The role of mutations in uncomplicated *Plasmodium falciparum* malaria and sulfadoxine pyrimethamine efficacy in Mpumalanga Province, South Africa.

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

Johannesburg, 2005

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

…….

……..day of ……2005.

DEDICATION

In memory of my mother

Sonile Loster

1920 – 1991

ABSTRACT

The antifolate combination of sulfadoxine and pyrimethamine (SP) is one of few remaining affordable drug combinations available for wide-scale treatment of uncomplicated *Plasmodium falciparum* malaria in Africa. *In vivo* studies of SP efficacy conducted during 1998, 2000 and 2002 at the Naas sentinel site in Mpumalanga province, South Africa, demonstrated a gradual non-significant increase in late treatment failure (LTF) and early treatment failure (ETF) resistance to SP, while gametocyte carriage increased significantly between 1998 and 2002 ($p < 0.0001$). This study aimed to determined and compare the frequency of dihydrofolate reductase (*dhfr*) and dihydropteroate synthetase (*dhps)* resistant haplotypes in *P. falciparum* parasites from patients treated with SP in three consecutive standardized *in vivo* therapeutic efficacy studies in Mpumalanga province, since implementation of SP as first line treatment in 1998, and to investigate associations between the presence of mutations and treatment outcomes after SP treatment. Four hundred-and-three samples were studied and 358 yielded polymerase chain reaction products. A novel high throughput sequence-specific oligonucleotide probe-based approach was used to examine the resistance status of the three *in vivo P. falciparum* populations. Screening for the presence of all known point mutations in *dhfr* and *dhps* genes revealed that only five *dhfr* and three *dhps* allelic haplotypes were present. In all the samples investigated, point mutations were identified only at codons 108, 51 and 59 of the *dhfr* gene and at codons 347 and 540 of the *dhps* gene. The prevalence of *dhfr* resistant haplotypes was 35.4% in 1998, 38.7% in 2000, and 41.0% in 2002, while the prevalence of *dhps* resistant haplotypes was 9.7% in 1998, 7.2% in 2000 and

41.6% in 2002, the latter representing a significant increase ($p < 0.002$). The prevalence in both *dhfr* and *dhps* gene resistant haplotypes were selected gradually during the three *in vivo* studies in Mpumalanga province. Infection with parasites having triple *dhfr* mutations and double *dhps* mutations, "the quintuple mutant", was associated with SP treatment failure ($p < 0.001$). Mutations at both *dhfr* and *dhps* loci may be important predictors of SP resistance in Mpumalanga province.

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LIST OF ABBREVIATIONS

- ACPR- Adequate clinical and parasitological response.
- CSPD- Disodium 3-(4- methoxyspiro (1,2- dioxetane-3,2'- (5'-chloro)tricyclo(3.3.1.1^{3,7}) decan) 4-yl) phenyl phosphate.
- DDT- Dichlorodiphenyltrichloroethane.
- DHFR- Dihydrofolate reductase.
- DHPS- Dihydropteroate synthetase.
- DIG- Digoxigenin.
- DNA- Deoxyribonucleic acid.
- DOH- Department of Health.
- DUMP- Deoxyuridine monophosphate.
- DTMP- Deoxythymidylate monophosphate.
- EDTA- Ethylenediamine tetra-acetic acid.
- ETF- Early treatment failure.
- GLURP- Glutamate rich protein.
- GTP- Guanosine triphosphate.
- LCF- Late clinical failure.
- LPF- Late parasitological failure.
- LTF- Late treatment failure.
- MgCl- Magnesium chloride.
- MSP-1- Merozoites Surface Protein-1.
- MSP-2- Merozoites Surface Protein-2.
- NADPH- Nicotinamide adenine dinucleotide phosphate dehydrogenase.

- NaOH- Sodium hydroxide.
- P. - Plasmodium.
- PABA- *p*-aminobenzoic acid.
- PBS- Phosphate buffered saline.
- PCR- Polymerase chain reaction.
- PCT- Parasite clearance time.
- SDS- Sodium Dodecyl Sulfate.
- SNP- Single nucleotide polymorphism.
- SP- Sulfadoxine Pyrimethamine.
- SSOP- Sequence specific oligonucleotide probe.
- SSPE- di-sodium phosphate ethoxyacetic acid.
- TMAC- Tetramethylammonium chloride.

CHAPTER ONE

INTRODUCTION

1.1 Overview of malaria transmission and treatment.

Malaria remains the most prevalent and devastating parasitic disease in the tropics (WHO, 1993; 2001). Worldwide, it causes 300-500 million clinical cases and more than one million deaths per annum. More than 80% occur in sub-Saharan Africa and more than 90% of malaria deaths (approximately 3.000 deaths each day), and almost all the deaths are children younger than five years. In highly endemic countries, malaria during pregnancy is a leading cause of low birth weight, one of the primary causes of neonatal mortality (WHO, 2001). Malaria is transmitted to human by the bite of an infected female anopheles mosquito. Human malaria is an infectious disease caused by four species of the *Plasmodium* parasite namely: *Plasmodium falciparum* (*P. falciparum*), *Plasmodium malariae* (*P. malariae*), *Plasmodium ovale* (*P. ovale*) and *Plasmodium vivax* (*P. vivax*). In Sub-Saharan Africa over 90% of human malaria infections are due to *P. falciparum* infection. *P. falciparum* is the only species associated with severe morbidity and mortality (WHO, 2002). The other three species generally cause milder illness; however, infections with *P. ovale* and *P. vivax* may relapse months later if appropriate treatment is not provided.

Early diagnosis and prompt treatment is the principal component of the global strategy to control malaria (WHO, 1993; 2001). Its effectiveness is highly dependent on access to antimalarial drugs, which should be safe, effective,

affordable and acceptable to the population at risk. The emergence and rapid spread of *P. falciparum* resistance to widely used antimalarial drugs, such as chloroquine, poses a serious challenge to the effectiveness of global malaria control efforts (Bloland *et al.,* 1993; Bloland and Ettling, 1999; Marsh, 1998).

1.2 Treatment of malaria in South Africa.

In South Africa, malaria occurs in limited areas mainly in the low altitude areas of Limpopo, Mpumalanga and north - eastern KwaZulu-Natal (DOH, 1996). Historically malaria was a major cause of mortality and morbidity, claiming thousands of lives and causing massive economic losses (Le Sueur *et al*., 1993). The fight against malaria in South Africa dates from the early 1940s, beginning in a few localities of relatively greater economic and social importance, and consisted of efforts to eliminate breeding places of malaria vectors and chemotherapy with quinine. The advent of dichlorodiphenyltrichloroethane (DDT) and its widespread introduction for vector control in 1946 reduced the number of malaria cases and dramatically constricted the geographical area experiencing seasonal malaria to about 30% of the original (Le Sueur *et al*., 1993). Malaria is now limited to the north - eastern border areas of South Africa (Figure 1.1), including the Ehlanzeni Region of Mpumalanga (Figure 1.2) and Limpopo Province, and the north-eastern part of KwaZulu-Natal where it remains a major cause of illness, death and a major consumer of provincial health budgets (Strebel *et al*., 1988; Hansford and Muller, 1990; Hansford, 1994).

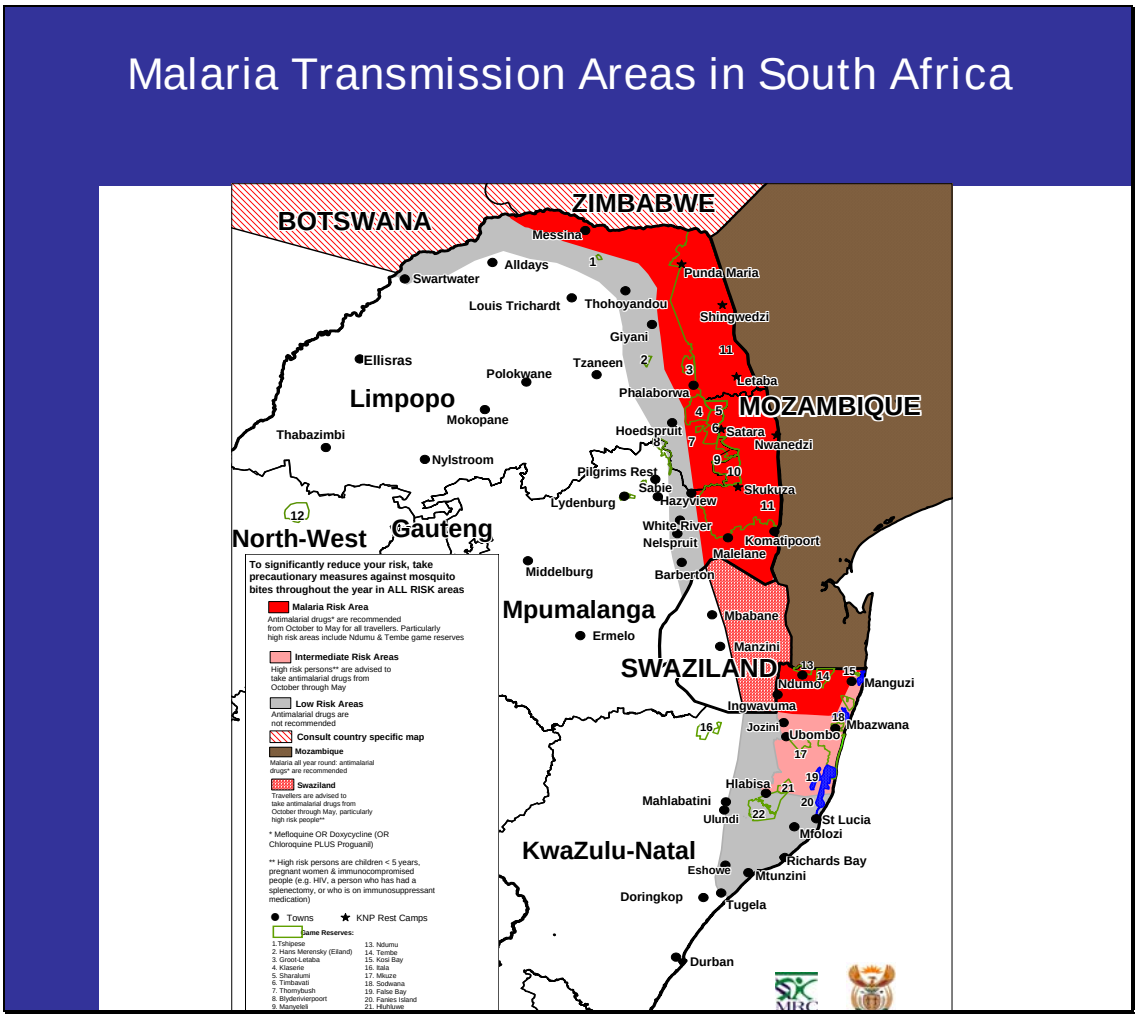


Figure 1.1: Malaria risk areas in South Africa.

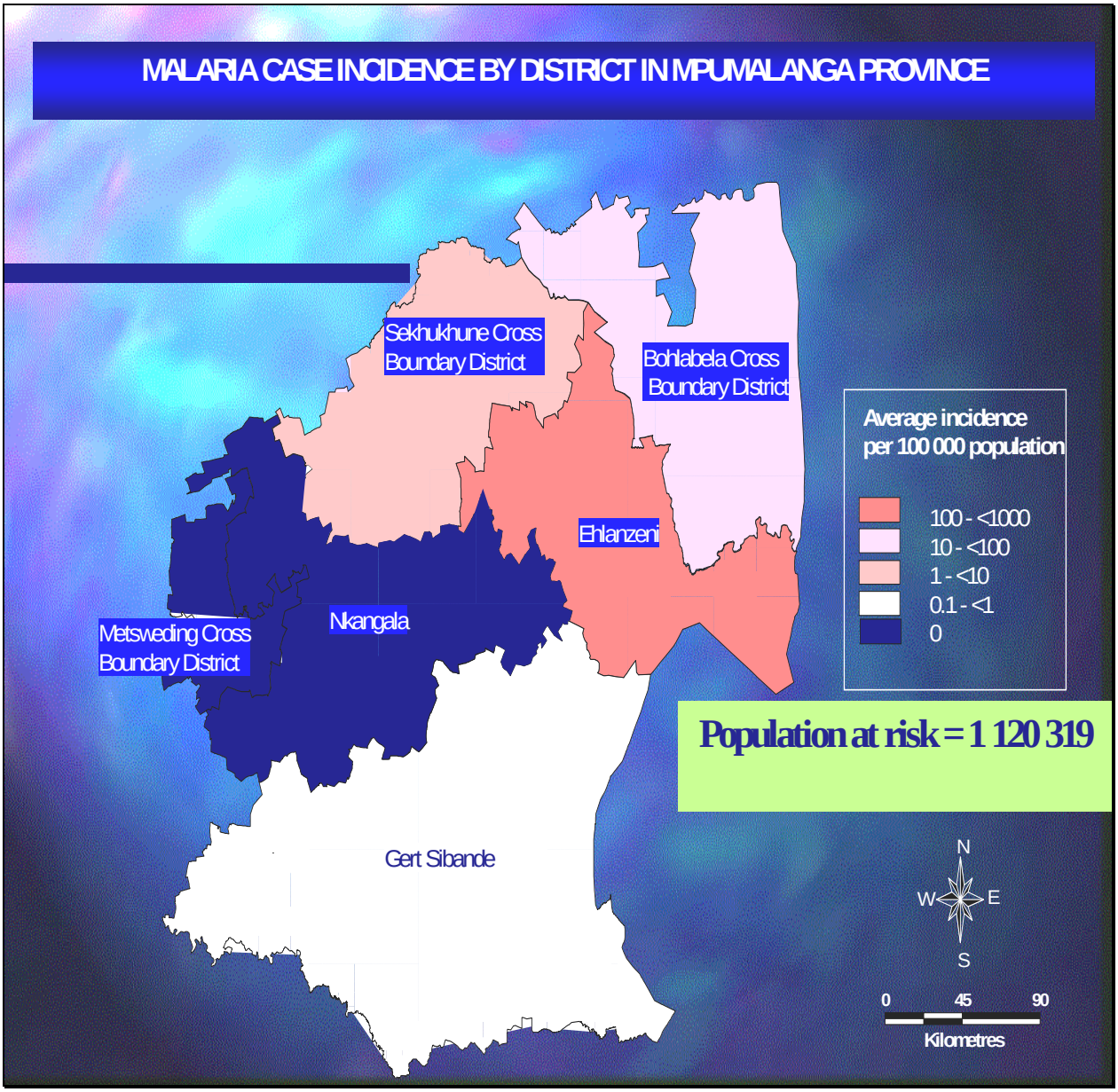


Figure 1.2: Malaria risk areas in Mpumalanga province.

Plasmodium falciparum was regarded as sensitive to chloroquine in South Africa until 1988, when *in vitro* (Deacon *et al.,* 1994; Freese *et al.,* 1994), unstandardised *in vivo* (Hansford, 1989) and opportunistic case follow-up (Kruger *et al.,* 1996) studies found evidence for, and documented resistance to, chloroquine. Unacceptably high levels of parasitological and clinical failure were found in a study in Mpumalanga province conducted according to a standardized WHO protocol in 1997, with a total RI/RII/RIII parasitological failure rate of 48.4% with 28/42 days of follow up. As a result, chloroquine was replaced with sulfadoxine/pyrimethamine (SP) as the first-line treatment of uncomplicated *P. falciparum* malaria throughout Mpumalanga (Govere *et al.,* 1999).

After five years of SP use, it is now a priority to study the nature and frequency of genetic determinants of *P. falciparum*, resistant haplotypes and their associations with *in vivo* SP therapeutic efficacy in Mpumalanga province. To investigate this issue, we carried out a genetic analysis of *P. falciparum* populations in passively-detected patients presenting with uncomplicated malaria. Specimens were collected during *in vivo* treatment cure rate (therapeutic efficacy) studies conducted in 1998, 2000 and 2002 at Naas health facility, the sentinel surveillance site for assessing malaria treatment cure rate in Mpumalanga province.

The work presented here gives first a literature review followed by aims, methods, results and discussion. A comprehensive bibliography is included.

CHAPTER TWO

LITERATURE REVIEW

2.1 The *de novo* selection of resistance

Patients with malaria generally have between 10^8 and 10^{12} haploid parasites circulating in their blood at the time of treatment, so even at the most conservative estimate of mutation rate new resistance mutations could be expected to rise and be selected for within one patient (White *et al*., 1999). In the emergence and spread of resistance to antimalarial drugs, the recrudescence and subsequent transmission of an infection, which generated *de novo* resistant malaria parasites, is necessary for resistance to be propagated (White *et al*., 1999). If resistance is low grade, or combination treatment is given that is highly effective, then resistance may confer only a very small increase in the treatment. Resistance to SP appears to arise *de novo* much less frequently, although it spreads very rapidly (Watkins *et al*., 1999). Sequence analysis of dihydrofolate reductase (*dhfr)* and dihydropteroate synthetase (*dhps*) in *P. falciparum* sampled worldwide has shown that resistance alleles with multiple mutations predominate in regions of high drug use where SP resistance problems are established (Wang *et al*., 1997a). Studies on genetic changes analysis have shown that gene flow rather than new mutations has been the most common originator of resistance in African countries (Roper *et al*., 2003).

2.2 Mode of sulfadoxine-pyrimethamine action

Folate synthesis is essential to parasites because they are unable to scavenge pyrimidines from their host (Ferone, 1977). Folate is an essential cofactor in one-carbon substitutions (Foote and Cowman, 1994), especially in the synthesis of thymidylate, acting with thymidylate synthase in the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidylate monophosphate (dTMP). For this reaction N^5, N^{10}–methylene tetrahydrofolate is converted to dihydrofolate and is cycled back to its original reduced form by dihydrofolate reductase and nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH) (Foote and Cowman, 1994). Folate itself is synthesized by the parasite and has as part of its precursor, *p*-aminobenzoic acid (PABA) that reacts with a pterin derivative in a reaction catalysed by dihydropteroate synthetase (Ferone, 1973). Dihydrofolate synthase converts this to dihydrofolate. The two major antimalarial drug targets in this pathway are dihydropteroate synthetase and dihydrofolate reductase. Sulfadoxine acts by inhibition of dihydropteroate synthetase (Triglia *et al*., 1997), whilst pyrimethamine inhibits dihydrofolate reductase (Ferone, 1977) and both of these enzymes are in the folate biosynthetic pathway (Figure 2.1). Figure 2.2 presents structures of both sulfadoxine and pyrimethamine antifolates. Blocking the synthesis of folate results in decreased synthesis of pyrimidines and consequently the arrest of DNA replication, as well as decreased methionine synthesis and reduced conversion of glycine to serine (Foote and Cowman, 1994). Depletion results in cell cycle arrest and finally death of the parasite.

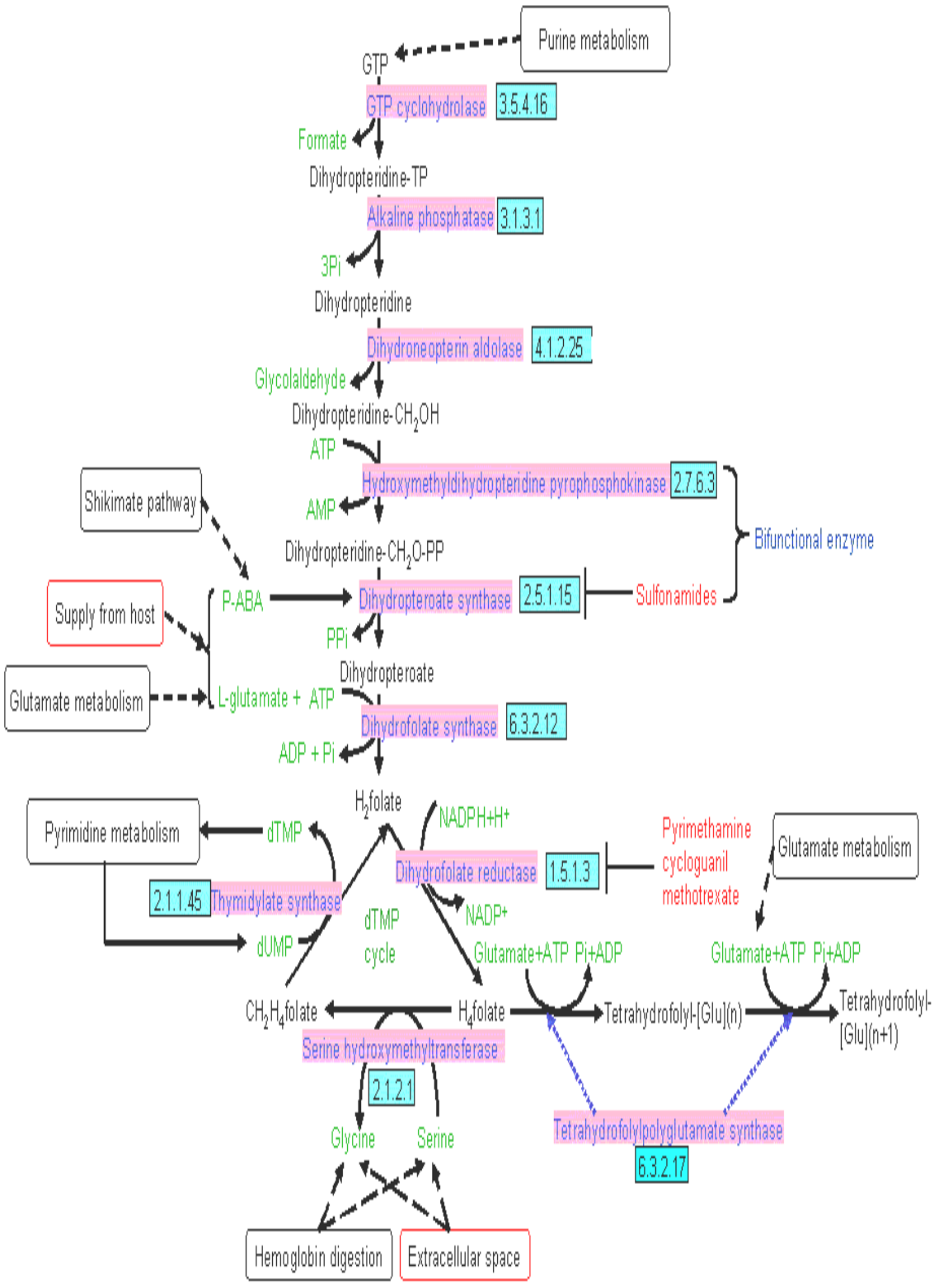
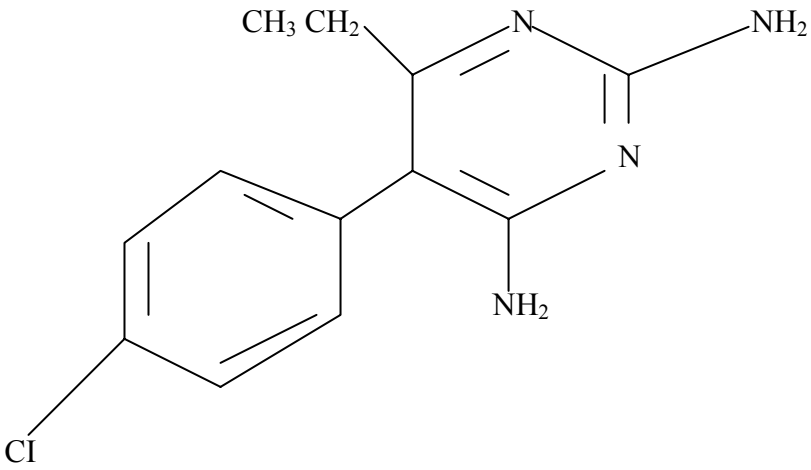
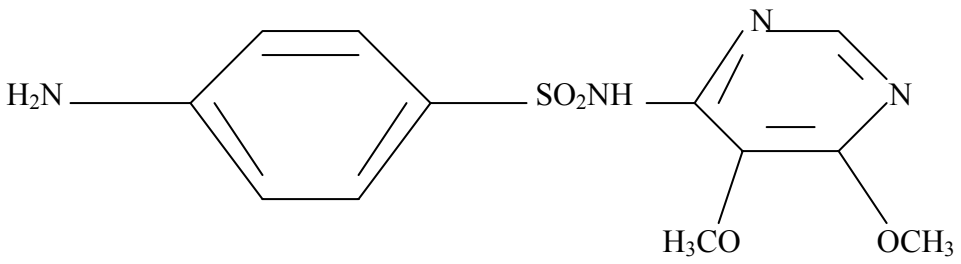


Figure 2.1 Summary of folate pathway



Pyrimethamine



Sulfadoxine

Figure 2.2 Antifolates structures of pyrimethamine and sulfadoxine.

2.3 Mechanism of sulfadoxine-pyrimethamine resistance

Sulfadoxine/pyrimethamine is a synergistic combination (Chulay *et al*., 1984). The drugs act as folate pathway blockers in the malaria parasite and thus interfere with the synthesis of DNA from guanosine triphosphate (GTP). Pyrimethamine inhibits *dhfr*, whereas sulfadoxine inhibits *dhps* (Bzik *et al*., 1987; Triglia and Cowman, 1994). A remarkable feature of *P. falciparum dhfr* mutations both in resistant and sensitive parasites is that the mechanism of resistance depends on the accumulation of point mutations (Cowman *et al*., 1988; Peterson *et al*., 1988; Zolg *et al*., 1989). The critical mutation is a serine to asparagine change at position 108, which is found in all resistant isolates. Altering Cys to Arg at position 59 and Asn to Ile at position 51 (Cowman *et al*., 1988; Peterson *et al*., 1988; Zolg *et al*., 1989) increases pyrimethamine resistance.

Resistance to sulfadoxine and sulfones results from mutations within *dhps* (Triglia and Cowman, 1994; Triglia *et al*., 1997; 1998; Brooks *et al*., 1994). Amino acid changes at four positions (Ser-436, Gly-437, Ala-581, and Ala-613) confer resistance to sulfadoxine and also cross-resistance to sulfones and sulfonamides (Triglia *et al*., 1997; 1998).

2.4 Monitoring sulfadoxine -pyrimethamine therapeutic efficacy

Sulfadoxine and pyrimethamine have long elimination half-lives of 116h and 81h, respectively (Winstanley *et al*., 1992; 1997; Mbongo *et al*., 1995), which was initially thought to be an advantage because of substantial prophylaxis conferred, but detailed follow-up has shown that for 15-52 days after SP treatment there is strong selective pressure, especially for pyrimethamine (Wang *et al*., 1997a; Watkins *et al*., 1997; Diourte *et al*., 1999, Watkins and Mosobo, 1993).

The SP combination is generally affordable and commonly used to replace chloroquine as first-line treatment in Africa, when the latter fails. Unfortunately, however, wherever SP has been widely used, the selection of resistant *P. falciparum* parasite populations has emerged (Wernsdorfer, 1994; White, 1992; White and Olliaro, 1996), thus reducing the useful therapeutic life of this drug. In East Africa, where SP has been the first-line treatment for less than five years, selection of parasites resistant to SP has already occurred, and the clinical effectiveness of the formulation already decreased (Curtis *et al.,* 1998; Jelinek *et al.,* 1998; 1999; Mberu *et al.,* 2000; Nzila *et al.,* 1998; 2000a; 2000b; Plowe *et al*., 2004). In KwaZulu-Natal, SP was introduced as the first-line treatment for *P. falciparum* malaria in 1988. Results of a small 1996 hospital-based study found a 23.5% ETF combined with LTF rate at 28 days (Medical Research Council, unpublished data) and 20% of patients with proven malaria infection treated with SP did not clear their parasites within 14 days of treatment. A subsequent study conducted in 2000 in the province revealed a 88% treatment failure rate with SP monotherapy (Bredenkamp *et al.,* 2001).

In vivo evaluation of SP's therapeutic efficacy as primary treatment for uncomplicated *P. falciparum* malaria, with 42 day follow up and PCR differentiation of recrudescence from reinfection, at introduction into the public health programme in Mpumalanga province revealed a 94.5% cure rate, and a combined ETF / LTF rates resistance of only 5.5% in 1998 (Govere *et al.,* 1999). A subsequent study, three years after introduction (2000), revealed a 93.6% cure rate (a non-statistically significant change), and combined ETF/LTF rates resistance of 6.3% (Mabuza *et al.,* 2000). A recent study conducted five years after SP introduction (2002) revealed a 90.5% cure rate, and a combined ETF/LTF rates resistance of 9.5%, indicating a steady but not statistically significant increase in resistance (Mabuza *et al*., 2005).

2.5 The effect of gametocytes to SP treatment response

Recrudescent infections are associated with increased gametocyte carriage rates, which provide a powerful selection pressure to the spread of resistance (Price *et al*., 1998). Significantly higher gametocyte densities on day 7 and day 14 were strongly recorded in SP treatment (Von Seidlein *et al*., 2000). A study in KwaZulu-Natal showed a similar peak in gametocyte carriers during the period day 8 and day 14 (Freese *et al*., in preparation). Sulfadoxine pyrimethamine has no known gametocytocidal properties (Sinden, 1983) and gametocyte generation and development appears to persist despite SP treatment.

2.6 The importance of mutations in *dhfr* and *dhps* to SP *in vitro*.

Both the *dhfr* and *dhps* mutations occur in a progressive, step-wise fashion, with higher levels of *in vitro* resistance occurring in the presence of multiple mutations (Plowe *et al*., 1997; Wang *et al*., 1997a). Distribution of mutations in the *dhfr* and *dhps* genes and their association with SP resistance have been assessed in different geographic areas, and it has been found that a greater number of mutations in both *dhfr* and *dhps* is a good predictor of drug failure (Zindrou *et al*., 1996; Plowe *et al*., 1997; Nagesha *et al*., 2001; Wang *et al*., 1997c).

Point mutation causing a Ser to Asn change at position 108 confers pyrimethamine resistance *in vitro* with only a moderate loss of susceptibility to the *dhfr* inhibitors cycloguanil and chlorcycloguanil. Transfection studies have implicated the Asn 108 mutation as playing a central role in pyrimethamine resistance (Brobey *et al*., 1996; Wu *et al*., 1996). However, several other studies have demonstrated that parasites with this mutation alone were cleared by SP (Wang *et al*., 1997b; Jelinek *et al*., 1997; Rallon *et al*., 1999).

Compared with the wild-type sequence, a point mutation in the *dhfr* gene resulting in a change from Ser 108 to Asn 108 (S108N), increases resistance to pyrimethamine about 100-fold (Cowman *et al.,* 1988). Additional mutations, altering Asn 51 to Ile (N51I), Cys 59 to Arg (C59R) and Ile 164 to Leu (I164L), progressively increase pyrimethamine resistance, with triple and quadruple mutants approximately an order of magnitude more resistant then single mutations (Wang *et al.,* 1997b; Hyde, 1990; Warhust, 1997).

Point mutations in the *dhps* domain are the predominant mechanism of sulfadoxine resistance development in *P. falciparum* (Wang *et al.,* 1997c; Triglia *et al.,* 1998). Changes in five different amino acids have been observed in *P. falciparum* in laboratory reference isolates: Ser 436 to Ala or Phe (S436A/F), Ala 437 to Gly (A437G), Lys 540 to Glu (K540E), Ala 581 to Gly (A581G) and Ala 613 to Ser or Thr (A613S/T). No mutations have yet been reported outside of these five codons. Although not all of the observed mutations have been tested in isolation for their individual contribution to parasite resistance, all of those tested have been associated with increased resistance to sulfadoxine, a number of other sulfonamides, and sulfone dapsone (Triglia and Cowman, 1999). Among these, Gly-437, followed by Glu-540, are the most strongly associated with SP treatment failure in Africa (Nzila *et al*., 2000a; Plowe *et al*., 1997).

2.7 The importance of mutations in *dhfr* and *dhps* to SP *in vivo* therapeutic efficacy.

The relative importance of mutations in *dhfr* and *dhps* to *in vivo* SP resistance has been debated (Sims *et al*., 1998; Watkins *et al*., 1999). Parasites with fewer than the three *dhfr* mutations Asn-108, Ile-51, and Arg-59 have been cleared by SP, regardless of *dhps* genotype (Wang *et al*., 1999). In other studies, the presence of multiple mutations in both *dhfr* and *dhps* was associated with resistance *in vivo* to SP (Plowe *et al*., 1998). In the presence of both *dhfr* and *dhps* genotypes, there is an increased likelihood of treatment failure. This is consistent with field studies that demonstrate an association between the prevalence of mutations in both genes and SP treatment failure rates, and selection for mutations in both genes

after SP treatment (Plowe *et al*., 1997; Curtis *et al*., 1998; Wang *et al*., 1997a; Kublin *et al*., 1998).

Other studies have associated particular point mutations that confer resistance to SP *in vivo* with treatment failure (Kublin *et al*., 2002; Omar *et al*., 2001), and found an overrepresentation of mutations in recrudescent infections after treatment (Basco *et al*., 2000; Brooks *et al*., 1994; Cortese *et al*., 1998; Curtis *et al*., 1998; Doumbo *et al*., 2000; Edoh *et al*., 1997; Jelinek *et al*., 1997; 1999; Khan *et al*., 1997; Nzila *et al*., 2000a). Interpretation of these studies is complicated by mixed infections. As the blood stage parasites are haploid, the occurrence of two or more genotypes in a specific infection means that variation at multiple sites cannot be assigned to an individual parasite line within the infection and, accordingly, that the predictive association with treatment outcome is rendered less straightforward. Acquired malaria immunity further adds to the complexity of describing the association between *dhfr* and *dhps* mutations with therapeutic outcome. In Africa, the *dhfr* triple mutant Asn-108, Ile-51 and Arg-59, and the *dhps* double mutant Gly-437 and Glu-540 have been most strongly associated with resistance to SP (Kublin *et al*., 2002; Nagesha *et al*., 2001). Various combinations of *dhfr* and *dhps* mutations have been associated with relatively high levels of SP therapeutic failure (Nzila *et al.,* 2000a; Basco *et al.,* 1998; Pearce *et al*., 2003). In most of sub-Saharan Africa, however, not only do *dhfr* and *dhps* mutations occur in many combinations, but most infections are polyclonal (Doumbo *et al*., 2000; Gupta *et al*., 1994; Farnert *et al*., 1999), and, therefore, whether mutations reside in the same parasites within a single infection

cannot be conclusively determined. Polyclonal infections may also be mixed in relation to the loci of interest, that is, individual infections carry some parasites with mutations at specific *dhfr* or *dhps* codons and other parasites without mutations at those codons.

2.8 The role of mutations *dhfr* and *dhps* to other antifolate drugs.

In response to the spread of SP resistance, alternative inexpensive antifolate drugs are being investigated. In some areas, proguanil has been used alone or combined with chloroquine for malaria prophylaxis (Winstanley, 2000). In most humans, proguanil is metabolized to cycloguanil, which is also a competitive inhibitor of *dhfr*. The affinity of cycloguanil for the wild-type enzyme is higher than that observed for pyrimethamine, but when the I51, R59 or L164 mutations are added to the N108 genotype, the affinity of cycloguanil for *dhfr* is progressively reduced (Sirawaraporn et al., 1997).

The combination of chlorproguanil and dapsone has been investigated as a therapeutic alternative to SP (Winstanley, 1997; Amukoye *et al*., 1997). Like proguanil, the biguanide chlorproguanil is metabolized to its active triazine form, chlorcycloguanil, which acts on *dhfr*. Dapsone, like sulfadoxine, inhibits the activity of *dhps*. Based on *in vitro* analysis, each drug has a higher affinity for its target enzyme than do pyrimethamine and sulfadoxine, respectively. Unfortunately, SP selects mutations in *dhfr* and *dhps* that also diminish the

affinity of chlorcycloguanil and dapsone (Nzila *et al*., 1998; Cortese *et al*., 1998; Triglia *et al*., 1997).

2.9 Molecular methods for detection of mutations in the *P. falciparum.*

The genes determining resistance of *P. falciparum* to some antimalarials have been identified, and molecular methods for the detection of alleles conferring drug resistance have been established (Peterson *et al*., 1988; Reed *et al*., 2000; Fidock *et al*., 2000). These methods include mutation-specific polymerase chain reaction (MS-PCR) (Zolg *et al*., 1990) and polymerase chain reaction (PCR) followed by restriction enzymes digestion of amplified DNA fragments (Duraisingh *et al*., 1998). The former method provides greater accuracy and sensitivity than the latter (Shaio *et al*., 1998), although reproducibility relies on having few copies of the DNA template. PCR coupled with restriction enzyme digestion is reproducible but relatively less sensitive in revealing minority parasite populations in multiple infections (Shaio *et al*., 1998), where constituent clones may vary in their response to antimalarials (Thaithong *et al*., 1984). Thus, the specificity and sensitivity of both MS-PCR and PCR coupled with restriction digestion may be compromised by quantitative variations of different *P. falciparum* clones in natural infections.

The sequence-specific oligonucleotide probes (SSOP) method has the advantange of high throughput, while retaining sensitivity and specificity equivalent to those methods used for detection of *dhfr* and *dhps* single nucleotide polymorphisms (SNP) (Adbel-Muhsin *et al*., 2002; Randford-Cartwright *et al*., 2002). It is a PCR high-throughput method, which uses sequence-specific oligonucleotide probes for detection of known single nucleotide polymorphisms to identify haplotypes. Haplotypes are combinations of SNP in the same gene in the same parasite, as distinct from associations of point mutations that co-occur because of a mixture of parasites of different genotypes within a single infection. Haplotypes are biologically meaningful, since they determine the resistance properties of parasites that are exposed to drugs at the time of treatment. When comparing populations, it is important to measure the frequency of haplotypes rather than the prevalence of each point mutation separately, because these haplotypes are the determinants of SP resistance levels.

2.10 Implications of literature review for this research

1. Sulfadoxine pyrimethamine resistance is conferred by the accumulation of point mutations in the *dhfr* and *dhps* enzymes. Drug pressure is considered the main causative factor in the selection and spread of resistant *P. falciparum* strains.
2. In three consecutive *in vivo* SP efficacy studies; the status of SP resistance in Mpumalanga province over a period of five years was monitored. The studies revealed a steady but non-significant increase in levels of SP resistance. The frequency and nature of *dhfr* and *dhps* resistant haplotypes and their association with *in vivo* SP efficacy are not known.
3. As blood stage parasites are haploid, the co-occurrence of two or more genotypes in an infection means that variation at multiple sites cannot be assigned to an individual parasite line within the infection and, accordingly, that the predictive association with treatment outcome is less straightforward. The introduction of SSOP in our study enabled us to assess the evolutionary origins of *dhfr* and *dhps* resistance determinants in Mpumalanga *P. falciparum* populations.
4. Thus, the contribution of allelic haplotypes of *dhfr* and *dhps* mutants need to be studied and compared with therapeutic outcome in the field studies conducted over the five years since the implementation of the SP malaria treatment policy. Larger sample sizes were also possible using this high-throughput method.

2.11 OBJECTIVES

2.11.1 Main objectives

This study aims to determine and compare the frequency of *dhfr* and *dhps* resistant haplotypes in *P. falciparum* parasites from patients treated with SP in three consecutive standardised *in vivo* therapeutic efficacy studies in Mpumalanga province, South Africa since implementation of SP as first line treatment in 1998, and to investigate associations between the presence of mutations and treatment outcomes after SP treatment.

2.11.2 Specific objectives

- To determine mutation frequencies in the *dhfr* and *dhps* haplotypes of *P. falciparum* isolates collected during three consecutive standardised *in vivo* SP drug efficacy studies using molecular methods.
- To correlate the frequency of resistant haplotypes to *in vivo* clinical and parasitological responses to SP in 1998, 2000 and 2002.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

The study was conducted in the Tonga health district (25°C 15'S, 32°C15'E) of Mpumalanga province, South Africa. Approximately 70% of the province's malaria cases occur in Tonga. The area has a population of 116 400 people and borders Mozambique in the east and Swaziland in the south. Malaria transmission in the area is seasonal, extending from October to May, with an annual peak from January to March coinciding with peak summer rainfall. Low intensity and seasonal pattern of malaria transmission preclude the development of any acquired malaria immunity, and malaria morbidity and mortality affects all age groups. *P. falciparum* accounts for in excess of 90% of reported malaria cases, while *P. malariae* and *P. ovale* make up the difference (Govere *et al*., 2001). Approximately 1938 malaria cases in 1998, 4212 in 2000 and 3190 in 2002 were considered to be imported from neighbouring Mozambique (DOH, 2003). Figure 2.1 presents malaria cases recorded during malaria seasons as from 1997/1998 till 2001/2002 inclusive. *Anopheles arabiensis* is the major malaria vector in the area and the cornerstones of control are annual indoor residual insecticide spraying performed by the provincial government's Malaria Control Programme, and early diagnosis and treatment provided at ten primary health care clinics and a district hospital. The climate in this area is sub-tropical, being warm and humid with daily temperatures ranging between 18°C and 35°C. Communities in the area are mainly economically dependent on subsistence farming or employment on commercial sugar cane, fruit and vegetable farms.

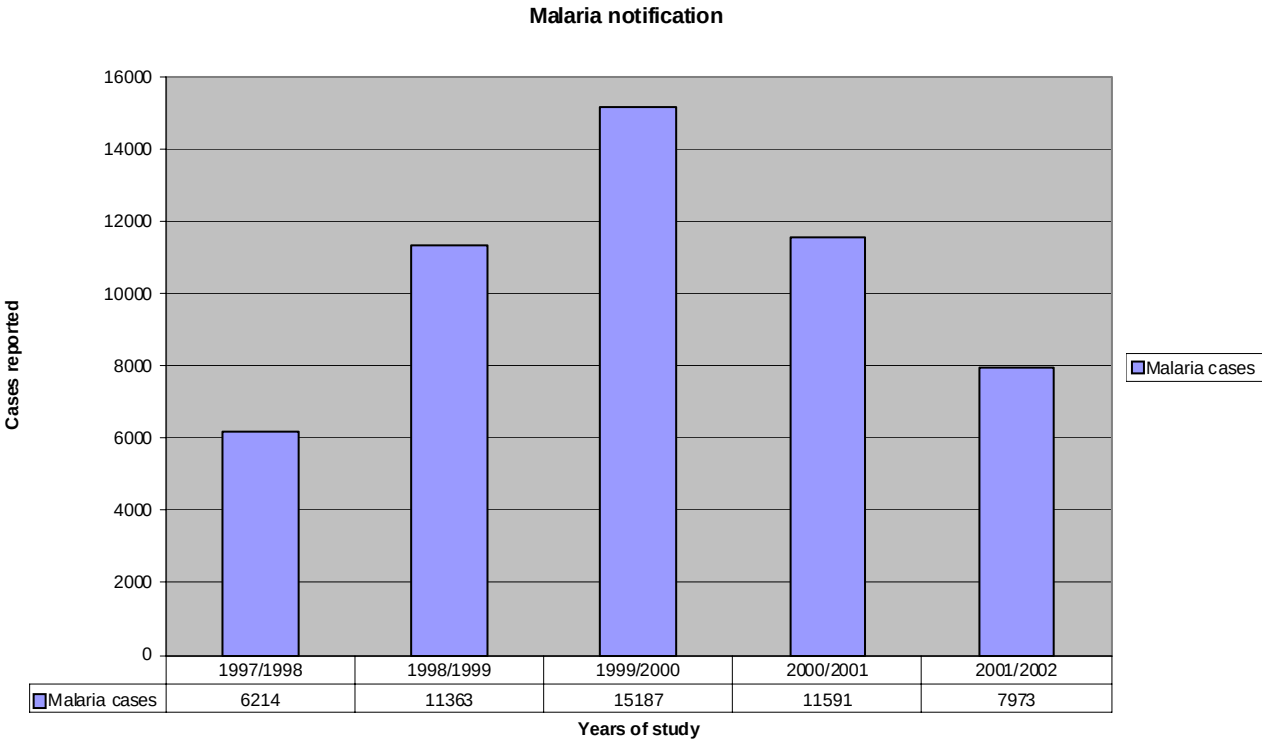


Figure 3.1 Representations of malaria notified cases between 1998 and 2002 malaria seasons in Mpumalanga province (DOH, 2003).

3.2 SP *in vivo* efficacy studies

Samples collected during standardised SP efficacy studies conducted during the year of SP introduction (1998) and then during the 2000 and 2002 malaria seasons at the same sentinel site, were used in this study. All patients with clinical features of malaria presenting at the clinic were tested for *P. falciparum* infection using a rapid immunochromatographic card test (ICT Malaria Pf^{TM} Diagnostics, Sydney, Australia for 1998 and 2000 and Core^{MT}Malaria Pf Diagnostics, Birmingham, B2 5HG, UK for 2002 samples). Positive patients were then recruited according to established World Health Organization (WHO, 1996) criteria, with inclusion criteria being:

- Age above two years.
- Symptomatic uncomplicated *P. falciparum* infection.
- *P. falciparum* asexual parasite density above 1000 parasites/µl blood.
- Proximity of patient's home for reliable follow up.
- Informed consent and history of fever or axillary temperature above 37.5°C.

Exclusion criteria included:

- Severe malaria.
- Inability to tolerate oral therapy.
- Children under two years and pregnancy.

Criteria for withdrawal included:

- Patient request.
- Clinical deterioration necessitating hospital referral.
- Loss to follow-up.

- Protocol violation.
- Self-administration of other anti-malarial drugs during follow-up.

Baseline data, including age, gender, weight and place of residence, were obtained from all study subjects by qualified healthcare providers on enrolment at the clinic.

Patients were treated according to the guidelines of the Mpumalanga Department of Health, with a single oral dose of SP, corresponding to 25mg/kg of sulfadoxine and 1.25mg/kg of pyrimethamine. After drug administration, patients were observed for one hour. If vomiting occurred within 30 minutes of drug administration, a full dose was repeated. If vomiting occurred between 30 and 60 minutes, an additional half dose was administered. Patients with clinical or parasitological treatment failure were referred to hospital for therapy with quinine according to South African guidelines for uncomplicated malaria treatment.

Clinical and parasitological assessments were conducted routinely on days 1, 2, 3, 7, 14, 21, 28 and 42 post-treatment. At each follow-up visit a thick blood smear was taken, body temperature was recorded and an assessment for adverse events was completed. Fever was defined as an axillary temperature exceeding 37.5°C. Blood samples were obtained by finger prick and blood spots were collected on filter paper strips (Whatman 3MM, Whatman International, Maidstone, UK) and allowed to air-dry before being carefully labelled and subsequently sealed in a small plastic bag, desiccated with silica gel and stored at room temperature. Parasitaemia was measured by counting the number of parasites against

300 leucocytes on a Giemsa-stained, finger-prick thick blood film and then multiplying this figure by 25, assuming a standard leucocyte count of 7500/μl blood to yield the approximate number of parasites/μl.

3.3 Classification criteria

Adequate clinical and parasitological response (ACPR) was defined as conversion from a positive smear at recruitment to a negative smear by day 7, and remaining negative until the end of the 42-day follow-up period. Recrudescence was defined as a negative blood film by day 7 and reappearance of parasites during the remaining follow-up period. Early treatment failure (ETF) was defined as warning (danger) signs or severe malaria on or before day 3, parasitaemia on day 2 higher than day 0 or parasitaemia on day 3 ≥ 25% of count on day 0 or fever and parasitaemia on day 3. Late clinical failure (LCF) was defined as development of danger signs or severe malaria after day 3 in the presence of parasitaemia; presence of parasitaemia and axillary temperature ≥ 37.5°C on any day from day 4 to day 28, without previously meeting any of the criteria of early treatment failure. Late parasitological failure (LPF) was defined as the presence of parasitaemia on any day from day 7 to day 28, and axillary temperature < 37.5°C, without previously meeting any of the criteria of early treatment failure or late clinical failure. Adequate clinical and parasitological response (ACPR) was defined as the absence of parasitaemia on day 28 irrespective of axillary temperature without previously meeting any of the criteria of early treatment failure or late clinical or parasitological failure. Parasite clearance time (PCT) was defined as the period from recruitment to the first of two successive thick smears with no asexual

parasites. Fever duration was the number of days from recruitment to the day when axillary temperature was recorded below 37.5°C without a subsequent recorded increase in temperature.

3.4 Extraction of deoxyribonucleic acid (DNA) from filter paper samples

DNA was extracted using the method of Plowe *et al*. (1995). In summary, the blood spot samples of 5mm diameter size were first soaked in 0.5% saponin in 1x phosphate-buffered saline (PBS), inverted 2-3 times in a 1.5-ml tube, and incubated overnight at room temperature. Blood spots were then washed twice in 1ml of 1x phosphate-buffered saline. Fifty microlitres of a stock solution of 20% Chelex-100 resin (Bio-Rad Richmond, CA) in water was added to 150 µl of water (pH 9.5) in a 1.5-ml tube and then boiled at 100°C for 8 minutes. After centrifugation at 10000-x g for 2 min, the supernatant was then collected into a new tube. The supernatant was used for polymerase chain reaction (PCR) investigation and the rest was stored at -20°C.

After unsuccessfully running samples with no positive DNA results, the Boom *et al.,* (1990) method for DNA extraction was attempted. This method provided good positive DNA results compared to the 0.5% saponin method. Thus Boom's method was adopted for use in this study. In summary, the blood spot segment of 5mm diameter contained in a 0.6ml Eppendorf reaction tube (Eppendorf type 3810) was first soaked in 50µl Proteinase K and incubated for 10min at 60°C in a water- bath. One hundred and fifty microlitres of lysis buffer L6 (made by dissolving 120g of GuSCN, Fluka Chemic AG. Buchs, Switzerland:

catalog no. 50990) in 100ml of 0.1M Tris hydrochloride. pH 6.4 (Boehringer GmbH. Mannheim. Federal Republic of Germany): subsequently, 22ml of a 0.2M EDTA solution (Titriplex: Merck. Darmstadt. Federal Republic of Germany) adjusted with NaOH to pH 8.0 and 2.6g of Triton X-100 (Packard Instrument Co.. Inc.. Downers Grove, III.) was added, mixed and further incubated for 10min in a 60°C water-bath. The base of the tube was repeatedly pierced with a lancet, and then placed into a 1.5ml Eppendorf reaction tube, which had its lid removed. After centrifugation for 3 min in an Eppendorf microfuge (fixed angle. 12.000-x g), the sample was transferred to a new, labeled 0.6ml Eppendorf reaction tube. After adding 25µl silica made by dissolving (60g of silicon dioxide. SiO_2: Sigma Chemical Co.. St. Louis. Mo.) in distilled water in a total volume of 500ml in a glass cylinder (height of aqueous column. 27.5 cm: width. 5 cm) the sample was kept at room temperature for ten minutes, with intermittent mixing. The specimen was then briefly centrifuged (4 sec) in an Eppendorf microfuge (fixed angle. 12.000-x g), the supernatant discarded, and 100µl of washing buffer L2 (made by dissolving 120g of GuSCN in 100ml of 0.1M Tris hydrochloride. pH 6.4) added before allowing resuspension. This process of centrifugation, adding washing buffer L2 and resuspension was repeated twice. Specimens were then washed twice with 250µl of 70% ethanol (add, resuspend, centrifuge and discard): and washed once with 250µl acetone (add, resuspend, centrifuge and discard). Thereafter, the tube was placed in an oven at 56°C to dry with open lids in an Eppendorf (Hamburg. Federal Republic of Germany) for 15min. Then 100µl TAE buffer (elution buffer made by dissolving 10mM Tris hydrochloride into 1 mM EDTA) pH 8.0 was added and resuspension allowed before incubating

for 10min in a 60°C water-bath with occasional mixing. Thereafter, the specimen was centrifuged for 2min at 12.000-x g and the supernatant containing pure DNA collected into a clean Eppendorf tube.

3.5 PCR amplification of *dhfr* and *dhps*

A 711-base pair fragment of *dhps* and a 594-base pair fragment of *dhfr* containing the polymorphic codons were independently amplified by nested PCR in a 96-well plate format. The PCR primer sequences and reaction conditions were: for *dhfr* Outer reaction, MI (5' TTTATGATGGAACAAGTCTGC 3') and 650bp, M7 (5' CTAGTATATACATCGCTAACA 3'); denaturation at 94°C for 3min, followed by 94°C for 1min, then followed by annealing at 52°C for 2min and extension at 72°C for 1min. This cycle was repeated 40 times and then followed by extension at 72°C for 10min. For *dhfr* Inner reaction, M3b (5'TGATGGAACAAGTCTGCGACGTT 3') and 594bp, M9 (5' CTGGAAAAAATACATCACATTCATATG 3'), denaturation at 94°C for 3min, then 94°C for 1min, then annealing at 44°C for 2min, followed by extension at 72°C for 1min. The cycle was repeated four times; followed by denaturation at 94°C for 1min, annealing at 44°C for 1min, and extension at 72°C for 1min. This cycle was repeated 34 times, followed by extension at 72°C for 10min.

For *dhps* Outer reaction, N1 (5' GATTCTTTTTCAGATGGAGG 3') and 770bp, N2 (5' TTCCTCATGTAATTCATCTGA 3'), denaturation at 94°C for 3min, then 94°C for 1min, annealing at 51°C for 2min, and extension at 72°C for 1min. This cycle was repeated 40 times and then followed by extension at 72°C for 10min.

For *dhps* inner reaction, R2 (5' AACCTAAACGTGCTGTTCAA 3') and 711bp, R (5' AATTGTGTGATTTGTCCACAA 3'); denaturation at 94°C for 3min, then 94°C for 1min, followed by annealing at 51°C for 2min and extension at 72°C for 1min. This cycle was repeated 40 times, and then followed by extension at 72°C for 10min.

The 25µl PCR mix contained primers at 0.25µM final concentration, 2mM $MgCl_2$, 250 µM each deoxynucleoside triphosphate, and 1x Bioline *Taq* polymerase. Template DNA (1 µl) was introduced to outer reaction mixtures. The *dhps* outer PCR product (1-µl) was introduced into a 25µl inner amplification mixture. Aliquots of 1µl of threefold-diluted *dhfr* outer PCR product were introduced into a 25µl inner amplification reaction mixture.

3.6 Preparation of DNA for blotting

The final PCR products were checked for successful amplification by running a few samples together with controls on a 1.5% agarose gel. If amplification was successful, then the final PCR products were used for blotting, by adding 20µl of PCR water to each well of the final plate. The diluted PCR products were then heat denatured at 94°C for 2min in a thermocycler, cooled, and finally spotted onto nylon membranes (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom) in 1µl volumes.

3.7 Preparation of nylon membranes for blotting

By using a pencil and a designated ruler, a rectangle of size 84mm x 70mm was drawn onto the nitrocellulose membrane, with 18 membranes prepared for *dhfr* and 14 membranes prepared for *dhps*. The 84mm length of each rectangle was divided into 12 columns by drawing lines 7mm apart. Using a surgical blade, the 84mm x 70mm rectangle nitrocellulose membranes were dissected out and transferred onto Whatman filter papers. The membranes were each labeled in the bottom left-hand corner, which then served as the tweezers corner, with membranes always handled during processing at that corner. Once the membranes were prepared, the PCR plate was aligned adjacent to the membrane with a corresponding label. The denatured PCR products were then dotted in 1µl volumes onto the membrane in the appropriate column using a multi-channel pipette, row for row and allowed to dry. The membranes were covered with Whatman filter paper and transferred into an oven for baking overnight at 80°C to fix the DNA onto the membranes. After fixing, the membranes were then transferred into 50ml Falcon tubes each labeled accordingly with the dotted side facing the inside of the tube. The membranes were then stored in the tubes at room temperature.

3.8 Probing

The hybridisation oven was set at 37°C and the water-bath at 53°C for the probing preparation. The blocking solution, di-sodium phosphate ethoxyacetic acid (SSPE) was prepared by (mixing 20ml 20 x stringency solution stock buffer, 1ml of 10% Laurylsarcosine and 1g of milk powder into 79ml of distilled water to make up 100ml final solution). A 1000ml tetramethylammonium chloride (TMAC) Hybridisation buffer was made by mixing 600ml of 5M TMAC stock solution, 50ml of 1M Tris (pH 8.0) solution, 5ml of 20% Sodium Dodecyl Sulfate (SDS) stock solution and 4ml of 0.5 EDTA (pH 8.0) solution with water added to make up 1000ml. The 1000ml of 2 x SSPE/0.1% SDS Wash Buffer was prepared by mixing 100ml of 20 x SSPE stock buffer and 5ml of 20% SDS stock solution with water added to make up 1000ml. The 1000ml of 10x Buffer 1 was made up by mixing 121.1g of Tris base (1M concentration) and 87.7g of NaCl (1.5 M concentration) with water added to make up 1000ml and the pH was adjusted to 7.5. The buffer was diluted 1:10 each time before use. The 20x SSPE stock was prepared by mixing 14.8g of EDTA, 350.6g of NaCl and 55.2g of $NaH_2PO_4.H_2O$ with water added to make up 2000ml and pH adjusted to 7.4.

The hybridisation buffer was always placed in a water-bath at 53°C. For stabilisation, 5ml of the blocking buffer was added to each 50ml tube containing membranes and this was placed on a rotisserie in a hybridisation oven for at least 30mins or overnight at 37°C. While the membranes were blocking, the probes (Table 3.1 and 3.2) were prepared by adding 10µl of the dig-oxygenin labeled probe into the appropriate 5ml solution of the prepared TMAC prehybridisation

buffer (2pmol/ml). When probes were already prepared at this concentration and had been stored in the freezer, they were refreshed by adding 1ul of stock probe. The probes primer sequences were obtained from Roche (PTY) LTD Diagnostic (Boehringer GmbH. Mannheim. Federal Republic of Germany) division and were as tabulated below:

Probe name	**Sequence**	**Amino acid**
436/437AA	GAATCCGCTGCTCCTTTT	ALA ALA
436/437AG	GAATCCGCTGGTCCTTTT	ALA GLY
436/437CA	GAATCCTGTGCTCCTTTT	CYS ALA
436/437FA	GAATCCTTTGCTCCTTTT	PHE ALA
436/437FG	GAATCCTTTGGTCCTTTT	PHE GLY
436/437SA*	GAATCCTCTGCTCCTTTT	SER ALA
436/437SG	GAATCCTCTGGTCCTTTT	SER GLY
540E	ACAATGGATGAACTAACA	GLU
540K*	ACAATGGATAAACTAACA	LYS
581A*	AGGATTTGCGAAGAAACA	ALA
581G	AGGATTTGGGAAGAAACA	GLY
613A*	GATTTATTGCCCATTGC	ALA
613S	GATTTATTTCCCATTGC	SER
613T	GATTTATTACCCATTGC	THR

Table 3.1 *dhps* probes, their names and sequences. * Indicate wild type probe.

Probe name	Sequence	Amino acid
16A*	CCATATGTGCATGTTGTA	ALA
16S	CCATATGTTCATGTTGTA	SER
16V	CCATATGTGTATGTTGTA	VAL
50/51CI	TGGAAATGTATTTCCCTA	CYS ILE
5051CN*	TGGAAATGTAATTCCCTA	CYS ASN
50/51CN2*	TGGAAATGTAACTCCCTA	CYS ASN
50/51RI	TGGAAACGTATTTCCCTA	ARG ILE
50/51RN	TGGAAACGTATTTCCCTA	ARG ASN
50/51RN2	TGGAAACGTAATTCCCTA	ARG ASN
59C*	AATATTTTTGTGCAGTT	LYS
59R	AATATTTTCGTGCAGTT	ARG
108N	AAGAACAAACTGGGAAAG	ASN
108S*	AAGAACAAGCTGGGAAAG	SER
108T	AAGAACAACCTGGGAAAG	THR
140L	ATGAAGATCTTTATATCA	LEU
140V*	ATGAAGATGTTTATATCA	VAL
164I*	GTTTTATTATAGGAGGTT	ILE
164L	GTTTTATTTTAGGAGGTT	LEU

Table 3.2 *dhfr* probes, their name and sequences. * Indicate wild type probe.

Prepared probes were then placed in the water-bath at 53°C together with the remaining hybridisation solution to warm. The blocking solution was poured off the membranes and 5ml of the remaining hybridisation buffer without probe was added to each tube. The tubes were then placed back on the rotisserie and the oven set at the critical hybridisation temperature of 53°C. When the hybridisation temperature was reached, the hybridisation solution was poured off and the relevant probe was added to the appropriate tube. The tubes were then incubated on the rotisserie at the hybridisation temperature for 90mins. While incubating the membranes, the temperature of the water-bath was set for high stringency wash at 59.5°C for both *dhfr* and *dhps* codons.

On completion of incubation, the membranes were then individually transferred into 500ml low stringency washing solution (2x SSPE, 0.1% SDS) in a plastic container at room temperature. The membranes were washed for 2x 10mins on an orbital shaker, making sure that membranes did not adhere to each other. Each membrane was transferred individually between washes. The warmed TMAC (about 500ml) was poured into a plastic container and the membranes transferred individually into the TMAC for the high stringency washes. Membranes were washed 2x 10mins and checked every 3-4mins to ensure that they did not stick together during washes. The membranes were then rinsed in buffer 1 for 5min and blotted onto filter paper. They were then placed, dot side in and not overlapping, in fresh Falcon tubes.

3.9 Detection

Five ml of buffer 2 was added into each tube-containing membrane and placed on the rotisserie at 37°C for at least 30mins or overnight. Buffer 2 was made up by mixing 100ml of buffer 1 with 1g of milk powder. A 1µl of anti-digoxygenin antibody Fab fragment conjugated with alkaline phosphatase (Cat. No.1093274 Roche Diagnostics GmbH, Roche Applied Science Nonnenwald 2, 82372 Penzberg, Germany) was carefully added to each tube, and put back onto the rotisserie at 37°C for 40min. Membranes were individually transferred from the tubes into buffer 1 contained in a plastic container. They were then washed 3 x 10mins on the orbital shaker at room temperature and transferred individually between each wash. After washes, membranes were rinsed for 5min in 500ml buffer 3, without shaking, at room temperature. Buffer 3 was prepared by mixing 50ml of 1M Tris (pH 9.5) solution, 10ml of 5M NaCl, with water added to make 500ml and give a final concentration of 0.1M. Membranes were then transferred individually, dot-side down, into the substrate solution contained in a plastic container. About 5 to 7 membranes were stacked on each other. The plastic container was then covered and membranes incubated for 5min at room temperature away from light, as the substrate is light sensitive. The substrate solution was prepared by adding 100 µl of CSPD (Disodium 3-(4-methoxyspiro (1.2-dioxetane-3.2'- (5'- chloro) tricyclo ($3.3.1.1^{3.7}$) decan)-4-yl) phenyl phosphate) stock into 10ml of buffer 3, filtered at 0.2 µm with a syringe and kept protected from the light in a foil-covered universal container.

A panel of four control haplotypes, PCR samples of known sequences representing all common sequence variants was spotted on every blot to act as positive and negative controls for probe specificity. These controls were obtained from Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, WCIE 7HT, United Kingdom. Their names and codons were:

For *dhfr*: -

Control Name	**Codon**					
	16	**50/51**	**59**	**108**	**140**	**164**
A	A	CI	R	N	V	I
B	A	CN	R	N	V	I
C	A	CI	C	N	V	I
D	A	CN	C	S	V	I

Table 3.3 *Dhfr* control names.

and for *dhps*: -

Control Name	**Codon**			
	436/437	**540**	**581**	**613**
1α	SA	K	A	A
2β	SA	K	A	A
3σ	SG	E	A	A
4Σ	SG	E	A	A

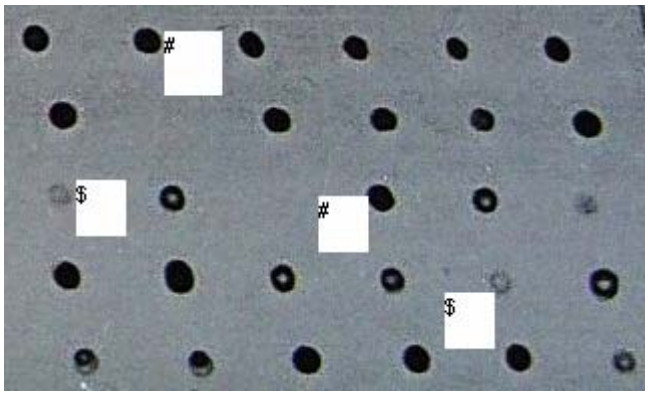
Table 3.4 *Dhps* control names.

Replicate blots were made of each array so that simultaneous probing with the oligonucleotide probes of the full panel for that gene could be conducted. After drying, the membranes were baked at 80°C for 2hrs or overnight in a hot air oven. Sequence-specific 18-bp oligonucleotide probes 3' end labeled with digoxigenin (DIG) (Roche Boehringer Mannheim, Germany) were each designed to complement the known sequence polymorphisms in *dhfr* and *dhps*. Single nucleotide polymorphism specific hybridization was followed by high-stringency TMAC washes, and alkaline phosphatase-conjugated anti-DIG fab fragments (Roche Boeringer Mannheim) were used to detect DIG labeled probes as described by Conway *et al*. (1999). These conditions allowed clear and accurate discrimination between alleles, including those differing at a single nucleotide position, as confirmed by the inclusion of allele-specific controls for typing in each assay.

3.10 Scoring

The presence or absence of the variant sequence polymorphism at each site was scored separately. Images of blots probed with variant sequences for a single locus were placed onto a UV light box reader and the standard control of each spot was defined with the intensity of chemifluorescence at a maximum wavelength of 477nm and then compared with the unknown. Background data were adjusted for by subtraction of the volume of the negative controls from the volume data. Thus, the volume of chemifluorescence for each spot was calculated as: Volume = (maximum intensity x spot area) – background. To determine the threshold of detection per se, the presence – flagging option was employed. By this method, the faintest spot considered present and not background was selected to set the flagging threshold value. To compare specific nucleotide probes (SNP) at a single site, Microsoft Excel bar charts were drawn comparing the volume data for each probe on every sample to the presence flagging result in each case. The following rules were used to determine whether a SNP was present or absent at each site: (a) A SNP was considered present in a PCR product when the volume value with a particular probe was higher than that of the background. When volume values were lower, presence flagging provided an internal control for avoiding possible biasing between probes; (b) A SNP was considered absent when all volume values below the internal control on the chart were rejected.

Samples were categorized into the single, majority, or mixed category at each site as follows. Samples were considered to be of mixed haplotypes when the volume value of the minority SNP was more than half the volume value of the majority SNP. Samples were considered to be mixed but containing a majority value SNP when the minority SNP value was less than half of the majority value. Samples were considered to be single when only one SNP was present at a site. Figure 3.2 shows examples of majority and minority SNP values of haplotypes.



= Majority haplotypes with dark coarse dots, $ = minority haplotypes with pale dots.

Figure 3.2: Presentation of SNP values of haplotypes as read during scoring.

3.11 Recrudescence infection.

Blood spot samples from patients who persistently showed the initial parasitaemia until day 3 or the reappearance of *P. falciparum* on or before day 42 were analysed for possible recrudescence or reinfection in all three *in vivo* studies. Recrudescent infection was distinguished from a newly acquired infection by comparing the genotypes of malaria parasites collected before and after SP treatment. Parasite DNA was extracted from the blood spots using the soponin lysis/ chelex extraction method developed by Wooden *et al*. (1993). Three parasite loci, that exhibit repeat number polymorphisms, were amplified by primary followed by nested rounds of PCR from the DNA of each isolate. These were the merozoites surface protein-1 and 2 (MSP-1) and (MSP-2) and the glutamate rich protein (GLURP), located on chromosomes 2, 9 and 10 respectively. Primers and amplification conditions for all three loci were used as described previously by Randford-Cartwright *et al*. (1993) and Paul *et al*. (1993, 1995). The PCR products were detected by electrophoresis of 8µl from each reaction on 1.5% agarose gels visualized by staining with ethidium bromide and UV fluorescence, and sized against a 100-basepair molecular weight marker (Gibco – BRL Life Technologies).

3.12 Statistical analysis.

All analyses were performed using both statistical packages; Stata 7.0, 2002, Stata Corporation, Texas, USA, and EpiInfo Version 6.0 (CDC Atlanta GA, USA) with cross-tabulation of categorical responses. The Fisher's exact test was used for testing associations between responses. Normally distributed continuous responses were summarised using means and standard deviations. Time to treatment failure was analysed using Kaplan-Meier Survival curves and compared using a log-rank test.

3.13 Ethical considerations.

Approval for the study protocol was obtained from the Mpumalanga Department of Health Ethics Committee and the Committee for Research on Human Subjects, University of Witwatersrand, Johannesburg and the University of Cape Town (Appendix 1.). Informed consent was obtained before enrolment from each patient or the guardians of minors. Treatment was given according to the Mpumalanga Department of Health guidelines and recommendation.

CHAPTER FOUR

RESULTS

4.1 *In vivo* therapeutic response

Between January and May in 1998, 2000 and 2002, 403 patients with uncomplicated *P. falciparum* malaria were enrolled in the *in vivo* studies in Mpumalanga province, South Africa. Of the 403 patients enrolled (64 female: 68 male in 1998; 60 female: 59 male in 2000 and 87 female: 65 male in 2002), 4 (3.7%) in 1998; 5 (4.6%) in 2000 and 12 (8.1%) in 2002 demonstrated initial clearance of parasites on blood microscopy but became microscopy positive after day 7 following SP treatment representing LTF. In 2 (1.8%) in 1998, 2 (1.8%) in 2000 and 2 (1.4%) in 2002, parasitaemia was reduced to < 25% of the pre-treatment value on day 3, but did not clear, representing ETF. The remaining patients remained aparasitaemic throughout the 42-day follow-up period after initial parasite clearance. Table 4.1 presents the geometric mean parasite density on day O observed among patients enrolled:

Year	No. of patients observed	Geometric mean	95% Confidence interval
1998	132	37694.27	34044.87 - 41734.85
2000	119	21803.56	19057.28 – 24945.61
2002	152	23278.06	18968.28 – 28567.07

Table 4.1: Geometric mean parasite density.

Gametocyte prevalence peaked on day 14 in 1998, 2000 and 2002 and an increase in prevalence was observed over the years of study. A recent study conducted five years after SP introduction (2002) revealed a significant increase in gametocyte carriage rates ($p < 0.001$) (Mabuza *et al*., 2005). Of the 132 patients who received treatment in 1998, 35.6% (47/132) had an inadequate dosage, while this was 23.5% (28/119), 27.05% (34/152) in 2000 and 2002, respectively.

4.1.1 Age distribution

The age ranges of subjects were 5 to 66 years in 1998, 2 to 79 years in 2000, and 4 to 71 years in 2002. The average ages were 20.26 (standard deviation (SD) = 13.83) in 1998, 22.63 (SD = 17.16) in 2000 and 23.07 (SD = 15.01) in 2002.

4.1.2 Parasite clearance times

	Treatment outcome				
Year	No. of patients observed on day O	ETF*	LTF#	Sensitive	Lost to follow-up
1998	132	2	4	103	23
2000	119	2	5	103	9
2002	152	2	12	134	4

* =Early treatment failure, # =Late treatment failure.

Table 4.2: Results of patients enrolled in the three *in vivo* SP therapeutic efficacy studies in 1998, 2000 and 2002.

Among patients enrolled in the three *in vivo* SP therapeutic efficacy studies, 17.4 %, 7.6% and 2.6% patients were lost to follow up in 1998, 2000 and 2002 respectively. Of the remainder, 78.0%, 86.6% and 88.2% became aparasitaemic and stayed so throughout 42-day follow up, while 3.0%, 4.5% and 7.9% resulted in late treatment failure and 1.5%, 1.7% and 1.3% were early treatment failure for the three years of studies respectively.

For all 3 years, 98% of patients survived without failure to at least day 7 (ETF was 2% in each of the 3 years). There was a decreasing trend in the percentage of patients who survived without failure for the entire 42-day period (95% in 1998, 94% in 2000 and 91% in 2002). However, there was no statistically significant difference between the survival function to recrudescence across the study period ($p > 0.4005$ Log-rank test).

4.1.3 Genotype frequencies

Samples pre- and post treatment from all three *in vivo* studies were genotyped for all three loci. The results showed that all (100%) had identical genotypes pre- and post treatment and recrudescence was detected up to 42 days post-treatment in all cases. None of the pre- and post treatment samples showed evidence of new infection. In addition, alleles detected in the pretreatment samples were also found post –treatment, suggesting the presence of recrudescent parasites.

4.1.4 Sample collection and analysis

Blood spots were collected on day O from all 403 patients enrolled. Of the 403 bloodspots, 358 yielded PCR products, including 113/132 (85.6%) bloodspots in 1998, 111/119 (93.3%) bloodspots in 2000 and 134/152 (88.2%) in 2002. In all the samples investigated, point mutations were identified only at codons 108, 51 and 59 of the *dhfr* gene and at codons 347 and 540 of the *dhps* gene. In this study, no point mutations were identified in codons 16, 50, and 164 of the *dhfr* gene; and 436, 581 and 613 of the *dhps* gene. Tables 4.3 and 4.4 present the number of point mutations in both *dhfr* and *dhps* gene respectively from samples yielded PCR products on day O for all the three years of studies. Allelic haplotypes were recorded only from single or majority genotype infections.

Year	No. of samples analysed	Wild haplotypes	Mutant haplotypes
1998	113	73	40
2000	111	68	43
2002	134	79	55

Table 4.3: Number of point mutations in the *dhfr* gene observed at day O among patients enrolled in the three *in vivo* SP therapeutic efficacy studies in 1998, 2000 and 2002.

Among samples analysed for *dhfr* gene; 40 (35.40%), 43 (38.74%) and 55 (41.04%) were mutant haplotypes and 73 (64.60%), 68 (61.26%) and 79 (58.96%) were wild type haplotypes in 1998, 2000 and 2002 respectively. However, there was no statistically significant difference between the wild type haplotypes to mutant haplotypes across the study period ($p > 0.670$, Fisher's exact test).

Year	No. of samples analysed	Wild haplotypes	Mutant haplotypes
1998	113	102	11
2000	111	103	8
2002	134	105	29

Table 4.4: Number of point mutations in the *dhps* gene observed at day O among patients enrolled in the three in vivo SP therapeutic efficacy studies in 1998, 2000 and 2002.

Among samples analysed for *dhps* gene; 11 (9.73%), 8 (7.21%) and 29 (21.64%) were mutant haplotypes and 102 (90.27%), 103 (92.79%) and 105 (78.36%) were wild type haplotypes in 1998, 2000 and 2002 respectively. There was a statistically significant difference between the wild type haplotypes to mutant haplotypes across the study period ($p < 0.002$, Fisher's exact test).

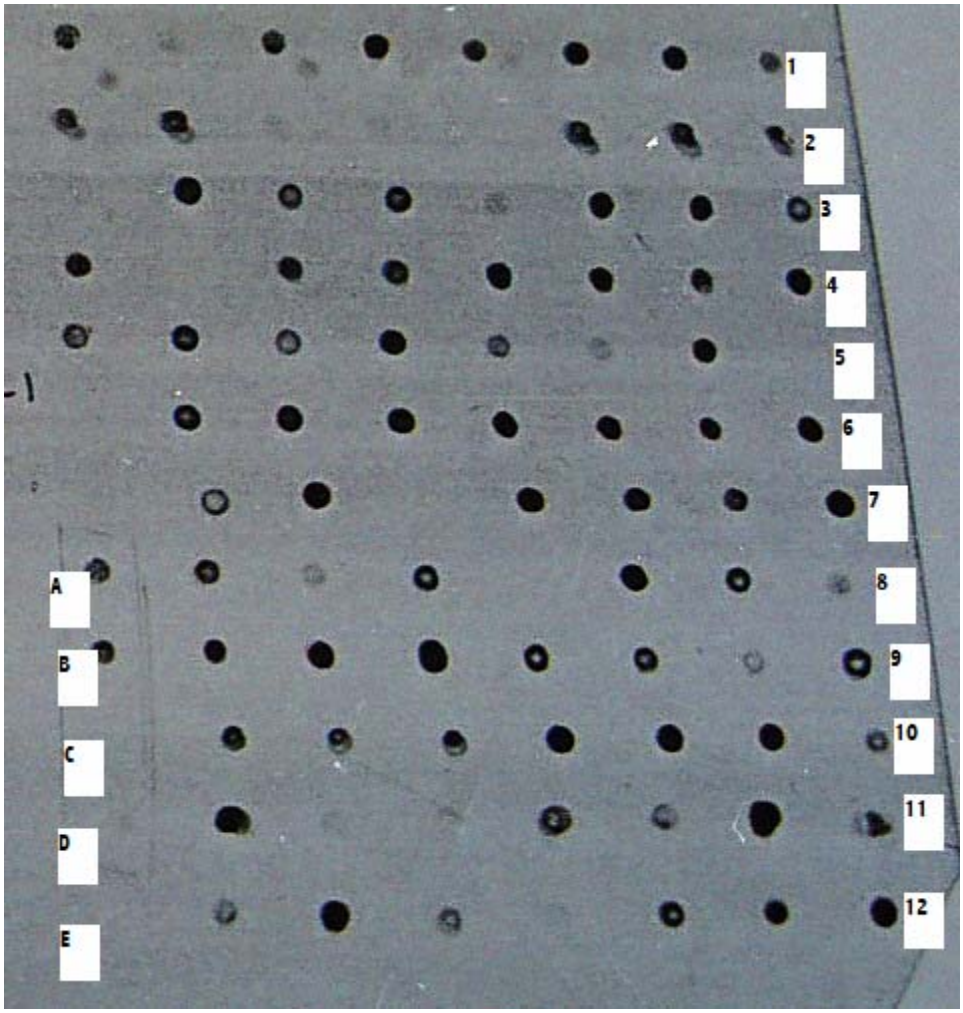


Figure 4.1 Nylon membrane showing haplotype results using the dot blot method-demonstrating readings of haplotypes as read for *dhfr* and *dhps* genes.

From the right: **First column:** numbers 1-4, 6-7, 9-12 show majority haplotype, number 5 negative and number 8 shows a minority haplotype. **Second column:** 1-8, 10-12 show majority haplotype, and number 9 shows a minority haplotype. **Third column:** 1-4, 6-10, 12 show majority haplotype, while numbers 5 and 11 show minority haplotypes. **Fourth column:** 1, 4-7, 9-11 show majority haplotype, while numbers 2, 8 and 12 are negative, number 3 shows a minority haplotype. **Fifth column:** 1, 3-6, 8-10, 12 show majority haplotype, while numbers 2, 7

and 11 are negative, no minorities found in this row. **Sixth column:** 1, 3-7, 9-10, 12 show majority haplotype, while numbers 8 and 11 show minority haplotypes and number 2 is negative. **Seventh column:** 2-3, 5-11-show majority haplotype, while number 12 shows a minority haplotype and numbers 1 and 4 are negative. **Eighth column:** 1, 2, 4, and 5 show majority haplotype, while numbers 3, 6 and 7 are negative. Labels A, B, C, D and E are controls (A and B) show positive majority haplotype, and (C, D and E) show negative controls.

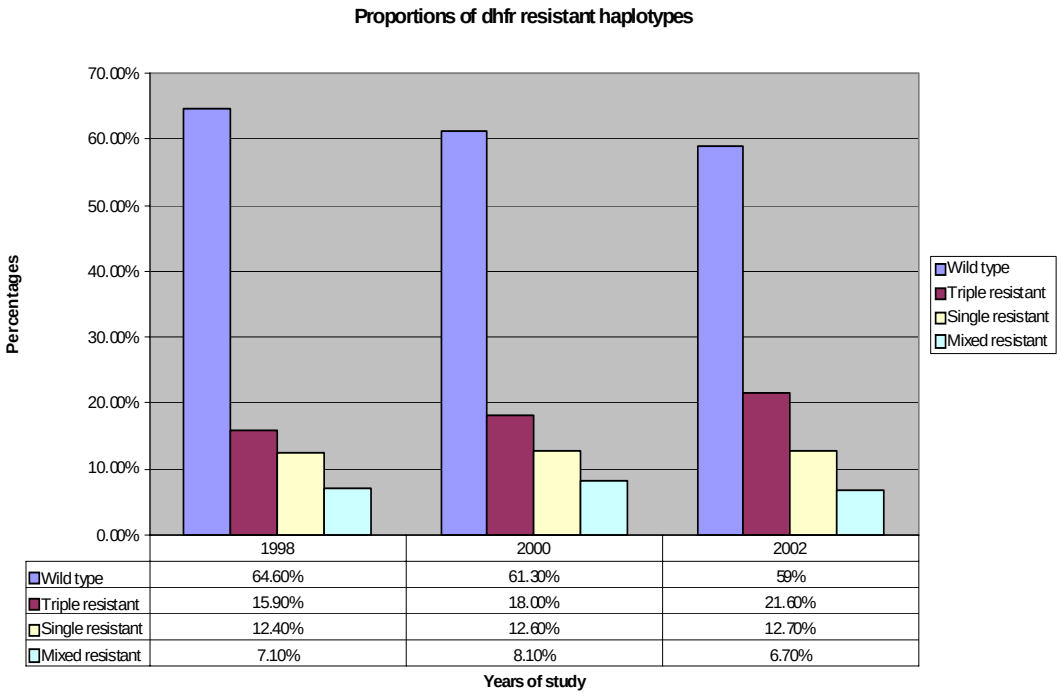


Figure 4.2: Frequency of relevant point mutation in the *dhfr* gene observed among patients enrolled in the three *in vivo* SP therapeutic efficacy studies in 1998, 2000 and 2002.

Figure 4.2 summarizes the frequency of point mutations in *dhfr* mutant haplotypes and their distribution among the three *in vivo* studies. Among 113, 111 and 134 isolates that were analysed, 18 (15.9%), 20 (18.0%) and 29 (21.6%) carried triple mutant *dhfr* haplotypes (S108N, N51I, and C59R) in 1998, 2000 and 2002 respectively. 18 (7.1%), 9 (8.1%) and 9 (6.7%) carried mixed *dhfr* mutant haplotypes (S108N + N51I or S108N + C59R) and 14 (12.4%), 14 (12.6%) and 17 (12.7%) carried single mutant haplotypes (S108N). The remaining 73 (64.6%), 68 (61.3%) and 79 (59.0%) carried wild type *dhfr* haplotypes.

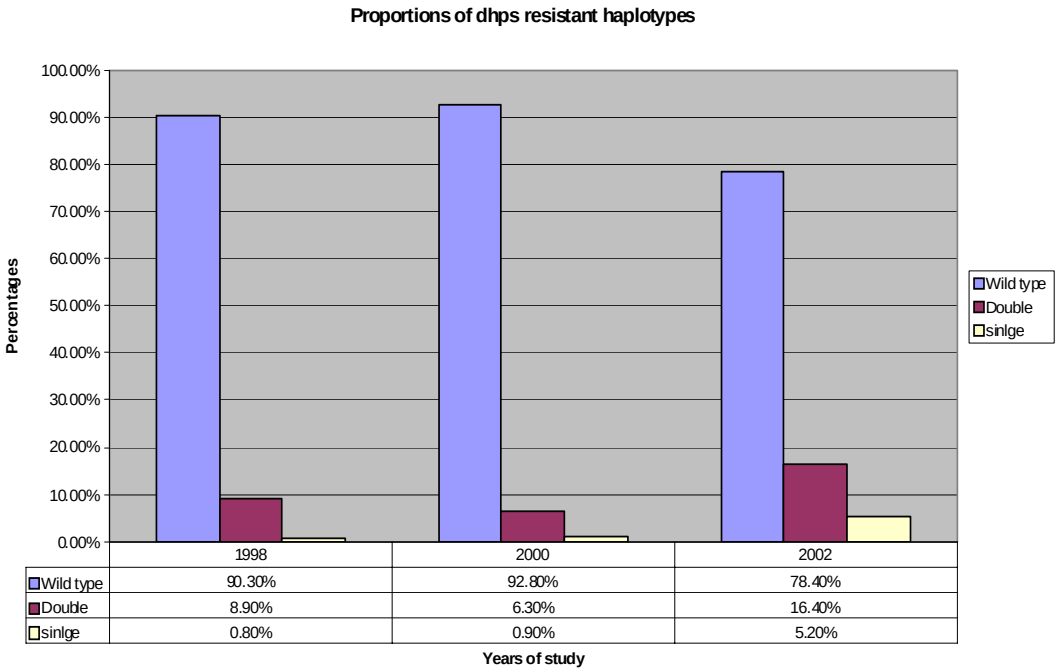


Figure 4.3: Frequency of relevant point mutation in the *dhps* gene observed among patients enrolled in the three *in vivo* SP therapeutic efficacy studies in 1998, 2000 and 2002.

Figure 4.3 summarizes the frequency of point mutations in *dhps* haplotypes and their distribution among the three *in vivo* studies. Among 113, 111 and 134 isolates that were analysed, 10 (8.9%), 7 (6.3%) and 22 (16.4%) carried double mutant *dhps* haplotypes (A437G + K540E) in 1998, 2000 and 2002 respectively. The 1(0.8%), 1(0.9%) and 7(5.2%) carried single *dhps* mutant haplotype (A347G). The remaining 102 (90.3%), 103 (92.8%) and 105 (78.4%) carried wild type *dhps* haplotypes.

	Parasitological outcome			
1998	No. of *dhfr* isolates	ACPR#	Resistant	LTFU*
Mutant haplotypes	40	25	6	9
Wild type haplotypes	73	61	0	12
Total	113	86	6	21
2000				
Mutant haplotypes	43	29	7	7
Wild type haplotypes	68	68	0	0
Total	111	97	7	7
2002				
Mutant haplotypes	55	39	14	2
Wild type haplotypes	79	77	0	2
Total	134	116	14	4

= Adequate clinical and parasitological response, * = Lost to follow-up.

Table 4.5: Correlation of *dhfr* haplotypes and parasitological outcome over time.

Table 4.5 summarizes the correlation between the *dhfr* haplotypes and the response to pyrimethamine antifolate. Among 113, 111 and 134 isolates that were analysed, 9 (22.5%), 7 (16.3%) and 2 (3.6%) of mutant haplotypes were lost to follow up in 1998, 2000 and 2002 respectively. Complete parasite resistance of mutant haplotypes was achieved in 6 (15.0%), 7 (16.3%) and 14 (25.5%) samples while 25 (62.5%), 29 (67.4%) and 39 (70.9%) were successfully cleared in 1998, 2000 and 2002 respectively. 12 (16.4%), 0 (0.0%) and 2 (2.5%) of wild type haplotypes were lost to follow up, while none of the wild type haplotypes were resistant to pyrimethamine antifolate in 1998, 2000 and 2002 respectively. 61

(83.6%), 68 (100.0%) and 77 (97.5%) of the wild type haplotypes were successfully cleared in 1998, 2000 and 2002 respectively.

	Parasitological outcome			
1998	No. of *dhps* isolates	ACPR#	Resistant	LTFU*
Mutant haplotypes	11	2	6	3
Wild type haplotypes	102	84	0	18
Total	113	86	6	21
2000				
Mutant haplotypes	8	1	7	0
Wild type haplotypes	103	96	0	7
Total	111	97	7	7
2002				
Mutant haplotypes	29	13	14	2
Wild type haplotypes	105	103	0	2
Total	134	116	14	4

= Adequate clinical and parasitological response, * = Lost to follow-up.

Table 4.6: Correlation of *dhps* haplotypes and parasitological outcome over time.

Table 4.6 summarizes the correlation between the *dhps* haplotypes and parasitological outcome response to sulfadoxine antifolate. Among 113, 111 and 134 isolates that were analysed, 3 (27.3%), 0 (0.0%) and 2 (6.9%) of mutant haplotypes were lost to follow up in 1998, 2000 and 2002 respectively. Complete parasite resistance of mutant haplotypes was achieved in 6 (5.5%), 7 (87.5%) and 14 (48.3%) while 2 (18.2%), 1 (12.5%) and 13 (44.8%) were successfully cleared

in 1998, 2000 and 2002 respectively. 18 (17.7%), 7 (6.8%) and 2 (1.9%) of wild type haplotypes were lost to follow up, while none of the wild type haplotypes were resistant to pyrimethamine antifolate in 1998, 2000 and 2002 respectively. 84 (82.4%), 96 (93.2%) and 103 (98.1%) of the wild type haplotypes were successfully cleared in 1998, 2000 and 2002 respectively.

Year	No. of cases	Amino acids at mutated positions			Response to antifolate
		51	59	108	Pyrimethamine
1998	61	Asn	Cys	Ser	ACPR
	25	**Ile**	**Arg**	**Asn**	ACPR
	6	**Ile**	**Arg**	**Asn**	Resistant
2000	68	Asn	Cys	Ser	ACPR
	29	**Ile**	**Arg**	**Asn**	ACPR
	7	**Ile**	**Arg**	**Asn**	Resistant
2002	77	Asn	Cys	Ser	ACPR
	39	**Ile**	**Arg**	**Asn**	ACPR
	14	**Ile**	**Arg**	**Asn**	Resistant

Table 4.7: *Dhfr* haplotypes and parasitological outcome over time.

Table 4.7 compares the *dhfr* genotypes found with parasitological outcome. All isolates analysed for *dhfr* in 1998 with wild type haplotypes, cleared successfully with SP, while only 25/31 (80.6 %) with mutant parasites cleared successfully. Similarly all those with wild type haplotypes cleared successfully with SP in 2000, while 29/36 (80.6%) with mutant haplotypes cleared successfully. A similar pattern was seen in 2002, with all patients with wild type haplotypes clearing successfully with SP, while 39/53 (73.6%) with mutant haplotypes cleared successfully.

Year	No. of cases	Amino acids at mutated positions		Response to antifolate
		437	540	Sulfadoxine
1998	84	Ala	Lys	ACPR
	2	**Gly**	**Glu**	ACPR
	6	**Gly**	**Glu**	Resistant
2000	96	Ala	Lys	ACPR
	1	**Gly**	**Glu**	ACPR
	7	**Gly**	**Glu**	Resistant
2002	103	Ala	Lys	ACPR
	13	**Gly**	**Glu**	ACPR
	14	**Gly**	**Glu**	Resistant

Table 4.8*: Dhps* haplotypes and parasitological outcome over time.

Table 4.8 compares the *dhps* genotypes found with parasitological outcome. All isolates analysed for *dhps* in 1998 with wild type haplotypes, cleared successfully with SP, while only 2/8 (25.0 %) with mutant parasites cleared successfully. Similarly all those with wild type haplotypes cleared successfully with SP

in 2000, while 1/8 (12.5%) with mutant haplotypes cleared successfully. A similar pattern was seen in 2002, with all patients with wild type haplotypes clearing successfully with SP, while 13/27 (48.2%) with mutant haplotypes cleared successfully.

Years	1998		2000		2002	
Haplotypes	ACPR#	Resistant	ACPR#	Resistant	ACPR#	Resistant
Wild type	100.00 (60)	0.00 (0)	100.00 (68)	100.00 (0)	100.00 (70)	0.00 (0)
Quintuple*	16.67 (1)	83.33 (5)	0.00 (0)	100.00 (6)	14.29 (2)	85.71 (12)
Mixed $	96.15 (25)	3.85 (1)	96.67 (29)	3.33 (1)	95.65 (44)	4.35 (2)

= % of adequate clinical and parasitological response, * = % of individuals with mutant haplotypes in both *dhfr* and *dhps* genes, $ = % of combination of mutant and sensitive haplotypes.

Table 4.9: Correlation of *dhfr / dhps* haplotypes and parasitological outcome over time.

Table 4.9 summarizes the presence of resistant haplotypes and parasitological outcome when combined in both *dhfr* and *dhps* codons. In comparison, in 1998, 2000 and 2002 with the presence of quintuple mutations, high levels of resistance prevailed whereas when mixed resistant and sensitive haplotypes were present, high successful cure rates were achieved.

CHAPTER FIVE

DISCUSSION

5.1 The *In vivo* SP therapeutic response

Sulfadoxine pyrimethamine was introduced in the malaria endemic areas of Mpumalanga province for treatment of uncomplicated malaria in 1997 (Govere *et al*., 1999; 2001). *In vivo* evaluation studies were conducted at baseline and then every two years (two studies) thereafter to monitor SP efficacy in the province (Mabuza *et al*., 2000). All these studies found that SP retained its effectiveness in providing a clinical cure in the vast majority of subjects within three days. However, nothing was known about the local prevalence of dihydrofolate reductase and dihydropteroate synthase mutations and their associations with parasitological outcomes.

It is not clear whether the selection for SP resistance in this study also applies to gametocytes, but the high frequency of gametocytaemia after SP treatment may explain the explosive spread of SP resistant strains in our study area. Again, the association of gametocytes with quintuple mutations in this study further increases suspicion of SP selection for treatment failure. SP has no known gametocytocidal properties and gametocyte generation and development appears to persist despite SP treatment (Sinden, 1983). This finding compares favorably with the previous study done in the Gambia, which found that 28.9% of patients treated with SP carried gametocytes at two week follow up (Von Seidlein *et al*., 1998).

A number of malaria control programmes in other African and South-East Asian countries have an established tradition of assessing the efficacy of their first-line malaria therapeutic regimens at sentinel surveillance sites on a regular basis to guide public health policy. The sentinel site, at which this study was conducted, has proven its value in this capacity in Mpumalanga (Durrheim *et al*., 2001). The seasonal nature of malaria transmission in Mpumalanga provides an opportunity for using the capacity developed at this site to the benefit of other public health programmes and also to field-test the accuracy and utility of rapid malaria diagnostic tests.

We report here on the association between *dhfr* and *dhps* mutation and SP efficacy in uncomplicated *P. falciparum* malaria in Mpumalanga province. The study was conducted among symptomic patients of uncomplicated *P. falciparum* monoinfection. Only local symptomatic patients were enrolled. In Mpumalanga province, where transmission and immunity are low, most infections are symptomatic and come (when treated with drugs) under drug pressure.

5.2 Parasite clearance times

We undertook 42-day follow-up in order to acquire an optimum duration for assessment of SP efficacy in our study. We were able to detect recrudescence in day 28 and day 42, which gave our study more value of success. In comparison, the widely used day 14 assessments could have missed between 66 and 85% of failures during the three *in vivo* studies. Assessment at day 28 could have missed between 33 and 28% failures. The situations in Mpumalanga province

when compared to that in Malawi differ. SP was introduced in Malawi as first line treatment in 1993. Contrary to expectations, SP was reported as retaining acceptable efficacy after 10 years as the first line antimalarial drug, although this has been questioned (Plowe *et al*., 2004). Assessment was done at day 14 and 28 in Malawian study. This could mean that certain percentages of failure could have been missed should they have had 42-day follow-up as we saw in our study. However, it should be remembered that malaria transmission in Malawi is holoendemic and chances of reinfection are good.

5.3 Genotype frequencies

In Mpumalanga province, where transmission is low, recrudescent infection up to 42 days after SP treatment have been identified using PCR of three polymorphic antigen loci. Overall, all samples from the three *in vivo* recurrent infections followed were identified as the same parasite genotype pre- and post- treatment. The use of this method as a tool for distinguishing recrudescent from novel infections in paired samples allows antimalarial drug efficacy assessment to be conducted.

Multiple clones are frequently found in natural *P. falciparum* infections (Babiker *et al*., 1997), and drug sensitive and resistant parasites often co-exist in the same infection (Thaithong *et al.,* 1984). The SSOP method with its high throughput and retained sensitivity and specificity equivalent is immensely valuable in understanding this phenomenon (Abdel-Muhsin *et al*., 2002; Randford-Cartwright *et al*., 2002). An assay should be sensitive enough to detect low-level

resistant clones in a mixture, as these are likely to increase in density through selection following treatment. The detection of minority parasite genotypes is of importance for accurate estimates and monitoring frequencies of drug resistance genes following changes in drug policy. Using a PCR high-throughput method (Conway *et al*., 1999), we studied point mutations at codons 16, 51, 59, 108 and 164 of the *dhfr* gene and point mutations at codons 436, 437, 581 and 613 of the *dhps* gene to detect mutations in both genes.

5.4 Sample collection and analysis.

Using single and majority genotype infections, point mutation haplotypes that were present in the three consecutive *in vivo* studies were determined. Five haplotype alleles at *dhfr*; the sensitive-allele haplotype, the single-mutant allele S108N haplotype, the double-allele S108N + N51I haplotype, the double-allele S108N + C59R haplotype, and the triple-allele S108N + N51I + C59R haplotype were found. Three haplotypes in *dhps*, the sensitive-allele haplotype, the single-mutant allele A437G haplotype and the double-mutant allele A437G + K540E haplotype were found. These results are similar to those reported from elsewhere (Jelinek *et al*., 1997; 1998; Kublin *et al*., 2002; Mutabingwa *et al*., 2001; Nzila *et al*., 2000a; 2000b; Wang *et al*., 1997a).

Our data show that, within the 42 days following SP treatment, some isolates were composed of parasites that had undergone point mutation associated with pyrimethamine resistance in *dhfr* and that there was a significant increase of sulfadoxine resistance in *dhps* resistant haplotypes. The study showed that *dhfr*

mutations were found with a relatively high overall prevalence in all three consecutive *in vivo* studies: 35.4% in 1998; 38.7% in 2000 and 41.0% in 2002. A low overall prevalence of *dhps* mutations was found for the first two studies; 9.7% in 1998 and 7.2% in 2000, but this increased dramatically in the 2002 study (41.6%). This argues against use of individual components of this combination.

We found resistant haplotypes in codons 51 and 59 of *dhfr* that have been associated with high levels of pyrimethamine resistance. These haplotypes were often combined with 108-asparagine resistant haplotypes in our study. The importance of an Asn 108-asparagine haplotype in the *dhfr* gene for resistance against pyrimethamine and amplification of this effect in combination with 51-isoleucine or 59-arginine have previously been described (Curtis *et al*., 1996; Sirawarapon *et al*., 1997; Basco and Ringwald 1998; Basco *et al*., 1998).

From this study, the presence of triple resistant haplotypes in the *dhfr* gene suggests a role played by *dhfr* in determining treatment failure. Previous studies have shown that three mutants in the *dhfr* domain can be used as a marker to predict the clinical outcome of SP treatment failure (Watkins *et al*., 1997; Basco *et al*., 1998). If this is correct, the three resistant haplotypes in the *dhfr* gene that we observed might be used to predict the clinical outcome of SP in our situation where SP has been used only sparingly.

Thr 108, Val 16 and Leu 164, which are associated with resistance to pyrimethamine and cycloguanil, were not detected in this study, confirming the rarity of these genotypes in field isolates. The *dhfr* 164 mutation has been found in areas of South East Asia and South America, where resistance to sulfadoxine/pyrimethamine is well established (Nzila *et al*., 2000a).

The 437 Gly variant mutations in the *dhps* gene, which is predominantly described previously by Wang *et al*. (1997a) and other authors (Basco *et al*., 1998; Brooks *et al*., 1994; Diourte *et al*., 1999; Plowe *et al*., 1997; Triglia and Cowman, 1994; Triglia *et al*., 1997) was present in our study. According to Wang *et al*. (1997a), isolates bearing the 437 Gly plus other mutations like the 581 Gly, 613 Ser, 613 Thr and 540 Glu had higher sulfadoxine resistance. This led them to postulate that the 437 Gly mutation was an intermediate step towards higher resistance in combination with further mutations elsewhere in the *dhps* gene. Thus, A437G may be the closest equivalent in *dhps* to the S108N alteration in *P. falciparum dhfr* that is almost always present in pyrimethamine-resistant parasites (Cowman *et al*., 1988; Peterson *et al*., 1988; Wang *et al*., 1997a). If the hypothesis of Wang *et al*. (1997a) is correct, according to the present study's results, most *P. falciparum* are already moderately resistant to sulfadoxine.

The double resistant haplotypes A437G+ K540E in *dhps* present in our study suggests that this may be sufficient to influence the outcome of SP therapy in our situation. Such findings are common in Thailand (Triglia *et al*., 1997) and parts of South America (Plowe *et al*., 1997), where there is seasonal malaria occurrence. When these double resistant haplotypes paired with triple resistant haplotypes in *dhfr*, treatment failure resulted, unlike in KwaZulu-Natal, where only one resistant *dhps* allele was found (Roper *et al*., 2003).

No amino-acid substitution was detected at positions 436, 581 and 613, unlike that found in Central African and South American isolates (Wang *et al*., 1997a; Plowe *et al*., 1998; Basco and Ringwald, 2000).

5.6 Correlation of *dhfr* and *dhps* genes and parasitological outcome.

In our study, SP easily cleared parasites with only *dhfr* or *dhps* mutant haplotypes regardless of their prevalencies. A significantly high cure rate by SP in all wild types of both *dhfr* and *dhps* was associated with ACPR. The relative importance of mutations in *dhfr* and *dhps* to *in vivo* SP resistance has been debated (Sims *et al*., 1998; Watkins *et al*., 1999). Our study showed that whenever there is wild, single or in some cases double resistant haplotypes in any *dhfr* or *dhps* codons, SP was able to clear those parasites.

We have shown that parasites that carried a triple mutant allele haplotypes in *dhfr* combined with double mutant allele haplotypes in *dhps* (quintuple) were the most likely to escape drug action. The presence of quintuple haplotype was strongly associated with SP treatment failure. Previous studies confirm that the presence of all three *dhfr* mutations together with the two *dhps* mutations before treatment is an important predictor of SP failure (Omar *et al*., 2001; Kublin *et al*., 2002). Our results are thus consistent with other studies that demonstrated that the *dhfr* triple mutant Asn-108, Ile-51 and Arg-59 and *dhps* double mutant Gly-437 and Glu-540 have been predominantly associated with SP resistance (Nzila *et al*., 2000a; Kublin *et al*., 2002; Nagesha *et al*., 2001).

CHAPTER SIX

CONCLUSION

Based on the findings of this study, we can conclude:

- That prevalence in both *dhfr* and *dhps* gene resistant haplotypes were selected gradually during the three *in vivo* studies in Mpumalanga.
- The combination of resistant haplotypes in both *dhfr* and *dhps* gene frequencies confer resistance to SP in Mpumalanga, quintuple being the main source of resistance.
- The triple resistant haplotypes found in *dhfr* were not sole determinant that accounts for therapeutic failure.
- Mutations at both *dhfr* and *dhps* loci may be important predictors of SP resistance in Mpumalanga province.
- A priority for future planning for management of resistance is further research into the extent of spread of the selected genetic determinants of resistance, and the potential of combination therapy for arresting or slowing the spread of these genes.
- The development of molecular techniques to detect and map the evolution of drug-resistant malaria is of major importance.
- This study has shown that genetic analysis of resistance genes can be incorporated into drug-resistance monitoring as an integral component of a malaria control programme.

APPENDIX 1.

Ethical clearance certificates

A. University of Cape Town approval certificate.

B. Mpumalanga Department of Health approval certificate.

C. University of Witwatersrand approval certificate.

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