CHAPTER FOUR

4 RESULTS

4.1 Treatment efficacies

A total of 158 malaria patients were monitored post-treatment for at least a month from the day of recruitment to the study. Of these, twenty-seven patients were excluded from the *in-vivo* analysis because they could not be traced. Ninety-three patients below the age of five and 38 patients above the age of five years were analyzed for *in-vivo* treatment regimen efficacies. Out of the 131 patients, 74 (57%), 54 (41%) and 3 (2%) patients were prescribed fansidar®, quinine and coartem®, respectively. Out of the 74 that were prescribed fansidar®, 20 (27%) had failed treatment. Fourteen (35%) of the 54 patients prescribed quinine, had the treatment repeated with either quinine or fansidar® during the period of the study. All the 3 patients prescribed coartem® got cured (Table 4.1).

Table 4.1 Summary of *in-vivo* malaria treatment efficacies to sulfadoxine /pyrimethamine (fansidar®), quinine and lumefantrine/artemether (coartem®)

Age group (yrs)	Treatment efficacies					
	Fansidar® (n = 74)		Quinine (n = 54)		Coartem® (n = 3)	
	Cured	Failed	Cured	Repeat *T	Cured	Failed
< 5	41 (55%)	18 (24%)	32 (59%)	12 (22%)	1 (33%)	0 (0%)
> 5	13 (18%)	2 (3%)	8 (15%)	2 (4%)	2 (67%)	0 (0%)
Total	54 (73%)	**20 (27%)**	40 (74%)	14 (26%)	3 (100%)	0 (0%)

NOTE: Repeat *T = repeated treatment; n = total number of patients prescribed that drug

Of the 20 patients who failed fansidar® treatment (Table 4.1), 12 (60%) were treated and cured using quinine, 4 (20%) using coartem®, and 4 (20%) with a repeat of fansidar® treatment (Table 4.2).

Table 4.2 Summary of the drugs used to treat the 20 patients with failed sulfadoxine/pyrimethamine (fansidar®)

Age group (yrs)	**Sanative drugs**		
	Quinine	**Coartem®**	**Fansidar® (repeated)**
< 5	11 (5%)	3 (15%)	4 (20%)
> 5	1 (55%)	1 (5%)	0 (0%)
Total	12 (60%)	4 (20%)	4 (20%)

4.2 Molecular analysis for drug resistance

Out of 158 blood spot samples, 112 (71%) and 103 (65%) were analyzed at *dhfr* codons 108 and 59, respectively, for mutations associated with pyrimethamine resistance. Figure 4.1 shows the GeneRuler™ 100 bp DNA ladder (pME-80J3 DNA – *Eco*1 471 and *Pvu*1 digest) in lane 1. The secondary PCR amplified sample product bands resulting from use of the primer pair of M3 and F/ for the *dhfr* codons 108 and 59 with band size of 522 bp are shown in lanes 2, 3, and 4 (Figure 4.1). Of the 158 blood spot samples, 124 (79%) and 116 (73%) were successfully analyzed for mutations on codons 437 and 540, respectively, of the *dhps* associated with sulfadoxine resistance. Figure 4.3 shows the secondary PCR amplified sample product bands in lanes 5, 6, and 7 resulting from use of the primer pair of K and K/

for the *dhps*, with a band size of 438 bp. Lane 6 contains a band preceded by a 438 bp band, which is a result of non specific amplification. In such cases, where non specific bands were obtained, the amplification for such samples had to be repeated because restriction digestion of such samples would result in multiple unexpected bands.

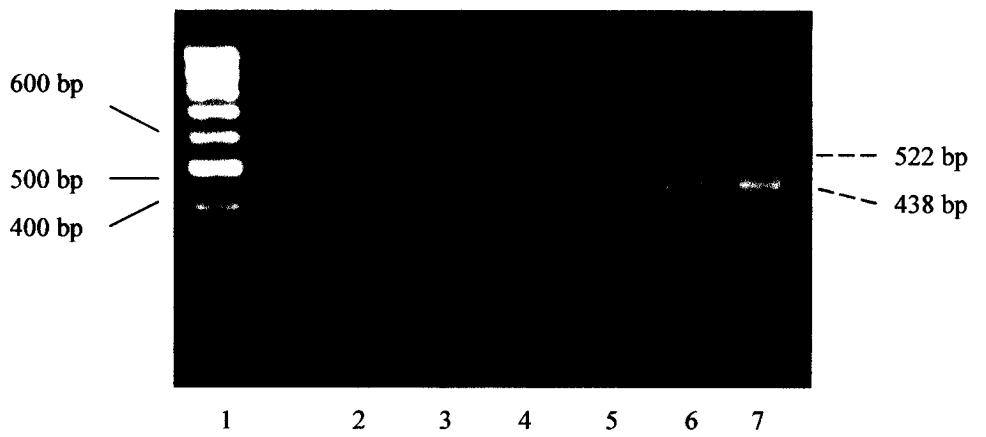

Figure 4.1 Secondary PCR amplified product of codons 108 and 59 of the dihydrofolate reductase gene (*dhfr*) (lanes 2, 3 and 4) and codons 437 and 540 of the dihydropteroate synthase gene (*dhps*) (lanes 5, 6 and 7) on 2.5% agarose serva gel [Solid lines used to label the DNA size marker bands and long dash lines the gene products]

The band in lane 7 of figure 4.2, of 404 bp size, was obtained from the secondary PCR product of 438 bp size in lane 6 after being subjected to restriction digestion with enzyme *Ava* II at codon 437. This result indicates a mutant parasite with mutation associated with resistance to sulfadoxine. Figure 4.2 shows in lane 5 the secondary PCR amplification product band for *pfcrt* codon 76 with size 145 bp. The supporting mutation at *pfcrt* codon 75 was detected in 153 (99%) of the 155 successfully analyzed samples (Table 4.6). The band in lane 3 with size 53 bp

resulted from restriction digestion with the enzyme *BspH* I of sample band in lane 2 with size 84 bp, indicating a wild type parasite for codon 75 (Figure 4.2).

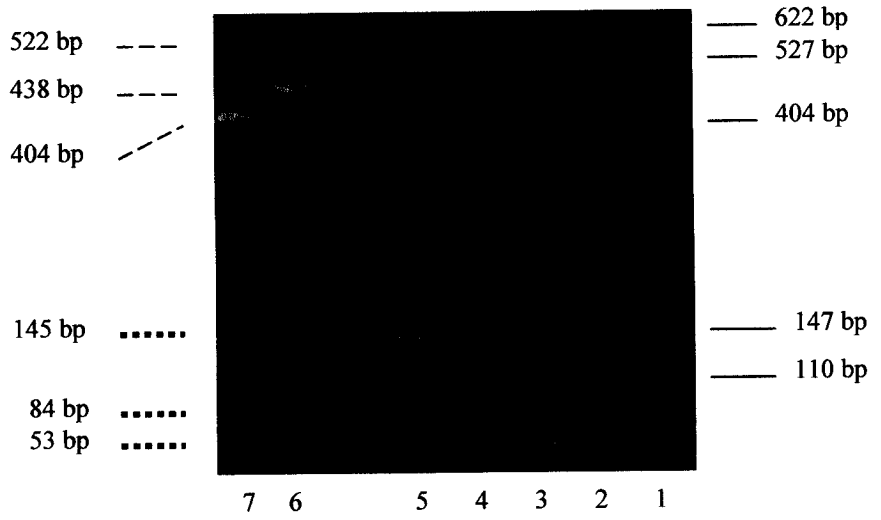

Figure 4.2 PCR amplified products of codon 75 and 76 of the *Plasmodium falciparum* chloroquine resistance transporter gene (*pfcrt*) (lanes 2, 3 and 5) and undigested (lane 6) and digested (lane 7) products of codon 437 of the dihydropteroate synthase gene (*dhps*) on 2.5% agarose serva gel. [Solid lines, square dot lines and long dash lines, used to label the DNA ladder bands, *pfcrt* bands and *dhps* bands, respectively]

4.2.1 Prevalence of mutations associated with sulfadoxine/pyrimethamine (fansidar®) resistance

Out of 124 samples analyzed for mutation at *dhps* codon 437, associated with resistance to sulfadoxine, 44 (36%) were positive (Table 4.3). There was no significant association between parasite genotype, mutant or wild type, at *dhps* codon 437 and the corresponding infected patients' *in-vivo* response to fansidar® treatment (χ^2, P = 0.143). The supporting mutation for resistance to sulfadoxine, at *dhps* codon 540, was detected in 19 (16%) samples, out of 116 successfully amplified samples (Table 4.3). There was also no significant association between the parasite genotype

at *dhps* codon 540 and the corresponding infected patients' *in-vivo* fansidar® treatment responses (χ^2, P = 0.205). No mixed wild/mutant parasites were detected at these *dhps* codons.

Table 4.3 Number of wild and mutant samples at codons 437 and 540 of the dihydropteroate synthase gene (*dhps*) associated with sulfadoxine resistance

Type of parasite detected	**Codon number**	
	437	**540**
Wild	80 (64%)	97 (84%)
Mutants	44 (36%)	19 (16%)
Total analyzed	124	116

Out of 112 samples successfully analyzed for mutation at *dhfr* codon 108, which confers pyrimethamine resistance, 24 (22%) samples were positive. The supporting mutation at *dhfr* codon 59 was detected in 36 (35%) samples, out of 103 samples successfully analyzed. There was no significant association between the parasite genotypes on the two codons and the *in-vivo* fansidar® treatment response (χ^2, P = 0.390 and 0.246, respectively). The prevalence of mixed wild and mutant type of parasites associated with pyrimethamine resistance was detected in 8 (7%) of 112 samples at codon 108 and 5 (5%) out of 103 samples at codon 59 (Table 4.4)

Table 4.4 Number of wild, mutant and mutant/wild samples at codons 108 and 59 of the dihydrofolate reductase (*dhfr*) associated with pyrimethamine resistance

Type of parasite detected	Codon number	
	108	**59**
Wild	80 (71%)	62 (60%)
Wild/mutants	8 (7%)	5 (5%)
Mutants	24 (22%)	36 (35%)
Total analyzed	112	103

A combination mutation occurring simultaneously on *dhps* codon 437 and *dhps* codon 540 in parasites associated with sulfonamides resistance was detected in 4 (4%) of the 102 samples successfully analyzed. Out of 101 samples successfully analyzed for double mutation on *dhfr* codons 108 and 59, 8 (8%) were positive (Table 4.5). However, there was no significant association between *in-vivo* fansidar® treatment response in infected patients and parasite genotypes at codon combinations of *dhps* 437 and 540 (χ^2, P = 0.454) and *dhfr* 108 and 59 (χ^2, P = 0.969).

Table 4.5 Mutants detected with two codon combinations on genes that confer resistance to sulfonamides and pyrimethamine and those with combined sulfadoxine/pyrimethamine resistance

Affected drugs	Gene	Codon combination	Mutants detected
Sulfonamides	*dhps*	437 + 540	4% (n=102)
Pyrimethamine	*dhfr*	108 + 59	8% (n=101)
Sulfadoxine/pyrimethamine	*dhps/dhfr*	437 + 108	11% (n=108)

n is the total number of samples analyzed

Mutations occurring simultaneously on *dhps* codon 437 and *dhfr* codon 108 in a single parasite, associated with both sulfadoxine and pyrimethamine resistance, was detected in 12 (11%) of the 108 samples successfully analyzed (Table4.5). There was a significant association between the presence of the two mutations and the *in-vivo* fansidar® treatment response in corresponding infected patients (χ^2, P = 0.001).

4.2.2. Prevalence of mutations associated with chloroquine resistance

Out of 158 samples collected, 152 (96%) and 155 (98%) were successfully amplified at *pfcrt* codons 76 and 75, respectively. Out of 152 samples analyzed, 147 (97%) samples had the prerequisite mutation for chloroquine resistance at codon 76 of the *pfcrt*, and 3 (2%) were found with a mixture of wild and mutant types (Table 4.6).

Table 4.6 Number of wild, mixed mutant/wild and mutant type of parasites detected at codons 75 and 76 of the *Plasmodium falciparum* chloroquine resistance transporter gene (*pfcrt*)

Type of parasite detected	**Codon number**	
	76	**75**
Wild	2 (1%)	2 (1%)
Wild/mutants	3 (2%)	0 (0%)
Mutants	147 (97%)	153 (99%)
Total analyzed	152	155

CHAPTER FIVE

5 DISCUSSION AND CONCLUSION

Malaria is the major cause of morbidity at Solwezi Urban Clinic (see Appendix 3). The *in-vivo* study of 74 *Plasmodium falciparum* infected patients at Solwezi urban clinic on fansidar® treatment revealed a treatment failure of 27% (20). While the molecular analysis gave 11% (n=108) prevalence of the mutations or molecular markers found simultaneously on *dhps* and *dhfr* of *P. falciparum* responsible for fansidar® resistance. Resistance to chloroquine has been associated with mutations at codons 72, 74, 75 and 76 of the *pfcrt* (Djimde *et al.*, 2001; Maguire *et al.*, 2001). This study focused on the mutations associated with codons 75 and 76, because the K76T mutation is the most important as it is the determinant of the resistance to chloroquine, while the other one plays a supporting role (Jelinek *et al*, 2002). Following the official withdrawal of the use of chloroquine in Zambia, fansidar® was made the first-line antimalarial drug (NMCC, 2003) and therefore, mutations on *dhfr* codons 108 and 59, and *dhps* 437 and 540 associated with fansidar® resistance (Duraisingh *et al*, 1998; Jelinek *et al*, 2002) were also studied.

The WHO recommended a treatment failure rate of 25% as the basis for possible drug change (WHO, 2001a). This resistance threshold recommended for treatment policy change has since been lowered to 10% to try and mitigate the drug resistance in Africa (WHO, 2006). Although no clear correlation between parasite genotype and *in-vivo* results has been documented in most field trials, the results obtained from such trials convincingly show that a selected parasite population, if properly used,

could help to correlate between point mutation and phenotypic signs of resistance (Jelinek *et al.*, 2002), such as treatment failure. A comparison of the *in-vivo* fansidar® treatment failure rate and the prevalence of the molecular markers for resistance to fansidar®, revealed a difference of 16% in the present study. The treatment failures observed for fansidar® in the *in-vivo* study were possibly not only due to the drug resistant parasites but also due to other factors. However, there was a significant association between the presence of the mutations at codon combination of *dhps* 437 and *dhfr* 108 in a parasite with the *in-vivo* fansidar® treatment response (χ^2, P = 0.001).

The factors that contribute to treatment failure and possible emergence of drug resistance have been identified in other studies. These factors include incorrect dosing, non compliance with duration of dosing regimen, poor drug quality, certain drug interactions, poor or erratic absorption and misdiagnosis (Bloland, 2001). Fansidar® is considered to be one of the few effective and cheap alternative antimalarial drug/s to chloroquine and it has the advantage of good tolerance, relatively few side effects and excellent single oral dose therapy (Basco *et al.*, 1998). Therefore, incorrect dosing and non compliance to duration of dosing regimen should not be a major cause of treatment failure in adults because of the single oral dose therapy which is easier to administer. However, the high fansidar® treatment failure rates observed at Solwezi urban clinic in the under five age group (Appendix 3), is possibly because treatment is mainly based on age instead of weight and has no standard treatment regimen (WHO, 2001a). Prescriptions for the under five age group are based on ¼, ½ or ¾, 1 and 2 tablet regimens. This apportioning of

prescribed fansidar® tablets was observed at Solwezi urban clinic during the study period. This kind of prescription can lead to incorrect dosing resulting in under-dosing and/or overdosing. Under-dosing is likely to cause treatment failure. Under-dosing and overdosing can result in unnecessary drug pressure on the parasite population, especially that presumptive treatment is also practiced. These factors are exacerbated by the non existence of or developing partial immunity to the disease in this age group (Mendis *et al.*, 1990). The absence of partial immunity to malaria in the under five age group makes them more vulnerable to infection than adults who would have had developed partial immunity to the disease due to previous exposures to the malaria parasite (Mendis *et al.*, 1990; WHO, 2001a). Absence or developing partial immunity in this age group coupled with the fact that Solwezi lies in the northern part of Zambia with the highest transmission rates of malaria (CBoH, 2003a) could explain why about 70% of the patients recruited for the study were below the age of five years. The higher fansidar® treatment failure rates observed in the under five age group in this study and probably elsewhere, could be attributed to the aforementioned besides those caused by the actual drug resistant *P. falciparum* infections.

It has been reported that partially immune patients, such as those individuals exposed to malaria since birth, respond to antimalarial chemotherapy better than non-immune individuals (Cravo *et al.*, 2001). Partial immunity has been known to diminish in the absence of *P. falciparum* challenge over a long period of time (Mendis *et al.*, 1990). It has also been reported that drug resistant parasites tend to behave like sensitive ones in the presence of partial immunity in experiments on *Plasmodium chabaudi*

(Cravo *et al.*, 2001). *Plasmodium chabaudi* is a rodent parasite used in laboratory models to help understand the biology of drug resistant *P. falciparum* infections in humans. The effect of partial immunity on drug resistance was attributed to the combined effect of the drug and immunity as being sufficient to limit the parasite population growth to virtually zero, whereas drugs or immunity alone may be insufficient to stop growth of parasites equipped with drug resistance mechanisms (Cravo *et al.*, 2001). The other practice observed that could compromise the future use of fansidar® is the switch that is made from quinine to fansidar® whenever the former is assumed to have failed. This was observed in 5% of the total 131 patients recruited for this study in the *in-vivo* analysis. Ideally, a treatment failure with a second line drug can not be rescued with a first-line drug because that would lead to exposure of the parasite to a drug which it may already be resistant to, and thus enhancing its resistance. Indiscreet drug use practices can lead to extensive use of the drugs which may contribute to drug pressure. Drug pressure has been cited as the major factor that is responsible for rapid emergence of drug resistant parasites (Basco *et al.*, 1998).

Mutations associated with chloroquine and fansidar® resistance were detected in *P. falciparum* isolates from Solwezi. This study detected 22% (n=112) and 35% (n=103) mutation prevalence at codons 108 and 59 of the *dhfr*, respectively. A study by Jelinek *et al.* (2002), based on total of 337 imported isolates of *P. falciparum* into Europe, found a prevalence of the mutations at codons 108 and 59 of this same gene to be 68.9% and 58.8%, respectively, for Southern Africa (n=20) and 72.9% and 5%, respectively, for East Africa (n=53). In a study carried out in Mali by Diourte *et al.*

(1999), a three fold increase in the prevalence of these two *dhfr* mutations at codon 108 and 59 was observed in post treatment parasites and selection for mutant parasites was attributed to drug pressure

The present study found the prevalence of the mutations at *dhps* codons 437 and 540, associated with sulfadoxine resistance of 36% (n=124) and 16% (n=116), respectively. The study by Jelinek *et al.* (2002) found prevalence of mutations on these codons of 26.7% (n=20) and 0% (n=20), respectively, for Southern Africa and for East Africa they were 30.8% (n=53) and 7.5% (n=53), respectively. Prevalence rates of mutation in this study are therefore relatively much higher than the aforementioned. Other than for the incorrect use of fansidar®, comparison of the prevalence of the various mutations associated with fansidar® resistance observed in this study, with those from East Africa, could to some extent be attributable to resistant parasites coming into Zambia from other parts of Africa, especially from the neighbouring countries in East Africa where the prevalence of these mutations are relatively high (Jelinek et al., 2002). However, emergency of drug resistant parasites from within the country can not be overlooked.

The prevalence of the individual mutations associated with resistance to individual component drugs of fansidar® such as the sulfonamides have been found to be higher than the prevalence of the various combinations of mutations required to occur simultaneously in a parasite associated with resistance. This study found a prevalence of mutations in the codon combination of 437 and 540 of the *dhps* associated with sulfonamide resistance to be 4% (n=102) and for pyrimethamine

resistance involving codon combination of 108 and 59 of the *dhfr* as 8% (n=101). The study by Jelinek *et al.*, (2002) found 0% (n=20) for *dhps* 437 and 540 and 33.3% (n=20) prevalence of the *dhfr* 108, 51, and 59 triple combination from South African isolates. It has been deduced from epidemiological studies in settings of increasing use of sulfadoxine/pyrimethamine and where its resistance exist, that prevalence of mutations in the DHFR and DHPS correlate relatively well with the drug treatment failure (Diourte *et al.*, 1999). In Zambia, there has been an increased use of fansidar® ever since it was made the first-line drug replacing chloroquine. The *in-vivo* studies have been showing increasing fansidar® treatment failure rates for different places in Zambia where such studies have been done (Appendix 4). Therefore, the mutations that have been detected in samples collected from Solwezi are likely to also indicate the extent of treatment failure due to fansidar® resistant *P. falciparum*. The high prevalence values obtained in this study for individual mutations and their effect on the sensitivity to individual drugs, pyrimethamine and sulfadoxine, treatment failure rates were expected to be relatively high. However there was no significant association between individual mutations at codons *dhps* 437 and *dhfr* 108 of the parasites and the *in-vivo* fansidar® treatment response in this study (χ^2, P = 0.143 and 0.390, respectively). The prevalence of the combined mutations that are supposed to render the parasites more resistant was even lower. It is perhaps because of the low prevalence of the mutation involving codon combinations of *dhps* 437 and *dhfr* 108, associated with fansidar® resistance that fansidar® seems to be more effective in treatment of malaria that may be resistant to the individual drugs that make up fansidar®. The effectiveness of fansidar®, results from the synergistic interaction effect of the two drugs on *P. falciparum* (Basco *et al.*, 1998). Thus antifolate

combination drugs may still be very effective in the treatment of malaria if used correctly, especially in the under five age group.

To improve drug efficacies in the under five age group, it may be necessary to introduce a directly observed therapy (DOT) plan. The plan is to administer a prescribed drug to a patient admitted at a well established health institution under close monitoring and supervision by a health worker. It is an idea or practice being considered for introduction in antimalarial treatment, as is the case with TB treatment in many different parts of the world. This idea is being advocated especially in the under five age group for both the admitted and non admitted malaria patients at Solwezi urban clinic (personal communication with the Sister-in-charge for Solwezi urban clinic, 2003). World Health Organisation is also advocating for the introduction of prepackaged antimalarial drug doses for use in the under five age group in order to improve compliance to treatment regimens. Dissemination of public information on malaria is also encouraged to facilitate correct home treatment (WHO/UNICEF, 2003). Improving the treatment cure rates in the under five age group would certainly help in reducing both morbidity and mortality and enhancing the overall control of malaria. This would happen by reducing the number of infections that may be witnessed from one period of noted low transmission to that of high transmission and *vice versa*. This is possible because most of the asymptomatic infections have been known to occur mostly in this age group. Unlike periods of low seasonal transmission with unsuitable conditions for mosquito vector breeding, high seasonal transmission periods have suitable conditions for mosquito vector breeding (Briët *et al.*, 2005). However, it is the asymptomatic infections of *P. falciparum* that

provide the parasite reservoir for bridging periods from low seasonal transmission to high seasonal transmission of malaria (Briët *et al.*, 2005).

The prevalence of the K76T, a mutation in the *pfcrt* that is associated with resistance to chloroquine by *P. falciparum,* was detected in 97% (n=152) of individuals in the current study. The prevalence of the supporting mutation at codon 75 was found to be 99% (n=155). In 1999, prevalence of the K76T observed in a study that was conducted in Malawi and extended into Zambia was 92% (Plowe, 2003). In another molecular surveillance study on drug resistance in imported isolates of *P. falciparum* into Europe from Southern and East Africa detected a prevalence of the K76T in 64.7% (n=20) and 89.6% (n=53) of the samples tested, respectively, (Jelinek *et al.*, 2002). Although the existence of the mutation at codon 75 in the parasites does not on its own make the parasite resistant to chloroquine, it is reported that the parasite attains enhanced resistance to the drug if found in combination with K76T mutation. The accompanying or supporting mutations at codon 75 and many more vary in different geographical regions (Plowe, 2003). The high mutation prevalence observed at codons 76 and 75 of the *pfcrt* could be attributed to the prolonged use of chloroquine even after it was known to have lost its efficacy against malaria. By the year 2000 the National Malaria Control Centre had already reported clinical malaria treatment failure cases of chloroquine as high as 52.7% in Northern Province and 34% in Northwestern Province (see Appendix 5). In another study conducted in 2000 and 2001 in Nakonde district of the Northern Province of Zambia, it was reported that there was poor compliance to the standard chloroquine therapeutic doses (Kaona and Tuba, 2003). The prolonged use and the poor compliance to therapeutic doses of

chloroquine is likely to have exerted enhanced drug pressure on the parasite population resulting in a high prevalence of K76T mutation and hence low chloroquine efficacies (Appendices 4 and 5). The use of chloroquine was thus, discontinued in Zambia in 2003 (CBoH, 2003b). However, there is need to continue monitoring the K76T mutation circulating in malaria patients because it has been found that susceptible *P. falciparum* re-emerge following the withdrawal of chloroquine use on resistant strains. In Cambodia, it was observed that susceptible *P. falciparum* re-emerged 20 years after the withdrawal of chloroquine (Lim *et al.*, 2003). A similar observation in Malawi indicated that the prevalence of K76T mutants dropped from 85% to 13% after eight years of chloroquine withdrawal and that a parasite clearance rate of 100% in asymptomatic children was achieved (Mita *et al.*, 2003). Continued monitoring of the K76T mutation is therefore necessary to determine the re-emergence of the susceptible *P. falciparum* strains and to indicate as to when re-introduction of chloroquine can be recommended for re-use in a possible rotational antimalarials therapy, especially given that there are very few new drugs with known safety and of low cost as chloroquine (Plowe, 2003). The different prevalence levels of K76T mutations from different regions can be attributed to drug pressure differences which in turn impact differently on *pfcrt* stability (Mita *et al.*, 2003). *Plasmodium falciparum* has been reported to have a high plasticity in its genome which allows it to adapt to adverse environments by regulating its gene expression through gene alterations (Sharma, 2005). The high prevalence of K76T mutation in this study may be attributed to the late official withdrawal of chloroquine use in Zambia. Chloroquine treatment failure rates in Zambia had reached as high as 52.7% in 2000, which was far above the 25% WHO criteria for possible drug change,

but its withdrawal was only effected in 2003. The apparent substantial recovery of chloroquine sensitivity by *P. falciparum* in Malawi gives hope that mutations associated with chloroquine resistance can be reversed in the absence of drug pressure (Mita *et al.*, 2003). It is also reported that removal of the antimicrobial drug pressure may or may not compromise the fitness of drug resistant microorganisms and result in selection of drug – sensitive phenotypes (Plowe, 2003). Judicious use of chloroquine in rotation with other antimalarials in the control of malaria is, therefore, still feasible but with appropriate tools in place.

It is worth noting that new mutations in the *pfcrt* have been linked to halofantrine and amantadine resistance in *P. falciparum* (Johnson *et al.*, 2004). These drugs are used to treat mild to moderate cases of chloroquine resistant malaria. This discovery was observed in parasite strains which were experimentally engineered to become resistant to the two drugs. As resistance to the two drugs increased, the parasites became susceptible to chloroquine (Johnson *et al.*, 2004). A similar competitive resistance establishment was also observed in drug resistant trypanosomes to the trypanocides berenil (diminazene aceturate) and samorin (isometamidium) (Chitambo and Arakawa, 1992). Lumefantrine, which is related to halofantrine, has been introduced in chloroquine resistant areas of Africa and Asia as a combination drug with artemether. This combination drug is being deployed in Zambia as coartem® (CBoH, 2003b; NMCC, 2003). This discovery of the possible central role of *pfcrt* in malaria drug resistance could help in the development of new therapeutic strategies that could be effective against chloroquine resistant parasites (Johnson *et al.*, 2004). One such hopeful strategy is the possible alternation of other drugs and

chloroquine, where the withdrawal of one drug that has become less effective is replaced with the other. It has also been discovered that one of these new mutations can be found in a strain of malaria from Southeast Asia (Johnson *et al.*, 2004). Southeast Asia, Thailand and South America are well known chloroquine resistant malaria foci (Cowman and Foote, 1990). Malaria drug resistant parasites can easily be transmitted from one part of the world to the other considering how easily people move around the world these days. Perhaps this may call for possible monitoring and screening of the people for malaria before they travel especially if they have a history of malaria. The new mutations in the *pfcrt* and K76T would save as a standard screening procedure for resistant mutants of *P. falciparum.*

The 11% samples that had a combination of mutations at *dhfr* codon 108 and *dhps* codon 437 associated with resistance to fansidar®, also revealed the presence of K76T mutation associated with resistance to chloroquine. Such parasites with the three mutations would resist treatment with either drug. The presence of such parasites could be attributed to a poorly managed transition period of chloroquine withdrawal and its replacement with fansidar®. Before chloroquine was withdrawn as the first line drug, fansidar® was the second line drug used to treat persistent malaria infections after treatment failure with chloroquine. This brought about a period when these two drugs were still used side by side, even when a lot of chloroquine treatment failures were being recorded. There is also evidence of continued use of chloroquine in some parts of Zambia even after the official announcement of its withdrawal, as it could still be accessed from the private health facilities and profit oriented private pharmacies and drug stores, including illegal outlets. The simultaneous exposure of

the parasites to chloroquine and fansidar® must have selected for parasites that can tolerate both drugs, and hence the mutations at the three codons associated with resistance to them in the samples from Solwezi.

The *in-vivo* studies are simple methods which can easily be used to assess antimalarial drug efficacies and thus provide guidance, rational and evidence based malaria treatment policies. However, standard *in-vivo* techniques are expensive and time consuming (Plowe, 2003). The other problem with *in-vivo* assays is that treatment failures determined using this method may not reflect true drug resistance situation. This method tends to overestimate the rate of early treatment failure and/or high level resistance (Plowe, 2003), since various factors are implicated in treatment failures besides the presence of drug resistant parasites. Furthermore, the *in-vivo* studies do not have reliable provisions to discriminate the various causes of treatment failures that may be determined through them. Nevertheless, the assays can be used for early warning of impending treatment failures regardless of the particular cause. Detection of molecular markers associated with drug resistance in parasites is more confirmative for treatment failures caused by mutations in drug resistant parasites. Molecular analysis of *P. falciparum dhfr* and *dhps* has been identified as a useful technical tool that can enable researchers to advise clinicians to predict patient's response to antimalarial treatment (Basco *et al.*, 1998). Molecular assays are therefore superior to the *in-vivo* techniques, but require more skilled personnel and specialized equipment to perform. In this study, molecular assays successfully detected drug resistant parasites in samples that were collected from patients who

were prescribed quinine because of a high parasitemia that was perhaps due to treatment failure.

A combination of *in-vivo* and molecular techniques is therefore, a valuable tool that should be established to routinely screen treatment failure rates and to confirm suspected drug resistant parasites in malaria patients. When the two methods are concurrently applied on samples collected from patients that are recruited for the *in-vivo* study, especially those that fail treatment, some light can be shade on the other possible causes of treatment failures. This is possible where the treatment failure from *in-vivo* analysis does not correspond with presence of mutations that are associated with resistance to a particular drug in samples obtained from the same patients.

Recommendations

This study was able to establish that the collection of blood samples on filter papers for DNA extraction is cheap, simple and feasible and therefore ideal for collection of samples from remote areas. It was also confirmed that the use of restriction fragment length polymorphism is a reliable molecular technical tool that can successfully be used to identify *P. falciparum* mutants which are resistant to fansidar® and chloroquine. According to the results obtained in this study, the following is recommended:

1. A vigorous dissemination of information on the importance of appropriate and judicious use, and compliance to drug regimen of antimalarial drugs. This

will not only improve treatment of malaria but will also prolong the length of time that the drugs will be in use.

2. There is need for improved specific formulation of antimalarials for paediatric use to improve correct dosing and compliance to treatment regimens in this age group.

3. Sensitization and close monitoring of illegal importation and adherence to withdrawal of chloroquine and its derivatives should be strengthened by statutes.

4. Prevalence of chloroquine resistant mutants should be continuously monitored because their reduction indicates possible future re-use of chloroquine either alone or in rotation with other antimalarials, considering that only a few new antimalarial drugs are likely to be developed in the near future.

5. There should be a continued monitoring of the genetic mutations associated with drug resistance in *P. falciparum*. This will help in making timely and informed policy decision for possible early drug change and thus avoiding emergence of multidrug resistance development as observed for fansidar® and chloroquine in this study.

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APPENDICES

APPENDIX: 1

THE UNIVERSITY OF ZAMBIA
SCHOOL OF VETERINARY MEDICINE
DEPARTMENT OF BIOMEDICAL SCIENCES

Telephone: +260-1-293655
Fax: +260-1-293727
P.O. Box 32379
Lusaka, Zambia.

Informed consent form

Study DETECTION OF MUTATIONS IN THE *pfcrt*, *dhps*, AND *dhfr* IN *Plasmodium falciparum* ISOLATES ASSOCIATED WITH RESISTANCE TO CHLOROQUINE, SULFADOXINE AND PYRIMETHAMINE.

To The Parent/Guardian/Patient

We are interested in knowing how well the current treatment for malaria with chloroquine and fansidar is working and whether the treatment failures that have been observed are due to drug resistant parasites. To do this we are recruiting malaria patients and they will be followed for 14 days to monitor the progress of treatment. We also wish to collect 3 – 6 droplets of blood on filter papers for a detailed study of the parasite.

The child (patient) providing the blood will;

a) *give blood only once during the entire project period*
b) *be encouraged to report back to the medical centre for review (not for another blood collection exercise)*

Parent or guardian or patient
*I*_____, *of*_____
have read and understood the statements on this consent form and therefore give permission to the trained nurse or medical officer to collect 3 – 6 droplets of blood from my child/me to be used only for the purpose outlined on this form.
*Sign*_____, *Medical Centre*_____
*Date*_____

APPENDIX: 2

PREPARATION OF TBE BUFFER AND LOADING DYE STOCK SOLUTIONS AND CONSTITUTION OF dNTPs MIXTURE STOCK SOLUTION

Preparation of 5× TBE

To 500 ml volumetric flask, add

27.00 gm Tris base

13.75 gm Boric acid

1.86 gm $Na_2EDTA.2H_2O$

Bring to the mark with distilled water (Store at room temperature)

Preparation of 6× Bromophenol blue / Sucrose loading dye

To 100 ml volumetric flask, add

40% Sucrose

0.25% Bromophenol blue

Bring to the mark with distilled water and Aliquot to 500 µl (Store at 4 °C)

Constitution of 2 µM dNTPs

20 µl 100 µM dATP

20 µl 100 µM dCTP

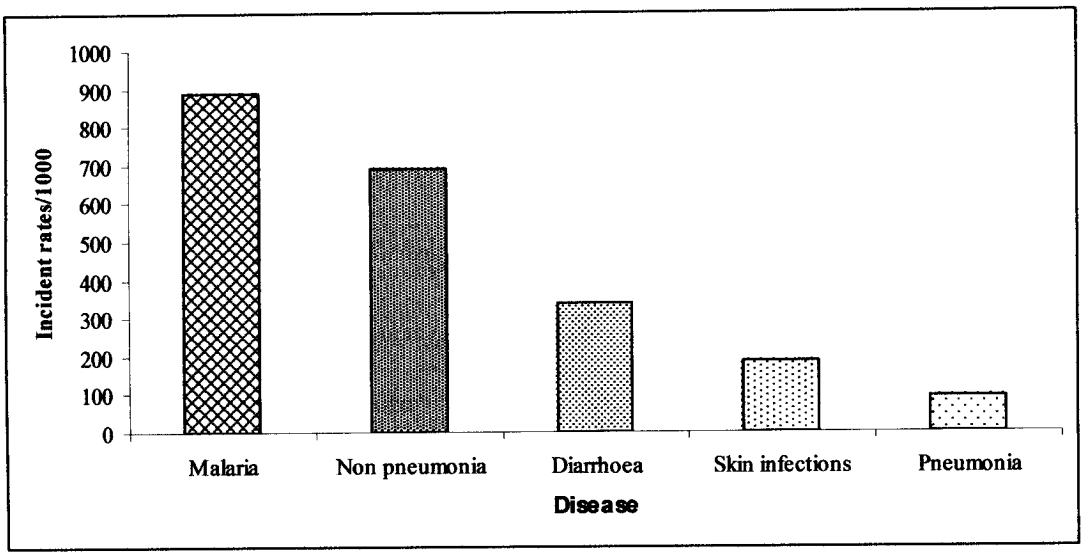
20 µl 100 µM dGTP

20 µl 100 µM dTTP

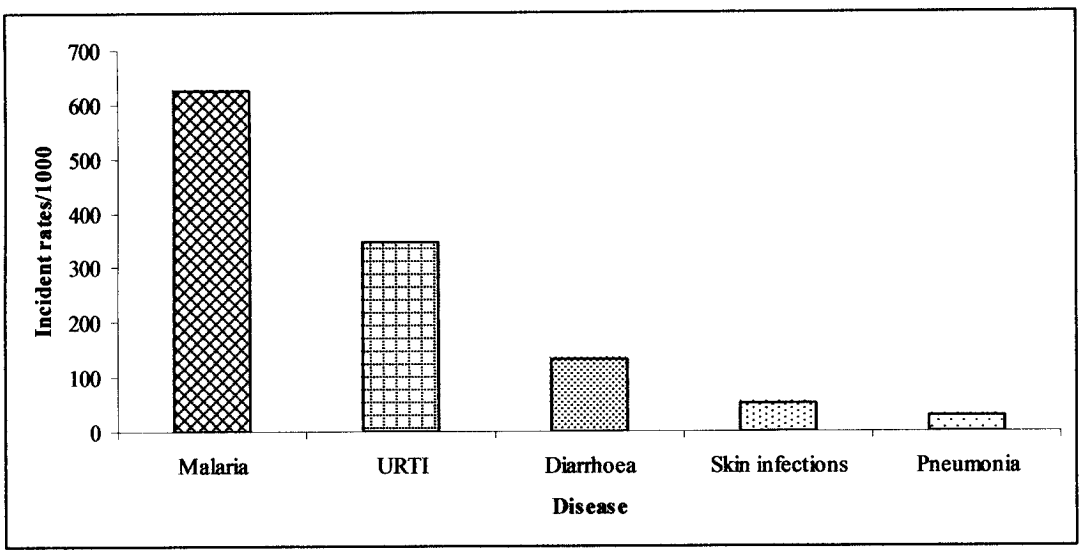
920 µl ddH_2O, Aliquot to 500 µl (Store at -20 °C)

APPENDIX: 3

Solwezi urban clinic catchment population is about 19 900, 20% of which are children under five years of age. Malaria is the leading cause of morbidity among the top five diseases in all age group and under five age group categories.



Top five causes of morbidity for all age groups at Solwezi urban clinic, Zambia in 2004.



Top five causes of morbidity in the under five age group for Solwezi urban clinic, Zambia in 2004, (URTIs - Upper Respiratory Tract Infections).

Source: Solwezi District Health Management Team, 2004

APPENDIX: 3 (continued)

Recommended treatment regimens based on *Plasmodium* parasitemia for Solwezi urban clinic

Score	Parasite density per M* field (x 100)	Recommended drug
1+	1 – 9 / 100	Fansidar® or Coartem®
2+	10 – 99 / 100	Quinine
3+	1 – 9 / 1	Quinine
4+	>10 / 1	Quinine

Note: M* - microscope (magnification of objective x 100 of eye piece)

Source: Solwezi District Health Management Team, 2004

APPENDIX: 4

TREND OF DRUG SENSITIVITY RESPONSE PATTERNS IN 11 SENTINEL SITES USING A CLINICAL CLASSIFICATION APPROACH DEVELOPED BY EXPERTS IN GENEVA, 1994 (WHO/MAL 94. 1070, 1994) From 1995 to 2000

SITE	YEAR MONTH	CLINICAL RESPONSE TO TREATMENT				ESS	Reported by: -
		ETF (%)	LTF (%)	TTF (%) (ETF+LTF)	ACR (%)		
Katete mission Hos.	June 95	5.1 CQ	8.5 CQ	13.6 CQ	86.4	(74) CQ	NMCC/TDRC
Chipata (Kapata & Prisons HC)	May 95	1.8 CQ	3.6 CQ	5.4 CQ	94.5 CQ	(70) CQ	NMCC
	Jan 97	0 SP 16.3 CQ	0 SP 12.2 CQ	0 SP 28.5 CQ	100 SP 71.4 CQ	(48) SP (49) CQ	NMCC
	Nov 99	11.9 SP 14.3 CQ	2.4 SP 11.9 CQ	15.3 SP 26.2 CQ	85 SP 73.8 CQ	(42) SP (42) CQ	NMCC
Chipata (Prisons HC)	**Apr. 2002**	**16.1 SP 22.6 CQ**	**1.8 SP 20.8 CQ**	**17.9 SP 43.4 CQ**	**82.1 SP 56.6 CQ**	**(56) SP (53) CQ**	**NMCC/TDRC**
Chongwe RHC	March 96	21.7 CQ	21.7 CQ	43.4 CQ	56.5 CQ	(46) CQ	TDRC
Chongwe RHC	**Apr. 2002**	**4.9 SP 30 CQ**	**2.4 SP 14 CQ**	**7.3 SP 44 CQ**	**93 SP 56 CQ**	**(48) SP (47) CQ**	**NMCC/TDRC**
Isoka Dis.Hos.	Apr. 96	32 CQ	22 CQ	54 CQ	46 CQ	(50) CQ	NMCC
	Nov. 99	10 SP 27 CQ	5 SP 17 CQ	15 SP 44 CQ	81 SP 46 CQ	(59) SP (59) CQ	TDRC
Isoka Dis.Hos.	**Apr. 2002**	**10 SP 42 CQ**	**0 SP 8 CQ**	**10 SP 50 CQ**	**90 SP 50 CQ**	**(50) SP (50) CQ**	**TDRC/TDRC**
Mansa (Central Clinic)	**Apr. 2002**	**6.3 SP 25 CQ**	**1.6 SP 17.9 CQ**	**7.9 SP 42.9 CQ**	**92 SP 57.1 CQ**	**(63) SP (56) CQ**	**NMCC/TDRC**

APPENDIX: 4 (continued)

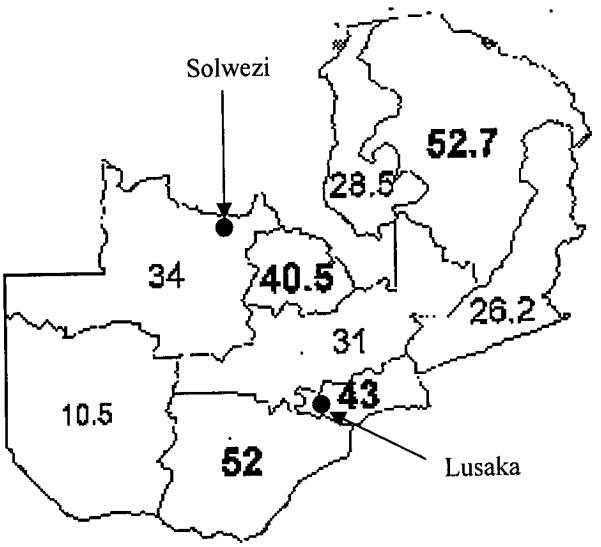
Mansa Dis. Hos.	March 96	2.4 SP 21 CQ	0 SP 12.2 CQ	2.4 SP 33 CQ	97.6 SP 65.8 CQ	(42)CQ	NMCC/USAID
	Nov. 99	7.4 SP 14.8 CQ	0 SP 9.3 CQ	7.4 SP 24.1 CQ	92.6 SP 75.9 CQ	(54) SP (54) CQ	NMCC
Lundazi Dis. Hos.	Feb. 96	2.7 CQ 0	21.4 CQ 2.4 SP	24.1 CQ 2.4 SP	77 CQ 97.6 SP	(52) CQ (41) SP	NMCC/USAID
	May 97	8.9 CQ	16.1 CQ	25 CQ	75 CQ	(56)CQ	NMCC/USAID
Choma Dis. Hos.	March 96	22 CQ	30 CQ	52 CQ	48 CQ	(50) CQ	NMCC/USAID
Mpongwe Mission Hos.	Jan. 96	7.1 CQ	21.4 CQ	28.5 CQ	71.4 CQ	(55) CQ	NMCC/TDRC
	Feb. 97	0 CQ	6.6 VCQ	6.6 CQ	93.4 CQ	(60) CQ	NMCC/TDRC
	May 97	4.2 SP 31.5 CQ	0 SP 20.4 CQ	4.2 SP 51 .9 CQ	95.8 SP 48.1 CQ	(48) SP (54) CQ	NMCC/TDRC
Mpongwe Mission Hos.	**Apr. 2002**	**6 SP 4 CQ**	**2 SP 22 CQ**	**8 SP 26 CQ**	**90 SP 74 CQ**	**(52) SP (54) CQ**	**TDRC/NMCC**
Mwinilunga Gen. Hos.	Apr. 96	14.2 CQ	19.9 CQ	34.1 CQ	66.1 CQ	(59) CQ	TDRC
Sesheke Dis. Hos.	Apr. 96	2.4 CQ	4.2 CQ	8.4 CQ	91.1 CQ	(47) CQ	NMCC/TDRC
	Jan 2000	1.9 SP 8.5 CQ	0 SP 4.3 CQ	1.9 SP 12.8 CQ	98.1 SP 87.2 CQ	(53) SP (47) CQ	NMCC
Chibombo	Jun, 97	0 CQ	30.6 CQ	30.6 CQ	69.4 CQ	(49) CQ	NMCC/TDRC

Abbreviations: - ETF=Early Treatment Failure, LTF=Late Treatment Failure, ACR=Adequate Clinical Response, ESS=Variable Sample size, CQ=Chloroquine, S/P= Sulfadoxine/Pyrimethamine, Dis=District, Hos=Hospital, RHC=rural Health Center, Miss=Mission. ?=Data not available see report above. **NB. All figures represent percentages except for ESS values in brackets.**

Source: National Malaria Control Centre, Lusaka Zambia, 2003.

APPENDIX: 5

CHLOROQUINE CLINICAL TREATMENT FAILURE RATES FOR THE NINE PROVINCES OF ZAMBIA



Chloroquine Clinical Treatment Failure: 1995 – 2000, standardized protocol (all values shown are in percentage). Source: National Malaria Control Centre, Lusaka Zambia, 2003.