parasite Clone	*DHFR*						
	Codon 108			**Codon 51**		**Codon 59**	
	Ser (W)	Asn (M)	Thr (M)	Asn (W)	Ile (M)	Cys (W)	Arg (M)
3D7	**positive**	negative	negative	**positive**	negative	**positive**	negative
Dd2	negative	**positive**	negative	negative	**positive**	negative	**positive**
HB3	negative	positive	negative	positive	negative	positive	negative
V1/S	negative	negative	negative	negative	positive	negative	positive

W= wild type, M= mutant

Controls labelled in bold were the major ones used for the experiments.

The amplification program for AMP1/AMP2 PCR was as follows: denaturation for 30 seconds at 92°C, annealing for 45 seconds at 45°C, extension for 45 seconds at 72°C for forty five cycles. The last extension was carried out for 3 minutes at 72°C.

2.6.2. SP1/SP2 PCR amplification

In the nested PCR amplification, the reaction took place in total volume of 30 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP (dGTP, dATP, dTTP, dCTP), primers at a final concentration of 1 µM each and 1 unit of Taq DNA Polymerase. Two microlitres of the primary PCR product was used as template in the nested PCR reaction. The amplification program for SP1/SP2 PCR was as follows: denaturation for 30 seconds at 92°C, annealing for 30 seconds at 45°C, extension for 30 seconds at 72°C for thirty cycles. The last extension was carried out for 3 minutes at 72°C. Five microlitres of nested PCR product were mixed with a 1-2 µl of 10X loading dye and analysed by agarose gel electrophoresis on a 1.5% agarose gel stained with ethidium bromide (0.8 µg/ml) in 1X Tris-acetate-

EDTA buffer. DNA was visualised by ultraviolet transillumination and the expected nested PCR product band size of 700 base pair was confirmed by comparison with a standard 100-base pair DNA molecular weight ladder.

2.6.3. Preparation of dot-blot

For each sample that gave a positive result by nested PCR, 20 µl of PCR product was denatured with EDTA and NaOH to a final concentration of 10mM EDTA and 0.4 M NaOH, and incubated for 10 minutes at 100°C. Each denatured PCR product was then neutralized with an equal volume of 2 M ammonium acetate, pH 7 and blotted in duplicate onto a Genescreen nylon membrane (New England Nuclear, Houndslow, United Kingdom) using a dot-blotting apparatus (Bio-Rad, Hemel Hempstead, United Kingdom) according to the manufacturer's instructions. Half of the denatured PCR product (equivalent to 10 µl of PCR product) was placed in each of two wells of the dot-blotter. The duplicates were placed in different parts of the blot. Following transfer of the DNA, the membrane was rinsed in 2 X SSC (0.15 M sodium chloride, 15 mM Trisodium citrate) for 1 minute, denatured in 0.4 M NaOH for 1 minute and finally neutralized in a mixture of 1 M Tris-HCl and 1.5 M NaCl, pH 8 for 30 seconds. The blotted PCR products were fixed on the membrane by exposure to ultraviolet light in a UV cross-linker (Stratalinker, Stratagene, Amsterdam, The Netherlands) according to the manufacturer's instructions.

2.6.4. Oligonucleotide probe labelling and hybridisation

In order to detect the *DHFR* alleles associated with changes at amino acid positions 108, 51 and 59 by the dot-blot hybridisation technique, specific probes were designed and labeled with [γ-^{32}P]-ATP (Abdel-Muhsin *et al.*, 2002). The sequences of the probes and the specific hybridisation conditions are given in Table 4. The *DHFR* Thr108 probe was not used because of the same reason given above.

Ten picomoles (pmol) of each probe were labelled using a single Ready-To-

Go polynucleotide kinase reaction mix (Amersham Pharmacia Biotech Inc, Little Chalfont, United Kingdom) containing 10 units of T4 polynucleotide kinase, 50 mM Tris-HCl, (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA (pH 8.0), 0.2 µM ATP and stabilizers in a total volume of 50 µl, to which 0.37 MBq (10 µCi) of [γ-^{32}P]-ATP was added. Each probe was incubated with the labelling mixture at 37°C for 30 minutes and the unincorporated [γ-^{32}P]-ATP was removed using Microspin G-25 columns (Amersham Pharmacia Biotech, United Kingdom) according to the manufacturer's instructions. Labelled probe was stored at -20°C until required.

The nylon blot was prehybridised with hybridisation buffer [5 X SSPE (0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA), 5 X Denhardt's reagent, 0.5% sodium dodecyl sulphate (SDS), 0.02 mg/ml of sonicated salmon sperm DNA] using 0.25-0.125 ml of hybridisation solution per cm^2 of membrane. The membrane and buffer were incubated in a hybridisation oven with rotisserie (with agitation) (Grant Boekel HIR 12, Cambridge, England) for ≥ 30 minutes at a temperature specific for each probe used (Table 4). The labelled and purified oligonucleotide probe was added to the hybridisation buffer (1µl probe for every 1ml of the hybridisation buffer) and the blot hybridised overnight at a temperature specific for the probe used (Table 4). The blot was washed once with 2 X SSC for 20 minutes and twice for 10 minutes in 1 X SSC/ 0.1% SDS or 0.5 X SSC/ 0.1% SDS, at the temperature specific for each probe (Table 4).

Table 4

Oligonucleotide probes for the detection of *DHFR* alleles of *P. falciparum* at positions 108, 51 and 59 and their dot-blot hybridisation conditions.

Probe	Sequence (shown 5' to 3')	Hybridisation temperature	Stringent washes
***DHFR*-108**			
Asn-specific (mutant)	AACAAACTGGGAAAACATTCCAA	54.5°C	[1XSSC/0.1%SDS]10min x2
Ser-specific (wild type)	AACAAGCTGCGAAAGCATTCCAA	50°C	[1XSSC/0.1%SDS]10min x2
***DHFR*-51**			
Ile-specific (mutant)	ATGGAAATGTATTTCCCTAGAT	50°C	[0.5XSSC/0.1%SDS]10min x2
Asn-specific (wild type)	ATGGAAATGTAATTCCCTAGAT	50°C	[0.5XSSC/0.1%SDS]10min x2
***DHFR*-59**			
Arg-specific (mutant)	GAAATATTTTCGTGCAGTTAC	52°C	[0.5XSSC/0.1%SDS]10min x2
Cys-specific (wild type)	GAAATATTTTTGTGCAGTTAC	50°C	[0.5XSSC/0.1%SDS]10min x2

The blot was wrapped in clingwrap and taped into an autoradiography cassette complete with intensifying screens. Location markers (Glogos II markers, Stratagene, Amsterdam, The Netherlands) were used to allow accurate positioning of the autoradiograph and blot. Each blot was exposed to Kodak MXB X-ray film overnight at –70°C after which the X-ray film was developed using an automatic developer

(X-OGRAPH Imaging system compact x 4). The probe was stripped from the blot with two washes in 0.1 M NaOH for 15 minutes each at room temperature. The blot was then rinsed briefly in 5 X SSC, air-dried, and then re-hybridised with other probes or kept at room temperature until required.

Each blot was hybridised with each of the probes shown in Table 4.

The blots were scored based on the specificity of the controls. Experimental samples and controls were dot-blotted in duplicate to reinforce the correct scoring. Blots that had autoradiograph results with controls showing non-specific hybridisation were given an extra stringent wash to ensure specificity. If this was not successful in removing the non-specific hybridisation, then the hybridisation and washing steps were re-optimised.

2.7. Statistical analysis methods

The results obtained were analysed using the Fisher's Exact statistical test.

The R X C package (downloadable free software) [http://bioweb.usu.edu/mpmbio/]) for the analysis of contingency tables (Mark P. Miller, 1997) was used for Fisher's Exact statistical test instead of the chi-squared test because most of the numbers were small (less than 5 in the expected groups). The results were analysed to determine if;

(a) there was an association between the presence of the mutation and treatment failure due to SP and a combination of CQ and SP.

(b) there was an association between the number of mutations and time to recrudescence following unsuccessful treatment with SP and a combination of CQ and SP.

The Chi-squared test was used to analyse if there was an association between age and clearance of parasites with *Pfcrt* T76 mutation following treatment with CQ and SP.

3. RESULTS AND DATA ANALYSIS:

3.1. SP/CQ efficacy trial

3.1.1. Drug efficacy results

Of 106 patients recruited and treated with a combination of SP and CQ, 53 were judged to have adequate clinical response or parasites which were sensitive to the drug (S). Treatment failed in 48 patients, and the remaining 5 patients were excluded at day 14 because they developed complications or changed treatment (supplied elsewhere) during the course of follow-up.

(i) Monitoring the response to treatment by measuring parasitaemia, 52% of patients had sensitive parasites, 40% had parasites exhibiting an RI response, 6% an RII response and 2% an RIII response. This indicated a drug failure rate of 48% (Table 5).

(ii) Measuring treatment outcome using clinical signs and symptoms, 92% of patients were judged to have an adequate clinical response, 2% an early treatment failure and 6% a late treatment failure. In this case, the total treatment failure rate was 8% (Table 6).

Table 5

SP/CQ drug resistance rate among evaluable cases of uncomplicated *P. falciparum* malaria in Tororo. Outcome of treatment was evaluated by parasitological examination.

	SP/CQ	
Parasitological	Age 6- 59months	
Outcome	n	(%)
Sensitive	53	(52.5)
RI	40	(39.6)
RII	6	(5.9)
RIII	2	(2.0)
Total	101	(100)

Table 6

SP/CQ clinical outcomes among evaluable cases of uncomplicated *P. falciparum* malaria in Tororo.

	SP/CQ	
Therapeutic	Age 6- 59months	
Efficacy	n	(%)
ACR	93	(92.1)
LTF	6	(5.9)
ETF	2	(2.0)
Total	101	(100)

ACR= adequate clinical response, LTF= late treatment failure, ETF= early treatment failure

3.1.2. Genotyping results at MSP1 *and* MSP2 *loci*

Amplification of DNA from 47 pairs of isolates (day 0 and R) for the recrudescent or treatment failure group (RI-RIII) was performed for the *MSP1* and *MSP2* loci. One RIII isolate was lost. The sample pairs were classified as follows, based on the combined results from both loci typed:

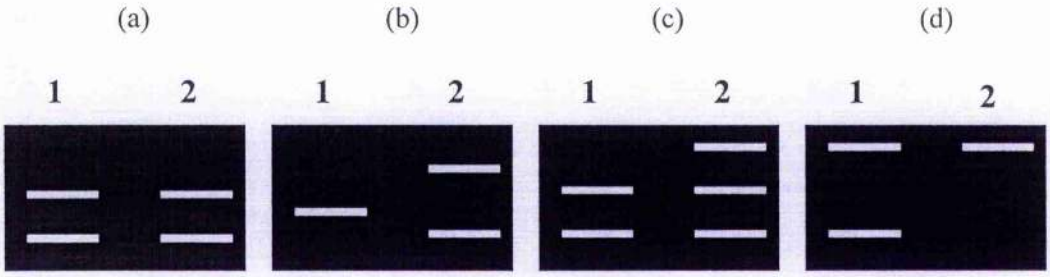
(a) If all PCR products in the day 0 sample were also present in the R sample and there were no additional fragments for both loci, this was considered a genuine recrudescence with all initial parasites recrudescing (all resistant) (Figure 3a).

(b) If PCR products in the day 0 sample were all completely different to those in the R sample this was considered evidence of reinfection during follow-up (Figure 3b).

(c) If all PCR products in the day 0 sample were present in the R sample but the R sample also had new PCR products this was considered a 'mixed' result in the R sample with mixtures of drug resistant and new parasites (Figure 3c).

(d) If the day 0 sample had some PCR products not in the R sample but the R sample had PCR products that were all present in the day 0, this was considered a recrudescence but with mixtures of resistant and sensitive parasites in the day 0 sample (Figure 3d).

Figure 3. Representation of gels of *MSP1* PCR products. Each box represents a gel. Lane 1 on each gel shows PCR products obtained after amplification of the *MSP1* locus of the day 0 DNA sample and lane 2 shows PCR products obtained after amplification of *MSP1* locus of the day of recrudescence (R) DNA sample. (a)-(d) represent different possible scenarios, where (a) and (d) are genuine recrudescences, (b) represents a reinfection and (c) a combination of recrudescence and reinfection. Results for *MSP2* PCR products were similarly classified.



Of the 47 pairs of isolates analysed, 10 (21%) had genuine recrudescent parasites as classified in Figure 3(a) and 3(d), none were entirely new infections, as shown in Figure 3(b), and 36 (77%) had both recrudescence and re-infections in the R sample as classified in Figure 3(c). One pair of isolates did not amplify for both loci (Table 7).

Table 7

Classification of *MSP1* and *MSP2* PCR results for the responses in 47 children under the SP/CQ treatment group, in which the success of the drug treatment was measured by examination of parasitaemia.

Parasitological	**SP/CQ**				
Outcome	n	Rec.	New	Both	Unknown
RI	40	9	0	30	1
RII	6	0	0	6	0
RIII	1	1	0	0	0
Total	47	10	0	36	1

n refers to number of children examined.
Rec. refers to genuine recrudescence as classified in Figure 3(a) and (d).
New refers to entirely new infection as classified in Figure 3(b).
Both refers to recrudescence plus re-infection as classified in Figure 3(c).
Unknown refers to the excluded samples that either did not amplify at one locus but did amplify at another locus or did not amplify at both loci.

3.1.3. Genotyping at the Pfcrt 76 *locus*

DNA from 47 pairs of isolates for the clinical treatment failures or from parasites classified as RI to RIII was amplified at the *Pfcrt* locus to identify the alleles at codon 76 of *Pfcrt*. One isolate in the RIII class went missing, hence a total of 47 pairs (RI-RIII) were analysed instead of 48 pairs as indicated in Table 5. DNA from 53 pre-treatment isolates from the patients who responded adequately to the drug was also subjected to PCR analysis.

Of the 53 patients whose parasitaemia cleared totally less than 7 days following SP/CQ treatment, 41 (77%) of their pre-treatment isolates were found to have parasites with the *Pfcrt* T76 (mutant) only, and none were found with the wild type allele only i.e. *Pfcrt* K76 (0%). The remaining 12 patients had parasites with both T76 and K76 alleles simultaneously (Table 8).

Table 8

Pfcrt allele prevalence in samples obtained from patients before SP/CQ treatment and patients with recurrent infection. M= mutant allele (T76), W= wild type allele (K76)

	SP/CQ No. observed / total no. of samples (%)		
Allele at *Pfcrt* 76	Before Treatment (S)	Before Treatment (RI-RIII)	After Treatment (RI-RIII)
Pfcrt T76 pure (M)	41/53 (77.4)	39/47 (83.0)	42/47 (89.4)
Pfcrt K76/T76 (W/M)	12/53 (22.6)	6/47 (12.8)	3/47 (6.4)
Pfcrt T76 Total	53/53 (100)	45/47 (95.7)	45/47 (95.7)
Pfcrt K76 pure (W)	0	0	0
Pfcrt K76 Total	12/53 (22.6)	6/47 (12.8)	3/47 (6.4)
Did not amplify	0	2/47 (4.3)	2/47 (4.3)

Of the 47 patients who did not clear their parasites within 7 days following SP/CQ treatment, 39 (83%) had pure *Pfcrt* T76 in their pre-treatment sample. This increased to 42 (89%) in the samples taken at recrudescence. Six patients (13%) had parasites present simultaneously with the T76 and with the K76 allele in the pre-treatment samples, and this reduced to 3 (6%) in the samples taken at recrudescence. No samples pre-treatment and/or post-treatment contained only parasites with the *Pfcrt* K76 allele (Table 8). DNA extracted

from two of the pre-treatment samples and from two samples taken at recrudescence did not amplify.

Following genotyping at the *MSP1* and *MSP2* loci, only ten of the 47 patients with apparent resistant parasites were found to have genuine recrudescences, with the remaining samples revealing the presence of some novel parasites in the R sample, indicating that these patients had mixtures of recrudescence and reinfection. The *Pfcrt*76 alleles found in the ten genuine recrudescence only samples were as shown in Table 9.

Table 9

Pfcrt allele prevalence in samples obtained from patients before SP/CQ treatment and patients with genuine recrudescence infection only. M=mutant allele (T76), W=wild type allele (K76).

Allele at *Pfcrt* 76	**SP/CQ** No. observed / total no. of samples (%)	
	Before treatment (D0) (RI-RIII)	After treatment (R) (RI-RIII)
Pfcrt T76 pure (M)	7/10 (70)	7/10 (70)
Pfcrt K76/T76 (W/M)	2/10 (20)	2/10 (20)
Pfcrt T76 Total	9/10 (90)	9/10 (90)
Pfcrt K76 pure (W)	0	0
Pfcrt K76 Total	2/10 (20)	2/10 (20)
Did not amplify	1/10 (10)	1/10 (10)

Seven patients (70%) had pure *Pfcrt* T76 in their pre-treatment isolates and seven (70%) had pure *Pfcrt* T76 in the recrudescent isolates. One (10%) patient had both the T76 and K76 alleles present simultaneously in both the pre-treatment sample and in the sample taken at recrudescence. No samples pre-treatment and/or post-treatment were found with *Pfcrt* K76 allele alone.

3.1.4. Statistical Analysis of results

The data from the 45 successfully typed treatment failure samples (Table 8) were analysed using Fisher's Exact statistical test to see if there was an association between the presence of the *Pfcrt* T76 allele in the

pretreatment samples and treatment outcome measured by parasitaemia (Table 10). No correlation could be seen (Fisher's Exact P= 0.287). Excluding the samples with a mixture of K76 and T76, there was also no correlation (Fisher's Exact P= 1.000). When mixtures were grouped with the mutant class, there was also no correlation (Fisher's Exact P= 1.000). The data were also analysed with respect to time to recrudescence. In this case the samples were divided into groups based on the time post-treatment at which recrudescent parasites appeared. The data were then analysed using a Fisher's Exact test to see if there was an association between the presence of the *Pfcrt*T76 allele in the pretreatment samples and time to recrudescence (Table 11). Samples with a mixture or mutant and wild type alleles were not included as mutants but were analysed separately under the mixed (T76/K76) allele group. The results of the analysis (P= 0.114) indicated that there was no significant effect of the presence of the mutant allele *Pfcrt*T76 on time to recrudescence.

Table 10

Prevalence of *Pfcrt* alleles in the pretreatment samples and response to SP/CQ, in which the success of the drug treatment was measured by examination of parasitaemia. M=mutant allele, W=wild type allele.

Allele at *Pfcrt* codon 76	Sensitive (Pre-treatment)	RI-RIII (Pre-treatment)	Total (n)
T76 (M)	41	39	80
K76 (W)	0	0	0
K76/T76 (W/M)	12	6	18
Total	53	45	98

Fisher's Exact P= 0.287.

Table 11

Prevalence of *Pfcrt76* alleles in the pre-treatment samples within different time-to-recrudescence groups for patients treated unsuccessfully with SP/CQ (RI-RIII).

Time Intervals (days)	K76 allele (Mutant)	T76 allele (Wild type)	T76/ K76 (Mixed)	Total (n)
D0-D7	2	0	0	2
D8-D14	5	0	0	5
D15-D21	15	0	0	15
D22-D28	17	0	6	23
Total (n)	39	0	6	45

Fisher's Exact P= 0.114

The results (Table 8) show that all the children with the infection who cleared their parasites and those who did not clear mutant parasites were found to carry the *Pfcrt* 76 mutation following SP/CQ treatment. Statistical analysis shows no association between the presence of *Pfcrt* 76 mutation and treatment outcome measured by parasitaemia (Table 10). Host factors such as immunity are thought to contribute to clearance of parasites resistant to CQ (Djimde *et al.*, 2003, Djimde *et al.*, 2001, Omar *et al.*, 2001). Therefore age was used here as an indicator to estimate the level of antiparasite immunity. The results obtained for each individual were grouped according to age and analysed using the Chi squared test for the association between age and clearance of resistant parasites. When children of all ages were considered, children infected with parasites carrying the *Pfcrt*T76 mutation who cleared their parasites following SP/CQ treatment were not significantly different in age from those who did not clear mutant parasites (X^2=4.17, P=0.124) (Table 12). When children under one year old (6-12 months) were excluded from the analysis, because they were thought to be more likely to have maternal antibodies to the parasite, there was a significant association between clearance of resistant parasites and age (X^2=4.09, P=0.043) (Table 13).

Table 12

Individuals carrying parasites with the *Pfcrt* T76 mutation in all age groups who did not clear and those who cleared the infection following SP/CQ treatment.

Age group (months)	Did not clear infection	Cleared infection	Total (n)
6-12	14	15	29
13-24	19	14	33
25-49	12	24	36
Total (n)	45	53	98

X^2=4.17, P= 0.124

Table 13

Individuals carrying parasites with the *Pfcrt* T76 mutation in older age groups who did not clear and those who cleared the infection.

Age group (months)	Did not clear infection	Cleared infection	Total (n)
13-24	19	14	33
25-49	12	24	36
Total (n)	31	38	69

X^2=4.09, P= 0.043

3.1.5. PCR/dot-blot hybridisation at the DHFR *locus*

The 47 isolates from the RI-RIII group were typed at the *MSP1* locus to determine if they were genuinely recrudescent. By this method 22/47 isolates were classified as recrudescent and 22 were classified as having both recrudescent parasites and reinfections. Three did not amplify. The 22 isolates showing apparent recrudescence by *MSP1* were PCR/dot-blot hybridised at the *DHFR* locus to identify alleles at codons 108, 51 and 59 (Table 15). The remaining 25 isolates classified as having both recrudescent parasites and reinfections including those that did not amplify by PCR were intentionally not hybridised by PCR/dot-blot because one of the main aims of the work involved identifying drug resistant and sensitive alleles from isolates

classified as genuinely recrudescent. After the dot-blot hybridisation analysis had been carried out, it was discovered that 12 of the original 36 isolates were in fact not genuinely recrudescent. Subsequent analysis by typing at the *MSP2* locus revealed the presence of additional novel parasites in the R sample, indicating that these patients represented mixtures of recrudescence and reinfection. To take this into account, the statistical analyses were done on the total blotted samples (n = 22) and separately on the genuine recrudescents only (n = 10).

Table 14

DHFR allele prevalence in subset of samples obtained from 10 patients with genuine recrudescence before (D0) and after (R) SP/CQ treatment.

Parasitological outcome	Allele at *DHFR* 108 (D0)			Allele at *DHFR* 108 (R)			Allele at *DHFR* 51 (D0)			Allele at *DHFR* 51 (R)			Allele at *DHFR* 59 (D0)			Allele at *DHFR* 59 (R)		
	Ser	Asn	Ser + Asn	Ser	Asn	Ser + Asn	Asn	Ile	Asn+Ile	Asn	Ile	Asn + Ile	Cys	Arg	Cys + Arg	Cys	Arg	Cys + Arg
RI	0	9	0	0	9	0	0	9	0	0	8	0	1	1	7	3	3	3
RII	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RIII	0	1	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0
Total (%)	0 (0)	10(100)	0 (0)	0 (0)	10(100)	0(0)	0(0)	9 (90)	1(10)	0 (0)	8(100)	0 (0)	1 (10)	1(10)	8 (80)	3 (33)	3(33)	3 (33)

Table 15

DHFR allele prevalence in samples obtained from 22 patients before (D0) and after (R) SP/CQ treatment, including patients with genuine recrudescence and patients with both recrudescence and re-infections.

Parasitological outcome	Allele at *DHFR* 108 (D0)			Allele at *DIIFR* 108 (R)			Allele at *DHFR* 51 (D0)			Allele at *DHFR* 51 (R)			Allele at *DHFR* 59 (D0)			Allele at *DHFR* 59 (R)		
	Ser	Asn	Ser + Asn	Ser	Asn	Ser + Asn	Asn	Ile	Asn + Ile	Asn	Ile	Asn + Ile	Cys	Arg	Cys + Arg	Cys	Arg	Cys + Arg
RI	0	20	0	0	20	0	0	20	0	0	18	0	2	3	15	5	8	7
RII	0	1	0	0	1	0	0	1	0	0	1	0	0	0	1	0	0	1
RIII	0	1	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
Total (%)	0 (0)	22(100)	0 (0)	0 (0)	21(96)	0(0)	0(0)	21(96)	1(4.5)	0 (0)	19(86)	0 (0)	2 (9)	3(14)	17 (77)	5 (23)	8 (36)	8 (36)

3.1.6. DHFR *codon 108*

Of the 22 patient DNA samples analysed by PCR/dot-blot hybridisation, all (100%) were typed as having pure *DHFR* Asn 108 (mutant form) in their pre-treatment isolates, and 21 (representing 100% of the samples successfully typed) had pure Asn 108 in the samples taken at recrudescence (Table 15). One isolate from the post- treatment isolates (an RIII response) failed to amplify by SP1/SP2 PCR, and therefore it was not included on the dot-blot. No samples pre-treatment and post-treatment were found with the allele encoding DHFR Ser 108 (wild type) alone and no samples were found with both alleles encoding Ser 108 and Asn 108 in the pre-treatment and/or post treatment isolates. In summary, all the isolates possessed the allele encoding Asn 108 alone, regardless of time taken to recrudescence. Therefore there is no correlation between the presence of alleles encoding Asn 108 and time to recrudescence following SP/CQ treatment (Table 16).

Equally, for the 10 genuine recrudescent subset of samples, all the day 0 and R samples had the allele encoding Asn 108, (Table 14), and there was no correlation with the time to recrudescence (Fisher's Exact P= 1.000).

Table 16

Prevalence of *DHFR*108 alleles in the pre-treatment samples within different time to recrudescence groups for patients treated unsuccessfully with SP/CQ.

Time Intervals (days)	Asn 108 (Mutant)	Ser 108 (Wild type)	108 Asn + 108 Ser (Mixed)	Total (n)
D0-D7	1	0	0	1
D8-D14	1	0	0	1
D15-D21	7	0	0	7
D22-D28	13	0	0	13
Total (n)	22	0	0	22

Fisher's Exact, P= 1.000

3.1.7. DHFR *codon 51*

Twenty one (95.5%) patients out of 22 had only parasites with the allele encoding DHFR 51 Ile (mutant form) in their pre-treatment samples. Nineteen of these had only parasites with the allele encoding DHFR 51 Ile in their recrudescent samples, representing 100% of those successfully typed, as 2 isolates taken at recrudescence (RI) were negative by PCR. One patient, who had an RIII response, had a mixture of the alleles encoding 51 Asn (wild type) and 51 Ile present in their pre-treatment sample but DNA from their post treatment sample failed to amplify. No samples pre- and post-treatment were found to possess only parasites with the allele encoding 51 Asn.

In summary, all the isolates except one possessed the allele encoding mutant form (51 Ile) alone, regardless of time taken to recrudescence. There was no correlation between the presence of alleles encoding 51 Ile and time to recrudescence following SP/CQ treatment (Table 17). For the subset of these samples showing genuine recrudescence with *MSP1* and *MSP2* typing, nine out of ten had only alleles encoding 51 Ile, and there was no correlation between the presence of 51 Ile and the time to recrudescence (Fisher's exact P= 0.108).

Table 17

Prevalence of *DHFR* 51 alleles in the 22 pre-treatment samples within different time to recrudescence groups for patients treated unsuccessfully with SP/CQ.

Time Intervals (days)	51 Asn (Wild type alone)	51 Ile (Mutant alone)	51Ile + 51Asn (Mixed)	Total (n)
D0-D7	0	0	1	1
D8-D14	0	1	0	1
D15-D21	0	7	0	7
D22-D28	0	13	0	13
Total (n)	0	21	1	22

Fisher's Exact P= 0.093

3.1.8. DHFR *codon 59*

Three patients out of 22 had only parasites with the allele encoding DHFR 59 Arg (mutant form) in their pre-treatment isolates (Table 18). Two of these patients had the allele encoding DHFR 59 Arg in their post- treatment isolates; the remaining patient had both alleles 59 Arg and 59 Cys (wild type) present in their post-treatment isolate. Overall 8 post-treatment isolates had only parasites with the allele encoding DHFR 59 Arg; six of these had both alleles encoding DHFR 59 Arg and DHFR 59 Cys in their pre-treatment isolates, indicating selection for the *DHFR* 59 Arg allele following unsuccessful treatment with the SP/CQ combination. Seventeen (77.3%) patients had both the *DHFR* 59 Cys and *DHFR* 59 Arg alleles present simultaneously in their pre-treatment isolates. Two (9.1%) pre-treatment isolates and 5 (22.7%) isolates at recrudescence had only parasites with the allele encoding DHFR 59 Cys. One isolate taken at recrudescence (RIII) failed to amplify by SP1/SP2 PCR.

The results of *DHFR* 59 typing of the subset of 10 genuine recrudescent samples are shown in Table 19.

Table 18

Prevalence of *DHFR* 59 alleles in the 22 pre-treatment samples within different time to recrudescence groups for the patients treated unsuccessfully with SP/CQ.

Time Intervals (days)	59Cys (Wild type alone)	59Arg (Mutant alone)	59Cys + 59Arg (Mixed)	Total (n)
D0-D7	0	0	1	1
D8-D14	0	0	1	1
D15-D21	1	1	5	7
D22-D28	1	2	10	13
Total (n)	2	3	17	22

Fisher's Exact P=1.000 treating mixtures as a separate group

Fisher's Exact P=1.000 grouping mixtures with mutant group

Table 19

Prevalence of *DHFR* 59 alleles in the 10 genuinely recrudescent samples within different time to recrudescence groups for the patients treated unsuccessfully with SP/CQ.

Time Intervals (days)	59Cys (Wild type alone)	59Arg (Mutant alone)	59Cys + 59Arg (Mixed)	Total (n)
D0-D7	0	0	1	1
D8-D14	0	0	0	0
D15-D21	1	0	4	5
D22-D28	0	1	3	4
Total (n)	1	1	8	10

Fisher's Exact P=1.000 treating mixtures as a separate group
Fisher's Exact P=1.000 grouping mixtures with mutant group

Fisher's Exact tests were used to see if parasites in the 22 sample set carrying the double mutant forms *DHFR*108 Asn/51Ile and *DHFR*108 Asn/59Arg were found at different frequencies in the pre-treatment samples of patients who had early rather than late recrudescences (Table 20). No significant differences were observed. The same analysis was carried out looking at the triple mutant form (*DHFR*108Asn/51Ile/59Arg) (Table 21) with no significant difference observed.

Table 20

Prevalence of double mutant haplotypes of *DHFR*108/51 and *DHFR*108/59 in the 22 pre-treatment samples within different time-to-recrudescence groups for patients treated unsuccessfully with SP/CQ.

Time Intervals (days)	Pure double mutant form (108Asn/ 51Ile)	Other alleles	Pure double mutant form (108Asn/ 59Arg)	Other alleles	Total (n)
D0-D7	0	1	0	1	1
D8-D14	1	0	0	1	1
D15-D21	7	0	1	6	7
D22-D28	13	0	2	11	13
Total (n)	21	1	3	19	22

Fisher's Exact P=0.088 (108Asn/ 51Ile); P=1.000 (108Asn/ 59Arg).

Table 21

Prevalence of triple mutant haplotype of *DHFR*108/51/59 in the 22 pre-treatment samples within different time to recrudescence groups for patients treated unsuccessfully with SP/CQ.

Time Intervals (days)	Pure triple mutation (108Asn/51Ile/59Arg)	Other alleles	Total (n)
D0-D7	0	1	1
D8-D14	0	1	1
D15-D21	1	5	6
D22-D28	2	10	12
Total (n)	3	17	20

Fisher's Exact P=1.000

Considering the subset of 10 samples (genuine recrudescents), there was no significant difference in the frequency of the double mutant forms in early rather than late treatment failure groups (*DHFR*108Asn/51Ile:P=0.106; or *DHFR*108Asn/59Arg: P=0.508) (Table 22).

Table 22

Prevalence of double mutant haplotypes of *DHFR*108/51 and *DHFR*108/59 in the pre-treatment samples within different time-to-recrudescence groups for the subset of 10 patients treated unsuccessfully with SP/CQ.

Time Intervals (days)	Pure double mutant form (108Asn/ 51Ile)	Other alleles	Total (n)	Pure double mutant form (108Asn/ 59Arg)	Other alleles	Total (n)
D0-D7	0	1	1	0	1	1
D8-D14	0	0	0	0	0	0
D15-D21	5	0	5	0	5	5
D22-D28	4	0	4	1	3	4
Total (n)	9	1	10	1	9	10

Fisher's Exact P= 0.106 (108Asn/ 51Ile); P= 0.508 (108Asn/ 59Arg)

Table 23

Prevalence of triple mutant haplotype of *DHFR*108/51/59 in the pre-treatment samples within different time to recrudescence groups for the subset of 10 patients treated unsuccessfully with SP/CQ.

Time Intervals (days)	Pure triple mutation (108Asn/51Ile/59Arg)	Other alleles	Total (n)
D0-D7	0	1	1
D8-D14	0	0	0
D15-D21	0	5	5
D22-D28	1	3	4
Total (n)	1	9	10

Fisher's Exact P= 0.504

3.2. SP efficacy trial

3.2.1. Drug efficacy results

Out of 88 patients recruited and treated with SP alone, 31 were judged to have an adequate clinical response (ACR), or to have been infected with parasites which were sensitive to the drug. Treatment failed in 49 patients. Three

patients were excluded at day 14 because of changing treatment, 2 were lost to follow-up at day 14 and 3 were lost to follow-up between day 14 and 28.

(i) Monitoring response to treatment outcome measured by parasitaemia, 39% of the patients had sensitive parasites, 53% had parasites exhibiting an RI response, 1% an RII response and 8% an RIII response. This indicated a drug failure rate of 62% (Table 24).

(ii) Measuring treatment outcome using clinical signs and symptoms, 91% of the patients were judged to have an adequate clinical response, 6% an early treatment failure and 1% a late treatment failure. The total treatment failure rate, as measured by clinical symptoms and signs alone, was 7% (Table 25).

Table 24

SP drug resistance rate among evaluable cases of uncomplicated *P. falciparum* malaria in Tororo. Treatment outcome was evaluated by parasitological examination.

	SP	
Parasitological	Age 6- 59months	
Outcome	n	(%)
Sensitive	31	(38.8)
RI	42	(52.5)
RII	1	(1.3)
RIII	6	(7.5)
Total	80	(100)

Table 25

SP clinical outcome among evaluable cases of uncomplicated *P. falciparum* malaria in Tororo

	SP	
Therapeutic	Age 6- 59months	
Efficacy	n	(%)
ACR	73	(91.3)
LTF	1	(1.3)
ETF	6	(7.5)
Total	80	(100)

ACR= adequate clinical response, LTF=late treatment failure, ETF= early treatment failure

3.2.2. Genotyping results at the MSP1 *and* MSP2 *loci*

Amplification of DNA from 48 pairs of isolates (day 0 and R) for the recrudescent or treatment failure group (RI-RIII) was performed for the *MSP1* and *MSP2* loci. The sample pairs were classified in the same way as for the SP/CQ trial. One sample under the RI class went missing therefore a total of 48 pairs were analysed and not 49 as indicated in Table 24.

Of the 48 pairs of isolates analysed, 13 (27%) had genuine recrudescent parasites, three (6%) were entirely new infections and 32 (67%) were mixtures of some recrudescence but also with evidence of reinfection in the R sample (Table 26).

Table 26

Classification of *MSP1* and *MSP2* PCR results for the responses in 48 children under the SP treatment group, in which the success of the drug treatment was measured by examination of parasitaemia.

Parasitological		Characterisation of recrudescent infection			
Outcome	n	Rec	New	Both	Unknown
RI	41	8	3	30	0
RII	1	0	0	1	0
RIII	6	5	0	1	0
Total	48	13	3	32	0

n refers to number of children examined.
Rec. refers to recrudescence as classified in Fig 3 (a) and (d).
New refers to entirely new infection as classified in Fig 3 (b).
Both refers to recrudescence plus re-infection as classified in Fig 3 (c).
Unknown refers to excluded samples that did not amplify.

3.2.3. PCR/Dot-blot hybridisation at the DIIFR *locus*

The 48 isolates from the RI-RIII group were typed at the *MSP1* locus to determine if they were genuinely recrudescent. By this method 33/48 isolates were classified as recrudescent and eight (16.7%) were classified as having both recrudescence and reinfections. Six (12.5%) isolates were classified as reinfections. DNA from one isolate did not amplify. The 33 isolates showing apparent recrudescence by *MSP1* were PCR/dot-blot hybridised at the

DHFR locus to identify alleles at codons 108, 51 and 59 (Table 27). The remaining 15 isolates classified as having both recrudescent and reinfections, only reinfections and those that failed to amplify by PCR were intentionally not hybridised by dot-blot PCR for the same reason as stated for the SP/CQ drug trial. After the dot-blot hybridisation analysis had been carried out, it was discovered that 20 of the original 33 isolates were in fact not genuinely recrudescent. Subsequent analysis by typing at the *MSP2* locus revealed the presence of novel parasites in the R sample, indicating that these patients hadmixtures of recrudescence and reinfection. Having taken this into account, the statistical analyses were done on the total blotted samples (n= 33) and separately on the genuine recrudescents only (n=13).

Table 27

DHFR allele prevalence in samples obtained from 33 patients before (D0) and after (R) SP treatment, including patients with genuine recrudescence and patients with both recrudescence and reinfections.

Parasitological outcome	Allele at *DHFR* 108 (D0)			Allele at *DHFR* 108 (R)			Allele at *DHFR* 51 (D0)			Allele at *DHFR* 51 (R)			Allele at *DHFR* 59 (D0)			Allele at *DHFR* 59 (R)		
	Ser	Asn	Ser+Asn	Ser	Asn	Ser+Asn	Asn	Ile	Asn–Ile	Asn	Ile	Asn+Ile	Cys	Arg	Cys+Arg	Cys	Arg	Cys+Arg
RI	0	28	0	0	26	0	0	22	0	1	20	1	8	2	18	2	18	5
RII	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RIII	0	5	0	0	4	0	0	5	0	0	3	0	0	4	1	0	4	1
Total (prevalence)	0 (0)	33 (100)	0 (0)	0 (0)	30 (91)	0 (0)	0 (0)	27 (82)	0 (0)	1 (3)	23 (70)	1 (38)	8 (24)	6 (18)	19 (58)	2 (6)	22 (67)	6 (18)

Table 28

DHFR allele prevalence in subset of samples obtained from 13 patients with genuine recrudescence before (D0) and after (R) SP treatment.

Parasitological outcome	Allele at *DHFR* 108 (D0)			Allele at *DHFR* 108 (R)			Allele at *DHFR* 51 (D0)			Allele at *DHFR* 51 (R)			Allele at *DHFR* 59 (D0)			Allele at *DHFR* 59 (R)		
	Ser	Asn	Ser+Asn	Ser	Asn	Ser+Asn	Asn	Ile	Asn+Ile	Asn	Ile	Asn+Ile	Cys	Arg	Cys+Arg	Cys	Arg	Cys+Arg
RI	0	8	0	0	8	0	0	7	0	0	8	0	1	1	6	0	7	1
RII	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RIII	0	5	0	0	4	0	0	5	0	0	3	0	0	4	1	0	4	0
Total (prevalence)	0 (0)	13 (100)	0 (0)	0 (0)	12 (92)	0 (0)	0 (0)	12 (92)	0 (0)	0 (0)	11 (85)	0 (0)	1 (7.7)	5 (38)	7 (54)	2 (6)	11 (85)	1 (7.7)

3.2.4. DHFR *codon 108*

Of the patient isolates analysed by PCR /dot-blot hybridisation, all 33 (100%) had *DHFR* alleles encoding pure Asn 108 (mutant form) in their pre-treatment isolates, and 30 (representing 100% of the samples successfully typed) had *DHFR* alleles encoding pure Asn 108 in the samples taken at recrudescence (Table 27). No samples pre-treatment and post-treatment had *DHFR* alleles encoding Ser 108 (wild type) alone, and no samples were found with both alleles encoding Ser 108 and Asn 108 alleles in the pre-treatment and/or post-treatment isolates (Table 27). DNA from three isolates from the post-treatment group (two RI & one RIII responses) failed to amplify by SP1/SP2 PCR, and therefore they were not included on the dot-blot. All the isolates possessed the allele encoding Asn 108 alone regardless of time taken to recrudescence. Therefore there is no correlation between the presence of *DHFR* alleles encoding Asn 108 and time to recrudescence following SP treatment (Table 29).

Equally for the 13 genuinely recrudescent subset of samples, all day 0 and R samples had *DHFR* alleles encoding Asn 108 (Table 28), and again there was no correlation with time to recrudescence (Fisher's Exact P= 1.000).

Table 29

Prevalence of *DHFR* 108 alleles in the pre-treatment samples within different time-to-recrudescence groups for patients treated unsuccessfully with SP.

Time Intervals (days)	Asn 108 (Mutant)	Ser 108 (Wild type)	108 Asn + 108 Ser (Mixed)	Total (n)
D0-D7	5	0	0	5
D8-D14	0	0	0	0
D15-D21	13	0	0	13
D22-D28	15	0	0	15
Total (n)	33	0	0	33

Fisher's Exact P= 1.000.

3.2.5. DHFR *codon 51*

Twenty seven patients out of 33 had only parasites encoding DHFR 51Ile (mutant form) in their pre-treatment samples. This represents 100% of those successfully typed, as six isolates (RI) were negative by PCR. Twenty three (85%) of these had only parasites with *DHFR* encoding 51Ile in their recrudescent samples. One patient, who had an RI response, had parasites encoding both 51Asn (wild type) and 51Ile present simultaneously in the sample taken at recrudescence. No patient had both alleles present in the pre-treatment isolates. One patient, who had an RI response, had only parasites encoding DHFR 51Asn present in the sample taken at recrudescence. None of the isolates pre-treatment contained parasites encoding DHFR 51Asn (Table 27). Eight isolates (two RIII and six RI) taken at recrudescence were negative by PCR. In summary, all the D0 isolates possessed only parasites with the mutant form (*DHFR* 51Ile) regardless of time taken to recrudescence. There was no correlation between the presence of alleles encoding DHFR 51Ile and time to recrudescence following SP treatment (Table 30).

For the subset of 13 samples showing only genuine recrudescence patterns, all (100%) had parasites encoding DHFR 51Ile in the D0 sample and therefore there was no correlation between the presence of *DHFR* 51Ile and time to recrudescence.

Table 30

Prevalence of *DHFR* 51 alleles in the pre-treatment samples within different time-to-recrudescence groups for patients treated unsuccessfully with SP.

Time Intervals (days)	51 Asn (Wild type alone)	51 Ile (Mutant alone)	51 Asn + 51 Ile (Mixed)	Total (n)
D0-D7	0	5	0	5
D8-D14	0	0	0	0
D15-D21	0	10	0	10
D22-D28	0	12	0	12
Total (n)	0	27	0	27

Fisher's Exact P= 1.000

3.2.6. DHFR codon 59

Six patients out of the 33 had only parasites with the *DHFR* allele encoding 59Arg (mutant form) in their pre-treatment isolates. All six of these patients had parasites encoding DHFR 59Arg only in their post-treatment isolates. Out of the 33 patients, 19 (58%) had a mixture of parasites encoding DHFR 59Cys and DHFR 59Arg in the D0 samples; in the recrudescent samples, three (10%) remained mixed, two lost the parasites encoding DHFR 59Arg and became DHFR 59Cys only, and the remainder (12) lost the parasites encoding DHFR 59Cys and became DHFR 59Arg only, indicating selection of parasites encoding the mutant form at *DHFR* 59 following unsuccessful treatment with SP. Eight (24%) patients had parasites encoding DHFR 59Cys only at D0 but at recrudescence five of these samples became *DHFR* 59Arg only. The other two (7%) samples had a mixture of parasites encoding DHFR 59Cys and DHFR 59Arg at recrudescence (Table 27). Three (9%) isolates taken at recrudescence (one RIII and two RI) failed to amplify by SP1/SP2 PCR.

When the samples with mixed (wild type and mutant) forms of *DHFR* 59 were included in the mutant class (Table 31), there was no association between the allele present and time to recrudescence. When the mixed class was treated as a separate group (Table 32), there was an association between the allele present and time to recrudescence (P= 0.021).

Table 31
Prevalence of *DHFR* 59 alleles in the 33 pre-treatment samples within different-time-to recrudescence groups for the patients treated unsuccessfully with SP.

Time Intervals (days)	59 Cys (Wild type alone)	59 Cys/Arg (Mutant/ Mixed)	Total (n)
D0-D7	0	5	5
D8-D14	0	0	0
D15-D21	4	9	13
D22-D28	4	11	15
Total (n)	8	25	33

Fisher's Exact P= 0.486 grouping mixtures with mutant group

Table 32
Prevalence of *DHFR* 59 alleles in the 33 pre-treatment samples within different time to recrudescence groups for the patients treated unsuccessfully with SP.

Time Intervals (days)	59 Arg Mutant)	59 Cys (Mild type)	59 Cys/Arg (Mixed)	Total (n)
D0-D7	4	0	1	5
D8-D14	0	0	0	0
D15-D21	1	4	8	13
D22-D28	1	4	10	15
Total (n)	6	8	19	33

Fisher's Exact P= 0.021 treating mixtures as a separate group

For the subset of 13 samples that were genuine recrudescents, analysing the *DHFR* 59 pretreatment results (Table 33) by Fisher's Exact test indicated no association between the presence of the allele in the pretreatment sample and time to recrudescence if the mixtures (wild type and mutant forms of *DHFR* 59) were grouped with the mutant class. A slight association was observed if mixtures were treated as a separate group (P=0.055) but this was not quite significant at the 5% level.

Table 33
Prevalence of *DHFR* 59 alleles in the 13 genuinely recrudescent samples within different time-to-recrudescence groups for the patients treated unsuccessfully with SP.

	Pre-treatment				Post-treatment			
Time Intervals (days)	59 Cys (wildtype alone)	59 Arg (mutant alone)	59 Cys + 59Arg (mixed)	Total (n)	59 Cys (wildtype alone)	59 Arg (mutant alone)	59 Cys + 59Arg (mixed)	Total (n)
D0-D7	0	4	1	5	0	4	0	4
D8-D14	0	0	0	0	0	0	0	0
D15-D21	1	1	2	4	0	3	1	4
D22-D28	0	0	4	4	0	4	0	4
Total (n)	1	5	7	13	3	11	1	12

Fisher's Exact P=0.055 treating mixtures as a separate group

Fisher's Exact P=0.631 grouping mixtures with mutant group

Fisher's Exact tests were used to analyse if parasites in the 33 sample set carrying the double mutant (108Asn/51Ile or 108Asn/59Arg) were found at different frequencies in the pre-treatment samples of patients who had early rather than late recrudescence (Table 34). There was no significant effect observed for *DHFR* 108Asn/51Ile. The P value (0.0016) was highly significant for *DHFR* 108Asn/59Arg indicating a correlation between the presence of the double mutation and time to recrudescence, i.e. parasites with the double mutation recrudesced significantly earlier than those without.

The same analysis was carried out for the triple mutant (108Asn/51Ile/59Arg). A highly significant effect was observed (P= 0.0009) (Table 35).

Table 34

Prevalence of double mutant haplotypes of *DHFR*108/51 and *DHFR*108/59 in the 33 pre-treatment samples within different time-to-recrudescence groups for patients treated unsuccessfully with SP.

Time Intervals (days)	Pure double mutant form (108Asn/ 51Ile)	Other alleles	Total (n)	Pure double mutant form (108Asn/ 59Arg)	Other alleles	Total (n)
D0-D7	5	0	5	4	1	5
D8-D14	0	0	0	0	0	0
D15-D21	11	0	11	1	12	13
D22-D28	11	0	11	1	14	15
Total (n)	27	0	27	6	27	33

Fisher's Exact P= 1.000 (108Asn/ 51Ile); P= 0.0016 (108Asn/ 59Arg)

Table 35

Prevalence of triple mutant haplotype of *DHFR*108/51/59 in the 33 pre-treatment samples within different time-to-recrudescence groups for patients treated unsuccessfully with SP.

Time Intervals (days)	Pure triple mutation (108Asn/51Ile/59Arg)	Other alleles	Total (n)
D0-D7	4	1	5
D8-D14	0	0	0
D15-D21	0	10	10
D22-D28	1	11	12
Total (n)	5	22	27

Fisher's Exact P= 0.0009

The Fisher's Exact test was used to analyse the subset of 13 samples that were genuinely recrudescent only. Parasites carrying the double mutant (108Asn/51Ile) were not found at different frequencies in the pre-treatment samples of patients who had early rather than late recrudescence (P=1.000) (Table 36). Parasites carrying the double mutant form *DHFR*108Asn/59Arg were found at significantly higher frequencies in early rather than late

recrudescences (P=0.0479). Parasites with the triple mutation (*DHFR*108Asn/51Ile/59Arg) were also significantly more frequent in early rather than late recrudescences (P= 0.028) in the 13 sample subset (Table 37).

Table 36

Prevalence of double mutant haplotypes of *DHFR*108/51 and *DHFR*108/59 in the pre-treatment samples within different time-to-recrudescence groups for the subset of 13 patients treated unsuccessfully with SP.

Time Intervals (days)	Pure double mutant form (108Asn/ 51Ile)	Other alleles	Total (n)	Pure double mutant form (108Asn/ 59Arg)	Other alleles	Total (n)
D0-D7	5	0	5	4	1	5
D8-D14	0	0	0	0	0	0
D15-D21	3	0	3	1	3	4
D22-D28	4	0	4	0	4	4
Total (n)	12	0	12	5	8	13

Fisher's Exact P=1.000 (108Asn/ 51Ile); P= 0.048 (108Asn/ 59Arg)

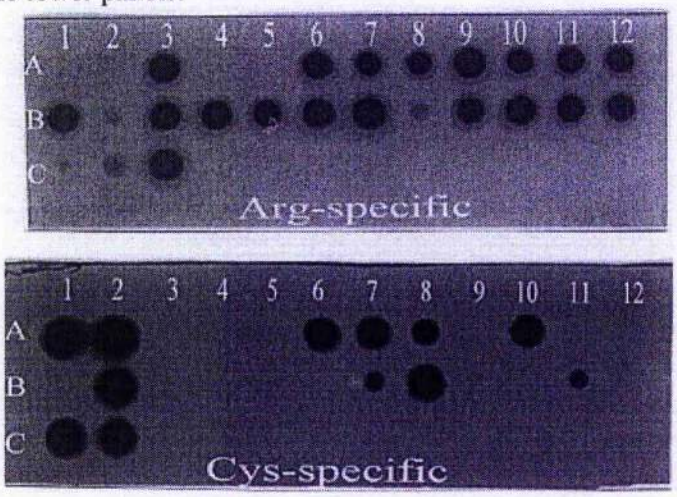
Table 37

Prevalence of triple mutant haplotype of *DHFR*108/51/59 in the pre-treatment samples within different time to recrudescence groups for the subset of 13 patients treated unsuccessfully with SP.

Time Intervals (days)	Pure triple mutation (108Asn/51Ile/59Arg)	Other alleles	Total (n)
D0-D7	4	1	5
D8-D14	0	0	0
D15-D21	0	3	3
D22-D28	0	4	4
Total (n)	4	8	12

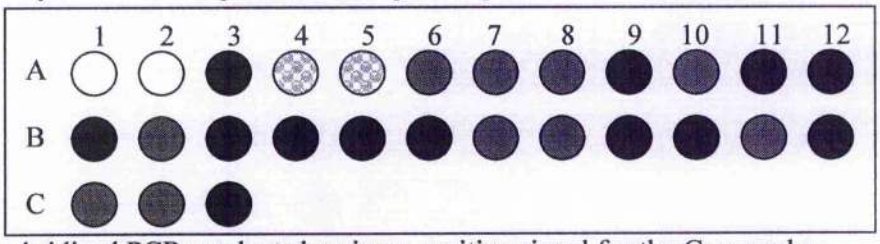
Fisher's Exact P= 0.028

Figure 4. Dot-blot of PCR products of *P. falciparum* pre-/ post-treatment isolates from patients treated with SP and SP/CQ hybridised with the DHFR 59 Arg-specific oligonucleotide probe in the upper and Cys-specific probe in the lower panels.



Positions A1 - A3 in both panels contain *P. falciparum* clones 3D7, HB3 & Dd2 respectively. Positions A4 - A5 in both panels contain negative controls (water). Positions A6- C3 in both panels contain patients *falciparum* isolates (sample numbers: 41 (R), 117 (D0), 119 (R), 121 (R), 124 (R), 137 (R), 158 (R), 177 (R), 201 (R), 230 (R), 254 (R), 73 (D0), 73 (R), 82 (R), 97 (D0), 156 (D0), 206 (R), 228 (D0) and 228 (R)- see Appendix 1). Below is a schematic showing the results for each spot.

Figure 5. Representation of the dot-blot of hybridised PCR products. Each spot is a hybridised PCR product for a specific probe.



○ - a hybridised PCR product showing a positive signal for the Cys- probe.

● - a hybridised PCR product showing a positive signal for the Arg- probe.

● - a hybridised PCR product showing a positive signal for both the Cys and Arg- probes.

◎ - a hybridised PCR product showing no signal for both the Cys and Arg probes.

4. DISCUSSION:

Several studies associating mutations in *DHFR*, *DHPS* and *Pfcrt* genes with treatment outcome following treatment of patients with SP and CQ respectively have been done. However, no study has been done to see whether there is an association between the number of mutations in the pyrimethamine resistance gene (*DHFR*) and the time to recrudescence or treatment failure following treatment with SP and the combination SP/CQ. Our work has therefore been the first to address this issue.

The work presented here has considered whether the number of mutations found in the parasite gene *PfDHFR* has an effect on the level of *in vivo* resistance, as measured by time to recrudescence of parasites or symptoms of malaria. The hypothesis was that parasites with two or three altered amino acids at the known key sites in this gene would have increased resistance to the drug, and would grow faster in the presence of therapeutic drug levels than parasites with only one altered amino acid. The children studied were taking part in two antimalarial drug efficacy trials, one of SP and one of SP plus CQ, the latter being the first line treatment for uncomplicated malaria in Uganda since 2001 (Communication from the Ministry of Health, Malaria Control Programme).

4.1. Clinical and Parasitological outcome

Treatment of children with a combination of SP/CQ was clinically effective with an adequate clinical response rate of 92%. At the level of parasite clearance rates, SP/CQ was less effective with only 53% of the children clearing their parasitaemia. Roughly 70% of the children had evidence of new infections with parasites during the follow-up period, accompanied by the persistence of parasites from the initial infection. Although the study was carried out in an area of high transmission, this may not indicate a high level of re-infection during the follow-up period. Only one sample was taken at admission, and therefore parasites that were sequestered, or in the liver stages, at the time of sampling could have been missed. Therapeutic blood levels of

CQ persist for 6-10 days (White, 1998) and those of SP following the usual dose persist for 15-52 days (Watkins and Masobo, 1993). Synergistic activity between sulfadoxine and pyrimethamine is lost after approximately 15 days and after this period, parasites respond only to pyrimethamine (Watkins and Masobo, 1993). Based on this, early recrudescences are therefore likely to be of drug resistant parasites as both drugs (SP/CQ) will still be at therapeutic levels; however shortly after 10 days for CQ and 15 days for SP, recrudescences could be of parasites with lower levels of resistance to CQ and pyrimethamine. Very late recrudescences could be of parasites sensitive to SP but probably resistant to pyrimethamine. It is also likely that the novel parasites appearing in early recrudescences will be resistant to SP, whereas those appearing later on could be sensitive to SP.

The clinical outcome following treatment with SP was very similar to that of the SP/CQ arm of the study. Adequate clinical response following treatment with SP was slightly lower than that following treatment with SP/CQ (91% ACR); and parasite clearance rates were also lower, with parasitaemia cleared in only 39% of the children treated with SP, compared to 53% with SP/CQ although this was not statistically significant (Fisher's Exact P= 0.3).

The level of overall recrudescence in the two drug treatment regimes was similar. In the SP/CQ arm of the study, there was no evidence of total parasite clearance followed by re-infection during the follow-up period. By contrast in the SP arm of the study, three children were typed with totally novel parasites that were present in the recrudescent sample but not in the initial sample, indicating the clearance of the original parasitaemia by the drug, but re-infection during the follow-up period. These three children were recrudescent at 16, 21 and 28 days post-treatment. Since the synergistic activity between sulfadoxine and pyrimethamine lasts for approximately 15 days after which the reappearing parasites respond only to pyrimethamine, then the reinfecting parasites in the first child are most probably resistant to SP. The reinfecting parasites in the other two children could have been SP-sensitive but most

probably pyrimethamine resistant. Although this difference between the two drug regimens may be due to chance alone, it is possible that the CQ remains at levels suppressive for sensitive parasites for 1-2 months (Krishna and White, 1996) which is much longer than for the synergistic effect of SP at 15 days (Watkins and Masobo, 1993) and therefore this could prevent the growth of reinfecting, drug-sensitive parasites. In the SP arm of the study once the levels of SP had dropped to sub-therapeutic levels, this then could allow reinfecting parasites to grow in treated children. In the SP/CQ arm, such parasites could have encountered therapeutic levels of both CQ and SP.

4.2. Mutations in *pfcrt76* and resistance to SP/CQ

The pre- and post- treatment samples from those in whom SP/CQ treatment failed to clear parasitaemia were genotyped at the *Pfcrt*76 locus. All (100%) of the pre- and the post-treatment samples possessed parasites with the mutant allele *Pfcrt*T76, which confers resistance to CQ *in vitro*. No infections in either the pre- or post-treatment samples were found to contain only wild type parasites. Of the post-treatment samples, 89% were infections with parasites carrying the mutant *Pfcrt*76 allele only, the remainder (6%) being multiple-clone infections of parasites carrying both mutant (T76) and wild type (K76) *Pfcrt* alleles. This was surprising, as it might have been expected that CQ would kill parasites carrying the K76 allele in multiple clone infections, but this was apparently not the case. One possible explanation for this could be poor drug absorption and/or fast metabolism of CQ in these children. It is also possible some clinical resistance to CQ could be the result of mutations in genes other than *Pfcrt*.

Typing of the pre-treatment samples of individuals in whom SP/CQ was successful in clearing parasitaemia showed that *Pfcrt*T76 is also present in 100% in samples (23% of these being multiple clone infections where the K76 is also present) (Table 8). The *Pfcrt*T76 allele therefore appears to be at very high prevalence in this region of Uganda. Despite this, treatment failure rates with CQ alone (the previous first line drug in Uganda until 2001, when it was replaced by SP/CQ) were estimated at 30 % (Ministry of Health)

[http://www.health.go.ug/malaria.htm]). It is obvious that in this area, the *Pfcrt*76 mutation is not predictive of CQ treatment failure, and it is likely that mutations in other genes, such as *pfmdr1* (Babiker *et al.*, 2001, Dorsey *et al.*, 2001, Mockenhaupt *et al.*, 2001) are necessary for clinical treatment failure.
Host factors such as immunity may also contribute to clearance of parasites resistant to CQ. The level of antiparasite immunity is difficult to measure, but age can be used as an indicator (Djimde *et al.*, 2003, Djimde *et al.*, 2001, Omar *et al.*, 2001). There was no significant difference in age between children infected with parasites carrying the *Pfcrt*76 mutation who cleared their parasites following SP/CQ treatment and those who did not clear mutant parasites (P=0.124) when children of all ages were considered. Excluding children under one year old (6-12 months) from the analysis, a significant association was observed between clearance of resistant parasites and age (P =0.043). Children in the age group 25-49 months are more likely to clear resistant parasites than younger children (6-12 months). However, it was observed that children aged 6-12 months old are better at clearing resistant parasites than children aged 13-24 months old though this was not statistically significant. This could be because younger children still retain some anti-parasite protective immunity from maternal antibodies. The maternal antibodies could have waned in children aged 13-24 months old and they have not had time to develop a significant and effective anti-parasite immunity themselves. Since malaria transmission in the study area is holoendemic, children were highly exposed. This could account for the increased rate of clearance of resistant parasites in the older age group (25-49 months old) due to age acquired immunity.

There appeared to be no correlation between the presence of parasites carrying the mutated form of *Pfcrt*76 and treatment failure with SP/CQ in this population. Overall for the data from this drug treatment regimen, it would appear that the presence of the *Pfcrt*T76 mutation had no influence on treatment failure with the drug combination SP/CQ. It is likely that the SP component of the drug combination is responsible for treatment outcome or

success. In support of this hypothesis prior to the introduction of SP/CQ as the first line drug, CQ treatment failure rates were in excess of 30 % (Talisuna *et al*., 2002), whereas the combination drug gave an adequate clinical response rate of 92 %.

4.3. The role of mutations in *DHFR* in resistance to SP and SP/CQ

Mutations in *DHFR* are clearly linked to resistance to pyrimethamine and to SP. The key mutation at position 108 (serine to asparagine) was present in all patients in whom treatment was unsuccessful, both pre- and post-treatment with SP/CQ and SP alone. None of the patients had parasites with the wild type allele *DHFR*108Ser.

Samples from patients in whom SP/CQ was successful in clearing infection have not been typed, but typing of other Ugandan samples from Tororo, Apac, and Kyenjojo suggests that the *DHFR*108Asn is at or close to fixation (≥ 90%) in this population (Unpublished data). There was obviously no correlation with time to treatment failure and the presence of different alleles at *DHFR 108* in the pre-treatment sample, since all pretreatment parasites carried the 108Asn-encoding allele. The same trend was observed with both pre- and post-treatment samples from SP arm treatment failures.

Two other mutations in *DHFR* in codons 51 and 59 have been linked to SP resistance.

For codon 51, the majority of parasites typed had the mutant form encoding 51Ile. Infections with purely the mutant form of codon 51 (51Ile) were found in over 95% of both pre- and post-treatment samples from patients in whom SP/CQ failed to clear parasitaemia, and in over 80% of pre- and post-treatment samples from patients in whom SP alone failed to clear parasitaemia. Mixtures of wild type (51Asn) and mutant (51Ile) parasites were found in two samples, one pre-treatment isolate in the SP/CQ arm, and one post-treatment isolate in the SP arm. A pure wild type infection was found in one patient with SP post-treatment failure. Because the majority of infections carried only the mutant allele, there was statistically no significant association between the presence of

different pre-treatment *DHFR* 51 alleles and the time to recrudescence or treatment failure in either SP/CQ or SP- treated patients.

For codon 59, the frequencies of wild type (59Cys) and mutant (59Arg) alleles were more evenly balanced: the majority of pre-treatment samples from SP/CQ and SP treatment failures had a mixture of wild type and mutant alleles, (17/22 and 19/33 respectively). Infections with only the wild type allele or only the mutant allele were found in a small number of pretreatment patients in both drug treatment regimes.

There was evidence for selection of parasites carrying the mutant genotype *DHFR*59Arg following treatment with SP/CQ. Of the 17 patients with both parasites with wild type (59Cys) and mutant (59Arg) alleles prior to treatment, half of them (8/17) had only parasites with the mutant allele at recrudescence, with the remainder unchanged as mixtures. The increase in prevalence of pure infections of *DHFR*59Arg, and decrease in mixed infections of wild type and mutant alleles was statistically significant (P=0.033). Further support for selection of parasites carrying *DHFR*59Arg was obtained in the SP arm of the study. Nineteen out of 33 patients had mixtures of wild type (59Cys) and mutant (59Arg) parasites prior to treatment, and 12 of these had only parasites with the mutant allele encoding 59Arg at recrudescence. It is also noteworthy that five of eight samples with apparently only parasites carrying *DHFR*59Cys at admission were found with parasites carrying only *DHFR*59Arg at recrudescence. These samples appeared to be genuinely recrudescent by *MSP1* and *MSP2* genotyping. One explanation for this is that parasites carrying 59Arg were present in the patient at admission, but were either at too low a parasitaemia to detect, or were not in periphery at time of sampling. Selection following SP treatment favored 59Arg parasites, so that their number increased to detectable levels by the time of clinical or parasitological treatment failure.

Analysis of time to recrudescence showed that patients with parasites carrying pure *DHFR*59Arg in the D0 sample recrudesced significantly earlier than those with a mixture of *DHFR* 59Cys + 59Arg or those with pure

*DHFR*59Cys. This was true only for the SP arm of the study; it was not observed with the SP/CQ arm of the study, possibly because of the low numbers of parasites recrudescing early (< 14 days) in the latter arm.

It has been reported that combinations of mutations in *DHFR* are associated with SP treatment failure *in vivo*. Patients carrying infections with pure 108Asn/51Ile did not suffer recrudescence of parasitaemia significantly earlier than those with mixtures of wild type and mutant parasites following treatment with SP/CQ or with SP alone. However, the low P value of 0.088 may indicate a possible association which could be investigated further with a larger sample size, although in this case the trend was for patients with the double mutant 108Asn/51Ile to suffer recrudescences later than patients with parasites with other combinations.

Patients carrying infections of pure *DHFR*108Asn/59Arg double mutant parasites had significantly earlier recrudescences following SP treatment than those with other alleles (P=0.0016). There was no significant difference in the patients treated with SP/CQ, possibly because very few patients in this drug treatment arm had early treatment failure (recrudescence before day 14 post treatment). Patients carrying infections of pure triple mutant (*DHFR*108Asn/51Ile/59Arg) recrudesced significantly earlier than patients with other infections in the SP drug treatment arm (P=0.0009), but not in the SP/CQ treated patients (P=1.000), for the same reasons discussed above. For the SP-true recrudescent subset, even with the low numbers, there was still an association with the triple mutant and early recrudescence.

4.4. Conclusions

The aim of this study was to tackle the question 'why do some patients progress to treatment failure much earlier than others?' by investigating if the number of mutations in the pyrimethamine resistance gene *DHFR* had any influence on how fast a patient progressed to treatment failure.

It is thought that mutations accumulate in *DHFR*, starting with changes to codon 108, and that each additional codon change increases the level of resistance (Peterson *et al.*, 1990). This study has attempted to investigate the role of double and triple mutant forms of *DHFR* in clinical treatment failure. The results show that parasite infections of pure double mutant (*DHFR*108Asn/59Arg) are associated with significantly earlier recrudescence following treatment with SP. Infections with parasites carrying the triple mutant form alone (*DHFR*108Asn/51Ile/59Arg) are even more significantly associated with early treatment failure.

The research presented here provides the first evidence that the number of mutations present in *DHFR* has a significant effect on the type of treatment failure (early vs. late, or RII/RIII vs RI) following SP chemotherapy. This has been previously assumed to be the case, but had not been formally demonstrated, although the association between any treatment failure and mutations in *DHFR* is well known. Mutations in *DHPS* have also been shown to be implicated in resistance to SP *in vivo* in several places (Triglia *et al.*, 1997, Plowe *et al.*, 1997, Wang *et al.*, 1997a). There is also an observation that the emergence of *DHPS* mutations on a background of pre-existing *DHFR* mutations coincided with the development of increased SP resistance. (Roper *et al.*, 2003, Mberu *et al.*, 2000, Nzila *et al.*, 2000, Curtis *et al.*, 1998, Plowe *et al.*, 1997). Due to financial constraints, this study did not investigate the association between mutations in *DHPS* and clinical treatment failure following treatment with SP. It is therefore not possible to tell whether the observed treatment outcomes based on the presence of the double or triple mutant in *DHFR* are independent/ or dependent on mutations in *DHPS*. Therefore, future studies using larger sample sizes and including *DHPS* genotyping should be undertaken in order to investigate the effect of *DHPS* mutations on clinical treatment failure in patients possessing double or triple mutations in *DHFR*.

The timing of recrudescence or treatment failure could also be due to different proportions of drug-resistant and -sensitive parasites in the initial infection. The different types of recrudescence seen could be the result of within-host parasite population changes, or may reflect differences in the level of resistance. RI (late) recrudescence could be a result of infection with weakly resistant parasites, which are affected by the drug so that they replicate more slowly, or only a proportion of them survive. An RIII recrudescence could be due to highly resistant parasites which are unaffected by drug treatment.
Alternatively, RI recrudescence could reflect an initial infection with both drug-sensitive and drug-resistant parasites, where the drug-resistance parasites represent the minority of the parasites present. After treatment with the drug, only the resistant parasites in the RI infection would survive, the overall parasitaemia would be markedly reduced, and it would take more time for the parasitaemia to reach detectable levels and/or the threshold for recurrence of clinical symptoms. A patient suffering a rapid RIII recrudescence could have an infection where all or the majority of parasites are resistant to the drug used for treatment. Infections with a majority of resistant parasites might be expected to recrudesce sooner than those where the majority of parasites are not resistant, and are killed by the drug. This has not been investigated in this study, but will form the basis of future work using techniques such as *in situ* PCR (Ranford-Cartwright and Walliker, 1999) and quantitative PCR (Bell and Ranford-Cartwright, 2002).

Additional findings of this work are that the drug combination SP/CQ had a lower treatment failure rate and a higher parasite clearance rate than SP alone in the population studied. This was unexpected given the high treatment failure rates previously observed using CQ as monotherapy, and the high prevalence (100%) of the *Pfcrt* mutation linked to CQ treatment failure in these patients. The most probable explanation of this finding is that there are mutations in other genes, which are associated with CQ resistance in this area. Mutations in such genes would need to be coupled with mutations in *DHFR*

for resistance to the combination of both drugs, and where this is not the case the SP/CQ drug combination will be relatively more effective than SP alone.

The findings as discussed above, will help in laying strategies that will lead to the delay of the spreading of antimalarial drug resistance in Uganda. The Ministry of Health in conjunction with the Malaria Control Programme will be able to use this information to adequately modify the national drug policy or distribute the available resources properly in order to preserve their efficacy. These findings will eventually help in the designing of an affordable and effective therapy for the treatment of malaria not only in Uganda but also in the rest of sub-Saharan Africa.

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APPENDIX 1: Raw data

SP TRIAL DATA FOR UNCOMPLICATED P. FALCIPARUM MALARIA IN TORORO (UGANDA)

Sample identification number (U01)	Clinical outcome	Parasitological outcome	MSP1/MSP2 PCR results	Parasite count (D0)	DHFR 108 Ser/Asn (D0)	DHFR 51 Asn/Ile (D0)	DHFR 59 Cys/Arg (D0)	Parasite count (R)	DHFR 108 Ser/Asn (R)	DHFR 51 Asn/Ile (R)	DHFR 59 Cys/Arg (R)
18	ACR	RI (D21)	Both	2,400/ul	Asn (mutant)	Ile (mutant)	Mixed	2,240/ul	Asn	No result	Mixed
21	ACR	RI (D21)	Both	1,760/ul	Asn	No result	Cys (wild)	240/ul	PCR failed	PCR failed	PCR failed
23	ETF	RIII (D3)	Recrudescent	19,360/ul	Asn	Ile	Mixed	480/ul	PCR failed	PCR failed	PCR failed
41	ACR	RI (D28)	Both	2,240/ul	Asn	Ile	Cys	5,440/ul	Asn	Mixed	Mixed
43	ACR	RI (D28)	Recrudescent	5,440/ul	Asn	Ile	Mixed	2,400/ul	Asn	Ile	Arg
47	ACR	RI (D28)	Both	7,040/ul	Asn	No result	Mixed	960/ul	PCR failed	PCR failed	PCR failed
52	ACR	RI (D28)	Both	3,760/ul	Asn	No result	Cys	16,640/ul	Asn	No result	Arg
61	ACR	RI (D28)	Both	4,960/ul	Asn	Ile	Cys	480/ul	Asn	Asn	Arg
64	ACR	RI (D18)	Recrudescent	18,880/ul	Asn	No result	Arg (mutant)	38,400/ul	Asn	Ile	Arg
67	ACR	RI (D21)	Both	1,280/ul	Asn	No result	Cys	2,400/ul	Asn	Ile	Arg
80	ACR	RI (D21)	Recrudescent	18,160/ul	Asn	Ile	Mixed	23,040/ul	Asn	Ile	Arg
85	ACR	RI (D28)	Recrudescent	7,520/ul	Asn	Ile	Mixed	24,960/ul	Asn	Ile	Arg
99	ACR	RI (D28)	Both	17,920/ul	Asn	No result	Mixed	2,240/ul	Asn	Ile	Arg
102	ACR	RI (D28)	Both	3,040/ul	Asn	Ile	Cys	1,120/ul	Asn	Ile	Arg
105	ACR	RI (D28)	Both	1,600/ul	Asn	Ile	Mixed	1,600/ul	Asn	No result	Arg
113	ETF	RIII (D3)	Recrudescent	22,080/ul	Asn	Ile	Arg	11,680/ul	Asn	Ile	Arg
117	ACR	RI (D22)	Recrudescent	3,440/ul	Asn	Ile	Mixed	33,120/ul	Asn	Ile	Arg
119	ACR	RI (D21)	Both	124,640/ul	Asn	Ile	Mixed	179,200/ul	Asn	Ile	Mixed
121	ACR	RI (D21)	Both	2,240/ul	Asn	Ile	Mixed	960/ul	Asn	Ile	Arg
124	ETF	RIII (D3)	Recrudescent	53,920/ul	Asn	Ile	Arg	160/ul	Asn	Ile	Arg
134	ACR	RI (D28)	Both	3,350/ul	Asn	Ile	Mixed	9,600/ul	Asn	No result	Cys
137	ACR	RI (D28)	Recrudescent	8,000/ul	Asn	Ile	Mixed	5,600/ul	Asn	Ile	Arg
157	ETF	RIII (D3)	Recrudescent	9,120/ul	Asn	Ile	Arg	8,800/ul	Asn	Ile	Arg
159	ACR	RI (D20)	Recrudescent	14,880/ul	Asn	Ile	Cys	91,920/ul	Asn	Ile	Mixed
176	ETF	RIII (D1)	Recrudescent	15,680/ul	Asn	Ile	Arg	27,840/ul	Asn	No result	Arg
177	ACR	RI (D21)	Recrudescent	15,520/ul	Asn	Ile	Mixed	27,040/ul	Asn	Ile	Arg
181	ACR	RI (D28)	Both	44,160/ul	Asn	Ile	Arg	9,440/ul	Asn	Ile	Arg
184	ACR	RI (D21)	Both	3,200/ul	Asn	Ile	Mixed	7,680/ul	Asn	Ile	Arg
197	ACR	RI (D28)	Both	7,040/ul	Asn	Ile	Mixed	11,040/ul	Asn	Ile	Arg
201	ACR	RI (D21)	Both	20,480/ul	Asn	Ile	Cys	1,120/ul	Asn	Ile	Arg
230	ACR	RI (D21)	Both	20,160/ul	Asn	Ile	Mixed	71,040/ul	Asn	Ile	Arg
254	ACR	RI (D28)	Both	3,680/ul	Asn	Ile	Mixed	12,160/ul	Asn	Ile	Mixed
261	ACR	RI (D21)	Both	3,040/ul	Asn	Ile	Mixed	3,520/ul	Asn	Ile	Cys
25	ACR	RI (D28)	Both	4,320/ul	Not blotted	Not blotted	Not blotted	4,480/ul	Not blotted	Not blotted	Not blotted
29	ACR	RI (D28)	Both	7,200/ul	Not blotted	Not blotted	Not blotted	4,320/ul	Not blotted	Not blotted	Not blotted
55	ACR	RI (D28)	Both	64,000/ul	Not blotted	Not blotted	Not blotted	22,400/ul	Not blotted	Not blotted	Not blotted
78	LTF	RII (D14)	Both	1,960/ul	Not blotted	Not blotted	Not blotted	6,080/ul	Not blotted	Not blotted	Not blotted
89	ACR	RI (D21)	Both	17,840/ul	Not blotted	Not blotted	Not blotted	5,440/ul	Not blotted	Not blotted	Not blotted
115	ACR	RI (D28)	Both	28,640/ul	Not blotted	Not blotted	Not blotted	16,800/ul	Not blotted	Not blotted	Not blotted
135	ETF	RIII (D3)	Both	48,000/ul	Not blotted	Not blotted	Not blotted	1,760/ul	Not blotted	Not blotted	Not blotted
187	ACR	RI (D21)	Both	16,160/ul	Not blotted	Not blotted	Not blotted	1,440/ul	Not blotted	Not blotted	Not blotted
190	ACR	RI (D21)	Both	44,480/ul	Not blotted	Not blotted	Not blotted	4,640/ul	Not blotted	Not blotted	Not blotted
225	ACR	RI (D16)	New	17,600/ul	Not blotted	Not blotted	Not blotted	33,600/ul	Not blotted	Not blotted	Not blotted
242	ACR	RI (D28)	Both	5,440/ul	Not blotted	Not blotted	Not blotted	20,480/ul	Not blotted	Not blotted	Not blotted
264	ACR	RI (D28)	New	2,600/ul	Not blotted	Not blotted	Not blotted	67,200/ul	Not blotted	Not blotted	Not blotted
267	ACR	RI (D21)	New	257,120/ul	Not blotted	Not blotted	Not blotted	1,600/ul	Not blotted	Not blotted	Not blotted
270	ACR	RI (D28)	Both	12,240/ul	Not blotted	Not blotted	Not blotted	4,800/ul	Not blotted	Not blotted	Not blotted
275	ACR	RI (D26)	Both	8,160/ul	Not blotted	Not blotted	Not blotted	3,200/ul	Not blotted	Not blotted	Not blotted

All the DHFR results were obtained by dot-blot hybridisation
No result implies the result was negative for both wild and mutant alleles at that locus
PCR failed implies there was no amplification by the dot-blot SP1/SP2 PCR
Not blotted implies the samples were not SP1/SP2 PCR amplified hence they were not hybridised

SP/CQ TRIAL DATA FOR UNCOMPLICATED P. FALCIPARUM MALARIA IN TORORO (UGANDA)

Sample identification number (U01)	Clinical outcome	Parasitological outcome	MSP1/MSP2 PCR results	Parasite count (D0)	PFCRT 76 Lys/Thr (D0)	DHFR 108 Ser/Asn (D0)	DHFR 51 Asn/Ile (D0)	DHFR 59 Cys/Arg (D0)	Parasite count (R)	PFCRT 76 Lys/Thr (R)	DHFR 108 Ser/Asn (R)	DHFR 51 Asn/Ile (R)	DHFR 59 Cys/Arg (R)
11	ACR	RI (D28)	Recrudescent	800/ul	Mixed	Asn (mutant)	Ile (mutant)	Mixed	2,960/ul	Mixed	Asn	Ile	Mixed
24	ACR	RI (D28)	Recrudescent	7,200/ul	Thr (mutant)	Asn	Ile	Mixed	2,280/ul	Thr	Asn	Ile	Mixed
60	ACR	RI (D28)	Both	5,600/ul	Thr	Asn	Ile	Arg (mutant)	489,600/ul	Thr	Asn	Ile	Mixed
73	ACR	RI (D21)	Recrudescent	2,400/ul	Thr	Asn	Ile	Mixed	8,800/ul	Mixed	Asn	Ile	Arg
82	ACR	RI (D28)	Both	39,520/ul	Thr	Asn	Ile	Mixed	17,760/ul	Thr	Asn	Ile	Arg
83	LTF	RII (D14)	Both	10,240/ul	Thr	Asn	Ile	Mixed	8,800/ul	Thr	Asn	Ile	Mixed
94	ACR	RI (D21)	Both	97,600/ul	Thr	Asn	Ile	Arg	4,160/ul	Thr	Asn	Ile	Arg
97	ETF	RIII (D3)	Recrudescent	9,720/ul	Thr	Asn	Mixed	Mixed	5,520/ul	PCR failed	PCR failed	PCR failed	PCR failed
103	ACR	RI (D28)	Both	30,400/ul	Thr	Asn	Ile	Mixed	6,400/ul	Thr	Asn	Ile	Arg
141	ACR	RI (D28)	Recrudescent	120,800/ul	Mixed	Asn	Ile	Mixed	1,280/ul	Thr	Asn	Ile	Cys
156	ACR	RI (D28)	Recrudescent	27,040/ul	Thr	Asn	Ile	Arg	91,520/ul	Thr	Asn	Ile	Arg
165	ACR	RI (D28)	Both	29,760/ul	Thr	Asn	Ile	Mixed	21,920/ul	Thr	Asn	Ile	Mixed
185	ACR	RI (D28)	Both	9,440/ul	Thr	Asn	Ile	Mixed	5,280/ul	PCR failed	Asn	Ile	Cys
186	ACR	RI (D15)	Both	16,240/ul	Thr	Asn	Ile	Mixed	28,800/ul	Thr	Asn	Ile	Arg
204	ACR	RI (D28)	Both	24,320/ul	Thr	Asn	Ile	Mixed	32,480/ul	Thr	Asn	Ile	Arg
205	ACR	RI (D21)	Recrudescent	9,280/ul	PCR failed	Asn	Ile	Mixed	640/ul	Thr	Asn	Ile	Mixed
224	ACR	RI (D15)	Recrudescent	10,240/ul	Thr	Asn	Ile	Cys (wild)	16,960/ul	Thr	Asn	Ile	Cys
228	ACR	RI (D21)	Recrudescent	9,760/ul	Thr	Asn	Ile	Mixed	66,560/ul	Thr	Asn	Ile	Arg
235	ACR	RI (D28)	Both	28,320/ul	Thr	Asn	Ile	Mixed	20,960/ul	Thr	Asn	Ile	Mixed
239	ACR	RI (D28)	Both	3,360/ul	Mixed	Asn	Ile	Mixed	8,160/ul	Thr	Asn	Ile	Mixed
240	ACR	RI (D21)	Recrudescent	6,400/ul	Thr	Asn	Ile	Mixed	33,280/ul	Thr	Asn	No result	Cys
259	ACR	RI (D28)	Both	45,120/ul	Thr	Asn	Ile	Cys	4,000/ul	Thr	Asn	No result	Cys
4	LTF	RII (D14)	Both	1,560/ul	Thr	Not blotted	Not blotted	Not blotted	4,800/ul	Thr	Not blotted	Not blotted	Not blotted
13	ACR	RI (D17)	Both	19,200/ul	Thr	Not blotted	Not blotted	Not blotted	2,560/ul	Thr	Not blotted	Not blotted	Not blotted
34	LTF	RII (D7)	Both	32,400/ul	Thr	Not blotted	Not blotted	Not blotted	15,840/ul	Thr	Not blotted	Not blotted	Not blotted
40	ACR	RI (D21)	Both	2,240/ul	Thr	Not blotted	Not blotted	Not blotted	2,800/ul	Thr	Not blotted	Not blotted	Not blotted
44	ACR	RI (D24)	Both	2,080/ul	Thr	Not blotted	Not blotted	Not blotted	12,000/ul	Thr	Not blotted	Not blotted	Not blotted
45	ACR	RI (D28)	PCR failed	800/ul	Thr	Not blotted	Not blotted	Not blotted	7,680/ul	Thr	Not blotted	Not blotted	Not blotted
46	ACR	RI (D28)	Both	36,160/ul	Thr	Not blotted	Not blotted	Not blotted	2,880/ul	Mixed	Not blotted	Not blotted	Not blotted
63	ACR	RI (D21)	Both	18,240/ul	Thr	Not blotted	Not blotted	Not blotted	3,360/ul	Thr	Not blotted	Not blotted	Not blotted
68	LTF	RII (D14)	Both	20,800/ul	Thr	Not blotted	Not blotted	Not blotted	1,320/ul	Thr	Not blotted	Not blotted	Not blotted
72	LTF	RII (D14)	Both	7,400/ul	Thr	Not blotted	Not blotted	Not blotted	6,880/ul	Thr	Not blotted	Not blotted	Not blotted
85	ACR	RI (D28)	Both	11,040/ul	Thr	Not blotted	Not blotted	Not blotted	2,240/ul	Thr	Not blotted	Not blotted	Not blotted
123	ACR	RI (D28)	Both	49,120/ul	Mixed	Not blotted	Not blotted	Not blotted	160,000/ul	Thr	Not blotted	Not blotted	Not blotted
127	ACR	RI (D27)	Both	5,120/ul	PCR failed	Not blotted	Not blotted	Not blotted	1,120/ul	Thr	Not blotted	Not blotted	Not blotted
132	ACR	RI (D21)	Both	3,680/ul	Thr	Not blotted	Not blotted	Not blotted	8,320/ul	Thr	Not blotted	Not blotted	Not blotted
136	ACR	RI (D28)	Both	6,360/ul	Thr	Not blotted	Not blotted	Not blotted	6,880/ul	Thr	Not blotted	Not blotted	Not blotted
154	ACR	RI (D18)	Both	20,160/ul	Thr	Not blotted	Not blotted	Not blotted	78,080/ul	Thr	Not blotted	Not blotted	Not blotted
161	ACR	RI (D28)	Both	14,880/ul	Thr	Not blotted	Not blotted	Not blotted	17,920/ul	Thr	Not blotted	Not blotted	Not blotted
179	ACR	RI (D28)	Both	11,040/ul	Mixed	Not blotted	Not blotted	Not blotted	9,440/ul	Thr	Not blotted	Not blotted	Not blotted
189	ACR	RI (D21)	Both	20,480/ul	Thr	Not blotted	Not blotted	Not blotted	9,600/ul	Thr	Not blotted	Not blotted	Not blotted
191	ACR	RI (D28)	Both	8,320/ul	Mixed	Not blotted	Not blotted	Not blotted	6,720/ul	Thr	Not blotted	Not blotted	Not blotted
192	ACR	RI (D21)	Both	34,560/ul	Thr	Not blotted	Not blotted	Not blotted	26,080/ul	Thr	Not blotted	Not blotted	Not blotted
203	ACR	RI (D21)	Both	1,600/ul	Thr	Not blotted	Not blotted	Not blotted	800/ul	Thr	Not blotted	Not blotted	Not blotted
212	ACR	RI (D28)	Both	8,320/ul	Thr	Not blotted	Not blotted	Not blotted	14,720/ul	Thr	Not blotted	Not blotted	Not blotted
213	ACR	RI (D21)	Both	9,120/ul	Thr	Not blotted	Not blotted	Not blotted	65,600/ul	Thr	Not blotted	Not blotted	Not blotted
258	LTF	RII (D14)	Both	4,480/ul	Thr	Not blotted	Not blotted	Not blotted	25,600/ul	Thr	Not blotted	Not blotted	Not blotted

All PFCRT results were obtained by MSPCR
All the DHFR results were obtained by dot-blot PCR hybridisation

Dedication

This thesis is dedicated to my loving husband John Fred Kazibwe

and

our son Jotham

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