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Characterisation of Southern African strains of the malarial parasite plasmodium falciparum

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CHARACTERISATION OF SOUTHERN AFRICAN STRAINS OF THE MALARIAL PARASITE *PLASMODIUM FALCIPARUM*

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ABSTRACT

This thesis describes the characterisation of southern African isolates of *Plasmodium falciparum* according to isoenzyme type, antigen variants and drug sensitivity. Nineteen southern African isolates and a Gambian reference isolate were cultured *in vitro* in gassed tissue culture flasks. Polyacrylamide gel electrophoresis with use of 5 enzymes revealed little variation amongst the isolates and the frequencies of enzyme forms were similar to those of isolates from other parts of the world. Antigenic diversity was demonstrated using a panel of 9 monoclonal antibodies in the indirect fluorescent antibody test. The antigenic composition of 70% of isolates was markedly different to any obtained in other geographical areas. Both characterisation techniques revealed a mixture of parasite types in some isolates. However, the characteristics of most of these heterogeneous isolates were not stable with time in culture. Most isolates proved to be resistant to chloroquine in a 48-hour growth inhibition test. A wide range in pyrimethamine susceptibilities was detected although most isolates exhibited a low level of resistance to this drug. In the radioisotope uptake assay, the halofantrine IC_{50} values obtained were comparable with

those of West African isolates but were higher than the IC_{50}s of South-East Asian isolates. All of the isolates resistant to chloroquine in the 48-hour test, were resistant in the radioisotope assay. However, 1 isolate shown to be sensitive to chloroquine in the first test was found to be resistant in the ^{3}H-hypoxanthine incorporation method. This was assumed to be the result of selection of resistant clones. Southern African isolates were shown to be fully susceptible to mefloquine, but had reduced susceptibility to quinine and sulphadoxine/ pyrimethamine. Most isolates were sensitive to amodiaquine. A field study of 31 KwaZulu isolates of *P. falciparum* obtained in 1987 and 1988 showed 29 to be resistant to chloroquine in the 24-hour microtechnique, with the majority being highly resistant. Chloroquine resistance was confirmed in this area in a field study carried out in 1989 but in 1990 all 4 isolates tested were sensitive to the drug. In 1989, 12 out of 13 KwaZulu isolates were resistant to amodiaquine and most isolates showed reduced susceptibility to quinine and sulphadoxine/ pyrimethamine.

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Signed at *DURBAN* this *19TH* day of *MARCH 1993*

J. A. Freese

J. A. Freese

In memory of my grandmother

Grace Anne Buchanan

1904 - 1992

"Though much is taken, much abides; and though
We are not now that strength which in old days
Moved earth and heaven, that which we are, we are,
One equal temper of heroic hearts,
Made weak by time and fate, but strong in will
To strive, to seek, to find, and not to yield."

Alfred, Lord Tennyson: *Ulysses*.

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PREFACE

This work was funded by the South African Medical Research Council and was carried out at the MRC laboratories in Durban under the supervision of Professor M. B. Markus of the Department of Zoology, University of the Witwatersrand.

This thesis describes the characterisation of southern African isolates of *Plasmodium falciparum*. It is presented in the form of journal articles, a practice which is in the process of becoming accepted by Universities overseas and locally. Chapters 2 - 9 have either been published or have been submitted for publication as follows:

Chapter 2:

Freese JA, Sharp BL, Ridl FC, Markus MB, 1988. *In vitro* cultivation of southern African strains of *Plasmodium falciparum* and gametocytogenesis. *S Afr Med J*, 73: 720-722.

Chapter 3:

Freese JA, Markus MB, 1990. Enzyme typing of southern African isolates of *Plasmodium falciparum* by polyacrylamide gel electrophoresis. *Trans R Soc Trop Med Hyg*, 84: 519-520.

Chapter 4:

Freese JA, Markus MB, Golenser J, 1990. Typing of southern African isolates of *Plasmodium falciparum* using monoclonal antibodies. *Am J Trop Med Hyg*, 43: 19-24 .

Chapter 5:

Freese JA, Markus MB, Golenser, J. *In vitro* sensitivity of southern African reference isolates of *Plasmodium falciparum* to chloroquine and pyrimethamine. *Bull WHO*, 69: 707-712.

Chapter 6:

Freese JA, Rossouw EJ, Gouws E, Sharp BL, Markus MB. *In vitro* sensitivity of southern African isolates of *Plasmodium falciparum* to halofantrine. *Ann Trop Med Parasitol*, 87: 235-237.

Chapters 7 and 9:

Freese JA, Sharp BL, Rossouw EJ, Gouws E, Fay SA, Markus MB. The *in vitro* sensitivity of southern African isolates of *Plasmodium falciparum* to amodiaquine, chloroquine, mefloquine, quinine and sulphadoxine/pyrimethamine. *S Afr J Sci*, 90: 417-420.

Chapter 8:

Freese JA, Sharp BL, Ngxongo SM, Markus MB, 1988. *In vitro* confirmation of chloroquine-resistant *Plasmodium falciparum* malaria in KwaZulu. *S Afr Med J*, 74: 576-578.

The journal articles have been altered slightly to avoid repetition of description of materials and methods used. The article on which Chapter 2 has been based, has been extended to include information on isolates which were established in culture after preparation of the paper for the journal. The section on gametocytogenesis, which was based on work carried out by one of the co-authors, has been excluded from this chapter.

I would like to thank the following people:
My supervisor, Professor Miles Markus, for his encouragement and support and for his excellent critical evaluation of the articles resulting from this work;
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1. GENERAL INTRODUCTION

Malaria is the most common severe parasitic disease affecting man. In Africa, where approximately 80% of the world's malaria cases occur, there are over 100 million cases of clinical malaria and more than 1 million deaths from malaria each year.[1]

In the Republic of South Africa, where malaria is endemic in the northern and eastern Transvaal and in northern Natal and KwaZulu, the number of cases has increased dramatically over the past 10 years. In KwaZulu, the number of cases rose from 75 in 1982 to 4836 in 1987.[2] This upsurge in malaria has been attributed to rainfall patterns, drug resistance and the influx of refugees from Mozambique.

Research on *Plasmodium falciparum*, which is by far the most common species of malaria parasite in South Africa, is relatively new in this country. In order to carry out any meaningful investigations on local parasites in the laboratory, it is necessary to have a bank of well-characterised isolates.

Initially, characterisation of this species by malariologists overseas was limited to studies of morphology, infectivity and virulence.[3] However, the

development of methods for the cultivation of erythrocytic forms *in vitro*,[4] has enabled investigations of diversity within this species to be extended to include enzyme typing, 2-dimensional protein screening, antigen variation studies, drug susceptibility studies and DNA studies.[5]

The results of characterisation of *P. falciparum* from various endemic regions of the world show that isolates from different geographical areas share common characteristics, suggesting that this species exists as a single, interbreeding, worldwide population. However, the frequencies of occurrence of some variants have been found to differ from region to region. Drug sensitivity work has revealed geographical differences within this species but these are probably related to the extent of use of a given drug in a particular region. Characterisation has revealed that most, if not all, isolates are made up of at least 2 populations of genetically distinct organisms.

The aim of this study was to characterise southern African reference isolates of *P. falciparum* according to enzyme electrophoretic patterns, antigen types and *in vitro* drug sensitivity; and to determine the *in vitro* drug susceptibility of field isolates from KwaZulu.

Chapter 2 describes the techniques used to culture southern African strains of *P. falciparum* and lists the isolates used in this investigation. The typing of these isolates according to their isozymes and their reaction with a panel of monoclonal antibodies, are described in Chapters 3 and 4, respectively.

A large proportion of this characterisation study was devoted to the determination of the *in vitro* drug sensitivity of both the cultured southern African reference isolates and primary isolates from KwaZulu. Several methods were used to assess drug susceptibility *in vitro*. In Chapter 5, the use of microscopy to determine parasite growth in the 48-hour growth-inhibition test is described and the results obtained from a study of the sensitivity to chloroquine and pyrimethamine of the reference isolates, using this technique, are discussed. The susceptibility of the cultured isolates to halofantrine was determined using the radioisotope uptake assay, which is described in Chapter 6. This technique was also used to assess the *in vitro* sensitivity to amodiaquine, chloroquine, mefloquine, quinine and sulphadoxine/pyrimethamine, using WHO predosed drug plates. The findings of this study are contained in Chapter 7.

Field studies to determine *in vitro* drug sensitivity in northern KwaZulu were carried out in 4 consecutive years. In 1987 and 1988, field investigations were undertaken to determine chloroquine susceptibility in this region using the 24-hour microtechnique. The results obtained in this study are discussed in Chapter 8. In 1989, a study was carried out to assess the sensitivity of local isolates to amodiaquine, chloroquine, quinine and sulphadoxine/pyrimethamine using the 24-hour microtest. In 1990, the sensitivity of *P. falciparum* from this area to chloroquine and quinine was assessed using the radioisotope incorporation assay. The findings of these investigations are presented in Chapter 9.

The implications of drug resistance for the South African traveller in southern Africa are discussed in Chapter 10. An overall summary of the characterisation study and the conclusions which may be drawn from the findings, are contained in Chapter 11.

2. *IN VITRO* CULTIVATION OF *PLASMODIUM FALCIPARUM*

2.1 Introduction

The successful long-term culture of *P. falciparum* is central to many epidemiological, immunological, biochemical and genetic studies. Such studies provide information which is vital to the effective control of malaria. As this disease in endemic in parts of South Africa and several neighbouring states, it is essential that local isolates be maintained in culture to enable investigations on the parasite to be carried out. The petri dish - candle jar method of Jensen and Trager[5] for the cultivation of *P. falciparum* has proved successful in the establishment of many strains.[6-8] However, not all isolates adapt to these culture conditions[7,8] and in our experience the candle jar technique is unsuitable for the establishment of local isolates. The growth of 12 isolates was initiated in culture using this method but none of the isolates adapted to the culture conditions. The survival times of these isolates ranged from 2 to 44 days.

Scheibel *et al.*[9] have shown the gas concentration in the candle jar (1.3 - 3.3% CO_2 and 14.5 - 17.8% O_2) to

be far from ideal for the growth of *P. falciparum*. They found that the best growth rates were achieved at 3% O_2 and 1 - 2% CO_2. Enrichment of the culture medium with additional glucose has enabled cultures to be left unattended for up to 5 days[10] and addition of hypoxanthine to the medium has been shown to improve parasitic yields.[11] Cultivation of the parasite in suspension rather than in a static situation has likewise resulted in improved yields.[11-14]

The techniques used to establish and maintain southern African isolates *in vitro* are described in this chapter.

2.2 Materials and Methods

2.2.1 *P. falciparum* isolates

The culture-adapted Gambian FCR-3 strain[7] has been successfully maintained in the laboratory in Durban since 1984. Four cryopreserved culture-adapted KwaZulu isolates were obtained from Brian Sharp[a] and 5 North-eastern Transvaal isolates were obtained from Anton Stoltz[b] within one week of initiation of growth. Twelve southern African isolates were collected from patients who had contracted their infections between July 1984 and February 1987. Blood samples were

a Leader, National Malaria Research Programme, Medical Research Council, Durban
b M.Sc student, Department of Biochemistry, University of Pretoria, Pretoria

collected from the patients in EDTA or lithium-heparin and processed within 24 hours of collection. Isolates which were not cultured immediately were cryopreserved. The origins of infection and dates of collection of the isolates are shown in Table 1.

2.2.2 Cryopreservation of infected erythrocytes

Infected red blood cells were washed twice in 10 volumes of culture medium (without serum) and suspended in an equal volume of 28% glycerol in phosphate-buffered saline (PBS). Aliquots (0.5 ml) of the suspension were placed in cryotubes (Nunc, Denmark) and the tubes were plunged directly into liquid nitrogen. When required, the frozen isolates were thawed rapidly in a waterbath at 37°C and then washed successively in the following solutions:

i) 12% NaCl (0.2 ml / 1 ml packed red blood cells)
ii) 1.6% NaCl (9 volumes)
iii) 0.9% NaCl + 0.2% D-glucose (9 volumes).

The washed cells were mixed with at least an equal volume of fresh, washed, normal red blood cells before being placed in culture.

Table 1. Origins of infection and dates of collection of 22 southern African isolates of *Plasmodium falciparum*.

Isolate	Origin of Infection	Date of Collection
RSA 1	KwaZulu[a]	30-1-85
RSA 2	KwaZulu	28-1-85
RSA 3	KwaZulu	11-3-85
RSA 4	KwaZulu	12-3-85
RSA 5	KwaZulu	12-3-85
RSA 6	KwaZulu	10-7-84
RSA 7	KwaZulu[a]	26-6-86
RSA 8	KwaZulu[a]	2-9-86
RSA 9	KwaZulu[a]	2-9-86
RSA 10	KwaZulu[a]	2-9-86
RSA 11	KwaZulu[a]	2-9-86
RSA 12	KwaZulu[a]	2-9-86
RSA 13	Transvaal	6-2-87
RSA 14	Transvaal	4-2-87
RSA 15	Transvaal	15-2-87
RSA 16	Transvaal	16-2-87
RSA 17	Transvaal	4-2-87
RSA 18	KwaZulu	11-3-85
SL 1	Swaziland	4-7-85
MW 1	Malawi	24-1-85
MW 2	Malawi	22-7-86
FCR-3	The Gambia	3-8-76

[a] These isolates were collected from labourers employed on farms in the Richards Bay area of Natal (a non-malarious area). They became infected either in northern KwaZulu or in Mozambique.

2.2.3 Red blood cells and serum

Human O-positive cells, collected in lithium-heparin, were stored as whole blood at 4°C for up to a week after collection. Prior to use, they were washed twice in 10 volumes of culture medium (without serum) and centifuged at 1 500 rpm for 5 minutes in a Beckman J-6B centrifuge. Care was taken to remove the buffy coat after spinning. Human A-positive serum was used in routine cultures and AB-positive in newly-initiated cultures to avoid damaging the infected cells of the patient. Serum was stored at -20°C.

2.2.4 Culture media

RPMI 1640 medium (Flow Laboratories), buffered with HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) was prepared as described by Jensen and Trager[6] and supplemented with glucose (4 g/l), hypoxanthine (44 mg/l), gentamycin (50 mg/l) and human serum (10%). RPMI 1640 buffered with TES (N-tris (hydroxymethyl) methyl-2-amino-ethanesulphonic acid)[14] was supplemented with glucose (2 g/l), gentamycin (50 mg/l) and human serum (10%) and further diluted with distilled H_2O (170 ml/l).

2.2.5 Cultivation procedure

Isolates were cultured in 50 ml and 250 ml tissue culture flasks (Sterilin) containing 5 ml and 15 ml respectively of a 5% suspension of infected cells in

the culture medium. Flasks were gassed with a mixture[15] of 3% O_2, 4% CO_2 and 93% N_2 and the lids were tightly secured. The flasks were incubated at 38°C, either in a static position or agitated on a shaker (approximately 60 oscillations/min.). The shaker was switched off 2 hours before each daily medium change to allow the erythrocytes to settle. The flasks were gently tilted to allow spent medium to be aspirated off. Fresh medium, warmed to 38°C, was added: 5 ml to 50 ml flasks and 15 ml to 250 ml flasks. Cultures were diluted with fresh red blood cells every 3 or 4 days. Parasite growth was assessed by examination of Giemsa-stained smears prepared from the settled cells.

2.3 Results and Discussion

2.3.1 Growth conditions

A controlled experiment was carried out to determine which of the 2 culture systems, static or suspension, was the most suitable for the growth of *P. falciparum* under local conditions. The FCR-3 strain was grown at a 5% haematocrit in both HEPES and TES media in static and shaken flasks for 4 days with daily medium change. Suspension cultures proved to be superior to static cultures and in each system the use of HEPES medium resulted in better yields than did the TES medium (Fig. 1). The 72-hour multiplication rate was 23.7-

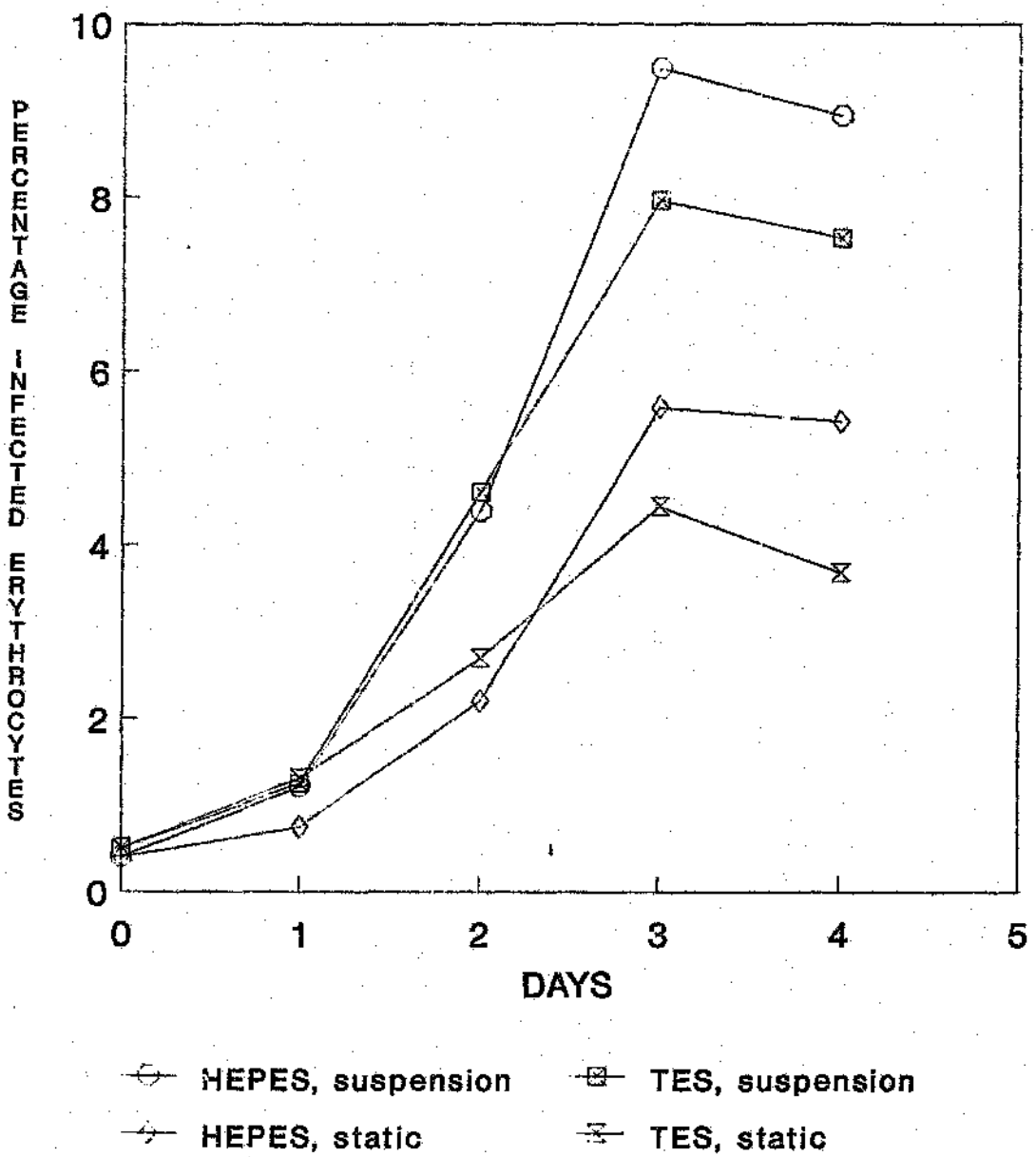

Figure 1. Effect of different culture conditions on the growth of the FCR-3 strain of *P. falciparum*.

fold in the HEPES medium and 15.92-fold in the TES medium in the agitated flasks, while in static flasks it was 13.95-fold and 8.88-fold in the HEPES and TES media respectively (mean of 3 flasks). The reason for the decline in parasite numbers in all flasks from day 3 to day 4 is unclear. It is possible that the isolate had reached its peak parasitaemia in all culture systems somewhere between day 3 and 4, with a resultant decrease in parasitaemia by day 4.

The FCR-3 strain was also grown at a 1% haematocrit in agitated flasks in both HEPES and TES media for 3 days without medium change. The 72-hour growth rate was 24.11-fold in the HEPES medium and 15.75-fold in the TES medium (mean of 3 flasks). These results are comparable with those obtained from the first growth experiment carried out at a 5% haematocrit with daily medium change.

2.3.2 Establishment and maintenance of cultures

Both static and suspension culture systems using either the HEPES or the TES medium were found to be suitable for the establishment and maintenance of *P. falciparum* in culture. However, the best growth rates were achieved for isolates grown in suspension in the HEPES medium. Four southern African isolates, namely RSA 6, RSA 18, SL 1 and MW 1, were established in culture by the static culture technique. Eight

isolates, namely RSA 3, 7, 8 - 12 and MW 2, were established in culture using the suspension system. It was found that suspension cultures generally peaked at higher parasitaemias than static cultures. The maximum number of infected cells ranged from 4 to 12% in isolates grown in static flasks and from 9 to 27% in isolates grown in shaken flasks. Growth rates were also found to differ in the 2 systems. The average 48-hour growth rate of individual isolates ranged from 3.2 to 4.2-fold in static cultures and from 3.9 to 9.5-fold in suspension cultures. These growth rates were measured once the isolates had passed the adaptive phase in which there is often negative growth accompanied by the presence of morphologically degenerate parasites.

The indications are that glucose supplementation of the culture medium is necessary for the establishment of southern African strains, although this has not been quantified. Culture of 6 isolates was attempted in static flasks using the Jensen and Trager[6] medium which contains 2 g glucose/l, and all failed to grow. Cryopreserved sub-samples of 3 of these isolates were later thawed and successfully grown in glucose-enriched HEPES medium (6 g/l). Consequently, all isolates that were established in culture were initiated and grown in the enriched medium. However, 2 of these isolates, once adapted to culture, were

successfully maintained in medium containing no additional glucose.

For the establishment of new isolates, it was found that it was advantageous to have higher starting parasitaemias although several isolates with initial parasitaemias as low as 0.01% were successfully established in culture. Furthermore, isolates appeared to adapt more readily to the culture conditions in shaken flasks. Seven isolates grown in shaken flasks with starting parasitaemias ranging from 0.01 - 0.7% all reached 1% within 6 days. The 4 isolates established in static flasks were placed in culture at parasitaemias ranging from less than 0.01% - 3.8% and the time taken for those isolates initiated at below 1% to reach this level ranged from 4 to 48 days.

Twenty established isolates are now being maintained *in vitro* at the Medical Research Council laboratory in Durban. Two isolates, RSA 18 and SL 1, were lost in culture as a result of bacterial contamination. Not all of the cultured isolates meet the criterion of Chin and Collins[8] for established strains (namely, a growth rate of greater than 10-fold in 96 hours). However, these strains are considered to be established as they have all been in culture for longer than 2 months and all have been frozen, thawed and successfully recultured at least once, meeting the

requirements of Campbell[16] for established isolates. Isolates are now routinely cultured in the HEPES medium at a 5% haematocrit from Monday to Friday with a daily medium change and at a 1% haematocrit from Friday to Monday with no medium change.

3. ENZYME TYPING

3.1 Introduction

Isoenzyme electrophoresis has proved to be a useful tool for determining genetic variation within and between populations of *P. falciparum*.[5] Several studies have shown that similar forms of enzymes occur in populations from different geographical regions suggesting that *P. falciparum* exists as a single interbreeding worldwide population. However, the frequencies of variants have been found to differ between regions. Furthermore, some isolates have been found to possess more than 1 form of a given enzyme. These are assumed to be clonal mixtures of parasites possessing different forms of the enzyme.

Most investigations of isozyme variation in *P. falciparum* have been carried out using starch gel electrophoresis. However, it was found that resolution of bands was improved by using polyacrylamide gels. The results of a study of the variation in 5 enzymes of 19 culture-adapted southern African isolates of *P. falciparum* are reported in this chapter.

3.2 Materials and Methods

The Gambian isolate, FCR-3,[7] which was characterized by Sanderson *et al.*,[17] was used as reference sample. All isolates were grown in suspension culture to a parasitaemia of at least 1% schizonts. Parasites were released from their host erythrocytes by saponin lysis as described by Sanderson *et al.*[17] and further lysed using 1% Triton-X-100 in EDTA-Tris-HCl buffer, pH 7.4.[18] Material for electrophoresis was prepared from each isolate on 3 separate occasions during 11 months of continuous cultivation. Lysates were stored in liquid nitrogen until required.

Electrophoresis was carried out on 7.5% polyacrylamide gels with a 2.8% cross-linkage. The enzymes studied were glucose phosphate isomerase (GPI) (EC 5.3.1.9), adenosine deaminase (ADA) (EC 3.5.4.4), lactate dehydrogenase (LDH) (EC 1.1.1.27), NADP-dependent glutamate dehydrogenase (GDH) (EC 1.4.1.4) and 6-phosphogluconate dehydrogenase (PGD) (EC 1.1.1.44).

The conditions of electrophoresis for GPI, LDH and GDH were basically as described by Carter[19] and for PGD and ADA as described by Harris and Hopkinson.[20] After electrophoresis, the agar enzyme solutions were poured over the surface of the gels and the gels were incubated at 37°C for approximately 1 hour. The

conditions of electrophoresis and the enzyme assay solutions are described below.

GPI

Electrode buffer: 0.2 M Tris-HCl, pH 8.0

Gel buffer: 0.05 M Tris-HCl, pH 8.0

Voltage: 200 V for 6 hrs (100 V for 1st 15 mins)

Enzyme assay solution:

- Fructose-6-phosphate 25 mg
- NADP 2.5 mg
- MgCl 10 mg
- Glucose-6-phosphate dehydrogenase 10 µl
- MTT 2.5 mg
- PMS 2.5 mg
- Electrode buffer 12.5 ml
- 2% agar 12.5 ml
- Glycerol 5 ml

ADA

Electrode buffer: 0.1 M phosphate, pH 6.5

Gel buffer: 0.01 M phosphate, pH 6.5

Reaction buffer: 0.05 M phosphate, pH 7.5

Voltage: 150 V for 2 hrs

Enzyme assay solution:

Adenosine 8 mg

Xanthine oxidase 15 µl

Nucleoside phosphorylase 15 µl

PMS 2.5 mg

MTT 2.5 mg

ADA reaction buffer 12.5 ml

2% agar 12.5 ml

Glycerol 5 ml

LDH

Electrode buffer: 0.22 M Tris - 0.0785 M Citrate, pH 6.2

Gel buffer: 0.065 M Tris - 0.0222 Citrate, pH 6.5

Voltage: 200 V for 4 hrs (100 V for 1st 15 mins)

Enzyme assay solution:

Lithium lactate 100 mg

NAD 5 mg

MTT 2.5 mg

PMS 2.5 mg

Electrode buffer 12.5 ml

2% agar 12.5 ml

GDH

Electrode buffer: 0.013 M Citrate - 0.155 M Na_2HPO_4, pH 7.0

Gel buffer: 0.0054 M Citrate - 0.032 M Na_2HPO_4, pH 7.0

Voltage: 200 V for 4 hrs (100 V for 1st 15 mins)

Enzyme assay solution:

Monosodium glutamate 50 mg

NADP 2.5 mg

MTT 2.5 mg

PMS 2.5 mg

Electrode buffer 12.5 ml

2% agar 12.5 ml

PGD

Electrode buffer: 0.1 M phosphate, pH 7.0 (add 10 mg NADP/500 ml buffer for cathodal tank)

Gel buffer: 0.01 M phosphate, pH 7.0 (add 4 mg NADP/ 200 ml gel mixture prior to pouring gel)

Reaction buffer: 0.5 M Tris - HCl, pH 8.0

Voltage: 200 V for 4 hrs (100 V for 1st 15 mins)

Enzyme assay solution:

$MgCl_2.6H_2O$ 200 mg

NADP (Na_2 salt) 2.5 mg

MTT 2.5 mg

PMS 2.5 mg

PGD reaction buffer 12.5 ml

2% agar 12.5 ml

3.3 Results and Discussion

The results for the 5 enzymes are shown in Table 2. There was little variation amongst the southern

Table 2. Distribution of enzyme forms in 19 southern African isolates of *Plasmodium falciparum*.[a]

Isolate	GPI	ADA	LDH	GDH	PGD
RSA 1	1+2[b]	1+2[c]	1	1	1
RSA 2	1	1	1	1	1
RSA 3	1+2[d]	1	1	1	1
RSA 4	1+2[d]	1	1	1	1
RSA 5	1	2	1	1	1
RSA 6	1+2[d]	1	1	1	1
RSA 7	1	1	1	1	1
RSA 8	1	1	1	1	1
RSA 9	1	1	1	1	1
RSA 10	1	1	1	1	1
RSA 11	1	1	1	1	1
RSA 12	1+2[b]	1	1	1	1
RSA 13	1+2	1	1+2	1	1
RSA 14	1+2[b]	1	1	1	1
RSA 15	1+2[b]	1	1	1	1
RSA 16	1	1	1	1	1
RSA 17	1	1	1	1	1
MW 1	1	1	1	1	1
MW 2	1	1	1	1	1
FCR-3	1	1	1	1	1

[a] Lysates were prepared from each isolate on 3 separate occasions during 11 months of continuous culture.

[b] GPI-1 and -2 were present in the 1st lysates prepared from these isolates but only GPI-1 was present in subsequent lysates.

[c] ADA-1 and -2 were present in the 1st and 3rd lysates prepared from RSA 1 but only ADA-2 was found in the 2nd lysate.

[d] GPI-1 and -2 were present in the 3rd lysate prepared from these isolates but only GPI-1 was present in the 1st and 2nd lysates.

African isolates and all enzymes showed similar forms in isolates from the different geographical regions. Ten of the 19 isolates exhibited the same electrophoretic pattern as the Gambian isolate, FCR-3. GDH and PGD revealed no variation amongst the isolates. This has been the experience of other workers.[5] GPI exhibited the most variation while ADA and LDH showed limited variation.

The enzyme frequencies of the southern African isolates were similar to those of isolates from other parts of the world.[5] GPI-1 occurred in 11 of the 19 isolates tested while the remaining 8 had both GPI-1 and -2. The ADA frequencies were similar to those found in the Gambia and Thailand where over 90% were ADA-1. Eighteen of the isolates studied had ADA-1 while the remaining isolate had both ADA-1 and -2. LDH-2, which has only been found in African isolates, appeared with LDH-1 in 1 southern African isolate.

Of the 8 isolates possessing both GPI-1 and -2, only 1 retained both variants throughout the test period. In 4 of these isolates, GPI-2 was only seen in the 1st lysate but not in lysates prepared subsequently. A further 3 isolates exhibited only GPI-1 in the 1st and 2nd tests but both GPI-1 and -2 in the 3rd test. Similarly, 1 isolate possessed both ADA-1 and -2 in the 1st and 3rd lysates but only ADA-2 in the 2nd lysate. The ADA-2 band in the 1st and 3rd lysates was

much darker than the ADA-1 band, thus indicating a higher proportion of parasites with ADA-2.

These changes in the electrophoretic pattern of an isolate with time may be ascribed to the relative numbers of parasites possessing the different forms of the enzymes at the time of preparation of lysates. Thus, parasites exhibiting a variant which appears after some time in culture may have grown from an undetectable number of organisms to a number sufficient to produce a band on the stained gel. Parasites characterized by a variant which disappears after some time in culture may be present in undetectable numbers or may even have died out in culture.

The results of this study lend further support to the conclusion that *P. falciparum* isolates from different geographical regions constitute a single species.

4. TYPING WITH MONOCLONAL ANTIBODIES

4.1 Introduction

The ever-increasing problem of drug resistance shown by malarial parasites[21] has made the development of a vaccine a top priority. Information on antigenic diversity within and between parasite populations is obviously essential for research aimed at vaccine production. A contribution in this regard has been made by an indirect immunofluorescent test using monoclonal antibodies[22] which demonstrated antigenic diversity amongst culture-adapted isolates of *P. falciparum* from different endemic regions.[22-24] The results of Schofield and others[25] following the raising of monoclonal antibodies against two isolates from Papua New Guinea suggested that parasites from a given geographical region are antigenically similar. As the antigenic diversity within the southern African population of *P. falciparum* has not previously been determined, a study was conducted in which 19 culture-adapted isolates from this region were serotyped using a panel of nine monoclonal antibodies. The results are presented in this chapter.

4.2 Materials and Methods

4.2.1 *P. falciparum* isolates

Nineteen culture-adapted southern African isolates of *P. falciparum*, namely RSA 1 - 17 and MW 1 and 2, were used. The Gambian FCR-3 isolate[7] was used as a reference strain throughout this study.

4.2.2 Preparation of antigen slides

The time *in vitro* between initiation of a culture and preparation of antigen slides for the first test ranged from 3 days to 19 weeks for the 19 isolates investigated. All isolates except RSA 8, 9, 11 and MW 2 had been cryopreserved at least once before preparation of the first set of antigen slides. Subsequent antigen slides were prepared from cultures within 11 weeks following preparation of the first set; and during this time only 4 isolates, namely RSA 3, 4, 5 and 13, were not cryopreserved, thawed and recultured.

Antigen slides were prepared from each isolate on 3 different occasions between July 1986 and October 1987. Parasites were harvested when at least 1% of erythrocytes contained schizonts. Infected red blood cells were washed twice in serum-free RPMI 1640 medium before being suspended to 10% in PBS, pH 7.2. Aliquots (5 µl) of the suspensions were spotted on to multiwell

slides and allowed to dry at room temperature before being stored in a desiccated state at -20°C. Immediately prior to use, antigen slides were fixed in acetone at -20°C for 30 minutes and washed 3 times in PBS.

4.2.3 Monoclonal antibodies

The antibody D5 was raised in rats against *P. berghei*, and it cross-reacts with *P.falciparum* antigen.[26] The other monoclonal antibodies used are specific for *P. falciparum* and were raised in mice against Thai isolates of the parasite.[22,27,28] Antibody 12.3 recognises a strain-specific epitope on a polymorphic 35-48 kDa glycoprotein from the merozoite surface.[28] Monoclonal antibody 5.1 recognises a variant of an internal 23 kDa protein of asexual blood forms, and cross-reacts with the circumsporozoite protein of sporozoites.[29] The other antibodies are all specific for polymorphic schizont antigen, the polymorphic 185-205 kDa schizont precursor of merozoite surface antigens.[27,30] Monoclonal antibody 9.8 is directed against a conserved epitope, while antibodies 7.3 and 7.6 or 9.2, 10.3 and 12,1 mark mutually exclusive groups of strain-specific epitopes localised in different regions of the molecule.[27,31] In this chapter, the antigenic epitopes are referred to by the same code numbers as the monoclonal antibodies which recognise them.

4.2.4 Indirect fluorescent antibody (IFA) test

The IFA procedure was as described.[24,27] Briefly, 20 µl of a predetermined dilution of monoclonal antibodies (1:100 to 1:5000 for different reagents) was applied to the wells and the slides were incubated for 30 minutes in a humid chamber at room temperature. After 3 washes in PBS, 20 µl of a 2.5% dilution of fluorescein-conjugated rabbit anti-mouse immunoglobulin (Bio-Yeda, Israel) in PBS containing 0.01% Evans Blue was added to each well. The slides were again incubated for 30 minutes in a humid chamber at room temperature. The slides were then washed 3 times in PBS and mounted in 50% glycerol in PBS. Parasites were observed under white and ultra-violet light using a fluorescence microscope. The reaction of each isolate with a particular monoclonal antibody was measured by estimating the proportion of fluorescent schizonts relative to the number of pigment granules observed under white light. Reactions were rated as positive if the majority of parasites (≥50%) reacted and negative if none reacted. Isolates with a minority of fluorescent parasites (<50%) were assumed to comprise a mixture of antigenically distinct clones.

4.3 Results

The reactions of 20 isolates with the panel of 9 monoclonal antibodies are summarised in Table 3. All 20 isolates reacted positively with monoclonal antibodies D5 and 9.8. Eighteen isolates reacted to some degree with monoclonal antibodies 5.1, 7.3, 7.6 and 12.3. Schizonts of 15 isolates reacted with monoclonal antibodies 9.2 and 10.3 and only 4 isolates reacted to any extent with monoclonal antibody 12.1.

Only 4 isolates, RSA 5, RSA 10, MW 1 and the control, FCR-3, appeared homogeneous in their reaction with the monoclonal antibodies and their patterns of reactivity remained stable with time in culture. These isolates contained either a majority of organisms which reacted with a given antibody or none at all. In this study, the reference isolate, FCR-3, gave the same pattern of reactivity with the 9 monoclonal antibodies as that obtained in a previous investigation.[22]

Sixteen isolates appeared to comprise at least 2 parasite populations with different immunological reactivities. These isolates contained a minority of schizonts which reacted positively with at least one of the antibodies while the majority were non-reactive. Some of these isolates were found to differ in their proportions of reactive schizonts (from 0% to

Table 3. Reactions of 9 monoclonal antibodies with 19 southern African isolates (and a control isolate, FCR-3) of Plasmodium falciparum.

ANTIGEN	P. berghei	gp 185-205						gp 35-48	p23	ANTIGENIC PATTERN AT FIRST TEST	ANTIGENIC PATTERN AT THIRD TEST
MONOCLONAL ANTIBODY-DEFINED EPITOPES	D5	9.8	7.3	7.6	9.2	10.3	12.1	12.3	5.1		
ISOLATE ORIGIN											
RSA 13 Transvaal	+	+	±	±	±	±	±	±	+	I	I
RSA 3 KwaZulu	+	+	±	±	±	±	-	±	+	II	II
RSA 2 KwaZulu	+	+	+	+	±	±	-	-	+	III	III
RSA 1 KwaZulu/Mozambique	+	+	+	+	±	±	-	±	-	IV	IV
RSA 15 Transvaal	+	+	±	±	±	±	±	±	±	I	V
RSA 17 Transvaal	+	+	+	+	±	±	±	±	±	I	V
MW 2 Malawi	+	+	+	+	±	±	-	±	+	II	V
RSA 6 KwaZulu	+	+	±	±	±	±	-	+	+	II	V
RSA 7 KwaZulu/Mozambique	+	+	±	±	±	±	-	±	+	II	V
RSA 8 KwaZulu/Mozambique	+	+	±	±	±	±	-	±	±	II	V
RSA 9 KwaZulu/Mozambique	+	+	±	±	±	±	-	+	+	II	V
RSA 11 KwaZulu/Mozambique	+	+	±	±	±	±	-	±	+	II	V
RSA 14 Transvaal	+	+	±	±	±	±	-	±	+	II	V
RSA 10 KwaZulu/Mozambique	+	+	+	+	-	-	-	+	+	V	V
RSA 12 KwaZulu/Mozambique	+	+	+	+	-	-	-	±	+	V	V
RSA 16 Transvaal	+	+	+	+	-	-	-	±	+	V	V
MW 1 Malawi	+	+	+	+	-	-	-	+	+	V	V
FCR-3(Control) The Gambia	+	+	+	+	-	-	-	+	+	V	V
RSA 4 KwaZulu	+	+	-	-	+	+	±	±	+	VI	VI
RSA 5 KwaZulu	+	+	-	-	+	+	-	-	-	VII	VII

\+ positive reaction, i.e. ≥ 50% of parasites reacted.
\- negative reaction, i.e. none of the parasites reacted.
± mixed reaction, i.e. < 50% of parasites reacted. Isolates which contained different proportions of reacting parasites in the 3 tests were scored as for a mixed reaction.

≥50%) at different times during the test period. Such reactions were scored as <50% in Table 3, indicating a mixture of at least 2 antigenically distinct clones.

Nine of the isolates, namely RSA 6, 7, 8, 9, 11, 14, 15, 17 and MW 2, reacted with antibodies from both mutually exclusive sets (7.3/7.6 and 9.2/10.3) in the first test although most of them contained a minority of reactive schizonts (Table 4). However, by the time schizonts were harvested from these isolates for the third test, the majority reacted with antibodies 7.3 and 7.6 and none reacted with 9.2 and 10.3. All of these isolates were cryopreserved after antigen slides had been prepared for the first test and they were thawed and re-established in culture prior to harvesting of parasites for the second and third tests. A further 4 isolates, RSA 1, 2, 3, and 13, contained schizonts reactive in all 3 tests to both pairs of monoclonal antibodies. Only RSA 2 was frozen, thawed and recultured during the test period.

Isolates RSA 15 and 17 also varied in their reactions with antibody 12.1 in the 3 tests. Both these isolates contained a minority of parasites which reacted positively with monoclonal antibody 12.1 in the first test but were negative for this antibody in the second and third tests (results not shown in figures).

Seven reactivity patterns were observed within this

Table 4. Reactions of 13 southern African isolates of *Plasmodium falciparum* with monoclonal antibodies 7.3, 7.6, 9.2 and 10.3.

	MONOCLONAL ANTIBODIES											
	7.3			7.6			9.2			10.3		
	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
ISOLATE												
RSA 6	±	+	+	±	+	+	±	±	-	±	±	-
RSA 7	±	+	+	±	+	+	+	-	-	+	-	-
RSA 8	±	+	+	±	+	+	±	±	-	±	±	-
RSA 9	±	+	+	±	+	+	±	-	-	±	-	-
RSA 11	+	±	+	+	±	+	±	±	-	±	±	-
RSA 14	±	±	+	±	±	+	±	±	-	±	±	-
RSA 15	±	+	+	±	+	+	±	-	-	±	-	-
RSA 17	+	+	+	+	+	+	±	-	-	±	-	-
MW 2	+	+	+	+	+	+	±	±	±	±	-	-
RSA 1	±	±	±	±	±	±	±	±	±	±	±	±
RSA 2	+	+	+	+	+	+	±	±	±	±	±	±
RSA 3	±	±	±	±	±	±	±	±	±	±	±	±
RSA 13	±	±	±	±	±	±	±	±	±	±	±	±

\+ positive reaction, i.e. ≥50% of parasites reacted
\- negative reaction, i.e. none of the parasites reacted
± mixed reaction, i.e. <50% of parasites reacted

group of southern African isolates (Table 3). However, by the time parasites were harvested for the third IFA test, 70% of the isolates, including FCR-3, displayed an identical antigenic pattern, namely a positive reaction with antibodies D5, 5.1, 7.3, 7.6, 9.8 and 12.3 and a negative reaction with antibodies 9.2, 10.3 and 12.4 (designated antigenic pattern V in this study).

4.4 Discussion

Each of the southern African *P. falciparum* isolates reacted with at least 4 of the monoclonal antibodies raised against isolates of *P. falciparum* from Thailand. This lends further support to the view that antigenic determinants are shared by isolates from different parts of the world.[22] The 8 *P. falciparum* monoclonal antibodies revealed considerable diversity within this group of southern African isolates and 7 different patterns of reaction were observed. The positive response of all the southern African isolates to monoclonal antibody 9.8 was to be expected as this specificity has been detected in over 200 culture-adapted isolates from all over the world.[27]

The *P. berghei* monoclonal antibody, D5, has been successfully used to detect *Plasmodium* in infected blood by means of a radioimmunoassay.[32] The ubiquitous response of the southern African strains to this

antibody indicates that it may be suitable for the immunodiagnosis of malaria in this region.

The heterogeneous reactivities of 16 isolates with the monoclonal antibodies may be ascribed to the presence of more than one antigenically distinct clone in each isolate. The relative proportions of schizonts might be expected to vary with time in culture as a result of asynchronous growth of parasites. Thus, individual harvests of a cultured isolate would contain differing proportions of reactive schizonts in the IFA test. Similar heterogeneity was observed within this same group of southern African isolates with respect to isoenzyme electrophoretic patterns and *in vitro* chloroquine sensitivity (see Chapters 3 and 5).

McBride and others[27] found that isolates which reacted with antibodies 7.3 and 7.6 did not react with antibodies 9.2, 10.3 and 12.1 (and *vice versa*). These authors suggested that these combinations of antigenic epitopes were mutually exclusive and represented different forms of the same gp 185 - 205 K antigen.[27] They also found that individual clones derived from an isolate which reacted to some extent with both sets of antibodies, likewise reacted with either one or the other combination but not with both.

In this study, 13 of the southern African isolates

reacted to a limited degree with antibodies from both sets in the first test, suggesting that at the time of preparation of antigen slides, these isolates consisted of mixtures of organisms expressing alternative forms of the antigen. By the time schizonts were harvested for the third test (1 to 11 weeks later), 9 of these isolates contained a majority of schizonts which reacted with antibodies 7.3 and 7.6 but had completely lost parasites reacting with antibodies 9.2 and 10.3. It is possible that those clones possessing antigenic markers 7.3 and 7.6 were able to withstand the freezing/thawing process and/or the culture conditions while parasites possessing the alternative form were not. This could have resulted in loss of organisms specific for monoclonal antibodies 9.2 and 10.3 in culture. Four isolates possessed both sets of antigenic determinants during the entire test period. Only one of these was cryopreserved during the study period.

Only 2 of the 19 southern African isolates were found to be non-reactive to monoclonal antibodies 7.3 and 7.6. Furthermore, none of the 13 clonally heterogeneous isolates lost the antigenic markers for these antibodies. This was in contrast to 7 cultured isolates from Brazil which were all negative for monoclonal antibodies 7.3 and 7.6 and positive for antibodies 9.2 and 10.3,[33] as well as to primary isolates from Papua New Guinea, where the majority

reacted positively with 9.2 and 10.3 and negatively with 7.3.[24] Twelve out of 17 Thai isolates were found by McBride and others[22] to be specific for antibodies 9.2 and 10.3 and one isolate reacted with both sets of antibodies. It is possible that, through natural selection, parasites in a given geographical region may tend to develop towards a similar antigenic type. However, the various studies of cultured isolates were carried out using parasites which had been maintained *in vitro* under different conditions in different laboratories, each of these having an environment vastly different from the *in vivo* situation. The culture conditions in a given laboratory may have resulted in the selection of antigenically similar organisms in that laboratory. In the present study, 70% of all isolates tested exhibited an identical reaction pattern at the end of the test period after having been passaged many times in culture and, in some cases, after they had been subjected to at least one cryopreservation and thawing procedure.

Ideally, characterisation studies should be carried out on primary isolates and their clones in order to determine the antigenic constitution of the wild population of *P. falciparum*. A panel of monoclonal antibodies raised against isolates from various endemic regions of the world might additionally be used to provide the answer to the question of regional

variation within the worldwide population of *P. falciparum*.

5. *IN VITRO* SENSITIVITY OF REFERENCE ISOLATES OF *PLASMODIUM FALCIPARUM* TO CHLOROQUINE AND PYRIMETHAMINE. USE OF MICROSCOPY TO ASSESS PARASITE GROWTH IN A 48-HOUR GROWTH-INHIBITION TEST

5.1 Introduction

One of the major problems facing malaria control is the resistance which *P. falciparum* develops to antimalarial drugs. Until the late 1970s, chloroquine was used very successfully in Africa but since publication of the first reports of chloroquine resistance from the continent,[34,35] *in vivo* and *in vitro* resistance has been documented in many African countries including Mozambique, Angola, South West Africa/Namibia,[36] Zimbabwe[37] and Swaziland.[38] Chloroquine resistance was first noted in the Republic of South Africa (RSA) in 1985 but many malariologists overseas are unaware of this, although attention was drawn to it at the time in a local medical journal.[39,40,41] Resistance of *P. falciparum* to pyrimethamine is similarly widespread in Africa[42] but resistance to this drug has not been monitored as closely as has chloroquine resistance. The number of cases of *P. falciparum* malaria from northern KwaZulu (one of the endemic malarial areas of the RSA) which failed to respond to treatment with the chloroquine-

pyrimethamine combination, rose from 0% in 1983 to 16.1 and 21.2% in field and hospital cases respectively in 1987.[43] The *in vitro* chloroquine sensitivity of isolates from this region has previously been investigated to a limited extent but no pyrimethamine sensitivity studies have hitherto been carried out in the RSA.

As part of the characterisation study of southern African isolates, the *in vitro* sensitivity to chloroquine and pyrimethamine of 20 culture-adapted reference strains of *P. falciparum* was determined. Such data are particularly useful because these isolates have been well-characterised genetically (see Chapters 3 and 4). Some correlation has been found between the *in vitro* data and the *in vivo* sensitivity to chloroquine or a combination of chloroquine and pyrimethamine. The results are reported in this chapter.

5.2 Materials and Methods

5.2.1 Clinical data

Isolate RSA 2 was collected in January 1985 from a patient from the endemic area of KwaZulu who subsequently responded to treatment with 10 tablets Daraclor (1 tablet = 150 mg chloroquine base and 15 mg pyrimethamine base). RSA 3, 4 and 5 were obtained from

patients reporting to a clinic in KwaZulu during March 1985. Clinical data on these patients are unavailable. RSA 6, obtained in July 1884, was from a KwaZulu patient who failed to respond to chloroquine treatment. RSA 1, 7, 8, 9, 10, 11 and 12 were obtained from apparently healthy labourers employed on farms in a non-malarious area of Natal. The patients from whom RSA 8, 10 and 11 were collected all failed to respond to treatment with 4 tablets Daraclor but were cleared of parasites when treated with 10 tablets Daraclor. Those from whom RSA 7 and 12 were obtained did not respond to treatment with 10 tablets Daraclor. Clinical data on the patients from whom RSA 1 and 9 were collected are unavai' ble. Isolates RSA 13 - 17 were obtained from patients from the endemic area of the North-eastern Transvaal. Three of these isolates, RSA 15 - 17, were from patients who were known to have been cleared of their symptoms after treatment with Daraclor. However, the *in vivo* response of the parasites to the treatment is not known as blood smears were not taken after treatment. Clinical data on the remaining 2 patients from this area are unavailable. MW 1 and MW 2 were collected from patients who became infected while visiting Malawi. In both cases, the parasites were resistant to chloroquine treatment. FCR-3 was isolated from a patient in the Gambia.[7] Clinical data on the patient are not available.

5.2.2 *In vitro* test

The *in vitro* sensitivities of the culture-adapted isolates to chloroquine and pyrimethamine were determined using the 48-hour test of Nguyen-Dinh and Payne,[44] modified as follows.

Infected red blood cells were diluted with fresh, washed, O-positive erythrocytes to a parasitaemia of between 0.1% and 0.9% and a 2% suspension in the culture medium was prepared. 0.5 ml aliquots of this suspension were added to the wells of 24-well plates which had been predosed with chloroquine and pyrimethamine to give final concentrations of 0, 0.03, 0.06, 0.1, 0.3, 0.6 and 1.0 μmols chloroquine/l and 0, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0 and 3.0 μmols pyrimethamine/l. Each isolate was tested in 3 wells at each drug concentration. The plates were agitated for a few seconds to dissolve the drugs and were then placed in a desiccator which was gassed for approximately 3 mins. The desiccator was tightly sealed and placed in an incubator at 38°C for 48 hours. At 24 hours, the plates were agitated to resuspend the settled red blood cells and the desiccator was regassed. Parasite counts were made microscopically from Giemsa-stained thin films prepared at the beginning and the end of the test. The minimum inhibitory concentration (MIC) was defined as the lowest drug concentration at which no parasite growth took place. Each isolate was tested on 3

different occasions between August 1986 and September 1987. The period between initiation of a culture and the first *in vitro* test ranged from 1 to 29 months. Subsequent tests were carried out within 12 months of the first test.

5.3 Results

5.3.1 Typical *in vitro* response to chloroquine and pyrimethamine

Tables 5 and 6 show the typical *in vitro* response of *P. falciparum* to chloroquine and pyrimethamine,

Table 5. In vitro response of isolate RSA 7 to chloroquine in the 48-hour test

Chloroquine concentration (μM)	Parasitized erythrocytes/10^4 [a]					
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total
Time 0	23	8	1	-	-	32
At 48 hours:						
Control	229	150	21	15	-	415
0.03	234	148	22	16	-	420
0.06	269	120	17	13	-	419
0.1	273	128	5	7	-	413
0.3	151	29	2	2	-	184
0.6	1	2	-	-	-	3

[a] Mean of three wells.
[b] Schizonts with two nuclei.
[c] Schizonts with three or more nuclei.

respectively. The responses of the 20 isolates to chloroquine and pyrimethamine are shown in Appendixes A and B, respectively. At low chloroquine concentrations, parasite counts were similar to those in the control wells and in some cases were even higher (Table 5). However, at concentrations close to the MIC, the number of mature parasites decreased until only rings and trophozoites remained at the end-point. In the pyrimethamine test, the number of rings and trophozoites decreased as concentrations became inhibiting and only morphologically degenerate trophozoites and schizonts were seen at the inhibitory

Table 6. In vitro response of isolate MW 2 to pyrimethamine in the 48-hour test

Pyrimethamine concentration (μM)	Parasitized erythrocytes/10^4 [a]					
	Rings	Trophozoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gametocytes	Total
Time 0	7	3	-	5	-	15
At 48 hours:						
Control	334	38	6	18	-	396
0.003	330	37	5	18	1	391
0.01	309	42	8	25	1	385
0.03	201	17	6	16	-	240
0.1	-	1	-	2	-	3

[a] Mean of three wells.
[b] Schizonts with two nuclei.
[c] Schizonts with three or more nuclei.

concentration (Table 6). Neither drug affected the gametocyte counts.

5.3.2 Initial tests

The results of the initial *in vitro* tests are shown in Table 7. Four KwaZulu isolates, RSA 2, 3, 4 and 5, were inhibited at chloroquine concentrations of 0.1 μM or less, indicating sensitivity to the drug.[45] All other KwaZulu isolates, namely RSA 1, 6, 7, 8, 9, 10, 11 and 12 were chloroquine-resistant *in vitro*, having MICs of 0.3 or 0.6 μM. Only 1 of the isolates from the North-eastern Transvaal, RSA 13, was sensitive to chloroquine, having been inhibited at 0.06 μM. The remaining 4 isolates from this region were resistant *in vitro*, all having MICs of 0.3 μM or greater. Both clinically chloroquine-resistant Malawian isolates, were resistant *in vitro*. The Gambian FCR-3 strain was inhibited at 0.6 μM.

The pyrimethamine MIC values of the southern African isolates ranged from 0.01 μM to >3.0 μM with the majority of the strains tested having been inhibited at 0.1 μM. Three isolates were inhibited at 0.03 μM or less and 2 were not inhibited at the highest concentration tested (namely 3 μM).

5.3.3 Change in drug sensitivity over time

Table 8 shows the results of the three chloroquine and pyrimethamine *in vitro* tests. The chloroquine MIC

Table 7. Initial *in vitro* response of southern African isolates of *Plasmodium falciparum* to chloroquine and pyrimethamine in the 48-hour test.

Isolate	Origin	Minimum inhibitory concentration (μM)	
		Chloroquine	Pyrimethamine
RSA 1	KwaZulu	0.6	0.03
RSA 2	KwaZulu	0.06	0.1
RSA 3	KwaZulu	0.06	> 3.0
RSA 4	KwaZulu	0.06	0.03
RSA 5	KwaZulu	0.1	> 3.0
RSA 6	KwaZulu	0.3	0.1
RSA 7	KwaZulu	0.6	1.0
RSA 8	KwaZulu	0.6	0.1
RSA 9	KwaZulu	0.6	0.1
RSA 10	KwaZulu	0.6	0.1
RSA 11	KwaZulu	0.3	0.1
RSA 12	KwaZulu	0.6	0.1
RSA 13	Transvaal	0.06	0.01
RSA 14	Transvaal	0.6	0.1
RSA 15	Transvaal	0.6	0.1
RSA 16	Transvaal	1.0	0.1
RSA 17	Transvaal	0.3	0.1
MW 1	Malawi	0.6	0.1
MW 2	Malawi	0.6	> 3.0
FCR-3	The Gambia	0.6	0.1

Table 8. *In vitro* response of southern African isolates of *Plasmodium falciparum* to chloroquine and pyrimethamine in the 48-hour test - results of 3 tests.

	Minimum inhibitory concentration (μM)					
	Chloroquine			Pyrimethamine		
Isolate	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
RSA 1	0.6	0.3	0.3	0.03	0.1	0.03
RSA 2	0.06	0.06	0.06	0.1	0.1	0.1
RSA 3	0.06	0.06	0.1	>3.0	>3.0	>3.0
RSA 4	0.06	0.06	0.06	0.03	0.03	0.03
RSA 5	0.1	0.06	0.06	>3.0	3.0	>3.0
RSA 6	0.3	0.6	0.3	0.1	0.1	0.1
RSA 7	0.6	0.6	0.3	1.0	0.1	0.1
RSA 8	0.6	0.3	0.3	0.1	0.1	0.1
RSA 9	0.6	0.3	0.3	0.1	0.1	0.1
RSA 10	0.6	0.6	0.3	0.1	0.1	0.03
RSA 11	0.3	0.3	0.3	0.1	0.1	0.1
RSA 12	0.6	0.6	0.3	0.1	0.1	0.1
RSA 13	0.06	0.03	0.06	0.01	0.01	0.03
RSA 14	0.6	0.3	0.6	0.1	0.1	0.1
RSA 15	0.6	0.3	0.3	0.1	0.1	0.1
RSA 16	1.0	0.3	0.3	0.1	0.1	0.1
RSA 17	0.3	0.6	0.3	0.1	0.1	0.1
MW 1	0.6	0.3	0.6	0.1	0.1	0.1
MW 2	0.6	0.3	0.6	> 3.0	0.1	0.1
FCR-3	0.6	0.6	0.6	0.1	0.1	0.1

value of most strains was found to fluctuate between 2 concentrations during the test period but was constant for RSA 2, 4 and 11 as well as for FCR-3 over the 3 tests. This variability was not common in the pyrimethamine tests and only 6 cultured isolates, namely RSA 1, 5 , 7, 10 and 13 and MW 2, yielded varying pyrimethamine MICs at different times.

5.4 Discussion

5.4.1 Typical *in vitro* response to chloroquine and pyrimethamine

The effect of chloroquine and pyrimethamine on the numbers of parasites at the various developmental stages at or near their respective MICs demonstrates the different modes of action of the two drugs. Although the antimalarial action of chloroquine is not yet fully understood, it is known that this drug prevents the transformation of rings to trophozoites.[46] Pyrimethamine is an inhibitor of the enzyme dihydrofolate reductase and thus interferes with nuclear division.[47] The numbers of parasites at each stage of development, in addition to the total parasite count, therefore provided a good indicator of the MIC. In the chloroquine test, only rings and abnormal trophozoites were present at the MIC, while only degenerate trophozoites and schizonts remained at the MIC in the pyrimethamine test.

5.4.2 *In vivo/in vitro* relationships

All southern African isolates were inhibited at *in vitro* chloroquine concentrations of 0.6 μM or less except for 1 isolate from the North-eastern Transvaal which was inhibited at 1.0 μM in 1 of the tests. These MICs are comparable with those of other African isolates[48] but are lower than those of Thai isolates, found by Thaithong and others[49] to range from 0.1 to 1.5 μM with the majority at 1.0 or 1.5 μM.

Isolate RSA 2, which was sensitive to chloroquine *in vivo*, and isolates RSA 3, 4 and 5 were all chloroquine sensitive *in vitro*. These isolates were collected early in 1985 from patients reporting to a hospital and a clinic in the endemic malarial area of KwaZulu which borders on Mozambique. The first reports of *in vitro* chloroquine resistance from this area were recorded in the same year[40]. However, *in vitro* field studies carried out in KwaZulu in May 1987 and January 1988, after chloroquine had been extensively used in the area for prophylaxis and treatment, revealed a high percentage of resistant isolates (see Chapter 8). These resistant parasites were probably introduced into the local *P. falciparum* population by movement of people across the border from Mozambique. The problems associated with the influx of Mozambicans were highlighted in 1986 when a large proportion of malaria cases detected in Natal were recorded as having been

imported from Mozambique[50]. Six isolates included in this study, namely RSA 7, 8, 9, 10, 11 and 12, were obtained from labourers employed on sugar farms in this non-endemic area in 1986. RSA 1 came from a labourer working in the same area in 1985. Although these infections were recorded as having originated in KwaZulu, it is possible that they had, in fact, originated in Mozambique as refugees are often reluctant to admit that they are from Mozambique and prefer to state that they are residents of KwaZulu. Four of 5 isolates from the North-eastern Transvaal, which also lies adjacent to Mozambique, were shown by this study to be resistant to chloroquine *in vitro*. It is likely that these resistant strains had been imported into this endemic region. As was expected from the clinical histories of their hosts, the Malawian isolates proved to be resistant *in vitro*.

A wide range of pyrimethamine sensitivities (0.01 to >3.0 μM) was found amongst the southern African isolates as in Thailand, where sensitivities *in vitro* ranged from 0.001 to 100.0 μM.[49] As pyrimethamine was used in combination with chloroquine for treatment, it was not possible to determine the *in vivo* response of these southern African isolates to pyrimethamine. Most of these isolates were inhibited at 0.1 μM *in vitro* which, judging from the literature, would seem to indicate that these strains are not fully susceptible

to the drug. However, the relationship between the *in vivo* response to pyrimethamine and the *in vitro* MIC has not yet been clearly defined. Smalley and Brown[51] concluded, from a correlation of *in vivo* and *in vitro* responses of *P. falciparum* from 10 countries, that isolates inhibited *in vitro* at 0.04 μM are likely to be pyrimethamine-sensitive while those which grow in the presence 0.1 μM or more of the drug are likely to be resistant. However, Spencer and others[52] found 1 clinically pyrimethamine-sensitive Kenyan isolate to have an MIC of 0.1 μM in a 48-hour *in vitro* test, while 3 other isolates sensitive to the drug were inhibited at 0,03 μM or less. It is clear that further *in vivo* and *in vitro* pyrimethamine-sensitivity studies are required in order to resolve this controversy.

5.4.3 Stability of drug sensitivity in culture

It was found that 0.6 μM of chloroquine was required to inhibit the FCR-3 strain *in vitro*. This isolate, which was initially cultured in August 1976,[7] was found to be inhibited at 0.1 μg chloroquine /ml (approx. 0.3 μM) up to one year after initiation of growth *in vitro*.[53] After 4 years in continuous culture, however, an *in vitro* test carried out in a second laboratory showed that 0.3 μg/ml (approx. 0.9 μM) was required to completely inhibit growth.[54] This change in chloroquine sensitivity had taken place in culture in the absence of drug pressure, suggesting that varying conditions of culture may result in an

isolate which is maintained in several laboratories having different sensitivities, possibly as a consequence of selection of chloroquine-resistant clones. It is therefore not surprising that the MIC value of FCR-3 as determined in the Durban laboratory differed from that of the other 2 laboratories.

It was found that even under the same culture conditions, the sensitivities of the southern African isolates to chloroquine and, to a lesser extent, pyrimethamine, fluctuated during the test period. Thaithong and others[55] found that such variation in drug sensitivity occurred in two Thai isolates but not in their clones. They assumed that these fluctuations were due to the presence of clones which differed in their drug sensitivity and which, as a result of asynchronous growth, were present in culture in different proportions at different times.

5.4.4 Implications for control of malaria

The results of this investigation confirm the presence of both chloroquine and pyrimethamine resistance in the endemic malarial areas of South Africa. This has serious implications for the prophylaxis and treatment of *P. falciparum* malaria in the region. At present, the chloroquine-pyrimethamine combination is commonly recommended in this country for both prophylaxis and treatment, but the merits of this drug combination

have recently been questioned. The continued use of these drugs is likely to result in an increased prevalence of resistant malaria and may also give rise to a higher degree of stable resistance to both drugs. Widespread pyrimethamine resistance in South Africa might place in jeopardy the effectiveness of other pyrimethamine combinations such as pyrimethamine-sulphadoxine.

The use of pyrimethamine for its sporontocidal effect is questionable, as it has been shown that pyrimethamine does not prevent the development of oocysts in isolates resistant to the drug.[56] However, it is not known whether pyrimethamine would affect oocyst development in isolates with low levels of resistance (MICs of 0.1 μM) as the resistant isolates studied in the investigation of Teklehaimanot and others[56] had MICs of > 0.3 μM. Studies carried out in Papua New Guinea suggest that pyrimethamine resistance may be a good indicator of Fansidar (pyrimethamine + sulphadoxine) resistance.[57] Using a 24-hour pyrimethamine *in vitro* test, it was found that those *P. falciparum* isolates inhibited at 20.0 μM were sensitive to Fansidar *in vivo*, while 6 out of 7 isolates which grew in 20.0 μM or more of pyrimethamine were resistant to the drug combination *in vivo*. The southern African isolates were not tested at pyrimethamine levels greater than 3.0 μM but only 2 isolates were not inhibited at this concentration and

the majority of MICs were well below 3 μM. Fansidar resistance would therefore not be expected to be a problem in this part of Africa, at least not in the near future.

6. *IN VITRO* SENSITIVITY OF REFERENCE ISOLATES OF *PLASMODIUM FALCIPARUM* TO HALOFANTRINE. DESCRIPTION OF THE RADIOISOTOPE UPTAKE ASSAY

6.1 Introduction

With the rapid spread of drug resistance throughout Africa and the rest of the world,[58] the number of drugs available for the successful treatment of malaria is becoming extremely limited. The recently developed phenanthrene-methanol, halofantrine hydrochloride, has been shown in clinical trials to be highly effective against both chloroquine-resistant and multidrug-resistant forms of malaria,[59-63] although it has been suggested that there might be cross-resistance between halofantrine and mefloquine.[64] *In vitro* halofantrine sensitivity studies have been carried out in Thailand,[59,65,66] Madagascar,[67] West Africa[68] and Kenya.[60] However, nothing is known about its action in vitro against southern African isolates of *Plasmodium falciparum*. As this drug is soon to be released for use in South Africa, we decided to investigate the *in vitro* susceptibility to halofantrine of 2 primary isolates of *P. falciparum* collected in KwaZulu and of 18 southern African reference isolates.

6.2 Materials and Methods

6.2.1 *P. falciparum* isolates

The southern African reference isolates RSA 1-3, 5-17 and MW 1 and 2 have been characterised according to their sensitivity to chloroquine and pyrimethamine *in vitro* (see Chapter 5), as has the Gambian "control" strain, FCR-3.[7] The origins and dates of collection of these isolates are given in Table 1. Isolates N35 and N42 were obtained from malaria patients reporting to a clinic in the Ingwavuma district of KwaZulu, South Africa.

6.2.2 Preparation of isolates for the *in vitro* test

The 19 culture-adapted isolates were maintained in suspension culture as described previously (see Chapter 2). For the test, infected erythrocytes were diluted with fresh, washed, human type O-positive red blood cells to a parasitaemia of 0.2 to 0.9% and suspended to 3% in RPMI 1640 medium containing 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid), 32 mM $NaHCO_2$ and 10% non-immune human type A-positive serum.

Infected erythrocytes collected from the 2 malaria patients were washed twice in culture medium, diluted with O-positive red blood cells, where necessary, to a parasitaemia of 0.2 to 0.9% and suspended to 3% in

culture medium supplemented with non-immune human type AB-positive serum.

6.2.3 Preparation of drug plates

A stock solution of halofantrine was prepared by dissolving 10.31 mg halofantrine hydrochloride in 70 ml ethanol and adding 30 ml H_2O as described by Desjardins *et al.*.[69] The stock solution was diluted 1000 times with culture medium to a concentration of 192 nmol/l which was then double-diluted in culture medium to 3 nmol/l. 50 µl aliquots of the drug solutions were dispensed into the wells of a 96-well microtitre plate, 1 concentration per row of 12 wells. The first row was the positive control and contained culture medium only, 50 µl per well.

6.2.4 *In vitro* test

100 µl aliquots of the 3% red blo cell suspensions were added to the drug solutions in the wells so that each isolate occupied 3 columns of 8 wells on the microtitre plate, i.e. each isolate was tested in 3 wells at each drug concentration. The final halofantrine concentrations were thus 0, 1, 2, 4, 8, 16, 32 and 64 nmol/l and the final haematocrit was 2%. The final concentration of ethanol was less than 0.03%. Desjardins *et al.*[69] found that 0.1% ethanol had no effect on the growth of the parasite. The plates were incubated at 38°C in a desiccator gassed with 3%

O_2, 4% CO_2 and 93% N_2. After 24 hours, ^{3}H-hypoxanthine, 0.5 µCi in 10 µl culture medium, was added to each well. The plates were agitated and incubated for a further 18 hours. The contents of the wells were then harvested on to glass fibre filters using a Titertek cell harvester. The filters were washed with distilled water, dried and placed in scintillation vials containing 4 ml scintillation fluid and counted for 5 minutes in a Beckman LS 1800 liquid scintillation counter.

6.2.5 Calculation of MIC and IC_{50}

Percentage growth was calculated as

$$\text{PCGROWTH} = \frac{100 * [\text{CPM (ISOLATE)} - \text{CPM (NEG CONTROL)}]}{[\text{CPM (POS CONTROL)} - \text{CPM (NEG CONTROL)}]}$$

where CPM (ISOLATE) was the mean of 3 values corresponding to 1 drug concentration, CPM (NEG CONTROL) was the mean of 6 values corresponding to the negative control (non-parasitised erythrocytes) and CPM (POS CONTROL) was the mean of 3 values corresponding to the positive control.

The minimum inhibitory concentration (MIC) was defined as the lowest concentration at which growth of at least 95% of the parasites was inhibited. The IC_{50}, i.e. the concentration required to inhibit 50% of parasite growth, was calculated using 2 methods.

Method 1: In this simplified estimation of the IC_{50}, the 2 PCGROWTH values closest to 50%, 1 on either side, were selected, assuming linearity between these 2 points. The concentration corresponding to 50% growth was then calculated by linear interpolation between the 2 points and their corresponding concentration values, i.e.

$$\frac{(y_1 - y_2)}{(x_2 - x_1)} = \frac{(y_1 - 50)}{(x_3 - x_1)}$$

$$\text{or } x_3 = \frac{(y_1 - 50)}{(y_1 - y_2)} * (x_2 - x_1) + x_1$$

where y_1, y_2 = the 2 PCGROWTH values closest to 50%, 1 on either side,

x_1, x_2 = their corresponding concentrations

and x_3 = the concentration corresponding to 50% growth, i.e. the IC_{50}

This method is illustrated in Fig. 2.

Method 2: Using the SAS/STAT computer programme,[70] the sigmoid dose-response equation was used to fit a curve through the data points for each isolate.

This non-linear regression procedure requires an initial estimation of the IC_{50} value and the slope of

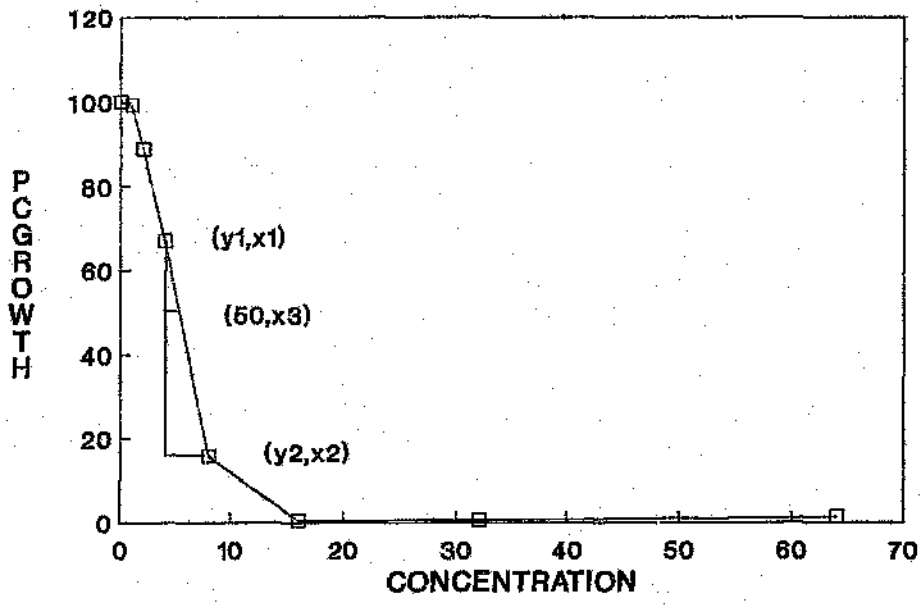

Figure 2. Determination of IC_{50} by linear interpolation.

the line. The best-fit IC_{50} value is then calculated from these estimates. For the initial estimates of IC_{50}, method 1 was used as a guideline.

6.3 Results

The mean counts per minute for each isolate tested are shown in Appendix C. No statistically significant difference was found between the 2 methods used for estimating the IC_{50}. The mean IC_{50} of the 21 isolates as determined by linear interpolation was 4.648 with a standard error of 0.876 and a 95% confidence interval

Table 9. ***In vitro*** **response of southern African isolates of** ***Plasmodium falciparum*** **to halofantrine**

	Halofantrine		Chloroquine
Isolate	IC_{50} (nM)	MIC (nM)	MIC (nM)*
RSA 1	1.265	4	300 - 600
RSA 2	2.458	8	60
RSA 3	15.000	32	60 - 100
RSA 5	2.381	8	60 - 100
RSA 6	2.460	8	300 - 600
RSA 7	7.272	16	300 - 600
RSA 8	2.667	8	300 - 600
RSA 9	3.000	8	300 - 600
RSA 10	6.789	16	300 - 600
RSA 11	6.217	8	300
RSA 12	2.023	8	300 - 600
RSA 13	9.151	16	30 - 60
RSA 14	1.268	8	300 - 600
RSA 15	4.792	16	300 - 600
RSA 16	9.902	32	300 - 1 000
RSA 17	11.261	16	300 - 600
MW 1	1.700	8	300 - 600
MW 2	1.282	4	300 - 600
FCR-3	6.007	16	600
N35	0.039	1	ND$
N42	0.060	1	ND

* From Chapter 5

$ Not determined

of 2.821 to 6.475. The mean IC_{50} calculated using the sigmoid dose-response procedure was 4.619 with a standard error of 0.884 and a 95% confidence interval of 2.775 to 6.462.

The results of the *in vitro* test are presented in Table 9. The IC_{50}s, as determined by the sigmoid dose-response procedure, ranged from 0.039 to 15.000 nmol/l. All isolates had halofantrine MICs of 32 nmol/l or less with a median of 8 nmol/l. The chloroquine MICs of the 18 southern African reference isolates and the "control" isolate, FCR-3, previously determined, ranged from 60 to 1 000 nmol/l with a median of 300 nmol/l (see Chapter 5). Fifteen of these reference isolates were resistant to chloroquine *in vitro*, having MICs of 300 nmol/l or more. The majority of these isolates exhibited low levels of resistance to pyrimethamine.

6.4 Discussion

This study has shown that linear interpolation provides a simple method for the determination of IC_{50} values. This method may also be used to provide estimates of the IC_{50} for the sigmoid dose-response method for determining the IC_{50}.

Southern African *P. falciparum* isolates are similar to those from West Africa in their sensitivity to halofantrine but are less susceptible to the drug than South-East Asian isolates. The mean IC_{50} value of the 21 isolates examined in the present study was 4.62 nM while that of six West African isolates, as determined by Oduola *et al.*,[71] was 2.79 ng/ml (5.57 nM). In contrast, the mean IC_{50} of six isolates from southern Thailand was 1.23 nM,[66] while that of isolates from four regions in Thailand ranged from 0.63 - 0.73 ng/ml (1.26 - 1.46 nM).[65]

Two cases of RI resistance to halofantrine treatment have been reported from West Africa. *In vitro* testing of the isolates revealed IC_{50} values of 7.5 nmol/l and 29.7 nmol/l (first and second malarial attack, respectively) for 1 isolate[68] and 14.0 nmol/l for the recrudescent parasites of the second isolate.[72] One of the 20 southern African isolates tested had an IC_{50} of 15 nmol/l and 4 of them had IC_{50}s greater than 8 nmol/l. Although the relationship between the *in vivo* response to halofantrine and the *in vitro* IC_{50} has not yet been determined, the results of this study suggest that some local isolates may have reduced susceptibility to halofantrine. However, further *in vitro* tests and *in vivo* evidence are required to confirm this.

The MIC results of this study compare very well with those of an investigation carried out in Kilifi, Kenya, where 32 out of 33 isolates had MICs of 32 nmol/l halofantrine or less and one isolate was not inhibited at 64 nmol/l,[60] being the highest halofantrine concentration tested. However, these Kenyan MICs are higher than those obtained by the same authors in Malindi, Kenya, where all 11 isolates tested had MICs of 16 nmol/l or less.[60] They are also higher than the MICs of 6 Thai isolates, which ranged from 94 to 7.79 nmol/l.[66]

In the present investigation, halofantrine was found to be equally active against chloroquine-sensitive and chloroquine-resistant isolates. In fact, the mean IC_{50} of the 4 chloroquine-sensitive isolates (RSA 2, 3, 5 and 13) was 7.248 nmol/l, which was higher than the mean IC_{50} (4.527 nmol/l) of the 15 chloroquine-resistant isolates. Halofantrine was also found to be active against pyrimethamine-resistant isolates in this study.

This *in vitro* investigation provides baseline data on the sensitivity of southern African isolates of *P. falciparum* to halofantrine. Although it is not possible to make direct comparisons between *in vitro* tests carried out by different laboratories, the results of various studies do at least give some

indication of the relative sensitivities to halofantrine of isolates from different geographical regions. The indications are that this drug would be suitable for treating *P. falciparum* malaria in southern Africa, particularly when there is resistance to chloroquine and pyrimethamine. However, further testing of halofantrine in southern Africa, both *in vivo* and *in vitro*, is required to confirm this observation.

7. *IN VITRO* SENSITIVITY OF SOUTHERN AFRICAN REFERENCE ISOLATES OF *PLASMODIUM FALCIPARUM* TO AMODIAQUINE, CHLOROQUINE, MEFLOQUINE, QUININE AND SULPHADOXINE/ PYRIMETHAMINE. USE OF PREDOSED DRUG PLATES IN THE RADIOISOTOPE UPTAKE ASSAY

7.1 Introduction

The epidemiology of drug-resistant *P. falciparum* is well documented.[58,73] Chloroquine resistance occurs in all southern African countries. However, apart from isolated reports of *in vivo* and *in vitro* resistance to sulphadoxine/pyrimethamine, quinine and mefloquine, little is known about the sensitivity of *P. falciparum* in this region to antimalarials other than chloroquine. The aim of this study was to characterise the southern African reference isolates of *P. falciparum* according to their *in vitro* susceptibility to amodiaquine, chloroquine, mefloquine, quinine and sulphadoxine/pyrimethamine.

7.2 Materials and Methods

7.2.1 *Plasmodium falciparum* isolates

Eighteen of the culture-adapted southern African reference isolates of *P. falciparum* (see Chapter 2),

collected during the period July 1984 to February 1987, and the Gambian reference isolate, FCR-3,[7] were grown in gassed tissue culture flasks to a parasitaemia of 0.2 to 0.9% before being tested.

7.2.2 *In vitro* tests

Using WHO predosed drug sensitivity plates, the sensitivity of these isolates to amodiaquine, chloroquine, mefloquine, quinine and sulphadoxine /pyrimethamine was determined as follows.

The isolates were washed twice with washing medium (culture medium without serum) and suspended in normal RPMI 1640 medium, for the testing of amodiaquine, chloroquine, mefloquine, and quinine, and in RPMI 1640 containing reduced levels of para-aminobenzoic acid (0.5 µg/l) and folic acid (10 µg/l), for the testing of sulphadoxine/pyrimethamine. Both media were buffered with HEPES (25 mM) and $NaHCO_3$ (32mM) and supplemented with the addition of 10% non-immune human type AB-positive serum. The parasitaemias of the reference isolates were reduced to ≤1% for the test.

The *in vitro* drug sensitivity was determined using a method based on that of Desjardins *et al.* [69] in which the uptake of [^{3}H]-hypoxanthine was used to measure the growth of the parasite. Briefly, erythrocytes were suspended to 2% in the 2 culture media and aliquoted

into the wells of the appropriate WHO predosed plates (100 µl/well; 3 wells per concentration). The plates were incubated as decribed above for 24 hours after which [^{3}H]-hypoxanthine, 0.5 µCi in 10µl culture medium, was added to each well. The plates were then incubated for a further 18 hours. After incubation, the contents of the wells were harvested, using a Titertek cell harvester, on to glass fibre filter discs which were washed with distilled water and allowed to dry. Each filter disc was placed in a vial containing 4 ml of scintillation fluid. The reactivity was counted using a Beckman LS liquid scintillation counter. Tests were considered successful when counts in the control wells exceeded 2000. The percentage growth relative to the control was calculated and the MIC was defined as the minimum concentration at which the growth of at least 95% of the parasites was inhibited. The IC_{50} was calculated as described in Chapter 6.

7.3 Results and Discussion

The mean counts per minute obtained in the investigation of the *in vitro* sensitivity of the southern African reference isolates of *P. falciparum* to amodiaquine, chloroquine, mefloquine, quinine and sulphadoxine/pyrimethamine in the radioisotope uptake assay, are shown in Appendixes D, E, F, G and H,

respectively. The MICs and IC_{50}s of these isolates are listed in Table 10. Table 11 is a summary of all the above results.

Table 10. Minimum inhibitory concentrations (MICs) and IC_{50} values (in µmol/l medium) of southern African reference isolates of Plasmodium falciparum

	Amodiaquine		Chloroquine		Mefloquine		Quinine		Sulphadoxine/ pyrimethamine	
Isolate	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC
KwaZulu, 1984-1986										
RSA 1	0.01	0.02	0.04	0.16	0.01	0.04	0.20	0.64	93.61	>100.0
RSA 2	0.01	0.02	0.03	0.04	0.02	0.08	0.20	0.64	4.56	30.0
RSA 3	0.01	0.02	0.01	0.01	0.13	0.32	0.21	1.28	1.30	30.0
RSA 5	0.01	0.02	0.01	0.02	0.02	0.08	0.09	1.28	14.57	>100.0
RSA 6	0.01	0.02	0.14	0.32	0.02	0.16	0.37	2.56	3.25	30.0
RSA 7	0.01	0.01	0.05	0.16	0.02	0.16	0.30	1.28	2.80	30.0
RSA 8	0.01	0.02	0.15	0.32	0.04	0.16	0.62	2.56	3.40	30.0
RSA 9	0.01	0.02	0.16	0.32	0.05	0.16	0.67	2.56	6.20	>100.0
RSA 10	0.01	0.02	0.08	0.16	0.06	0.16	0.51	2.56	2.53	30.0
RSA 11	0.01	0.02	0.21	0.64	0.08	0.16	0.80	>2.56	2.26	30.0
RSA 12	0.01	0.02	0.05	0.16	0.02	0.16	0.07	1.28	3.18	30.0
Transvaal, 1987										
RSA 13	0.02	0.02	0.08	0.16	0.05	0.16	1.09	2.56	2.19	10.0
RSA 14	0.06	0.08	0.06	0.16	0.03	0.16	0.50	2.56	2.30	30.0
RSA 15	0.11	0.16	0.09	0.32	0.04	0.16	0.59	>2.56	2.26	30.0
RSA 16	0.01	0.02	0.06	0.32	0.03	0.16	0.65	>2.56	2.10	10.0
RSA 17	0.01	0.01	0.08	0.32	0.07	0.16	0.59	2.56	2.30	30.0
Malawi, 1985-1986										
MW 1	0.01	0.02	0.04	0.08	0.02	0.04	0.99	>2.56	2.19	10.0
MW 2	0.01	0.02	0.05	0.16	0.01	0.08	0.90	2.56	3.20	>100.0
Gambia 1976										
FCR-3	0.01	0.02	0.05	0.16	0.03	0.16	0.41	2.56	2.47	10.0

Table 11. In vitro response of southern African isolates of Plasmodium falciparum to five antimalarial drugs

Origin and Collection Date		Amodiaquine	Chloroquine	Mefloquine	Quinine	Sulphadoxine/ Pyrimethamine[a]	Growth Assessment
KwaZulu	No. of isolates:	11	11	11	11	11	
South Africa	MIC:	0.01 - 0.02 (0.02)[b]	0.01 - 0.64 (0.16)	0.04 - 0.32 (0.16)	0.64 - >2.56 (1.92)	10 - >100 (30)	[^{3}H]-hypoxanthine
1984 to 1986	IC_{50}:	0.01	0.01 - 0.21 (0.08)[c]	0.01 - 0.13 (0.04)	0.07 - 0.80 (0.37)	1.3 - 93.61 (12.52)	incorporation
Transvaal	No. of isolates:	5	5	5	5	5	
South Africa	MIC:	0.01 - 0.16 (0.02)	0.16 - 0.32 (0.32)	0.16	2.56 - >2.56 (2.56)	10 - 30 (30)	[^{3}H]-hypoxanthine
1987	IC_{50}:	0.01 - 0.11 (0.04)	0.06 - 0.09 (0.07)	0.03 - 0.07 (0.04)	0.50 - 1.09 (0.68)	2.1 - 2.3 (2.23)	incorporation
	No. of isolates:	2	2	2	2	2	
Malawi	MIC:	0.02	0.08 - 0.16 (0.12)	0.04 - 0.08 (0.06)	2.56 - >2.56	10 - >100	[^{3}H]-hypoxanthine
1985 to 1986	IC_{50}:	0.01	0.04 - 0.05 (0.05)	0.01 - 0.02 (0.02)	0.90 - 1.00 (0.95)	2.19 - 3.2 (2.70)	incorporation
	No. of isolates:	1	1	1	1	1	
Gambia	MIC:	0.02	0.16	0.16	2.56	10.0	[^{3}H]-hypoxanthine
1976	IC_{50}:	0.01	0.05	0.03	0.41	2.47	incorporation

[a] Sulphadoxine/pyrimethamine concentration expressed as µmol sulphadoxine/l medium. Ratio of sulphadoxine:pyrimethamine = 80:1.
[b] Range and median in µmol/l medium.
[c] Range and mean in µmol/l medium.

7.3.1 Amodiaquine

All KwaZulu isolates, collected from 1984 to 1986, were sensitive to amodiaquine, having MICs of 0.01 to 0.02 µmol/l (Table 10), which are below the discriminating MIC of 0.04 µmol/l for sensitive isolates.[74] The median MIC value and the mean IC_{50} for this set of isolates were 0.02 and 0.01 µmol/l, respectively (Table 11).

Three of the Transvaal isolates collected in 1987 were sensitive to amodiaquine, with MICs of ≤0.04 µmol/l, and 2 were resistant, with MICs of ≥0.08 µmol/l (Table 10). The median MIC value was 0.02 µmol/l and the mean IC_{50} was 0.04 µmol/l (Table 11).

Both Malawian isolates had an MIC of 0.02 µmol/l and an IC_{50} of 0.01 µmol/l, indicating sensitivity to amodiaquine (Table 10). FCR-3 was also sensitive to this drug, having an MIC of 0.02 µmol/l and an IC_{50} value of 0.01 µmol/l.

7.3.2 Chloroquine

Eighteen of the 19 southern African reference isolates of *P. falciparum* examined previously for chloroquine sensitivity by the 48-hour growth inhibition assay (see Chapter 5), were retested using the [^{3}H]-hypoxanthine incorporation method. The 19th isolate (RSA 4), a chloroquine-sensitive isolate from KwaZulu,

was lost in culture prior to carrying out this present study. Resistance was confirmed (MICs ≥0.16 µmol/l) in all southern African isolates showing *in vitro* resistance in the previous study, and the 3 sensitive KwaZulu isolates were shown to have retained their sensitivity to chloroquine *in vitro* (MICs ≤0.114 µmol/l) (Table 10). However, the culture-adapted Transvaal isolate RSA 13, which was shown in the previous investigation to have an MIC of 0.03 to 0.06 µmol/l, showed a decrease in chloroquine sensitivity *in vitro* to an MIC of 0.16 µmol/l. This is probably the result of selection of chloroquine-resistant clones in this isolate.

The mean IC_{50}s of the reference isolates were 0.08, 0.07 and 0.05 µmol/l for those originating in KwaZulu, Transvaal and Malawi, respectively (Table 11). FCR-3 had an MIC of 0.16 µmol/l and an IC_{50} of 0.05 µmol/l.

The results of these investigations confirm the existence of chloroquine-resistant *P. falciparum* in southern Africa.

7.3.3 Mefloquine

All southern African isolates tested were sensitive to mefloquine, with 1 being inhibited at 0.32 µmol/l and the remaining 17 isolates having MICs of ≤0.16 µmol/l (Table 10). The median MIC and the mean IC_{50} for both

the KwaZulu and the Transvaal reference isolates were 0.16 and 0.04 µmol/l, respectively (Table 11). The 2 Malawian isolates had MICs of 0.04 and 0.08 µmol/l and IC_{50}s of 0.01 and 0.02 µmol/l, respectively (Table 10). The MIC and IC_{50} values for FCR-3 were 0.16 and 0.03 µmol/l, respectively.

In Zambia, *in vitro* resistance to mefloquine was observed in 1986 in 15.2% and 3.6% of isolates from Lubwe and Kalene, respectively.[75] 48.5% of isolates from Lubwe and 21.4% of isolates from Kalene had MICs ≥0.32 µmol/l.

It would seem that mefloquine might prove to be a useful drug for the treatment and prophylaxis of chloroquine-resistant *P. falciparum* malaria in southern Africa. However, the sensitivity to this drug needs to be monitored in this region.

7.3.4 Quinine

The KwaZulu reference isolates were shown to have a low sensitivity to quinine (median MIC of 1.92 and mean IC_{50} of 0.37 µmol/l (Table 11). The Transvaal and Malawian reference isolates were also found to have reduced sensitivities to quinine (a median MIC of 2.56 µmol/l for Transvaal and mean IC_{50}s of 0.68 and 0.95 µmol/l for Transvaal and Malawi, respectively). FCR-3 had an MIC of 2.56 µmol/l and an IC_{50} value of 0.41

μmol/l.

Sensitivity to quinine is defined in the WHO *in vitro* microtechnique as schizont inhibition at ≤2.56 μmol/l medium.[74] However, not all of the isolates studied using the radioisotope uptake method were inhibited at the highest concentration tested of 2.56 μmol/l. Ten of the 11 KwaZulu reference isolates were inhibited at 2.56 μmol/l or less (Table 10). Two of the 5 Transvaal reference isolates and 1 of the 2 Malawian reference isolates failed to be inhibited at 2.56 μmol/l. However, the discriminating concentration for resistant isolates in the [^{3}H]-hypoxanthine incorporation assay is not known.

Although quinine can normally still be used successfully for the treatment of chloroquine-resistant *P. falciparum* malaria in southern Africa, there have been isolated reports of resistance to this drug from Malawi[76] and Zambia.[77] The results presented in this chapter reveal reduced susceptibility to quinine amongst southern African isolates and although the situation is not nearly as serious as it is in South-East Asia, where cure rates have fallen to below 50% in some areas,[73] there is nevertheless cause for concern.

7.3.5 Sulphadoxine/pyrimethamine

The mean IC_{50} of reference isolates originating in KwaZulu 12.52 µmol/l (4.4 µmol/l if the outlying IC_{50} of 93.61 µmol/l of one of the isolates is excluded). The mean IC_{50}s of the Transvaal and Malawian reference isolates were 2.23 and 2.70 µmol/l, respectively (Table 11). The IC_{50} for FCR-3 was 2.47 µmol/l.

A wide range in MIC values (10 - >100 µmol/l) was also shown amongst the reference isolates, with the majority of these isolates being inhibited at ≤ 30 µmol/l (Table 10).

7.3.6 Cross resistance

Cross resistance was determined from the IC_{50} values obtained from the southern African reference isolates using Spearman's correlation coefficients (Table 12). A weak correlation between chloroquine and amodiaquine ($r = 0.37$, $p = 0.1101$) and a moderate correlation between chloroquine and quinine ($r = 0.51$, $p = 0.0244$) was shown. There was also a moderate correlation between chloroquine and mefloquine ($r = 0.54$, $p = 0.0166$) and a weak correlation between quinine and mefloquine ($r = 0.32$, $p = 0.1629$) for these isolates.

A moderate negative correlation was found between mefloquine and sulphadoxine/pyrimethamine, and quinine and sulphadoxine/pyrimethamine, for the reference

isolates (r values of -0.55 and -0.45 and p values of 0.0182 and 0.0521, respectively).

Table 12. Cross resistance within the southern African reference isolates of Plasmodium falciparum

Drug Comparison	Spearman's Correlation Coefficient	p value
Amodiaquine & chloroquine	0.37	0.1101
Amodiaquine & mefloquine	0.12	NS*
Amodiaquine & quinine	0.20	0.3548
Amodiaquine & sulphadoxine/pyrimethamine	-0.02	NS
Chloroquine & mefloquine	0.54	0.0166
Chloroquine & quinine	0.51	0.0244
Chloroquine & sulphadoxine/pyrimethamine	-0.09	NS
Mefloquine & quinine	0.32	0.1629
Mefloquine & sulphadoxine/pyrimethamine	-0.55	0.0182
Quinine & sulphadoxine/pyrimethamine	-0.45	0.0521

* Not significant

7.3.7 Comparison of the 48-hour growth inhibition assay with the radioisotope uptake assay

Using the data from the previous drug sensitivity study of the 18 reference isolates of *P. falciparum* (Chapter 5), the IC_{50}s were determined by the sigmoid dose-response method and compared with the chloroquine IC_{50}s obtained by the radioisotope uptake method in the present investigation (Table 13). Fifteen isolates had lower IC_{50}s in the [^{3}H]-hypoxanthine incorporation

assay than in the 48-hour growth inhibition method, while 3 isolates had higher IC_{50}s in the radioisotope uptake assay. The mean IC_{50} for the 48-hour growth inhibition test was 0.198 µmol/l and that for the radioisotope uptake assay was 0.076 µmol/l. The t-test showed a significant difference between the 2 means (p=0.0009).

Table 13. Comparison of IC_{50} values (µmol/l) obtained using the 48-hour growth inhibition assay and the [^{3}H]-hypoxanthine incorporation method

	48-hour growth inhibition assay[a]	[^{3}H]-hypoxanthine incorporation method
RSA 1	0.280	0.035
RSA 2	0.040	0.030
RSA 3	0.040	0.010
RSA 5	0.070	0.010
RSA 6	0.045	0.140
RSA 7	0.300	0.050
RSA 8	0.300	0.150
RSA 9	0.330	0.162
RSA 10	0.215	0.075
RSA 11	0.140	0.211
RSA 12	0.180	0.052
RSA 13	0.045	0.075
RSA 14	0.390	0.055
RSA 15	0.406	0.090
RSA 16	0.121	0.056
RSA 17	0.140	0.075
MW 1	0.320	0.042
MW 2	0.210	0.048
Mean	0.198	0.076

[a] Calculated from data presented in Chapter 5.

The reason for the differing results of the 2 assays is not clear. Although these assays were not carried out at the same time, it is doubtful whether this apparent decrease in IC_{50} values could be the result of a general decline in the sensitivity of the isolates during approximately 4 years of cryopreservation and culture. It is more likely that the difference is due to the effect of the drug being detected at a lower concentration in the radioisotope assay than in the growth inhibition assay. Morphologically degenerate parasites may not be distinguished by the microscopist in the growth inhibition assay and may therefore be included in the parasite count, while degenerate parasites may not incorporate as much [^{3}H]-hypoxanthine as healthy parasites, thus resulting in a lower IC_{50} value in the ^3H uptake assay.

Schapira *et al.*,[78] however, found that the IC_{50} values were lower in the 48-hour growth inhibition assay than in the radioisotope uptake assay and ascribed this difference to the longer incubation time of the former method.

It is clear that different techniques for the determination of drug sensitivity *in vitro* will yield conflicting results and that direct comparisons cannot be made between studies employing different assays. Nevertheless, whatever the method used, *in vitro*

studies give a good indication of the drug sensitivity of parasite populations.

8. *IN VITRO* CHLOROQUINE SENSITIVITY OF FIELD ISOLATES OF *PLASMODIUM FALCIPARUM* FROM KWAZULU, 1987-1988. DESCRIPTION OF THE 24-HOUR MICROTECHNIQUE

8.1 Introduction

Since 1978, when the first reports of chloroquine-resistant *P. falciparum* malaria in Africa were received from Kenya and the United Republic of Tanzania, chloroquine resistance has been found as far South as South West Africa/Namibia[36] on the West coast and Mozambique[79] and probably Swaziland[38] on the East coast. In 1985, chloroquine resistance was confirmed *in vitro* in 6 patients from Natal/KwaZulu, it being assumed that the infections had originated in Mozambique or areas immediately bordering on Mozambique.[40] The cure rate in KwaZulu following administration of the standard adult curative dose of chloroquine (1500 mg) had decreased from 100% in 1983 to 78.8% and 83.9% in hospital and field cases respectively by 1987.[43]

Chloroquine resistance has serious implications for the treatment of malaria and may severely hamper parasite control, resulting in a change in the whole epidemiological picture. A study was therefore undertaken in the endemic malarial area of KwaZulu to

determine the *in vitro* sensitivity of *P. falciparum* to chloroquine.

8.2 Materials and methods

8.2.1 Study area

The endemic malarious region of Natal/KwaZulu encompasses the districts of Ingwavuma, Ubombo and Hlabisa. During the first 5 months of 1987, 437 indigenous malaria cases were reported from the Mamfene area and 299 from the Ophansi area of Ubombo, these figures being considerably higher than the means of 12.6 for Mamfene and 21.5 for Ophansi recorded annually over the 11-year period from 1976 to 1986. Ubombo was therefore chosen as the study area for the chloroquine-resistance survey in May 1987. A second investigation was undertaken in January 1988 in the Ndumu area of Ingwavuma because of the relatively large number of early-season malaria cases reported from it.

8.2.2 Selection of samples

From 18 to 21 May 1987 venous blood was collected in lithium/heparin tubes from actively-detected malaria cases[a] identified during mass blood investigations

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[a] "Actively-detected" malaria cases are those detected during routine active surveillance, epidemiological investigations or by mass blood examinations.[50]

carried out in the Mamfene and Ophansi areas of Ubombo and from passively-detected[b] cases reporting to Bethesda Hospital and several clinics in the Ubombo district. From 18 to 21 January 1988 blood samples were obtained from suspected malaria patients reporting to Bethesda Hospital and to clinics in the Ingwavuma district. All subjects were questioned about their use of antimalarial drugs prior to the survey and urine samples were collected from the patients included in the 1987 investigation.

The blood specimens were transported on ice to the field laboratory at Jozini where they were processed within 12 hours of collection. Thick and thin smears were prepared from each sample, stained with Giemsa and examined microscopically for the presence of *P. falciparum*. Urine samples were tested for the presence of 4-aminoquinolines by the Dill-Glazko method.[80] Isolates were considered to be suitable for the *in vitro* chloroquine sensitivity test if they contained 500 - 100 000 parasites/µl. Samples were excluded if the patient had ingested antimalarial drugs in the previous 2 weeks and/or if the Dill-Glazko test was positive.

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[b] Malarious patients who present at clinics or hospitals are defined as "passively-detected" cases.[50]

8.2.3 *In vitro* tests

Chloroquine sensitivity *in vitro* was determined by a modification of the Rieckmann microtechnique.[81] Infected erythrocytes were washed twice in RPMI 1640 culture medium. Care was taken to remove the buffy coat during the washing process. A 5% red blood cell (RBC) suspension was prepared from each sample using the medium supplemented with 10% non-immune human AB serum. 50µl aliquots of these suspensions were added to the wells of microtitre plates predosed in the laboratory with 1, 2, 4, 5.7, 8, 16, and 32 pmols chloroquine. An additional concentration of 64 pmols/well was used in the January 1988 survey. At least 2 wells per chloroquine concentration were used for each isolate. The plates were agitated for a few seconds to dissolve the drug and then they were placed in a desiccator (a convenient air-tight container to use) which was gassed for approximately 3 mins with a mixture of 3% oxygen, 4% carbon dioxide and 93% nitrogen. After gassing, the desiccator was tightly sealed and incubated at 38°C for 24 to 26 hours. After incubation, a smear was prepared from each well, stained with Giemsa's solution and examined microscopically. The number of schizonts (i.e. parasites having more than two nuclei) per 200 asexual parasites was determined at each chloroquine concentration. Tests were considered "successful" when at least 5% of the trophozoites in the control

wells had developed to schizonts.[82] *In vitro* resistance was defined as schizont development at a concentration of ≥ 5.7 pmols chloroquine/well.[83]

8.3 Results

8.3.1 May 1987

Of 6 407 smears prepared and screened in the mass blood examinations carried out in the Mamfene and Ophansi areas, 34 proved to be positive. Parasites from 8 of the 11 samples found to be suitable for the test, grew in culture. The *in vitro* response of the isolates to chloroquine is summarised in Table 14. The parasite counts for each isolate tested are shown in Appendix I. Only 2 of these isolates were sensitive to chloroquine while the remainder were resistant in varying degrees. In addition, isolates from 4 patients who had received chloroquine in the previous 2 weeks grew successfully *in vitro* and their inhibitory concentrations demonstrated resistance.

Twenty-seven blood samples were collected from suspected malaria cases reporting to the hospital and several clinics; 14 were positive and 11 of these were found to be suitable for the sensitivity test. Of the 9 isolates which grew successfully, none was found to be sensitive and the majority were inhibited at 32 pmols/well (Table 14). In this group, the trophozoites of 1 patient who had chloroquine in his circulation,

developed to schizonts under the culture conditions and schizont inhibition was achieved at 32 pmols/well. The parasite counts for each isolate tested are shown in Appendix J.

Table 14. In vitro response to chloroquine of isolates of Plasmodium falciparum from KwaZulu

	May 1987		January 1988
Minimum Inhibitory Concentration (pmols/well)	Number of Actively-detected cases	Number of Passively-detected cases	Number of Passively-detected cases
5,7	2	0	0
8,0	3(+1)*	0	0
16,0	1(+1)	1	1
32,0	0(+2)	5(+1)	9(+2)
64,0 (>32,0)$	2	3	3(+1)
>64,0 #	-	-	1 #
Total cases	8(+4)	9(+1)	14(+3)

* Figures in parentheses indicate isolates from patients who were known to have received chloroquine in the two weeks prior to the test.

$ 32 pmols/well was the highest concentration used in the May 1987 survey. However, some isolates were not completely inhibited at this level.

Tests were not done at concentrations >64 pmols/well.

8.3.2 January 1988

Forty-nine blood specimens were collected from patients suspected of having malaria and 27 were found to contain *P. falciparum* of which 20 were suitable for the test. The parasite counts for each isolate tested are shown in Appendix K. All 14 isolates tested successfully were resistant *in vitro* with the majority being completely inhibited at 32 pmols/well (Table 14). One of these isolates failed to be inhibited at 64 pmols/well. In addition, the isolates from 3 patients who had ingested chloroquine in the 2 weeks prior to the investigation were found to be resistant. Four samples with more than 100 000 trophozoites/μl were diluted with fresh, washed, uninfected RBCs (O blood group) before being tested. Three of these isolates developed to the schizont stage. The fourth sample (parasites from which did not mature) was from a patient who had taken chloroquine 1 week prior to the test.

8.4 Discussion

The modified *in vitro* test has proved successful in the determination of chloroquine sensitivity of southern African isolates of *P. falciparum*. Changes to the Rieckmann microtest were made on the basis of experiments carried out on local strains of the parasite (see Chapter 2). It was found that southern

African isolates do not grow well in the candle jar but that they adapt readily to culture conditions in an atmosphere of 3% oxygen, 4% carbon dioxide and 93% nitrogen. It was also found that supplementation of RPMI 1640 medium with glucose and hypoxanthine promotes the growth of local isolates *in vitro*. Markus[84] suggested that replacement of patients' plasma with non-immune serum might result in a higher percentage of successful 24-hour drug sensitivity tests. Washing of the patients' erythrocytes effectively removed all plasma from the blood samples, following which growth factors for the development of trophozoites were provided by the non-immune AB serum. A success rate of 74% was achieved using this modified technique. This compares very favourably with the percentages, ranging from 20 to 80%, of effective tests achieved in other endemic regions of the world.[83]

Kouznetsov *et al.*[85] showed that the interpretation of the results of the microtest was dependent on parasite density and that isolates with more than 100 000 parasites/μl require more chloroquine to completely inhibit schizont maturation than isolates of the same sensitivity having smaller numbers of parasites. It was found that it was possible to include isolates in the test which would otherwise have been excluded on the basis of parasite count, by diluting the infected erythrocytes with washed, uninfected RBCs. Three out

of 4 isolates diluted in this manner were successfully cultured. Failure of the fourth isolate to grow under test conditions might have been due to the presence of chloroquine in the infected RBCs.

The results of these investigations confirm the presence of chloroquine-resistant *P. falciparum* malaria in northern KwaZulu. What is disturbing is that no sensitive strains were found amongst the isolates from passively-detected cases collected in either 1987 or 1988. Furthermore, a high level of resistance was recorded, with the majority of the isolates being inhibited at 32 pmols/well or more, thus indicating R III resistance.[47] These results compare favourably with those of Herbst *et al.*[86] These authors found that schizont inhibition in isolates from 15 malaria patients, who were assumed to have become infected in Mozambique or Ingwavuma between July 1985 and June 1986, occurred in wells containing >24 pmols chloroquine; and that schizogony occurred at 48 pmols/well in the majority of the samples. The response to chloroquine of isolates collected from clinics did not change markedly in the 8 months between May 1987 and January 1988. However, a change in chloroquine susceptibility *in vitro* has been observed since 1985 when 5 isolates collected from clinics in Ingwavuma were found to be sensitive in a 48-hour sensitivity test (see Chapter 5).

In comparison with the passively-detected cases, parasites from 2 of the 8 field samples collected in May 1987 were found to be chloroquine-sensitive and 6 were inhibited at 16 pmols/well or less (concentrations associated with R I and R II resistance). It should, however, be borne in mind that in both surveys, isolates were obtained shortly after extensive use had been made of chloroquine in the study areas for treatment and prophylaxis. It is therefore possible that chloroquine-susceptible parasites had been eliminated, leaving a reservoir of relatively resistant parasites.

The *in vitro* response of the 8 isolates from patients with a history of chloroquine ingestion and which grew under the test conditions, was found to mimic that of isolates free of the drug, in that the samples from actively-detected cases were generally inhibited at lower concentrations (≤32 pmols/well) than the samples collected from passively-detected cases (≥32 pmols/well).

The difference in *in vitro* chloroquine sensitivity between actively- and passively-detected isolates may be attributable to host immunity. Semi-immune residents of endemic areas often harbour parasites without showing symptoms, and resistance may only be detected as a result of *in vivo* and *in vitro*

investigations.[87] It is only as the level of chloroquine resistance increases that these infections become clinically apparent.[88] Thus, subjects harbouring highly resistant organisms would report to clinics while those with infections less resistant or chloroquine-sensitive parasites would only be detected by active surveillance.

The presence of a partially chloroquine-resistant *P. falciparum* population coupled with a large mosquito vector population, would most certainly lead to an upsurge in the number of cases of clinical malaria. This may have contributed to the situation in the endemic areas of KwaZulu where 4830 malaria cases were recorded in 1987, far in excess of the number of cases reported annually over the 11-year period from 1976 to 1986, which ranged from 75 to 1199 with a mean of 648. Furthermore, the number of cases in the off-season (July to November) of 1987 was 532, which was much higher than the mean number of off-season cases (118,5; range = 3 to 226) recorded over the same 11-year period.

During January 1986, a large population of *Anopheles arabiensis* was found breeding in the irrigational overflow from an agricultural development scheme at Mamfene (Brian Sharp, personal communication). The adult mosquitoes of this species, which is the

principal vector of malaria in numerous localities in Africa,[89] were found biting man both indoors and outdoors. However, in 1986 only 5 cases of malaria were reported in the Mamfene area. In 1987, *An. arabiensis* was again detected in large numbers in this locality, but over 500 malaria cases were recorded. This was considerably higher than the annual mean case rate for Mamfene of 12,6 (with a range of 0 to 34), calculated over the 11 years from 1976 to 1986. What needs to be considered is whether this upsurge in malaria could be the result of the introduction to the area, possibly through migrant labourers, of a large parasite reservoir which, as this study suggests, may consist of an increased proportion of chloroquine-resistant parasites. If this is the case, parasite control measures involving the use of chloroquine for treatment may have been or may soon be compromised in this region. As *An. arabiensis* is widely distributed within the endemic malarial areas of KwaZulu,[90] it may be that a similar situation to that in the Mamfene area also exists in the Ndumu area (where there is also chloroquine resistance as this study has shown) and possibly in all the endemic malarial areas of KwaZulu in which a sudden upsurge in the number of malaria cases was experienced. However, definite conclusions as to the reasons for the increased incidence of malaria in 1987 would be premature.

The findings of this study have serious implications

regarding the prophylaxis and treatment of malaria in Natal/KwaZulu. In view of the fact that parasite control is essential to containment of the disease, there is clearly an urgent need to consider whether or not the use of alternative antimalarial drugs might be appropriate in the future. The continued use of chloroquine on a long-term basis will result in an increased prevalence of malaria resistant to it and may also give rise to a higher degree of stable resistance.

9. *IN VITRO* SENSITIVITY TO AMODIAQUINE, CHLOROQUINE, QUININE AND SULPHADOXINE/PYRIMETHAMINE OF FIELD ISOLATES OF *PLASMODIUM FALCIPARUM* FROM KWAZULU, 1989-1990

9.1 Introduction

Although chloroquine has been reported from northern KwaZulu (see Chapter 8), nothing is known about the susceptibilty *in vitro* to other antimalarial drugs in this region. Since January 1988, sulphadoxine/ pyrimethamine has replaced chloroquine in the treatment of malaria in this area. However, the effect of this change in drug therapy on the *in vitro* susceptibility of the local *P. falciparum* population has not been investigated. Quinine is also used in this region and in the rest of South Africa for the treatment of complicated malaria. Amodiaquine is not available in this country, but it has been used with some success in the treatment of chloroquine-resistant malaria in some areas.[73]

The aim of this study was to assess chloroquine susceptibility in northern KwaZulu and to compare the results with those of the 2 previous field investigations, to determine the base-line sensitivity

to quinine and sulphadoxine/ pyrimethamine of *P. falciparum* in this area, and to assess the suitability of amodiaquine as an alternative antimalarial.

9.2 Materials and Methods

9.2.1 *Plasmodium falciparum* isolates

Blood samples were collected from suspected malaria patients reporting to clinics in the Ubombo district of KwaZulu during April 1989 and March 1990. Isolates were included in the study if at least 0.02% of the erythrocytes were infected with *P. falciparum* only and if patients had no recent history (< 14 days) of ingestion of 4-aminoquinolines and/or sulphonamides.

9.2.2 *In vitro* tests

Using WHO predosed drug sensitivity plates, the susceptibility of the 1989 field isolates to amodiaquine, chloroquine, quinine and sulphadoxine/ pyrimethamine was tested using a modification of the WHO *in vitro* microtechnique (see Chapter 8).

Briefly, the isolates were washed twice with washing medium and suspended in the appropriate culture media (as described in Chapter 7). Field isolates with parasitaemias of >2% were diluted with with human type O-positive erythrocytes to ≤1% before being suspended in culture medium. The tests were carried out as

described in Chapter 8. In the sulphadoxine/ pyrimethamine tests, the number of morphologically normal schizonts with 8 or more chromatin dots was counted. The minimum inhibitory concentration (MIC) was defined as the lowest concentration at which complete schizont inhibition took place. The IC_{50}, namely the concentration required to inhibit the growth of 50% of the parasites, was determined using the sigmoid dose-response equation, as described in Chapter 6.

The *in vitro* sensitivity to chloroquine and quinine of 4 field isolates collected in KwaZulu in 1990 was determined using WHO predosed drug plates in the radioisotope uptake assay, as described in Chapter 7.

9.3 Results and Discussion

Tables 15 and 16 show the chloroquine MICs and IC_{50}s of the KwaZulu field isolates tested in the May 1987 and January 1988 field surveys, respectively (see Chapter 8). The parasite counts from these investigations are listed in Appendixes I, J and K. The parasite counts obtained in the 1989 KwaZulu field study of amodiaquine, chloroquine, quinine and sulphadoxine/pyrimethamine in the *in vitro* microtechnique, are listed in Appendixes L, M, N, and O, respectively and Table 17 shows the MICs and IC_{50}

values of these isolates. The mean counts per minute of KwaZulu field isolates tested in March 1990 using chloroquine and quinine in the [^{3}H]-hypoxanthine incorporation assa are shown in Appendixes P and Q, respectively. The MIC and IC_{50} values determined from these results are listed in Table 18. All of the above results are summarised in Table 19.

Table 15. Minimum inhibitory concentrations (MICs) and IC_{50} values (in µmol/l medium) of KwaZulu field isolates of *Plasmodium falciparum* collected from active and passive cases in May 1987

Isolate	Chloroquine MIC	Chloroquine IC_{50}
Active cases		
A4	0.640	N.D.[a]
A6	0.160	0.10
A8	0.320	0.11
A51	0.320	N.D.
A53	0.640	N.D.
A56	0.114	0.02
A57	>0.640[b]	0.46
A58	>0.640	0.36
A59	0.160	N.D.
A61	0.114	0.09
A65	0.160	0.12
A67	0.160	0.09
Passive cases		
P2	0.640	0.03
P3	>0.640	N.D.
P11	0.320	0.16
P17	0.640	0.19
P18	0.640	0.27
P21	>0.640	0.37
P22	>0.640	0.51
P23	0.640	N.D.
P24	>0.640	0.12
P25	0.640	0.13
P26	0.640	0.12

[a] Not determined
[b] Some isolates were not inhibited at the highest concentration tested of 0.64 µmol/l

Table 16. Minimum inhibitory concentrations (MICs) and IC_{50} values (in µmol/l medium) of KwaZulu field isolates of *Plasmodium falciparum* collected from passive cases in January 1988

Isolate	Chloroquine MIC	Chloroquine IC_{50}
J3	1.28	N.D.[a]
J4	0.64	0.23
J8	1.28	0.24
J10	0.64	0.35
J11	0.64	0.30
J12	0.64	0.11
J13	0.32	N.D.
J14	0.64	0.18
J19	>1.28[b]	0.38
J20	0.64	N.D.
J27	0.64	N.D.
J34	0.32	N.D.
J36	0.64	0.48
J37	1.28	N.D.
J38	0.64	0.48
J40	1.28	0.48
J44	0.64	N.D.
J45	0.64	N.D.

[a] Not determined
[b] Not inhibited at the highest concentration tested of 1.28 µmol/l

9.3.1 Amodiaquine

Only 1 isolate from this area was found to be sensitive to this drug, having an MIC of 0.04 µmol/l, while the remaining 12 isolates tested were resistant, with MICs of 0.08 µmol/l or greater (Table 17). One isolate was not inhibited at the highest concentration tested (32 µmol/l). The median MIC and mean IC_{50} for the 1989 KwaZulu isolates were 0.16 and 0.06 µmol/l, respectively (Table 19).

Table 17. Minimum inhibitory concentrations (MICs) and IC_{50} values (in µmol/l medium) of KwaZulu field isolates of Plasmodium falciparum collected from passive cases in April 1989

	Amodiaquine		Chloroquine		Quinine		Sulphadoxine/ pyrimethamine	
Isolate	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC
P7	0.06	0.08	0.02	0.04	0.20	1.28	0.19	0.6
P9	0.13	>0.32	0.47	1.28	0.79	1.28	41.98	200.0
P10	0.03	0.16	0.05	0.16	0.21	0.64	3.80	60.0
P12	0.02	0.16	0.04	0.32	0.24	0.64	11.00	60.0
P13	0.06	0.08	N.D.	0.04	0.36	1.28	30.96	60.0
P14	0.04	0.16	0.10	0.64	0.51	1.28	N.D.	N.D.[a]
P15	0.03	0.16	0.32	1.28	0.85	1.28	2.00	200.0
P16	0.12	0.32	0.26	1.28	0.50	1.28	103.97	200.0
P18	0.12	0.16	0.42	1.28	0.55	1.28	10.00	60.0
P22	0.02	0.04	0.33	0.64	0.56	1.28	N.D.	N.D.
P32	0.03	0.16	0.32	1.28	0.69	1.28	3.40	60.0
P33	0.10	0.16	0.48	1.28	0.40	1.28	40.00	200.0
P35	0.03	0.08	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
P45	N.D.	N.D.	0.22	0.64	1.10	2.56	N.D.	N.D.
P46	N.D.	N.D.	0.11	0.32	0.40	1.28	N.D.	N.D.

[a] Not determined

Table 18. Minimum inhibitory concentrations (MICs) and IC_{50} values (in µmol/l medium) of KwaZulu field isolates of Plasmodium falciparum collected from passive cases in March 1990

	Chloroquine		Quinine	
Isolate	IC_{50}	MIC	IC_{50}	MIC
N35	0.02	0.04	0.03	0.16
N42	0.01	0.04	0.06	0.32
N69	0.02	0.04	0.06	0.64
N81	0.02	0.08	0.09	0.64

Table 19. In vitro response of southern African isolates of Plasmodium falciparum to five antimalarial drugs

Origin and Collection Date		Amodiaquine	Chloroquine	Mefloquine	Quinine	Sulphadoxine/ Pyrimethamine[a]	Growth Assessment
KwaZulu South Africa 1987[d]	No. of isolates:	0	17	0	0	0	
	MIC:		0.114 - 1.28 (0.64)				
	IC_{50}:		0.02 - 0.51 (0.19)				Microscopy
KwaZulu South Africa 1988[d]	No. of isolates:	0	14	0	0	0	
	MIC:		0.32 - > 1.28 (0.64)				
	IC_{50}:		0.11 - 0.48 (0.32)				Microscopy
KwaZulu South Africa 1989	No. of isolates:	13	14	0	14	10	
	MIC:	0.04 - >0.32 (0.16)	0.04 - 1.28 (1.28)		0.64 - 2.56 (1.28)	0.6 - 200 (60)	
	IC_{50}:	0.02 - 0.13 (0.06)	0.02 - 0.48 (0.24)		0.20 - 1.10 (0.53)	0.19 - 103.97 (24.73)	Microscopy
KwaZulu South Africa 1996	No. of isolates:	0	4	0	4	0	
	MIC:		0.04 - 0.08 (0.04)		0.16 - 0.64 (0.48)		
	IC_{50}:		0.01 - 0.02 (0.02)		0.03 - 0.09 (0.06)		[^{3}H]-hypoxanthine incorporation

[a] Sulphadoxine/pyrimethamine concentration expressed as µmol sulphadoxine/l medium. Ratio of sulphadoxine:pyrimethamine = 80:1.
[b] Range and median in µmol/l medium.
[c] Range and mean in µmol/l medium.
[d] From data presented in Chapter 8.

Resistance to this drug has been shown in other parts of southern Africa. In an investigation carried out in Maputo, Mozambique in 1986, 76% of the cases studied were found to be resistant to amodiaquine *in vivo*.[91] The mean IC_{50} of 17 isolates tested *in vitro* was 0.12 µmol/l blood (= 0.012 µmol/l medium). In Zambia, approximately 15% of the cases studied *in vivo* in 1986 were resistant to amodiaquine.[92]

9.3.2 Chloroquine

MIC values revealed a high proportion of resistant isolates (MIC ≥0.16 µmol/l) in the field isolates collected in KwaZulu; 88.2% (15/17) in 1987 (Table 15), 100% (14/14) in 1988 (Table 16) and 85.7% (12/14) in 1989 (Table 17). The MICs quoted for 1987 and 1988 are those obtained in a previous study (see Chapter 8). Curves could not be fitted to all of the *in vitro* data obtained from these field isolates. Thus, only 10 IC_{50} values were obtained from the 1988 data set. The mean IC_{50}s of the KwaZulu field isolates were 0.19, 0.32 and 0.24 µmol/l for 1987, 1988 and 1989, respectively (Table 19). Analysis of variance was used to compare the mean IC_{50}s of the 1987, 1988 and 1989 isolates and no significant differences were found.

In 1990, the 4 KwaZulu field isolates tested were all sensitive to chloroquine, having MICs, as determined by the radioisotope uptake assay, of ≤0.114 µmol/l and

a mean IC_{50} of 0.02 µmol/l (Table 18). Since January 1988, Fansidar has replaced chloroquine in the treatment of *P. falciparum* malaria in KwaZulu and these low MICs observed in 1990 may be a consequence of increased chloroquine sensitivity in the absence of chloroquine pressure. The 1990 KwaZulu data cannot be directly compared with the 1987 , 1988 and 1989 field results as different methods were used to determine drug sensitivity and only 4 isolates were successfully tested in 1990. However, it has been shown in Thailand that withdrawal of chloroquine for treatment of malaria coincided with an increase in sensitivity to the drug.[93] Further studies are required in the KwaZulu area to establish the current status of sensitivity to chloroquine.

9.3.3 Quinine

The KwaZulu reference isolates and the 1989 KwaZulu field isolates were shown to have a lower sensitivity to quinine (median MICs of 1.92 and 1.28 µmol/l and mean IC_{50}s of 0.37 and 0.53 µmol/l, respectively) than the 4 1990 KwaZulu field isolates (median MIC = 0.48 µmol/l and mean IC_{50} = 0.06 µmol/l) (Table 19). All of the 1989 and 1990 KwaZulu field isolates were inhibited at ≤2.56 µmol/l (Tables 17 and 18) indicating to sensitivity to this drug.

9.3.4 Sulphadoxine/pyrimethamine

The mean IC_{50} (determined by the radioisotope uptake method) of isolates originating in KwaZulu prior to 1987 was 12.52 µmol/l (4.4 µmol/l if the outlying IC_{50} of 93.61 µmol/l of one of the isolates is excluded) (see Chapter 7) but by 1989, the mean IC_{50} (determined using the modification of the WHO *in vitro* microtechnique) of 10 KwaZulu field isolates was 24.73 µmol/l (15.93 µmol/l if the outlying value of 103.97 µmol/l is excluded) (Table 19).

The same pattern of sensitivity to sulphadoxine /pyrimethamine in these isolates was reflected in the MIC values. 72.7% of the KwaZulu reference isolates collected from 1984 to 1986 had sulphadoxine MICs of ≤30.0 µmol/l (see Chapter 7) while only 10% of the 1989 KwaZulu field isolates were inhibited at 30 µmol/l or less (Table 17).

The apparent decrease in sensitivity to sulphadoxine/ pyrimethamine of KwaZulu isolates may be a consequence of the increased use of this drug combination in this area since January 1988. However, further investigations are required to confirm this.

9.3.5 Cross resistance

Cross resistance was determined from the IC_{50} values using Spearman's correlation coefficients (Table 20).

A weak correlation between chloroquine and amodiaquine ($r = 0.39$, $p = 0.2392$) and a moderate correlation between chloroquine and quinine ($r = 0.56$, $p = 0.0456$) was shown. A moderate correlation between amodiaquine and sulphadoxine/pyrimethamine ($r = 0.59$, $p = 0.0739$) and between chloroquine and sulphadoxine/pyrimethamine ($r = 0.43$, $p = 0.2520$) was shown for the 1989 KwaZulu field isolates.

Table 20. Cross resistance within the field isolates of Plasmodium falciparum collected in 1989

Drug Comparison	Spearman's Correlation Coefficient	p value
Amodiaquine & chloroquine	0.39	0.2393
Amodiaquine & quinine	0.02	NS*
Amodiaquine & sulphadoxine/pyrimethamine	0.59	0.0739
Chloroquine & quinine	0.56	0.0456
Chloroquine & sulphadoxine/pyrimethamine	0.43	0.2520
Quinine & sulphadoxine/pyrimethamine	0.15	NS

* Not significant

9.4 Conclusions

Most of the KwaZulu field isolates tested were found to be resistant to amodiaquine *in vitro* and the indications are that this drug would not be suitable as an alternative antimalarial agent in this region. Chloroquine resistance was shown to exist in northern

KwaZulu in the 1987 and 1988 field studies and was confirmed in the 1989 field investigation. However, the 1990 study of 4 isolates from this area using the radioisotope uptake assay, showed an increase in chloroquine susceptibility *in vitro*. This may have been a result of the replacement of chloroquine with sulphadoxine/pyrimethamine in northern KwaZulu but further tests are required to confirm this. The low susceptibility of isolates from this area to sulphadoxine/pyrimethamine in 1989 may have been a result of the increased use of Fansidar since January 1988. Although isolates from this region required high levels of quinine to inhibit schizont development in the *in vitro* test, most of them were inhibited within the sensitive range of concentrations. It is clear that there is a need to continue monitoring drug sensitivity in northern KwaZulu.

10. IMPLICATIONS OF DRUG RESISTANCE FOR THE SOUTH AFRICAN TRAVELLER IN SOUTHERN AFRICA: A DISCUSSION OF CHEMOPROPHYLAXIS AND CHEMOTHERAPY

10.1 Introduction

Until the late 1970s, a decision as to which antimalarial drug to take for prophylaxis when travelling in the southern African region, was simple. Chloroquine was cheap, well tolerated and no resistance to this drug had been reported on the continent. However, with the spread of chloroquine resistance to states in southern Africa, the issue has become more complex. This chapter includes a review of current recommendations for prophylaxis, comments on the availability of prophylactic drugs to the South African traveller in the southern African region and a discussion of the implications of drug resistance for chemoprophylaxis and chemotherapy in such travellers.

10.2 Distribution of *P. falciparum* in southern Africa

P. falciparum malaria occurs in all southern African countries.[94] In Botswana, Namibia, South Africa, Swaziland and Zimbabwe, only certain areas are affected, while in the remaining southern African

states, malaria occurs in the whole of the country. In Angola, Comores, Madagascar, Malawi, Mozambique, Swaziland and the Zambezi valley in Zambia and Zimbabwe, transmission takes place throughout the year, while in Botswana, Namibia, South Africa and in Zambia and Zimbabwe excluding the Zambezi valley, malaria occurs from November to June. In Mauritius, only *P. vivax* malaria occurs in the northern rural areas throughout the year.

10.3 Drug resistance in southern Africa

Chloroquine resistance exists in all southern African countries other than Mauritius.[94] Resistance to sulphadoxine/pyrimethamine (Fansidar) has been reported from Angola,[94] Mozambique, Zambia and Zimbabwe.[95] 6.9% of hospital and 0.4% of field cases of *P. falciparum* malaria in KwaZulu failed to respond to Fansidar treatment during March 1988.[43] Resistance to this drug combination has also been reported from Kenya and Tanzania[95] and, in view of the fact that pyrimethamine resistance is widespread in Africa,[42] it is not unreasonable to expect that Fansidar resistance will soon spread to all southern African states. Isolated cases of quinine resistance have occurred in Malawi[76] and Zambia.[77] Mefloquine resistance was observed *in vitro* in Zambia in 1986[75] and there have been cases of resistance to this drug in West Africa

and Tanzania.[73]

The results of the studies of southern African reference and field isolates of *P. falciparum* have confirmed the existence of chloroquine resistance in this region. Resistance to amodiaquine was shown to occur in northern KwaZulu. Southern African isolates were shown to be fully susceptible to mefloquine but reduced sensitivity to quinine and sulphadoxine/ pyrimethamine was found in some of these *P. falciparum* isolates.

10.4 Recommendations for prophylaxis

All southern African countries, with the exception of Mauritius, fall within the area designated "Zone C" by the World Health Organization (WHO).[94] The WHO recommendations for prophylaxis for this region are:

i. mefloquine
or ii. doxycycline
or iii. chloroquine + proguanil (if mefloquine or doxycycline is unavailable or contraindicated)
or iv. no prophylaxis (if the risk of contracting *P. falciparum* malaria is very low)

Mauritius falls within "Zone A" and the WHO recommendations for this area are:

i. chloroquine

or ii. where there is a low risk of infection,

nothing.

If chloroquine + proguanil are used for prophylaxis in "Zone C" or if no prophylactic drugs are taken, travellers are advised to carry either quinine, mefloquine or halofantrine for the treatment of malaria. These drugs are to be used under medical supervision or where there is no access to a doctor or a hospital. The stand-by drug for treatment of malaria when no prophylaxis is taken in "Zone A" is chloroquine.

The Centers for Disease Control, USA (CDC), recommend mefloquine for prophylaxis in all southern African countries, excluding Mauritius, where travellers are advised to take chloroquine alone.[96] The CDC-recommended alternatives to mefloquine are doxycycline or chloroquine. Proguanil is advised in combination with chloroquine for East Africa but not for southern Africa. The CDC advises travellers on chloroquine prophylaxis to carry Fansidar for treatment but advises against the use of mefloquine for self-treatment because of the risk of serious side effects at higher dosages. Neither the WHO nor the CDC recommends Fansidar as a prophylactic drug.

10.5 Prophylactic antimalarial drugs available in South Africa

At present, the prophylactic drugs available in South Africa are chloroquine (Nivaquine), pyrimethamine (Daraprim), chloroquine/ pyrimethamine (Daraclor), dapsone/pyrimethamine (Maloprim) and doxycycline (Vibramycin). Mefloquine is not yet registered for use in South Africa but it may be obtained on a clinical trial basis from selected doctors. However, the continued availability of this drug is not guaranteed. Proguanil may be obtained in Angola, Malawi, Mozambique, Zambia and Zimbabwe but it is not always freely available.[97]

Chloroquine alone is only recommended for Mauritius and the chloroquine/pyrimethamine combination is not recommended at all. Resistance to pyrimethamine is widespread in Africa and a recent study of cultured isolates of *P. falciparum* from northern KwaZulu, the north-eastern Transvaal, Mozambique and Malawi showed that southern African isolates have a low sensitivity to this drug (see Chapter 5). Dapsone/pyrimethamine is no longer recommended by the WHO because of the serious side effects of this drug combination.

The only available antimalarial recommended by WHO and CDC is doxycycline. Doxycycline may be obtained on

prescription but use of this drug may result in skin photosensitivity and it should therefore not be taken by those who are likely to be exposed to direct sunlight for extended periods of time unless they are protected by an effective sunscreen. Doxycycline should not be given to children under eight years of age and is contraindicated in pregnancy.

One option open to South African travellers to neighbouring countries is to start taking chloroquine one week before leaving home and then to purchase proguanil on arrival in one of the African states in which it is available. The traveller should thereafter take chlorquine in combination with proguanil.

Mefloquine may be obtained on a clinical trial basis but it is contraindicated in certain groups of people. It is not recommended for children under two years of age and it should not be taken by pregnant women. Women of child-bearing age should not ingest mefloquine during the three-month period before conception. Mefloquine should not be taken by those performing tasks requiring fine coordination and spatial discrimination (e.g. airline pilots) and it is contraindicated in people taking beta-blockers and in those with a history of epilepsy.

10.6 Treatment of malaria in southern Africa

Although chloroquine is still used with some success to treat malaria in semi-immune inhabitants of southern African states, chloroquine would be an unwise choice for chemotherapy in non-immune travellers in this region, considering that chloroquine-resistant *P. falciparum* is to be found in all southern African countries (with the exception of Mauritius).

The results of the *in vitro* studies suggest that quinine and sulphadoxine/pyrimethamine may be useful therapeutic drugs, although some of the southern African isolates tested showed reduced susceptibility to these antimalarials. Both quinine and Fansidar are available in South Africa. Mefloquine and halofantrine were found to be effective against southern African strains of *P. falciparum*. However, mefloquine is only available on special release and halofantrine is not yet registered for use in South Africa.

10.7 Conclusions

The choice of antimalarial prophylactic drugs available to the South African traveller is severely limited. None of the antimalarial drugs registered to be sold over-the-counter in this country is recommended by the WHO or CDC for southern Africa,

except for Mauritius. Doxycycline is available on prescription but is not suitable for certain groups of travellers, and mefloquine is not freely available. There is clearly an urgent need to have more prophylactic drugs registered for use in this country. Proguanil would be one obvious choice as the risk of serious side-effects associated with taking this drug in combination with chloroquine for prophylaxis is low; and this combination is safe for children and in pregnancy. More importantly, proguanil has been reported to be an effective prophylactic antimalarial in East Africa and there have been no reports of proguanil resistance in southern Africa.

Quinine and sulphadoxine/pyrimethamine are still effective in southern Africa in the treatment of *P. falciparum* malaria. However, it may only be matter of time before resistance to these drugs appears in this region.

11. OVERALL SUMMARY AND CONCLUSIONS

This thesis describes the characterisation of southern African isolates of *Plasmodium falciparum* according to isoenzyme type, antigen variants and sensitivity *in vitro* to chloroquine, pyrimethamine, amodiaquine, mefloquine, quinine, sulphadoxine/pyrimethamine and halofantrine. In addition to this laboratory-based study of established isolates, 4 field surveys were carried out in KwaZulu to determine the *in vitro* susceptibility to amodiaquine, chloroquine, quinine and sulphadoxine/pyrimethamine of primary isolates from this region.

Nineteen southern African isolates of *P. falciparum* and the Gambian reference isolate, FCR-3, were cultured *in vitro* in gassed tissue culture flasks using HEPES-buffered RPMI 1640 medium supplemented with glucose and hypoxanthine. Static cultures resulted in satisfactory multiplication rates but by growing isolates in shaken flasks, higher growth rates and peak parasitaemias were achieved. By reducing the haematocrit from 5% to 1%, it was possible to leave these suspension cultures unattended for 3 days, thus saving time, labour and culture medium.

Polyacrylamide gel electrophoresis involving 5 enzymes (GPI, ADA, LDH, GDH and PGD) revealed little variation amongst the isolates. The frequencies of enzyme forms

were similar to those of isolates from other malarial areas of the world. Eight of the isolates contained 2 forms of GPI, indicating clonal heterogeneity. One of these 8 isolates also contained 2 forms of ADA and another showed 2 forms of LDH.

Antigenic diversity was demonstrated using a panel of 9 monoclonal antibodies in the indirect fluorescent antibody test, which showed that southern African isolates share antigens with isolates from other malarial regions of the world. Both characterisation techniques revealed a mixture of parasite types in some patients. However, the characteristics of most of these heterogeneous isolates were not stable with time in culture and some isolates became homogeneous during the test period with respect to certain markers. By the end of the investigation, 70% of isolates displayed an identical antigen pattern, which was markedly different to any obtained in other parts of the world. Differences may be due to geographic origin of parasites or to variation in culture conditions in different laboratories.

Several methods were used to determine *in vitro* drug sensitivity in southern African *P. falciparum* isolates. In a 48-hour growth inhibition assay, 4 reference isolates collected in KwaZulu early in 1985 were found to be sensitive to chloroquine. One of these was known to be sensitive to the drug *in vivo*.

Eight isolates which originated in KwaZulu or Mozambique were resistant to chloroquine *in vitro*, having been inhibited at 0.3 μM or more. Six of these isolates were known to be chloroquine-resistant in varying degrees *in vivo*. Four of 5 isolates from the North-eastern Transvaal and 2 clinically chloroquine-resistant Malawian isolates were resistant to chloroquine *in vitro*. A wide range of pyrimethamine susceptibilities was detected (0.03 μM to > 3.0 μM), although most isolates were inhibited at 0.1 μM, thus indicating a low level of resistance. The sensitivities to chloroquine and, to a lesser extent, pyrimethamine, fluctuated during the test period, probably because of the heterogeneous nature of the isolates.

Using the radioisotope uptake assay, the sensitivity to halofantrine of 18 of the southern African reference isolates and 3 isolates obtained in March 1990 from KwaZulu malaria patients, was determined. This study showed that linear interpolation provides a simple method for the determination of IC_{50} values and that this procedure may also be used to provide estimates of the IC_{50} for the sigmoid dose-response method for assessing the IC_{50}. The IC_{50}s, as determined by the sigmoid dose-response procedure, ranged from 0.039 to 15.000 nmol/l. All isolates had halofantrine MICs of 32 nmol/l or less with a median of

8 nmol/l. Southern African *P. falciparum* isolates were found to be similar to those from West Africa in their sensitivity to halofantrine but were less susceptible to the drug than South-East Asian isolates. However, the results of this study suggested that some local isolates may have reduced susceptibility to halofantrine. Further *in vitro* tests and *in vivo* evidence are required to confirm this.

The [^{3}H]-hypoxanthine incorporation method was also used to determine the *in vitro* drug sensitivity of 18 southern African reference isolates of *P. falciparum* using microtitre plates predosed with amodiaquine, chloroquine, mefloquine, quinine and sulphadoxine/ pyrimethamine. Chloroquine resistance was confirmed in those isolates showing resistance in the 48-hour growth inhibition assay. However, 1 isolate, RSA 13, which was sensitive in the 48-hour assay, was found to be resistant to the drug in the radioisotope uptake assay. This is assumed to be a consequence of selection in culture of chloroquine-resistant clones in this isolate over time. All reference isolates, with the exception of 2 Transvaal isolates, were sensitive to amodiaquine *in vitro* and all of these isolates were found to be mefloquine-sensitive. However, these isolates were found to have reduced susceptibility to quinine, with 8 isolates being inhibited at only the highest concentration tested of 2.56 µmol/l. Four isolates were not inhibited at this

level. A wide range of sulphadoxine/pyrimethamine sensitivities (10 - >100 µmol/l) was shown amongst the southern African reference isolates. There was some cross resistance between chloroquine and each of amodiaquine, mefloquine and quinine and between quinine and mefloquine. A moderate negative correlation was found for these isolates between mefloquine and sulphadoxine/pyrimethamine and quinine and sulphadoxine/ pyrimethamine.

Four *in vitro* drug sensitivity st ies were carried out in northern KwaZulu from 1987 to 1990. In May 1987 and January 1988, the chloroquine sensitivity of *P. falciparum* in the Ubombo and Ingwavuma districts of KwaZulu was determined by a modified *in vitro* microtest in which the patients' plasma was replaced with non-immune human AB serum and the test plates were incubated in an atmosphere of 3% O_2, 4% CO_2 and 93% N_2. A success rate of 74% was achieved using this technique. All of 23 successfully tested isolates from malaria patients reporting to clinics and a hospital in these areas were found to be resistant to chloroquine, schizogony being inhibited at 32 pmol per well in the majority of tests. Six out of 8 isolates obtained through active surveillance in the Ubombo district were found to be resistant in varying degrees. Malarial parasites collected from clinics and a hospital in the endemic area did not change markedly

in their *in vitro* response to chloroquine during the 8-month period from May 1987 to January 1988.

Two field investigations were carried out in April 1989 and March 1990, using blood samples collected from suspected malaria patients reporting to clinics in the Ubombo district of KwaZulu. In the 1989 *in vitro* field survey, in which the 24-hour *in vitro* microtechnique was used to determine drug sensitivity, only 1 isolate from this area was found to be sensitive to amodiaquine, and 12 out of 14 isolates tested were resistant to chloroquine. Analysis of variance was used to compare the mean chloroquine IC_{50}s of the 1987, 1988 and 1989 field isolates and no significant difference was found. However, in the 1990 field investigation, in which the radioisotope uptake assay was used to assess drug susceptibility, the 4 isolates tested were all sensitive to chloroquine. It is possible that this apparent increase in chloroquine sensitivity may be a consequence of the replacement of chloroquine with Fansidar for the treatment of *P. falciparum* malaria in this area. However, the 1990 KwaZulu results cannot be directly compared with the 1987 , 1988 and 1989 field results as different methods were used to determine drug sensitivity and only 4 isolates were successfully tested in 1990. Further studies are required in the KwaZulu area to determine the current status of sensitivity to chloroquine. All of the 1989 and 1990

field isolates were inhibited at 2.56 µmol quinine/l but the 1989 KwaZulu field isolates were shown to have a lower sensitivity to quinine than the 1990 KwaZulu field isolates. Isolates collected in 1989 were found to have a low susceptibility to sulphadoxine/ pyrimethamine, with only 10% of these isolates being inhibited at ≤30 µmol/l. This may have resulted from the increased use of Fansidar in this region since January 1988.

For the 1989 KwaZulu field isolates, a weak correlation between resistance to chloroquine and amodiaquine and a moderate correlation between resistance to chloroquine and quinine was shown. Some cross resistance between amodiaquine and sulphadoxine/pyrimethamine and between chloroquine and sulphadoxine/pyrimethamine was also shown for these isolates.

In a comparison of the 48-hour growth inhibition assay with the radioisotope uptake assay, it was found that the [^{3}H]-hypoxanthine incorporation assay yielded lower IC_{50} values than the 48-hour assay in 15 of the 18 isolates compared. The difference in the mean IC_{50}s in the 2 assays was statistically significant. It is possible that this difference may be due to the effect of the drug being detected at a lower concentration in the radioisotope assay than in the growth inhibition

assay. It is clear that direct comparisons cannot be made between *in vitro* drug sensitivity studies employing different assays. However, regardless of the method used, *in vitro* studies give a good indication of the drug sensitivity of parasite populations.

Apart from 2 *in vitro* chloroquine sensitivity studies of Natal and KwaZulu isolates of *P. falciparum* in 1985[40] and 1987,[86] these laboratory- and field-based investigations are the first *in vitro* drug susceptibility studies of South African isolates. The results have serious implications for the South African traveller in southern Africa. At present, the prophylactic drugs available in this country are chloroquine (Nivaquine), pyrimethamine (Daraprim), chloroquine/pyrimethamine (Daraclor), dapsone/ pyrimethamine (Maloprim) and doxycycline (Vibramycin). The only available prophylactic antimalarial recommended by WHO and CDC for southern Africa, is doxycycline. This drug is not available over-the-counter and is not suitable for all travellers. The results of the *in vitro* studies suggest that quinine and sulphadoxine/pyrimethamine may be useful therapeutic drugs, although some of the southern African isolates tested showed reduced susceptibility to these antimalarials. Both quinine and Fansidar are available in South Africa. Mefloquine and halofantrine were found to be effective against southern African strains. However mefloquine is only available on

special release and halofantrine is not yet registered for use in South Africa. There is clearly an urgent need to have more prophylactic drugs registered for use in South Africa.

The results of this characterisation study of southern African isolates of *P. falciparum* revealed considerable diversity in the region, thus confirming the findings of similar investigations in other malarial areas of the world.[5] This is not surprising, as it now seems that most, if not all, isolates possess unique characteristics. The isozyme and antigenic studies showed that southern African isolates share variants with isolates from other malarial areas. However, the typing with monoclonal antibodies revealed an antigenic pattern in 70% of the southern African isolates which was markedly different from other geographic regions. Both characterisation techniques revealed clonal mixtures in some of the isolates. Drug susceptibility in this region could be correlated with the intensity of drug use and population migration. This study has provided a bank of well-characterised southern African isolates of *P. falciparum* for use in research on various aspects of malaria.

12. GENERAL DISCUSSION

The field of malaria parasite research is a dynamic one and there have been many new developments since the completion of this study. In this chapter, the various problems associated with *P. falciparum* isolate characterisation are discussed and findings are related to recent developments.

12.1 *In vitro* cultivation of *P. falciparum*

Because of the long-term nature of this project, it was necessary to first establish the *P. falciparum* isolates in culture before carrying out the characterisation studies. The adaptation of the southern African isolates to culture conditions formed a major part of the work. This section contains discussions on the problems associated with, and recent developments in, the establishment of strains, gametocytogenesis, cloning of isolates and cross-contamination between laboratory lines.

12.1.1 Establishment of southern African strains of *P. falciparum*

The establishment of strains *in vitro* was fraught with many difficulties. Of 75 isolates initiated in

culture, only 16 became adapted to the culture conditions. The main areas in which problems were experienced were in the transport of infected blood specimens, the culturing of cryopreserved field isolates, the optimising of culture conditions and contamination.

12.1.1.1 Collection of *P. falciparum* isolates

As malaria is under the control of the various health authorities within the Republic of South Africa, the control programmes being aimed at both the mosquito vectors and the parasite, the incidence of malaria is relatively low when compared with other malarial areas of the world.[50] Coupled with the low number of cases, was the problem of obtaining chloroquine-resistant, as well as chloroquine-sensitive, isolates. At the time when specimens were being collected for this project, i.e. between July 1984 and February 1987, chloroquine resistance was only just beginning to emerge in South Africa,[39,40,41] although resistance to this drug had been reported earlier from Mozambique.[98] The isolates collected in 1984 and 1985 were mainly chloroquine sensitive while those collected in 1986 and 1987 were resistant to the drug.

Most of the isolates were collected on field trips to the malarial areas of KwaZulu and the north-eastern Transvaal. Other isolates were obtained from

hospitals, mainly from patients who had failed to respond to chloroquine treatment. Because of the long distances between the collection points and the laboratory in some cases, many isolates lost viability during transport. Infected blood specimens were generally transported on ice and were normally delivered to the laboratory within 24 hours of collection. On examination of the specimens on arrival at the laboratory, many specimens were found to contain morphologically degenerate parasites which subsequently failed to grow in culture.

12.1.1.2 Culturing of cryopreserved isolates

In cases where it was not possible to deliver the infected blood sample to the laboratory within 24 hours of collection, isolates were cryopreserved in the field laboratory before being transported to the main laboratory in Durban. In an attempt to ensure viability of the isolates, infected erythrocytes were cryopreserved as soon as possible after collection. Although some success was achieved in culturing these frozen isolates, the success rate was relatively low. Of the 47 *P. falciparum* isolates cryopreserved, only six were successfully established in culture.

12.1.1.3 Culture conditions

There are many factors to consider when culturing *P. falciparum*. These include the type of culture vessel

used; whether the culture is held in suspension or whether it is static; medium, erythrocyte, serum and gas requirements; initial parasitaemia; and use of antimalarial drugs by the patient prior to collection of the blood specimen. The conditions for the cultivation of the southern African strains are described in Chapter 2. General cultivation procedures for erythrocytic stages are adequately described by Jensen.[99]

For the establishment of the southern African strains of *P. falciparum* it was advantageous to have high initial parasitaemias, although some isolates with as few as 0.1% parasitised erythrocytes were successfully cultured.

Many of the patients, especially those who were hospitalised or who had attended a clinic, had already been treated, usually with chloroquine, prior to collection of the infected blood specimen. This was found to adversely affect the success rate in establishing strains in culture, although eight of the southern African isolates, namely RSA 6, 7, 8, 10, 11, 12, MW 1 and 2, were all obtained from patients who had received chloroquine, yet were successfully cultured.

The quality of culture medium, serum and erythrocytes

was found to be very important in the establishment of growth of *P. falciparum in vitro*. RPMI 1640, obtained from Flow Laboratories, was routinely used in the cultivation of the southern African isolates. As funds were limited, it was decided to try a cheaper source of medium and RPMI 1640 was obtained from a local company. Unfortunately, this product consistently gave poorer growth rates in established isolates than the overseas product and was found to be unsuitable for the adaptation of new strains to culture conditions. However, this local medium was found to be suitable for short-term growth experiments e.g. *in vitro* drug sensitivity tests.

Many difficulties were experienced in obtaining serum for the supplementation of the culture medium and it was not always possible to use pooled sera from different donors, as is recommended.[99] Whole blood, normally A-positive, collected in a serum pack containing no anticoagulant and screened for the presence of malaria antibodies, was generally purchased from the local blood bank and the serum removed after coagulation of the red blood cells. However, not all batches of sera were able to support the growth of *P. falciparum in vitro*. In one experiment, an attempt was made to establish 34 isolates in culture but none grew. It was subsequently discovered that the serum used was not suitable for

cultivation as it was not able to sustain the growth of culture-adapted isolates. After this discovery, each new batch of serum was tested for its ability to sustain the growth of established strains before being used for the establishment of new isolates.

In an attempt to find an alternative source of serum, the ability of bovine serum to support the growth of *P. falciparum in vitro* was compared with that of human serum (results not shown). Isolate RSA 10 was cultured in suspension in gassed tissue culture flasks in the routine culture medium supplemented with either pooled human serum (group O-positive, 10 donors) or pooled bovine serum (10 animals) for 96 hours. The medium was replenished daily. After 96 hours, the growth rate in the human serum-supplemented medium far exceeded that in the medium enriched with bovine serum. Parasite numbers in the latter medium increased slightly in the first two days and then decreased and parasites became morphologically degenerate. It was concluded that bovine serum was not a suitable substitute for human serum in this culture system. Divo and Jensen[100] reported that bovine serum needs to be supplemented with 0.3 - 1.2 µmol/l hypoxanthine in order to adequately support the growth of *P. falciparum in vitro* and that growth rates in bovine serum-supplemented medium were lower than those in medium enriched with human serum. Other workers have reported

some success with rabbit, horse, sheep and bovine sera in the cultivation of *P. falciparum in vitro*.[101]

Recently Ofulla *et al.*[102] described a serum-free medium enriched with bovine albumin and lipids-cholesterol-rich mixture which was reported to adequately support the growth of *P. falciparum* in culture. However, it is not known whether this medium could be used in the establishment of primary isolates as only culture-adapted strains were used in their experiments. As human serum is costly and not readily available, the development of an effective serum-free medium would be a great advantage in the cultivation of *P. falciparum*.

Erythrocytes were obtained from donors working in the laboratory. Although this was never quantified, red blood cells from some donors consistently yielded higher growth rates for any one isolate than did erythrocytes from other donors.

Although every care was taken to work aseptically, one problem which frequently arose was that of bacterial and fungal contamination of the cultures. This was often due to contamination of the original sample but sometimes isolates become contaminated within the cultivation environment. This contamination arose in spite of the routine use of gentamycin (50 mg/l) in

the culture medium, presumably by bacteria resistant to this antibiotic.

In an evaluation of 18 antimicrobial agents, eight were found to be suitable for routine use in the cultivation of *P. falciparum*. These were streptomycin, gentamycin, kanamycin, chloramphenicol, chlortetracycline, nystatin and amphotericin B.[103] Although some success was achieved with the use of chloramphenicol[104] in the elimination of the contaminants in the southern African isolates, it was generally easier to discard the contaminated culture and begin again with a cryopreserved aliquot of the same isolate, as the use of this antibiotic adversely affected growth rates.

12.1.1.4 Use of feeder cells in the cultivation of *P. falciparum*

Some workers have reported on the use of feeder cells in the adaptation of primary isolates to continuous culture. Mazier *et al.*[105] found that by growing *P. falciparum* on monolayers of hepatocytes, the success rate of adaptation was greater than that obtained in a modification of the candle jar method and that multiplication rates in established strains increased. Phillips *et al.*[106] used cells washed from the mouse peritoneal cavity (MPWC) to establish strains that would not grow without these MPWCs. MPWCs also

increased the growth rates of established strains. Feeder cells were not used in the establishment of southern African isolates of *P. falciparum*.

12.1.2 Gametocytogenesis

The ability to produce gametocytes *in vitro* is one of the biological characteristics of *P. falciparum* isolates. Although this property was not one of the subjects of this study, some observations on gametocyte production in the southern African isolates were made during the establishment and maintenance of these strains *in vitro*.

Although gametocytes were not seen in all of the original blood samples, all of the southern African isolates produced gametocytes at some time during routine culture. Several workers have found that gametocytogenesis is reduced in isolates that have been cultured for long periods.[107] It has also been found that infectivity of gametocytes to mosquitoes may also be adversely affected in long-term culture. It is therefore recommended that aliquots of isolates found to be good producers of gametocytes should be cryopreserved as soon as possible after initiation in culture. Where possible aliquots of the original blood specimen should also be frozen down.

Stimulation of gametocytogenesis has been achieved by

the supplementation of the culture medium with hypoxanthine (50 mg/l).[107] The culture medium used in the cultivation of the asexual stages of the southern African isolates was routinely supplemented with hypoxanthine (44 mg/l) as this was reported to improve parasitic yields.[11] This enrichment of the medium may have promoted the production of gametocytes during routine culture of the southern African strains.

Many chemicals have been reported to induce gametocytogenesis in cultures of *P. falciparum*, the most recent of these being Berenil, a substance which inhibits the synthesis of DNA, RNA and polyamine.[108]

Using the method of Ponnudurai *et al.*,[109] in which gametocytogenesis was stimulated by adverse culture conditions produced by the non-addition of fresh erythrocytes to the cultures, gametocytes were produced by a colleague (B.L. Sharp) in four of the southern African reference isolates.[110] The rate of gametocyte production was high when compared with that of African and South American isolates reported by other workers.

Using the gametocyte-synchronisation method of Ponnudurai *et al.*,[111] mature gametocytes were successfully produced from RSA 2 on three separate occasions (J.A. Freese - unpublished data). Although

exflagellation was observed, attempts to infect colonised *Anopheles arabiensis* mosquitoes failed. It is not known whether the gametocytes were non-infective or whether the mosquitoes were refractory to infection. This aspect of malaria research is currently being further investigated by a Ph. D. student in the MRC laboratory in Durban using the southern African reference isolates and NF54.[112] The successful infection of mosquitoes by the southern African isolates would open up new avenues of research locally.

12.1.3 Cloning of *P. falciparum* isolates

It is widely accepted that most, if not all, isolates of *P. falciparum* are comprised of at least two clones.[5] When carrying out characterisation studies it is desirable to investigate pure clones rather than isolates. However, attempts at cloning two southern African isolates proved unsuccessful and, therefore, clones were not included in this study.

Clones may be produced from heterogeneous isolates by means of limiting dilution[113] or by micromanipulation.[114] The results of this study suggested that many of the southern African isolates were comprised of genetically diverse clones. For example, RSA 2 displayed a mixed reaction with monoclonal antibodies 9.2 and 10.3, while RSA 15 had a

mixed reaction to antibodies 7.3, 7.6, 9.2, 10.3, 12.1, 12.3 and 5.1, contained two forms of GPI (1 and 2) and varied in its chloroquine MIC at different times in culture. Attempts to clone these two isolates by limiting dilution proved unsuccessful. One of the possible reasons for failure to produce clones may have been that the cloning procedure was carried out in a static system whereas these isolates were routinely cultured in suspension. Indeed, it was subsequently found that the success rate in drug tests carried out on the southern African isolates, which were also performed in a static system, was improved by allowing the isolate to grow for several days in flasks that were not shaken, prior to carrying out the tests.

12.1.4 Cross-contamination of *P. falciparum* isolates

One of the problems associated with maintaining many isolates of *P. falciparum* in one laboratory is the danger of cross-contamination between strains.[115] This may arise through mislabelling of flasks and/or through use of the same pipette to add culture medium or erythrocytes to more than one isolate. Using several genetic markers, Robson *et al.*[115] found a number of isolates and clones used in many laboratories to be identical. They suspected that this had resulted from cross-contamination. They recommended that clones rather than mixed isolates be

used in laboratory investigations and that each clone (or isolate) be monitored regularly using a set of appropriate markers.

The variations over time in culture with respect to reactions with monoclonal antibodies, isozyme type and drug sensitivity in some of the southern African isolates has been ascribed to clonal mixtures. However there are some instances where contamination of one isolate with another is a distinct possibility.

In the enzyme typing of isolate RSA 3, 4 and 6, both GPI-1 and -2 were present in the third lysate prepared from these isolates but only GPI-1 was present in the first and second lysates. It is possible that GPI-2 was present in these isolates at the time of preparation of lysates 1 and 2 but that the number of parasites exhibiting this isozyme was so small, that it was not detectable on the stained gel. However, another possibility is that in the time between the preparation of the 2nd and 3rd lysates, these three isolates had become contaminated with one or more isolates containing both GPI-1 and -2 such as RSA 1, 12, 14 or 15. In addition to this, 10 of the 19 southern African isolates exhibited the identical isozyme pattern as FCR-3, indicating the possible contamination of these strains with FCR-3.

In the typing with monoclonal antibodies, 70% of all isolates tested exhibited an identical antigenic pattern at the end of the three tests. This was ascribed to the possible dominance of this particular antigenic pattern in the natural *P. falciparum* population which was selected for under the laboratory conditions. However, another explanation could be the contamination of these isolates with one or more isolates showing this particular antigenic pattern, namely RSA 10, 12 16, MW 1 and FCR-3.

Fluctuations over time in sensitivity to chloroquine and pyrimethamine of the southern African isolates were also ascribed to their heterogenic nature. This may have been a reasonable explanation in the majority of cases where the MIC of a given isolate differed by only one concentration increment in the three 48-hour tests. However, two isolates differed in their pyrimethamine MICs between the first and subsequent tests by more than one concentration increment. The MIC of RSA 7 in the initial pyrimethamine test was 1.0 μM but was found to be 0.1 μM in subsequent tests Similarly, MW 2 was not inhibited by 3 μM pyrimethamine (the highest concentration tested) in the 1st test, but growth was inhibited by 0.1 μM pyrimethamine in subsequent tests. These two isolates may have become contaminated by an isolate with a lower pyrimethamine MIC, which outgrew the original

isolate *in vitro*. RSA 13, which was found in the 48-hour growth inhibition assay to be sensitive to chloroquine, was subsequently shown in the radioisotope uptake assay to be resistant to this drug. This could be explained by the selection of resistant clones in this isolate or by the contamination of RSA 13 with a chloroquine-resistant isolate.

If the southern African reference isolates described in this thesis are to be used for further laboratory investigations, both locally and overseas, it is essential that they be checked for possible cross-contamination. Recently, Wooden *et al.*[116] developed a method for distinguishing between *P. falciparum* strains in the laboratory, based on the polymerase chain reaction (PCR) technique. Using primers that amplified unconserved repeat regions in the genes coding for four surface antigens, they were able to distinguish strains on the basis of the size of the amplified products in three of the four genes. Together with a simplified method for the isolation of DNA from the isolates, they have described the conditions for the PCR assay using a very small amount of culture material which yields a unique banding pattern for each strain. This method will make possible the rapid detection of cross-contamination between strains. It is the intention to use this

technique to determine whether mixing of the southern African isolates has occurred during routine cultivation.

12.2 Enzyme typing

Chapter 3 describes the isozyme characterisation of the southern African isolates of *P. falciparum* using polyacrylamide gel electrophoresis. This method was found to be superior to starch gel electrophoresis in the resolution of bands. In spite of this improvement, however, flat bed polyacrylamide gel electrophoresis of PGD did not produce discrete bands and it was found that a vertical electrophoresis system using a 4% stacking gel and a 7.5% running gel produced a better result. Lysates were mixed with an equal volume of 50% glycerol in PBS and the wells were loaded with 10 µl aliquots. The system was allowed to run for four hours at 200 V. This vertical system was also used to confirm the results obtained for GPI and ADA on the flat bed system.

It was found that freezing and thawing of lysates caused the enzyme activity to decrease, resulting in faint bands. To overcome this problem, lysates were aliquoted into several Nunc tubes to allow the enzyme pattern of each lysate to be examined several times without having to thaw and re-freeze the same sample

for each run.

This method of characterising *Plasmodium* parasites was first developed for rodent malaria species and revealed considerable inter- as well as intra-species diversity of enzyme forms.[117, 118] Enzyme typing of *P. falciparum* was first carried out on isolates from the Gambia[119] and from several regions in West and East Africa[120] and has since been carried out in several malarial areas of the world.[5] Although similar forms of each enzyme are found in different countries, some differences in frequencies of different isozymes have been observed.[5]

The technique of enzyme typing is not as discriminating as other characterisation methods but isozymes have provided useful markers for cloning[113] and genetic[121] experiments. Isolates exhibiting two forms of one or more enzymes may be cloned and the isozyme type may be used to identify the resulting clones.[113] Clones identified by enzyme pattern have been used in genetic studies in rodent malaria and in *P. falciparum*.[121] In a crossing experiment carried out between two clones of *P. chabaudi*, 1 exhibiting PGD-2 and LDH-3 and the other showing PGD-3 and LDH-2, four classes of progeny clones were obtained: two parental (PGD-2/LDH-3 and PGD-3/LDH-2) and two recombinant (PGD-2/LDH-2 and PGD-3/LDH-3) types. The ratio of

parental:recombinant types was close to the predicted ratio. This study also showed the blood forms to be haploid. Enzyme forms were also used as one of the markers in a crossing experiment between two cloned lines of *P. falciparum*.[121] This study revealed that cross-fertilisation occurs readily between parent clones, that the blood forms of *P. falciparum* are haploid and that some genomic rearrangement takes place during hybridisation.

12.3 Antigenic diversity and variation

12.3.1 Antigenic diversity

The typing of southern African strains of *P. falciparum* revealed antigenic diversity amongst parasite from this region (see Chapter 4). Antigenic diversity is of considerable relevance to the development of a vaccine as many of the molecules that exhibit this diversity are likely to be targets of protective immunity.[5]

Antigenic diversity of *P. falciparum* has been demonstrated by a number of workers using various techniques and many of the antigens studied have been well characterised (for reviews see refs 5, 122-125). Diversity has been revealed in the following antigens: S-antigen;[5,122,123,124,125] merozoite surface antigen-1 (MSA-1, also known as the 195K protein, P190, the

200 kDa protein, the polymorphic schizont antigen (PSA), the precursor to the major merozoite surface antigens (PMMSA) or gp185);[5,122,123,124] merozoite surface antigen-2 (MSA-2);[122,123,124] the CS protein-related antigen (CRA, also known as Exp-1);[5,122] histidine-rich proteins (HRP-I (or KAHRP), HRP-II, HRP-III (or SHARP));[122,125] the ring-infected erythrocyte surface antigen (RESA, also known as Pf155);[122] and the circumsporozoite (CS) antigen.[5,123,125]

The S-antigen is a soluble, heat-stable protein which is secreted into the parasitophorous vacuole and released upon schizont rupture. S-antigens are found in the sera of individuals who have been exposed to *P. falciparum* parasites. Immunodiffusion tests revealed a large number of antigenic specificities and studies suggest that there are over 50 distinct serotypes of S-antigens. These immunologically distinct types have been shown to have a world-wide distribution. Molecular studies have shown S-antigens to vary considerably in size and sequence. They have been shown to possess similar N- and C-terminal regions but their central regions, which consist of tandem repeats, differ markedly. There is evidence to suggest that S-antigens may be a target for protective immunity. Firstly, studies in the Gambia revealed that malaria patients exhibited different S-antigen

serotypes in subsequent infections suggesting that they were protected from infection by parasites bearing the original S-antigen type. Secondly, monoclonal antibodies directed against S-antigens inhibited erythrocyte invasion *in vitro*. However, the exact function of these proteins is not known.

MSA-1 is a polypeptide of approximately 200 kDa which is broken into smaller fragments at the time of schizont rupture. The MSA-1 sequence has been determined for seven isolates of *P. falciparum* and the antigen has been shown to be dimorphic. This molecule is composed of 17 blocks, including the highly conserved N- and C-terminals which flank regions differing in their degree of variability. Recently, Miller *et al.*[126] re-analysed all published MSA-1 sequences and found the dimorphic region to be a patchwork distribution from the 5' region of block 3, missing block 4, through blocks 5 to 16. Based on studies of inhibition of parasite invasion *in vitro*, it is thought that the repeat regions of MSA-1 may not be very immunodominant. However, some epitopes at the N- and C-terminal regions are thought to play a role in protective immunity.

MSA-2 is a 45-52 kDa membrane protein located on the merozoite surface. This antigen consists of conserved N- and C-terminal regions with a centrally-located

domain of repeat sequences. These repeats are flanked by non-repetitive variable regions which define two major MSA-2 gene families. MSA-2 is thus dimorphic. Isolates studied were shown to be members of one of the two allelic families with the exception of the gene of one isolate which was an apparent recombination between the two forms. MSA-2 may be immunologically important since monoclonal antibodies directed against this molecule partially inhibited invasion of erythrocytes *in vitro*.

CRA is a small protein located in the parasitophorous vacuole and membrane structures of the infected erythrocyte. Two forms of this antigen are recognised by their reaction with monoclonal antibody 5.1 which also reacts with the CS protein (see below). Antibody 5.1 was shown to be directed against the sequence NADP in CRA, which is closely related to the NANP sequence in the CS protein. Isolates negative for 5.1 were shown to contain the sequence NAGP, which resulted from a single amino acid substitution. This cross-reactivity with the CS protein suggests that CRA may be immunologically important.

The **CS-antigen** is the major antigen found on the surface of the sporozoite. It consists of N- and C-terminal non-repetitive sequences flanking a central domain of repeats. The repeat region is highly

immunodominant and consists of two types of repeat sequences, namely NANP, which is the most repetitive, and NVDP. The number of repeats has been found to differ between isolates. It is against the sequence NANP that the sporozoite vaccines have been directed.

Some culture-adapted isolates have lost the ability to express antigens *in vitro*.[122,123] These antigens include the histidine-rich proteins HRP-I, HRP-II and HRP-III, RESA and MESA. However, this failure of gene expression is not thought to be a cause of antigenic diversity in naturally occurring populations of *P. falciparum*.

HRP-I, the knob-associated histidine-rich protein (KAHRP), is a 80-120 kDa protein associated with the surface of erythrocytes infected with mature parasites. These knobs mediate cytoadherence of infected red blood cells to endothelial cells. However, HRP-I is not directly involved in cytoadherence as it is located on the cytoplasmic side of the erythrocyte plasma membrane. HRP-II (65-85 kDa) is excreted from parasitised erythrocytes into the serum and is not associated with knobs or cytoadherence. This protein serves as an indicator of infection in the newly-developed diagnostic technique, Parasight.[127] HRP-III is a small histidine- and alanine-rich protein (SHARP) of 30-50 kDa which is

closely related to HRP-II. It is found within the infected erythrocyte but is not associated with knobs or cytoadherence. The failure to express HRP-I *in vitro* has been shown in several different isolates to result from the partial or total deletion of the HRP-I gene on chromosome 2. Deletions on chromosomes 13 and 8 have similarly been shown to result in the failure to express HRP-II and HRP-III, respectively.

The lack of expression of RESA *in vitro* by the isolate FCR-3 was shown to result from a deletion on chromosome 1. This antigen is a merozoite-derived polypeptide, incorporated into the erythrocyte membrane at invasion, which has been shown to induce protective immunity. It contains two regions of tandemly repeated amino acid sequences which are immunodominant. Antibodies to several of the repeat sequences were shown to inhibit invasion *in vitro* and a study carried out in the Gambia showed a significant association between the presence of antibodies to the 3′ repeat sequence, $(EENV)_6$, and resistance to clinical malaria. These studies suggest that RESA may be a suitable target antigen for vaccine development.

The monoclonal antibodies used in the characterisation of southern African isolates of *P. falciparum* were directed against three blood form antigens, namely MSA-1, MSA-2 and CRA. Monoclonal antibody 9.8 was

directed against a conserved epitope on MSA-1 while antibodies 7.3, 7.6, 9.2, 10.3 and 12.1 recognised variant epitopes on this molecule. Antibody 12.3 was directed against MSA-2 while 5.1 was directed against CRA. The different patterns of reactivity with these monoclonal antibodies indicated different allelic forms of the antigens.

12.3.2 Antigenic variation

In addition to antigenic diversity in *P. falciparum* due to the expression of polymorphic allelic genes, further diversity results from antigenic variation within clonal parasite populations (reviewed in refs 124, 128). Studies have shown that the antigenic and adhesive phenotype of *Plasmodium*-infected erythrocytes are linked. Biggs *et al.*[129] examined a large erythrocyte surface antigen, known as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), in clones prepared from a cloned parental line of *P. falciparum* *in vitro* and found it to vary between clones in its agglutination to clone-specific antisera and its antigenicity. They concluded that these differences were due to antigenic variation in PfEMP1. Roberts *et al.*[130] showed that surface antigens in cloned lines can vary at the rate of 2% per generation and that this variation is associated with a change in the agglutination phenotype.

This antigenic variation appears to be a major factor in the evasion of the immune system by *Plasmodium*.[128] However, in spite of the large number of variant antigenic types, clinical immunity does develop after limited exposure to infection. Roberts *et al.*[128] suggest that because of the high rate of switching in antigenic phenotypes, individuals are exposed to many variants in each infection, resulting in immunity to a large number of variant antigens after only a few episodes of malaria.

12.3.3 Target antigens for vaccine development

Much progress has been made in recent years towards the development of a malaria vaccine. Many antigens have been identified as being appropriate vaccine targets and the advances in the field of malaria immunology together with promising results from vaccine trials in monkeys and humans provide hope for the development of a successful vaccine in the near future. Because immunity appears to be stage specific, a successful vaccine will most likely be a cocktail of antigens from the different developmental stages of the parasite.

12.3.3.1 Preerythrocytic vaccines

Experiments in rodents, monkeys and humans have shown that immunization with irradiated sporozoites can provide protection against infection.[131,132] The

circumsporozoite (CS) protein was identified as the major component of the sporozoite surface coat and monoclonal antibodies directed against the B epitope of this protein were shown to be protective *in vivo*.[133] In *P. falciparum*, this B epitope consists of tandem repeats of the sequence Asn-Ala-Asn-Pro (NANP). Subunit vaccine trials using the $(NANP)_n$-B cell epitope have been carried out in mice and humans with varying levels of antibodies being produced resulting in different degrees of protection.[132] However, the level of antibodies to the CS repeates did not always correlate with protection. One approach being considered to boost antibody levels is to include in the CS vaccine T helper epitopes which are present in the CS protein.[133] Whether or not this approach will be successful remains to be seen. There is evidence that effector T cells play a major role in sporozoite immunity and the site of this cell-mediated immunity is the exoerythrocytic form (EEF) of the parasite found in the hepatocyte.[133] The effector mechanism involved is thought to be either cytotoxic T lymphocytes (CTLs) that destroy infected hepatocytes, or cytokines that inhibit EEF development. Vaccines that induce CTLs are currently being developed.

12.3.3.2 Sexual stage vaccines

Vaccines aimed at the sexual stage of the parasite would provide an effective means of blocking

transmission. One advantage of such a vaccine is that it would be aimed at parasitic forms in the midgut of the mosquito which are free of the erythrocyte and are therefore exposed to antibodies in the bloodmeal. Sexual stage-specific antigens have recently been reviewed.[134] In *P. falciparum*, the 230 and 48/45 kDa proteins (Pfs230 and Pfs48/45) are exposed on the gamete surface and some monoclonal antibodies directed against these antigens were able to block transmission when mixed with gametocytes before fertilisation. Only a proportion of individuals exposed to these proteins develop antibodies.

Pfs25, a 25 kDa protein, is synthesized after fertilisation and is located on the zygote and ookinete surface and is also a target of transmission-blocking antibodies. It is thought to have a protective function for the ookinete in the midgut and is also implicated in the migration of the ookinete through the epithelium. Pfs25 has been shown to be a good immunogen in mice.

Other surface antigens include 40 and 10 kDa proteins, which elicit restricted immune responses in mice, and Pfs16 which exhibited amino acid sequence variation in geographic isolates. Pf155/RESA, which has amino acid repeats present in both sexual and asexual parasites, is present in protein aggregates in the erythrocyte

cytoplasm when macrogametes are formed, as is a 260-350 kDa protein, 11.1, which is antigenically related to Pf155/RESA.

The internal sexual stage antigens include Pfg27/25, which is a good immunogen. Sera from most patients exposed to *P. falciparum* malaria contain antibodies to this protein.

Vaccine trials in animals have been encouraging.[132] Vaccination with whole gametocytes or gametes resulted in transmission-blocking immunity. A 42 kDa antigen on the surface of *P.yoelli* microgametes was the basis for a vaccine which successfully reduced transmission in mice. In humans, there has been considerable variability in responsiveness to sexual stages amongst exposed individuals.[132] A greater understanding of this difference in immune response should lead to the development of a sexual stage vaccine in humans.

12.3.3.3 Asexual stage vaccines

The candidate antigens for asexual vaccine development include both soluble and surface membrane antigens.[132,135] Some soluble antigens, e.g. SERA, Ag2 and Ag7, are released when the schizont-infected erythrocyte ruptures. Other soluble antigens, e.g. HRP-II, are liberated when the parasite reaches maturity. Soluble proteins from *P. falciparum* culture

supernatent, purified SERA from cultures, as well as recombinant forms of SERA provided varying degrees of protection against infection in monkeys.

Target antigens on the surface of the infected erythrocyte include PfEMP1, Rosettin, HRP-II and Ag332. Monkeys vaccinated with HRP-II recombinant proteins were partially protected against infection. Ag332 is thought to be exposed on the infected red blood cell surface and parts of the Ag332 gene have been cloned.

Candidate antigens from the merozoite surface membrane include MSA-1, MSA-2, RESA, AMA-1, QF3 (RAP-1/RAP-2), EBA-175 and the peptide polymer, SPf66. AMA-1 is a 62 kDa protein found on the surface of *P. falciparum* merozoites. EBA-175 is a 175 kDa protein which is thought to function as a bridge between the merozoite and the uninfected erythrocyte during the invasion process. The EBA-175 gene has been cloned and studies on its immunogenicity are now being carried out. QF3 (RAP-1/RAP-2) is an 80/42 kDa protein complex associated with the merozoite rhoptries. SPf66 is a hybrid polymer consisting of amino acid sequences derived from the 83 kDa N-terminal fragment of MSA-1 and from a 35 kDA and a 55 kDa merozoite protein and the NANP repeat sequence derived from the CS protein.[136]

Monoclonal antibodies against MSA-1 and QF3 inhibited growth *in vitro* and *P. knowlesi* monoclonal antibodies blocked merozoite invasion *in vitro*.[135] Anti-RESA antibodies were also shown to be inhibitory *in vitro*. Antibodies to EBA-peptide 4, encoding a sequence from the C-terminus of EBA-175, inhibited erythrocyte binding and merozoite invasion.

Immunization experiments in animals showed MSA-1, AMA-1, QF3, RESA, SPf66 and conserved peptide sequences of MSA-2 to be protective against challenge infections.[135] Human vaccination trials in South America with SPf66 showed this polymer to be both immunogenic and protective.[137] However, some doubt has been expressed regarding the choice of peptides in the construct, the adjuvant used and the correlation of immunogenicity with protection.[138] The results of a trial of SPf66 currently being carried out in Tanzania are awaited with interest.

12.4 Chemotherapy

The bulk of this thesis deals with the sensitivity of southern African isolates to various antimalarial drugs (see Chapters 5 - 9). This aspect of parasite characterisation was warranted as very little work on drug susceptibility had previously been carried out in

southern Africa and, with the advent of chloroquine-resistant *P. falciparum* malaria, it was necessary to determine which compounds could serve as a suitable replacement for chloroquine in the treatment and prophylaxis of malaria in this region.

This section contains comments on various practical aspects of the drug sensitivity tests, including new techniques, and a discussion of the genetics of drug resistance and the development of new antimalarial drugs.

12.4.1 Drug sensitivity tests

Drug sensitivity of *P. falciparum* may be determined both *in vivo* and *in vitro*. In the *in vivo* test, the parasite response to a standard dose is determined in the patient. The result of this test is greatly affected by the immune status of the infected person. The *in vitro* test measures the susceptibility of the parasite to various drug concentrations in a cultivation system and is not subject to the influence of host factors, although in some tests, patients' sera are included in the test system. Ideally, *in vitro* studies should be backed up by *in vivo* investigations, particularly where the results of such a study are to be used in the formulation of a drug policy, the former to determine the degree and extent of drug resistance in the parasite population and the

latter to determine the effectiveness of a drug in controlling infection in local subjects.

Unfortunately, it was not possible to carry out *in vivo* tests on the patients from whom the southern African reference strains were isolated, as most of the patients had begun therapy by the time the isolates were received in our laboratory. Furthermore, most of these subjects were out-patients at clinics or hospitals and it was not practical to follow them up for the required seven or 28 days. Where possible, clinical data on the patients were obtained and related to the *in vitro* findings (see Chapter 5).

It was also not feasible to carry out *in vivo* investigations in KwaZulu at the time that the *in vitro* field studies were being undertaken as we did not have the facilities to hold patients during such a study in order to draw daily blood samples for the determination of parasitaemias and to ensure that reinfection did not occur. In addition to this, the relatively low prevalence of malaria in South Africa, relative to other malarial countries, would have necessitated an extended study period in order to obtain a suitable number of patients, thereby increasing costs even further.

Recently, an *in vivo* drug sensitivity investigation

was carried out in KwaZulu by the KwaZulu Health Department, in collaboration with the Medical Research Council, to determine the efficacy of chloroquine and Fansidar in this area. In this study, patients were visited in their homes for treatment and preparation of blood smears. Parasitaemias were monitored daily for the first seven days and thereafter on days 14, 21 and 28. The results of this study are presently being analysed. The disadvantages of such a study are that it requires a large team of workers to administer the drugs and to monitor the parasitaemia, it is subject to non-compliance as patients are often not at home when the observers visit, and there is a danger that patients may become reinfected during the study period as they are not removed from the malarial area. However, it does have the advantage that patients are disturbed as little as possible in that they are treated in their home environment thus avoiding the high cost of hospitalisation.

The *in vitro* tests used in this study may be divided into two categories: laboratory-based studies using culture-adapted *P. falciparum* isolates and field-based studies using primary isolates. The advantage of using reference isolates is that tests may be repeated several times on the same isolates whereas field isolates may only be tested once, unless they are established in culture and tested at a later stage.

However, the disadvantage of using culture-adapted strains is that they are unlikely to possess the full repertoire of clones present in the blood of the patient at the time of isolation because of the selection pressures placed on them in culture. The result of this is that faster growing clones will tend to predominate over, or even completely outgrow, less viable clones in a given isolate. Primary isolates are also subject to selection pressure under test conditions, although probably not to the same extent as isolates being established in culture, and are therefore also not truly representative of the parasite population. Nevertheless, *in vitro* tests are invaluable in the identification and monitoring of drug resistance in *P. falciparum* populations.

In the laboratory, two types of test were used. The first test was the 48-hour test in which inhibition of total growth of the parasite by chloroquine and pyrimethamine was determined. This type of test is more suited to the slower-acting compounds such as antifolates. Growth of the parasite was assessed by microscope counts of parasites on Giemsa-stained smears. Using this method, the action of chloroquine and pyrimethamine on the various erythrocytic stages could be determined by the effect of these drugs on the morphology of these stages at inhibitory concentrations. However this method of growth

assessment proved laborious and time-consuming.

The second laboratory test used was the radioisotope uptake assay in which growth of the parasite in the presence of increasing concentrations of antimalarial compounds was assessed by the uptake of ^{3}H-hypoxanthine. Isolates were allowed to grow in medium containing the drugs for 24 hours before being pulsed with the radioisotope for a further 18 hours. Use was made of predosed drug plates supplied by the WHO which, it appears from the literature, has not been done before with this technique. Although this method was more expensive than the microscopic method and required the use of a cell harvester, the harvesting of parasites and reading of counts was far less time consuming. This technique also eliminated operator error and the incorporation of the isotope was more sensitive than microscopy in the assessment of parasite growth. However a disadvantage of this technique is the handling and disposal of the radioisotope. Recently Makler *et al.*[139] showed that parasite lactate dehydrogenase activity could be used as effectively as the ^{3}H-hypoxanthine uptake in the determination of drug sensitivity. This technique would eliminate the problems associated with the use of radioisotopes.

All of the KwaZulu field drug resistance surveys, with

the exception of one, were carried out using the WHO *in vitro* microtechnique, in which inhibition of schizont development was determined by microscopy. The radioisotope uptake assay was used in the small field study done in 1990. Many of the problems experienced in these drug sensitivity surveys were the same as those experienced in the collection of infected blood samples such as the low number of malaria cases, the prior treatment of patients with antimalarial drugs and the contamination of blood specimens. The low prevalence of malaria in the study area necessitated the screening of a large number of samples. For example in the May 1987 survey, 6 407 people from Ubombo were screened for malaria in a mass blood examination and only 34 were positive. Of these, only 11 were suitable for the test and isolates from 8 of these patients grew under the test conditions i.e. only 0.125% of those screened were successfully tested. However, the screening of passive cases generally resulted in a higher yield. For example, in the same survey, of the 27 blood samples collected from patients reporting to clinics with malaria symptoms, 14 were positive for *falciparum* malaria, 11 were found suitable for testing and 9 tested successfully i.e. 33% of the total passive cases screened.

At present, the standard method used in this country

for the detection of malaria parasites is the microscopic examination of Giemsa-stained bloodsmears. Although this procedure is relatively cheap and simple, at low levels of infection it becomes time consuming and labour intensive. Recently, new techniques have been developed for the rapid diagnosis of malaria which make use of fluorescent dyes. These include the "QBC" method,[140] in which parasites are concentrated by centrifugation in a capillary tube and stained with acridine orange, and the staining of parasites using benzothiocarboxypurine (BCP).[141] Both of these procedures require the use of a fluorescence microscope in order to be able to view the parasites although the recent development of the "Makler" objective has enabled BCP-stained parasites to be viewed with a standard light microscope.[142] An interference filter system for acridine orange has been described which also enables fluorescing parasites to be detected using a standard light microscope.[143] Recently, a dip-stick method, called Parasight,[127] has been developed for the diagnosis of malaria in which HRP-II in whole blood is detected by means of an antibody-coated dipstick. This technique is rapid and simple to perform with good specificity and high specificity. A comparative study of the above microscopic diagnostic tests has recently been completed by an M. Sc. student at the MRC laboratory in Durban using both cultured material and blood from

infected patients from KwaZulu. The results of this investigation are currently being analysed.

Non-microscopic methods have also been successfully used in the diagnosis of malaria. These methods include immunological tests and genetic probes.[144] Golenser *et al.*[145] recently developed an ELISA test for the detection of *P. falciparum* in infected blood based on the monoclonal antibody, D5.[26] This monoclonal antibody, which was raised against a *P. berghei* antigen which cross-reacts with *P. falciparum*, reacted with all 19 southern African isolates (see Chapter 4) confirming the usefulness of this antibody for diagnostic tests. D5 has been successfully used in a radioimmunoassay to detect *Plasmodium* in infected blood.[32] These diagnostic techniques have been shown to be comparable to the standard Giemsa method and have been shown to be useful in epidemiological studies.

Another problem experienced in the field drug sensitivity investigations was the presence of non-*falciparum* parasites in a large proportion of the isolates. These were usually *P. ovale* although some *P. malariae* parasites were also seen, and they generally occurred in the same isolate with *P. falciparum*. It is difficult to distinguish between these species when only the ring stages are present and unless schizonts

or gametocytes are present, it is impossible to identify these other species with any degree of certainty. The development of serological tests[146] and PCR[147] techniques for the identification of the four human *Plasmodium* species have eased this task.

12.4.2 Molecular genetics of drug resistance

In recent years, much work has been carried out on the molecular biology of resistance of *P. falciparum* to chloroquine and, to a lesser extent, pyrimethamine and cycloguanil.

There is much controversy surrounding the mechanism of chloroquine resistance in *P. falciparum*.[148-150] Many researchers believe this resistance to be similar to the multidrug resistance (MDR) phenotype of mammalian tumour cells which is mediated by a P-glycoprotein molecule which acts as a pump to remove the drug from the cell. The first evidence of this was the demonstration by Martin *et al.*[151] of the reversal of chloroquine resistance *in vitro* by the calcium channel blocker, verapamil. Since this discovery, a large number of calcium channel blockers, calmodulin antagonists and lysosomotropic compounds have been shown to modulate chloroquine resistance and new compounds are continually being added to the list. Recently, dexniguldipin, an anti-cancer drug, was found to reverse chloroquine resistance in the

southern African reference isolates of *P. falciparum* (J.A. Freese - unpublished data). In the same study, the reversal activity of verapamil was confirmed in these isolates. Krogstad *et al.*[152] showed the rate of efflux of chloroquine from resistant parasites to be 40-50 times faster than that from sensitive parasites and the accumulation of this drug to be similar in both sensitive and resistant parasites. However, Bray *et al.*[153] found the accumulation of chloroquine to be greater in sensitive than in resistant isolates with no difference in efflux rates.

Two *P. falciparum* mdr genes (*pfmdr-1*[146] and *pfmdr-2*[155]) encoding P-glycoprotein homologues (pgh-1 and pgh-2, respectively) have been identified. *Pfmdr-1*, which is present on chromosome 5, was amplified in some chloroquine- and mefloquine-resistant isolates.[154] However, *pfmdr-2* was not amplified in any isolates examined and recent work has revealed that there is no evidence for the involvement of this gene in drug resistance.[156] Chloroquine resistance has been linked to two alleles of the *pfmdr-1* gene supporting the hypothesis that chloroquine resistance is a multigenic event. However, there is evidence that there is no relationship between the *pfmdr* genes and chloroquine resistance. This was revealed in a genetic cross between chloroquine-sensitive and resistant cloned lines of *P. falciparum* in which the

amplification of *pfmdr-1* gene in the resistant parent used in the cross, segregated separately from resistance in the progeny clones.[157] In a separate study, moderately drug-resistant clones subjected to drug pressure were shown to have decreased copies of *pfmdr* and less pgh-1 with increasing levels of chloroquine resistance.[158] Recently, Frean *et al.*[159] described a PCR technique for the detection of *pfmdr-1* mutations in chloroquine-resistant isolates. However, these mutations were shown to be absent in chloroquine-resistant isolates from Sudan.[160] In an investigation currently being carried out at the MRC laboratory in Durban on the southern African reference isolates of *P. falciparum*, preliminary results have indicated that the local isolates also lack these mutations (B. Bredenkamp - unpublished data). Wellems *et al.*[161] have identified a single genetic locus on chromosome 7 which is linked to the chloroquine-resistance phenotype. All of this evidence suggests that chloroquine-resistant *P. falciparum* parasites do not represent the MDR phenotype. There is undoubtedly much work to be done before the mechanism of chloroquine resistance is clearly understood.

The mechanism of resistance to dihydrofolate reductase (DHFR) inhibitors has been shown to be mutations in DHFR.[162] A single point mutation (Ser-108 → Asn-108) in the DHFR active site was shown in a genetic cross

between pyrimethamine-sensitive and pyrimethamine-resistant parasites to be linked to pyrimethamine resistance.[163] This mutation was also found in wild pyrimethamine-resistant isolates from various geographic origins.[162] Two secondary mutations in the presence of Asn-108 were found to be associated with increased pyrimethamine resistance. The point mutations responsible for resistance to cycloguanil were found to be different from those involved in pyrimethamine resistance, while parasites resistant to both pyrimethamine and cycloguanil had three DHFR mutations in common.[162] These mutations apparently prevent binding of these compounds to the active site of DHFR and would explain why proguanil (of which cycloguanil is the active metabolite) is sometimes effective against pyrimethamine-resistant strains while in some cases it is not. A PCR technique based on the DHFR point mutations has recently been developed to identify pyrimethamine- and cycloguanil-resistant isolates.[164]

Cryopreserved samples of all of the southern African reference isolates have been sent to the laboratory of Dr Thomas Wellems of the National Institutes of Health in Bethesda, Maryland, U.S.A., where DHFR point mutation data will be generated. The chromosome 7 markers for chloroquine resistance will also be examined by restriction analysis and DNA probe

hybridisations. These investigations will provide more characterisation data on the southern African isolates. Markers for drug resistance will be of great value in determining the origin and spread of such resistance.

12.4.3 Recent developments in chemotherapy

This investigation has concentrated on the sensitivity *in vitro* of southern African isolates to drugs readily available, or soon to be made available, in this region, namely chloroquine, quinine, mefloquine, pyrimethamine, sulphadoxine/pyrimethamine, amodiaquine and halofantrine. The study includes discussions on drugs recommended for use, namely proguanil and doxycycline. As a consequence of the ever-increasing list of compounds to which *P. falciparum* is developing resistance, much work has been done in recent years on the development of new drugs and the revival of some older compounds.[165] Many of these formulations have been shown to act synergistically in combination and the recent discovery that resistance to chloroquine and other quinoline-containing drugs can be reversed may allow for the continued use of these antimalarials in areas of resistance.

There are many new antimalarials which show promise in the field of chemotherapy.[165] Four drugs are now in the advanced stage of development and have undergone

extensive clinical trials. These are halofantrine,[59-63] artimisinin,[166] pyronaridine,[167] and enpiroline.[165] Halofantrine is now available for use in southern Africa but resistance to this drug has recently been reported from the Congo[168] although this has been disputed.[169]

The Chinese plant, Qinghao (*Artimisia annua*), has been used as a herbal medicine in China for over 2000 years and its antimalarial properties are well-known. The active compound is a sesquiterpene lactone called Qinghaosu or artimisinin. Both water-soluble derivatives (artesunate and artelinate) and oil-soluble derivatives (artemether and arteether) as well as artimisinin have been shown to be the most rapidly acting of all antimalarial drugs and are effective against multidrug-resistant *P. falciparum* malaria. *In vitro* studies of three African reference clones and 36 primary isolates from the Comoros Islands and Central and West Africa have demonstrated the derivatives artelinate, artemether and arteether to be twice as potent as artimisinin.[170] Results from this investigation suggested that sensitivity to artimisinin may be inversely related to chloroquine sensitivity and directly related to the susceptibility to mefloquine and halofantrine. Unfortunately, neither artimisinin nor its derivatives are generally available outside of Asia. However, the World Health

Organization is currently funding the development of arteether towards registering its use in western countries.

Pyronaridine, a derivative of mepacrine, is a blood schizonticide which was developed in the People's Republic of China in the early 1970s.[165,167] It is highly effective against both chloroquine-sensitive and chloroquine-resistant isolates of *P. falciparum* and shows no cross-resistance with 4-aminoquinolines or quinolinemethanols. However, Warsame *at al.*[171] demonstrated a positive relationship between the response to chloroquine and pyronaridine and suggested that resistance to chloroquine may tend to result in the fast development of pyronaridine resistance. As resistance to pyronaridine was shown to develop rapidly, combinations with other antimalarials were tested.[167] Pyronaridine/sulphadoxine/pyrimethamine was shown to be a promising combination in clinical trials.

Enpiroline, a pyridinemethanol developed in 1972, is a blood scizonticide effective against both *P. vivax* and *P. falciparum*.[165] In clinical trials enpiroline was found to be active against parasites resistant to chloroquine, pyrimethamine and quinine and there were minimal side effects. However, the drug was not shown to have any advantage over mefloquine or halofantrine.

Many candidate antimalarials are in an advanced preclinical stage of development.[165] These drugs include 4-aminoquinolines and Mannich bases (e.g. Dabechin, piperaquine, hydoxypiperaquine, tripiperaquine and dichlorquinazine), 8-aminoquinolines, 4-quinolinemethanols quinolones, napthoquinolones, quinazolines, dihydrotriazines. Many formulations from these groups show potential as alternatives to currently-used antimalarials. (For more detail, see Ref. 165).

Many other compounds are seen as potential antimalarials. Several antibiotics have been shown to have antimalarial activity.[165] For example, tetracycline is widely-used with quinine in the treatment of complicated *P. falciparum* malaria[165] and doxycycline is one of the recommended prophylactic drugs for areas of chloroquine resistance.[94,96] Cyclosporin A, an undecapeptide and metabolite of the fungus *Tolypocladium inflatum* has been shown to be effective against *P. falciparum in vitro*.[165] Although this compound was shown in a study carried out on the southern African reference isolates not to be an effective reverser of chloroquine resistance, some antimalarial activity was demonstrated, albeit much lower than that of chloroquine (J.A. Freese - unpublished data). Many plant extracts have exhibited

antiplasmodial activity and are seen as potential sources of antimalarial drugs.[165, 172] The possible antimalarial activity of extracts of *Artimisia afra* is currently being investigated by workers at the Department of Chemical Pathology, University of Cape Town Medical School using both chloroquine-sensitive and chloroquine-resistant reference isolates of *P. falciparum* and the results have been encouraging (P. Berman - personal communication).

Another approach to malaria chemotherapy is the use of biochemical targets.[165] A good example of this is the development of the folate pathway antagonists, sulphonamides, sulphones and dihydrofolate reductase inhibitors. However, the biochemistry of the malaria parasite is poorly understood and until our knowledge in this area is increased, this approach to chemotherapy will be limited. Some of the biochemical pathways in which this approach might be possible are those of energy metabolism, protein synthesis, nucleic acid synthesis, folate metabolism and lipid biosynthesis. Other biochemical targets showing promise are the pathway of haemoglobin degradation[173] and nucleoside transport pathways.[174] The use of iron chelators is another chemotherapeutic strategy.[175] Desferrioxamine has been shown to be effective both *in vitro*[176] and *in vivo*[177] and it is thought that in addition to the chelation of iron in the parasite

which is essential to plasmodial growth, desferrioxamine chelates iron from the haemozoin crystal, resulting in free radical generation and parasite death.[178] Reactive oxygen species within the infected erythrocyte are known to be deleterious to the survival of the parasite.[179] It is possible that this natural defence mechanism may be utilised in the development of antimalarial drugs. However, the parasite is well-adapted to oxidant stress[180] and therefore this approach to malaria control may not prove to be effective.

Armed with these recently-developed chemotherapeutic strategies, the successful control of malaria seems more accessible than it did a decade ago. However, the ease with which the parasite develops resistance signals the importance of a continual search for new and improved antimalarial drugs.

12.5 Other characterisation methods

There are several methods available for the characterisation of *P. falciparum* which were not used in this investigation. These procedures are based on variations in proteins and DNA.

The 2D-PAGE technique separates proteins by their isoelectric point in one dimension and by their

molecular weight in the second dimension resulting in a pattern of protein spots on the gel which are made visible by autoradiography.[181] This procedure has been successfully applied to malaria parasites[182] and has revealed considerable diversity amongst isolates.[5] However, a disadvantage of this method is that it is time-consuming and the interpretation of results requires a high degree of skill and experience.[5]

P. falciparum may be typed according to the presence or absence of protein-rich knobs on the surface of erythrocytes infected with trophozoites and schizonts.[5] These knobs have been shown to have an antigenic function and strain variation in this antigen has been demonstrated. The binding of knob-positive erythrocytes to endothelial or melanoma cells was blocked by sera from immunised monkeys, homologous sera being more active that heterologous sera. Cytoadherence, as measured by the melanoma cell binding assay, has been shown to differ in isolates from different geographic areas.[183]

DNA variations have been demonstrated using a number of different techniques.[5] Pulse field gel electrophoresis, in which *P. falciparum* chromosomes were separated, revealed considerable chromosome size variation amongst isolates.[184] Repetitive DNA sequences have also been used to type malaria strains.

For example, the rep-20 sequence has been shown to be a good marker of diversity.[185] Variations in DNA sequences have also revealed variation amongst *P. falciparum* isolates.[5] These differences have been demonstrated in Southern blot hybridisations of gene probes to restricted genomic DNA (e.g. Ref. 186) and in gene sequencing. PCR was recently used to analyse several genetic loci in parallel and showed promise as a useful tool for the typing of *P. falciparum* isolates.[187]

Many of these techniques were developed fairly recently and therefore were not used in the study of southern African strains of *P. falciparum*. However, it is intended to use some of these molecular biological methods to further characterise the reference isolates and some of their clones. DNA has already been extracted for this purpose. The more extensive the characterisation of isolates, the more useful they will be in malaria parasite research.

In vitro response to chloroquine of 20 southern African reference isolates of *Plasmodium falciparum* in the 48-hour growth inhibition test

RSA 1

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a] Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	5	3	1	0	0	9	
At 48 hours:							
Control	30	3	0	0	0	33	48.53
0.03	22	30	0	0	0	52	76.47
0.06	31	36	0	0	0	68	100.00
0.1	36	30	0	0	0	66	97.06
0.3	25	1	0	0	0	26	38.24
0.6	1	0	0	0	0	1	1.47
1.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	14	5	2	3	0	24	
At 48 hours:							
Control	95	32	9	7	0	144	89.44
0.03	118	19	10	6	0	154	95.65
0.06	120	23	12	8	1	161	100.00
0.1	105	35	6	4	1	150	93.17
0.3	22	2	0	0	0	24	14.91
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	9	4	1	0	0	13	
At 48 hours:							
Control	14	14	0	3	0	31	88.57
0.03	18	17	0	1	0	35	100.00
0.06	16	12	0	0	0	29	82.86
0.1	8	8	0	0	0	16	45.71
0.3	1	0	0	0	0	1	2.86
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached.
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 2

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a] Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	15	0	0	0	1	16	
At 48 hours:							
Control	77	3	1	5	0	86	100.00
0.03	52	2	1	5	0	60	69.77
0.06	4	4	0	0	0	8	9.30
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	18	1	2	2	0	22	
At 48 hours:							
Control	112	13	9	7	1	142	100.00
0.03	34	12	4	0	0	50	35.21
0.06	10	2	0	0	0	12	8.45
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	10	9	2	2	1	22	
At 48 hours:							
Control	178	66	5	15	6	270	100.00
0.03	180	55	7	14	5	261	96.67
0.06	15	4	0	0	2	21	7.78
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 3

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a] Rings	Trophozoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gametocytes	Total	Percentage control growth[d]
Test 1							
Time 0	29	3	0	1	0	32	
At 48 hours:							
Control	165	28	3	5	4	205	100.00
0.03	135	12	3	2	1	153	74.63
0.06	17	5	2	1	0	24	11.71
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	18	4	1	1	0	23	
At 48 hours:							
Control	55	6	3	7	1	72	100.00
0.03	18	3	2	6	0	30	41.67
0.06	5	2	2	1	0	11	15.28
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	7	1	1	3	0	11	
At 48 hours:							
Control	35	33	2	5	0	75	100.00
0.03	47	23	2	2	0	74	98.67
0.06	23	9	1	1	0	33	44.00
0.1	7	2	0	0	0	9	12.00
0.3	-	-	-	-	-	-	0.00
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 4

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a]						
	Rings	Trophozoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gametocytes	Total	Percentage control growth[d]
Test 1							
Time 0	25	3	4	4	12	47	
At 48 hours:							
Control	40	12	3	5	3	63	100.00
0.03	22	12	5	6	7	52	82.54
0.06	14	6	0	1	1	21	33.33
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	34	11	3	2	0	49	
At 48 hours:							
Control	97	10	5	4	2	118	100.00
0.03	73	10	2	1	1	87	73.73
0.06	10	7	1	2	0	20	16.95
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	24	4	1	1	1	30	
At 48 hours:							
Control	142	24	4	5	4	179	100.00
0.03	100	27	2	2	2	133	74.30
0.06	7	6	1	1	1	15	8.38
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 5

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	19	13	14	4	1	49	
At 48 hours:							
Control	132	49	126	55	9	372	100.00
0.03	147	61	96	56	5	365	98.12
0.06	176	42	9	4	5	237	63.71
0.1	8	5	1	0	1	14	3.76
0.3	-	-	-	-	-	-	0.00
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	5	6	6	3	0	19	
At 48 hours:							
Control	43	31	3	1	0	78	100.00
0.03	52	8	0	0	0	60	76.92
0.06	6	1	0	0	0	7	8.97
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	47	1	1	2	1	51	
At 48 hours:							
Control	112	12	7	57	1	189	100.00
0.03	94	10	15	22	1	139	73.54
0.06	22	6	0	0	0	28	14.81
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 6

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a]						Percentage control growth[d]
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	
Test 1							
Time 0	17	3	1	3	0	23	
At 48 hours:							
Control	44	32	3	4	2	86	100.00
0.03	41	24	3	2	3	73	84.88
0.06	29	9	4	1	2	45	52.33
0.1	12	12	3	1	1	29	33.72
0.3	8	4	0	0	1	13	15.12
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	11	5	0	1	0	16	
At 48 hours:							
Control	211	32	2	3	0	248	100.00
0.03	207	27	3	5	0	242	97.58
0.06	193	37	2	3	0	236	95.16
0.1	199	28	2	2	0	231	93.15
0.3	18	5	0	2	0	25	10.08
0.6	4	2	0	0	0	6	2.42
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	21	5	2	2	0	30	
At 48 hours:							
Control	167	9	3	12	0	191	83.77
0.03	203	6	3	17	0	228	100.00
0.06	186	8	1	14	0	209	91.67
0.1	93	7	1	8	0	110	48.25
0.3	10	3	0	0	0	14	6.14
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 7

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	8	6	4	3	1	21	
At 48 hours:							
Control	43	9	1	1	1	55	100.00
0.03	49	4	0	0	1	55	100.00
0.06	39	4	0	0	0	43	50.91
0.1	35	2	1	1	0	39	70.91
0.3	25	2	0	0	0	28	50.91
0.6	4	1	0	0	0	5	9.09
1.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	23	8	1	0	0	31	
At 48 hours:							
Control	229	150	21	15	0	415	98.81
0.03	234	148	22	16	0	420	100.00
0.06	269	120	17	13	0	420	100.00
0.1	273	128	5	7	0	413	98.33
0.3	151	29	2	2	0	185	44.05
0.6	1	2	0	0	0	4	0.95
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	19	1	0	7	1	28	
At 48 hours:							
Control	151	6	8	18	1	183	49.86
0.03	323	17	8	17	1	367	100.00
0.06	255	10	5	21	0	290	79.02
0.1	161	4	9	17	1	192	52.32
0.3	14	0	0	0	0	14	3.81
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 8

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a] Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	7	22	13	2	0	44	
At 48 hours:							
Control	33	68	123	51	0	275	98.92
0.03	28	45	133	72	0	278	100.00
0.06	32	44	115	57	0	248	89.21
0.1	46	63	105	36	0	250	89.93
0.3	80	59	0	0	0	139	50.00
0.6	14	9	0	0	0	23	8.27
1.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	30	9	0	1	0	39	
At 48 hours:							
Control	170	127	20	19	0	336	94.92
0.03	109	112	43	49	2	315	88.98
0.06	109	149	42	48	1	349	98.59
0.1	152	138	39	24	1	354	100.00
0.3	12	8	1	0	0	21	5.93
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	10	3	0	1	0	14	
At 48 hours:							
Control	112	54	4	2	0	172	97.73
0.03	112	58	3	3	0	176	100.00
0.06	104	65	2	2	0	173	98.30
0.1	126	43	0	3	0	172	97.73
0.3	1	3	0	0	0	4	2.27
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 9

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	5	8	7	0	1	21	
At 48 hours:							
Control	43	60	93	41	0	237	70.33
0.03	54	58	143	82	0	337	100.00
0.06	47	58	130	72	0	307	91.10
0.1	61	63	105	45	0	274	81.31
0.3	90	40	1	0	0	130	38.58
0.6	8	8	1	0	0	16	4.75
1.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	21	29	3	0	0	53	
At 48 hours:							
Control	62	168	109	173	0	512	90.62
0.03	47	105	125	288	0	565	100.00
0.06	46	130	100	211	0	487	86.19
0.1	56	167	133	115	0	471	83.36
0.3	18	17	3	0	0	38	6.73
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	6	15	11	4	0	36	
At 48 hours:							
Control	43	62	301	58	0	464	99.36
0.03	35	37	314	65	0	451	96.57
0.06	30	48	324	65	0	467	100.00
0.1	26	40	299	27	0	392	83.94
0.3	10	12	1	0	0	23	4.93
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 10

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	7	2	3	4	1	17	
At 48 hours:							
Control	178	29	2	2	0	211	100.00
0.03	181	22	2	1	0	205	98.57
0.06	182	20	2	2	0	206	97.62
0.1	173	15	4	2	2	196	93.81
0.3	36	7	3	0	0	46	21.90
0.6	5	5	0	0	0	10	4.76
1.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	6	5	1	4	0	16	
At 48 hours:							
Control	43	8	1	8	0	60	98.36
0.03	44	8	3	6	0	61	100.00
0.06	45	7	1	2	0	55	90.16
0.1	44	5	3	2	0	54	88.52
0.3	26	3	0	2	0	31	50.82
0.6	5	0	0	0	0	5	8.20
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	19	1	0	1	0	21	
At 48 hours:							
Control	153	7	1	4	0	165	96.49
0.03	146	17	2	6	0	171	100.00
0.06	149	17	2	1	1	170	99.42
0.1	120	6	1	4	0	131	76.61
0.3	11	4	1	0	0	16	9.36
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 11

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	17	2	1	1	0	21	
At 48 hours:							
Control	45	39	27	3	2	116	73.89
0.03	53	45	28	5	3	134	85.35
0.06	47	62	35	6	7	157	100.00
0.1	46	34	24	1	2	107	68.15
0.3	1	1	1	1	0	4	2.55
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	41	33	1	0	0	75	
At 48 hours:							
Control	144	39	3	1	0	187	100.00
0.03	118	48	1	3	1	171	91.44
0.06	132	35	0	2	0	169	90.37
0.1	148	15	1	1	0	165	88.24
0.3	13	6	1	0	0	20	11.23
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	73	9	0	4	0	86	
At 48 hours:							
Control	630	178	11	32	0	851	100.00
0.03	644	147	10	26	0	827	97.18
0.06	676	137	5	31	0	849	99.76
0.1	682	112	4	22	0	820	96.36
0.3	22	32	0	1	0	55	6.46
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 12

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a]						
	Rings	Trophozoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gametocytes	Total	Percentage control growth[d]
Test 1							
Time 0	13	3	1	1	0	18	
At 48 hours:							
Control	247	3	2	3	0	255	98.84
0.03	246	4	1	7	0	258	100.00
0.06	235	6	1	4	0	246	95.35
0.1	211	4	1	4	0	220	85.27
0.3	19	5	2	2	0	27	10.47
0.6	9	5	0	0	0	14	5.43
1.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	17	1	0	0	0	18	
At 48 hours:							
Control	278	22	4	5	0	311	86.63
0.03	275	29	4	5	0	313	87.19
0.06	302	44	4	9	0	359	100.00
0.1	281	36	6	5	0	328	91.36
0.3	99	2	0	2	0	103	28.69
0.6	11	0	0	0	0	11	3.06
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	10	4	1	0	0	15	
At 48 hours:							
Control	90	27	2	2	0	120	98.36
0.03	93	26	2	1	0	122	100.00
0.06	94	24	2	2	0	122	100.00
0.1	81	10	0	2	0	93	76.23
0.3	1	2	0	1	0	4	3.28
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 13

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a] Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	7	7	13	2	1	30	
At 48 hours:							
Control	15	24	12	3	2	56	100.00
0.03	30	17	0	0	1	49	87.27
0.06	2	4	0	0	0	6	10.91
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	30	2	1	0	0	33	
At 48 hours:							
Control	49	12	3	3	0	67	100.00
0.03	5	19	2	0	0	26	39.39
0.06	3	5	0	0	0	8	0.00
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	4	8	2	1	1	16	
At 48 hours:							
Control	16	29	8	14	1	68	100.00
0.03	21	23	3	2	1	50	73.53
0.06	0	1	0	0	0	1	2.94
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 14

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	22	24	4	0	0	50	
At 48 hours:							
Control	112	29	23	17	3	184	88.04
0.03	125	18	19	15	6	183	87.56
0.06	97	17	13	17	11	155	74.16
0.1	131	33	22	17	6	209	100.00
0.3	89	32	4	2	3	130	72.20
0.6	11	11	0	0	0	22	10.53
1.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	71	4	0	1	0	76	
At 48 hours:							
Control	584	160	15	6	1	766	100.00
0.03	496	182	21	10	0	709	92.29
0.06	481	159	13	7	0	660	86.27
0.1	555	159	10	5	0	729	95.29
0.3	43	11	4	1	0	59	8.10
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	13	15	2	3	0	33	
At 48 hours:							
Control	162	260	40	259	1	722	100.00
0.03	158	227	46	278	1	710	98.47
0.06	146	195	55	206	0	602	83.63
0.1	107	188	37	133	0	465	64.49
0.3	57	13	0	1	0	71	9.57
0.6	9	2	0	0	0	11	1.53
1.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 15

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	19	17	9	0	0	45	
At 48 hours:							
Control	137	34	22	4	2	199	100.00
0.03	106	36	20	6	2	170	86.36
0.06	93	29	21	4	10	157	79.80
0.1	105	29	9	6	7	156	78.28
0.3	115	24	0	1	5	145	73.74
0.6	7	6	0	0	0	13	6.57
1.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	27	1	0	1	0	29	
At 48 hours:							
Control	470	66	3	3	0	542	100.00
0.03	403	107	1	3	0	514	94.83
0.06	449	82	5	6	0	542	100.00
0.1	460	51	1	4	0	516	95.20
0.3	8	15	1	1	0	25	4.61
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	1	6	2	5	0	14	
At 48 hours:							
Control	221	18	4	8	1	252	98.82
0.03	226	18	4	7	0	255	100.00
0.06	202	20	3	6	0	231	90.59
0.1	183	12	2	4	0	201	78.82
0.3	9	4	0	0	0	13	5.10
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 16

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	39	7	3	17	0	66	
At 48 hours:							
Control	653	48	18	126	0	845	95.37
0.03	671	45	17	153	0	886	100.00
0.06	612	42	21	145	0	820	92.55
0.1	443	40	16	103	0	602	67.95
0.3	89	25	0	0	0	114	12.87
0.6	41	31	0	0	0	72	8.13
1.0	39	26	0	0	0	65	7.34
Test 2							
Time 0	15	3	1	2	0	21	
At 48 hours:							
Control	266	34	9	29	0	339	100.00
0.03	218	27	5	23	0	273	80.53
0.06	232	29	6	18	0	285	84.07
0.1	209	24	2	12	0	247	73.16
0.3	13	3	0	0	0	16	4.72
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	37	1	1	1	0	40	
At 48 hours:							
Control	340	39	16	4	0	399	84.57
0.03	377	70	20	5	1	473	100.00
0.06	310	53	27	7	0	397	83.93
0.1	362	63	14	2	0	441	93.23
0.3	14	12	1	0	0	27	5.71
0.6	20	9	0	0	0	23	0.00
1.0	17	7	0	0	0	24	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

KSA 17

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a]						
	Rings	Trophozoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gametocytes	Total	Percentage control growth[d]
Test 1							
Time 0	28	3	2	6	0	39	
At 48 hours:							
Control	369	20	2	14	1	406	100.00
0.03	362	21	4	9	1	397	97.78
0.06	370	17	2	13	0	402	99.01
0.1	291	16	2	16	0	325	80.05
0.3	14	10	0	0	0	24	5.91
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	50	20	16	0	0	88	
At 48 hours:							
Control	728	10	8	53	0	800	96.27
0.03	726	21	9	50	7	813	97.83
0.06	742	16	7	61	5	831	100.00
0.1	423	15	15	45	5	503	60.53
0.3	77	15	0	0	0	92	11.07
0.6	62	17	0	0	0	79	9.51
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	12	4	0	1	0	17	
At 48 hours:							
Control	303	33	1	2	0	339	97.41
0.03	324	24	0	0	0	348	100.00
0.06	299	20	0	1	0	320	91.95
0.1	217	6	0	1	0	224	64.37
0.3	9	1	0	0	0	10	2.87
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

MW 1

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	9	7	0	0	0	16	
At 48 hours:							
Control	43	64	11	6	0	124	100.00
0.03	22	37	5	7	0	71	57.26
0.06	22	36	7	4	0	69	55.65
0.1	21	35	9	3	0	68	54.84
0.3	26	32	5	10	0	73	58.87
0.6	6	6	0	0	0	12	9.68
1.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	29	25	0	9	0	63	
At 48 hours:							
Control	324	4	10	47	1	386	66.90
0.03	404	9	8	28	2	451	78.16
0.06	516	9	9	40	3	577	100.00
0.1	188	6	20	43	1	258	44.71
0.3	40	16	0	0	0	56	9.71
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	16	1	0	1	0	18	
At 48 hours:							
Control	538	13	1	16	0	568	100.00
0.03	520	11	1	13	0	545	95.95
0.06	505	10	2	12	0	529	93.13
0.1	280	11	1	6	0	298	52.46
0.3	18	4	0	0	0	22	3.87
0.6	11	2	0	0	0	13	2.29
1.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

MR 2

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	3	2	2	3	0	10	
At 48 hours:							
Control	13	11	8	18	2	52	94.55
0.03	13	8	7	10	4	42	76.36
0.06	32	6	7	8	2	55	100.00
0.1	13	8	7	10	2	40	72.73
0.3	6	5	0	0	0	11	20.00
0.6	5	2	0	0	0	8	14.55
1.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	36	17	2	1	0	56	
At 48 hours:							
Control	130	183	67	78	0	458	98.28
0.03	103	175	67	107	0	452	97.00
0.06	116	192	69	89	0	466	100.00
0.1	142	174	43	30	0	389	83.48
0.3	9	19	0	0	0	28	6.01
0.6	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	6	3	0	5	0	14	
At 48 hours:							
Control	309	37	4	22	1	373	99.73
0.03	329	23	4	18	0	374	100.00
0.06	240	24	4	15	0	283	75.67
0.1	146	17	2	16	0	181	48.40
0.3	14	6	0	0	0	20	5.35
0.6	6	1	0	0	0	7	1.87
1.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

FCR-3

Chloroquine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes[a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	15	4	0	1	0	20	
At 48 hours:							
Control	61	65	0	1	0	127	86.99
0.03	61	82	1	2	0	146	100.00
0.06	46	79	1	2	0	128	87.67
0.1	26	84	1	3	0	114	78.08
0.3	17	18	0	0	0	35	23.97
0.6	2	10	0	0	0	12	8.22
1.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	8	5	0	0	0	13	
At 48 hours:							
Control	110	48	7	2	0	167	89.30
0.03	70	67	5	1	0	143	76.47
0.06	86	88	9	4	0	187	100.00
0.1	77	63	5	4	0	149	79.68
0.3	37	1	0	0	0	38	20.32
0.6	0	2	0	0	0	2	1.07
1.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	14	6	0	0	0	20	
At 48 hours:							
Control	200	32	2	1	0	235	100.00
0.03	196	28	1	1	0	226	96.17
0.06	198	32	1	0	0	231	98.72
0.1	199	31	2	1	0	233	99.15
0.3	124	4	0	1	0	129	54.89
0.6	0	1	0	0	0	1	0.43
1.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

In vitro response to pyrimethamine of 20 southern African reference isolates of Plasmodium falciparum in the 48-hour growth inhibition test

RSA 1

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a] Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	5	3	1	0	0	9	
At 48 hours:							
Control	34	25	0	0	0	61	100.00
0.003	32	24	0	0	0	56	91.80
0.01	21	28	0	0	1	50	81.97
0.03	2	2	0	0	1	5	8.20
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	14	5	2	3	0	24	
At 48 hours:							
Control	95	32	9	7	0	144	91.72
0.003	114	23	7	2	1	143	91.08
0.01	123	19	10	4	1	157	100.00
0.03	41	9	6	3	1	60	38.22
0.1	2	2	2	9	0	14	8.92
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	8	4	1	0	0	12	
At 48 hours:							
Control	12	12	0	0	0	24	92.31
0.003	9	11	0	0	0	21	80.77
0.01	12	13	0	1	0	26	100.00
0.03	1	3	1	2	0	6	23.08
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 2

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a] Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	15	0	0	0	1	16	
At 48 hours:							
Control	62	2	2	3	1	70	74.47
0.003	74	1	1	6	0	82	87.23
0.01	85	3	0	6	0	94	100.00
0.03	18	1	0	8	0	27	28.72
0.1	0	1	0	9	0	10	10.64
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	18	1	2	2	0	22	
At 48 hours:							
Control	114	15	8	7	0	144	100.00
0.003	94	13	10	4	3	124	86.11
0.01	108	11	11	10	3	143	99.31
0.03	44	5	2	4	1	56	38.89
0.1	1	0	2	6	1	10	6.94
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	9	8	2	2	1	22	
At 48 hours:							
Control	188	55	5	14	5	267	98.52
0.003	177	64	10	14	6	271	100.00
0.01	176	59	6	14	7	262	96.68
0.03	99	34	3	15	8	159	58.67
0.1	0	4	1	9	7	21	7.75
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 3

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	28	3	0	1	0	32	
At 48 hours:							
Control	140	2	3	3	0	148	87.72
0.003	182	0	2	4	0	188	100.00
0.01	64	2	2	3	0	71	37.77
0.03	57	5	3	6	1	72	38.30
0.1	47	4	4	10	1	66	35.11
0.3	67	5	9	3	0	84	44.68
1.0	70	4	5	15	1	95	50.53
3.0	25	1	2	20	0	48	25.53
Test 2							
Time 0	18	4	1	0	0	23	
At 48 hours:							
Control	55	6	3	7	1	72	91.14
0.003	71	4	0	3	1	79	100.00
0.01	35	3	1	6	1	46	58.23
0.03	28	1	1	7	1	38	48.10
0.1	34	2	1	9	0	46	58.23
0.3	39	3	2	8	1	53	67.09
1.0	34	3	1	11	1	50	63.29
3.0	16	1	0	11	0	28	35.44
Test 3							
Time 0	6	1	1	3	0	11	
At 48 hours:							
Control	32	35	4	5	1	77	97.47
0.003	36	33	4	5	1	79	100.00
0.01	29	34	6	4	0	73	92.41
0.03	25	28	2	4	0	59	74.68
0.1	22	27	3	4	0	56	70.89
0.3	21	30	2	4	0	57	72.15
1.0	22	25	3	4	0	54	68.35
3.0	3	22	1	3	1	30	37.97

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 4

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	25	3	4	4	12	47	
At 48 hours:							
Control	41	8	7	8	5	69	66.99
0.003	78	7	3	10	5	103	100.00
0.01	34	4	3	9	7	57	55.34
0.03	5	1	4	14	11	35	33.98
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	34	11	2	2	0	49	
At 48 hours:							
Control	107	16	5	6	2	136	100.00
0.003	113	11	4	3	4	135	98.54
0.01	51	8	3	4	2	68	48.91
0.03	1	2	3	12	2	20	14.60
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	24	3	1	1	1	30	
At 48 hours:							
Control	146	26	1	2	4	179	100.00
0.003	146	21	2	5	3	177	98.88
0.01	51	14	2	3	2	72	40.45
0.03	0	1	0	7	3	11	6.18
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 5

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	19	13	14	4	1	49	
At 48 hours:							
Control	168	69	121	53	13	[illegible]	100.00
0.003	123	56	103	72	10	[illegible]	85.88
0.01	136	68	86	61	14	365	85.88
0.03	116	57	92	87	10	362	85.18
0.1	121	87	89	65	11	373	87.76
0.3	93	60	67	80	9	309	72.71
1.0	123	63	77	68	11	342	80.24
3.0	48	21	18	12	14	113	26.59
Test 2							
Time 0	4	6	6	3	0	19	
At 48 hours:							
Control	29	37	9	3	0	78	100.00
0.003	20	43	3	2	1	69	83.95
0.01	25	43	6	3	0	77	96.30
0.03	26	36	6	3	1	72	87.65
0.1	11	24	2	0	0	36	45.68
0.3	9	19	2	1	1	32	40.74
1.0	11	18	0	1	1	31	37.04
3.0	6	8	0	1	1	16	20.99
Test 3							
Time 0	47	1	1	1	1	51	
At 48 hours:							
Control	123	11	12	27	2	175	76.75
0.003	184	8	8	24	3	228	100.00
0.01	137	9	8	15	0	169	74.12
0.03	125	10	16	26	1	178	78.07
0.1	113	11	10	22	2	159	69.74
0.3	170	6	10	23	1	209	91.67
1.0	128	3	9	17	2	159	69.74
3.0	21	7	9	31	1	68	29.82

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 6

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a] Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	17	3	1	3	1	23	
At 48 hours:							
Control	31	26	18	10	2	87	100.00
0.003	19	19	20	13	2	73	84.09
0.01	9	15	17	10	5	56	62.50
0.03	0	7	13	9	5	34	38.64
0.1	0	1	8	6	5	20	23.86
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	11	4	0	1	0	16	
At 48 hours:							
Control	205	31	1	2	0	239	100.00
0.003	199	32	3	3	0	237	98.33
0.01	191	37	2	6	0	236	98.75
0.03	182	29	1	3	0	215	89.58
0.1	0	0	0	4	0	4	1.67
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	21	5	2	2	1	31	
At 48 hours:							
Control	167	9	3	12	0	191	82.68
0.003	199	5	3	14	0	221	95.67
0.01	205	9	1	16	0	231	100.00
0.03	195	8	3	11	0	217	93.94
0.1	4	1	0	1	0	6	2.60
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 7

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a] Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	7	6	4	2	1	20	
At 48 hours:							
Control	47	10	1	2	1	61	100.00
0.003	45	4	0	2	1	52	86.67
0.01	44	12	0	2	1	59	98.33
0.03	43	9	2	3	1	58	96.67
0.1	25	3	1	0	2	31	51.67
0.3	39	8	1	1	0	49	81.67
1.0	15	3	0	1	1	20	33.33
3.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	22	8	1	0	0	31	
At 48 hours:							
Control	218	156	23	20	0	417	98.12
0.003	217	157	28	21	0	423	99.53
0.01	204	178	19	23	1	425	100.00
0.03	151	49	1	5	0	206	48.47
0.1	0	1	1	5	0	7	1.65
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	19	1	1	7	1	28	
At 48 hours:							
Control	229	15	7	14	2	267	77.39
0.003	310	18	3	11	3	345	100.00
0.01	281	21	7	20	1	330	95.65
0.03	191	15	3	12	0	221	64.06
0.1	0	0	0	1	6	7	2.03
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 8

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	7	22	13	2	0	44	
At 48 hours:							
Control	40	41	118	91	0	290	86.57
0.003	48	59	151	77	0	335	100.00
0.01	43	43	126	107	0	319	95.22
0.03	23	32	62	43	0	160	47.76
0.1	0	1	0	7	1	9	2.69
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	30	9	0	1	0	40	
At 48 hours:							
Control	170	127	20	19	1	337	90.11
0.003	96	167	45	63	1	372	90.47
0.01	94	166	54	59	1	374	100.00
0.03	97	132	25	8	0	262	70.05
0.1	0	1	1	11	0	14	3.74
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	10	4	0	1	0	15	
At 48 hours:							
Control	125	43	3	2	0	173	98.86
0.003	110	60	1	3	0	174	99.43
0.01	99	70	3	3	0	175	100.00
0.03	80	46	0	1	0	127	72.57
0.1	1	0	0	2	0	3	1.71
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 9

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	5	8	7	0	1	21	
At 48 hours:							
Control	62	78	126	67	0	333	98.23
0.003	49	62	135	68	0	314	92.63
0.01	54	67	148	70	0	339	100.00
0.03	24	29	28	2	0	83	24.48
0.1	1	1	0	2	1	5	1.47
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	21	29	3	0	0	53	
At 48 hours:							
Control	62	168	110	173	0	513	97.34
0.003	52	129	118	228	0	527	100.00
0.01	60	120	103	231	0	514	97.53
0.03	45	113	90	188	0	436	82.73
0.1	0	0	0	17	0	17	3.23
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	6	15	11	4	0	36	
At 48 hours:							
Control	4	47	347	20	0	418	80.38
0.003	43	52	339	72	0	506	97.31
0.01	34	55	354	77	0	520	100.00
0.03	39	45	284	47	0	415	79.81
0.1	0	0	0	5	0	5	0.96
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 10

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	7	3	2	3	1	16	
At 48 hours:							
Control	165	13	4	5	0	187	85.78
0.003	190	20	3	4	1	218	100.00
0.01	163	20	3	4	1	191	87.61
0.03	52	4	2	2	0	60	27.52
0.1	3	0	2	6	0	11	5.05
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	6	5	1	4	0	16	
At 48 hours:							
Control	48	8	1	4	0	61	96.83
0.003	46	7	2	4	0	59	93.65
0.01	49	7	1	6	0	63	100.00
0.03	30	5	1	3	0	39	61.90
0.1	0	1	0	4	0	5	7.94
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	19	[illegible]	0	1	0	21	
At 48 hours:							
Control	184	9	2	5	1	201	98.53
0.003	164	13	2	3	0	182	89.22
0.01	181	16	4	3	0	204	100.00
0.03	11	2	0	3	1	17	8.33
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 11

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	17	2	1	1	0	21	
At 48 hours:							
Control	137	53	28	4	0	222	100.00
0.003	117	44	23	10	0	194	89.04
0.01	81	64	36	11	0	192	87.67
0.03	29	33	15	3	1	81	37.44
0.1	0	0	1	2	0	3	1.37
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	40	33	1	0	0	74	
At 48 hours:							
Control	144	39	3	1	0	187	100.00
0.003	125	19	1	2	0	147	79.14
0.01	113	44	0	1	0	158	83.96
0.03	103	25	2	2	0	132	70.59
0.1	2	4	0	13	0	19	10.70
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	70	10	0	2	0	82	
At 48 hours:							
Control	630	178	11	32	0	851	98.50
0.003	621	169	16	41	0	847	98.03
0.01	591	209	19	26	0	845	97.80
0.03	701	135	6	22	0	864	100.00
0.1	26	4	1	25	0	56	6.48
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 12

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a]						
	Rings	Trophozoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gametocytes	Total	Percentage control growth[d]
Test 1							
Time 0	13	3	1	1	0	18	
At 48 hours:							
Control	263	4	2	5	0	274	92.57
0.003	286	7	1	2	0	296	100.00
0.01	258	3	2	5	0	268	90.54
0.03	190	4	1	10	0	205	69.26
0.1	2	1	1	11	2	17	5.74
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	17	1	0	0	0	18	
At 48 hours:							
Control	278	27	4	5	0	314	78.30
0.003	292	46	4	7	0	349	87.03
0.01	300	76	13	12	0	401	100.00
0.03	207	48	4	5	0	264	65.84
0.1	2	0	0	11	0	13	3.24
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	10	3	1	0	0	14	
At 48 hours:							
Control	93	27	2	3	0	125	95.42
0.003	93	27	3	4	0	127	96.95
0.01	92	34	2	3	0	131	100.00
0.03	52	12	0	4	0	68	51.91
0.1	0	1	0	5	0	6	4.58
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 13

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	7	7	13	2	1	30	
At 48 hours:							
Control	6	17	42	17	1	83	100.00
0.003	4	11	34	17	2	68	81.93
0.01	1	4	9	10	2	26	30.12
0.03	-	-	-	-	-	-	0.00
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	30	2	1	0	0	33	
At 48 hours:							
Control	50	9	4	4	1	68	100.00
0.003	47	13	3	5	0	68	100.00
0.01	11	5	1	4	1	22	29.41
0.03	-	-	-	-	-	-	0.00
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	4	8	2	1	1	16	
At 48 hours:							
Control	16	32	7	12	1	68	100.00
0.003	11	25	8	12	2	59	85.51
0.01	3	13	4	9	2	31	44.93
0.03	0	0	0	0	1	1	1.45
0.1	-	-	-	-	-	-	0.00
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 14

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	22	24	4	0	0	50	
At 48 hours:							
Control	213	34	19	24	3	293	77.31
0.003	235	60	40	40	4	379	100.00
0.01	221	53	43	40	6	363	95.78
0.03	111	20	7	9	5	152	40.11
0.1	1	0	0	8	8	17	4.49
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	71	4	0	1	0	76	
At 48 hours:							
Control	584	160	15	6	1	766	99.87
0.003	588	158	13	7	1	767	100.00
0.01	561	166	10	7	1	745	97.13
0.03	195	24	0	5	0	224	29.20
0.1	1	1	0	29	0	31	4.04
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	9	13	4	1	0	27	
At 48 hours:							
Control	159	212	31	106	0	508	95.85
0.003	155	182	27	166	0	530	100.00
0.01	129	181	26	186	3	525	99.06
0.03	98	101	13	45	1	258	48.68
0.1	0	0	0	4	0	4	0.75
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 15

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	19	17	9	0	0	45	
At 48 hours:							
Control	145	48	17	5	9	224	100.00
0.003	77	35	12	4	10	138	60.89
0.01	67	41	21	8	10	147	65.33
0.03	36	16	3	5	12	72	32.00
0.1	3	2	0	1	7	13	5.33
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	27	1	0	1	0	29	
At 48 hours:							
Control	470	66	3	3	0	542	100.00
0.003	416	96	8	4	0	524	97.40
0.01	427	94	5	3	0	529	98.33
0.03	225	23	0	1	0	249	46.20
0.1	1	0	1	12	0	14	1.67
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	1	6	3	5	0	15	
At 48 hours:							
Control	225	15	2	8	0	250	98.04
0.003	226	15	3	4	0	248	97.25
0.01	216	28	3	7	1	255	100.00
0.03	121	11	2	5	0	139	54.51
0.1	0	0	1	3	0	4	1.57
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 16

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	13	11	4	13	0	41	
At 48 hours:							
Control	621	45	21	102	0	789	86.23
0.003	630	49	12	135	0	826	90.27
0.01	749	33	11	121	1	915	100.00
0.03	503	35	37	47	0	622	67.98
0.1	2	1	0	31	0	34	3.72
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	15	3	1	2	0	21	
At 48 hours:							
Control	306	19	7	27	0	359	93.98
0.003	322	17	8	27	0	374	97.91
0.01	333	17	6	26	0	382	100.00
0.03	285	18	6	19	0	327	85.60
0.1	3	0	0	4	0	7	1.83
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	15	5	1	0	0	21	
At 48 hours:							
Control	383	41	18	6	0	448	94.12
0.003	395	50	23	8	0	476	100.00
0.01	342	65	26	8	0	441	92.65
0.03	350	61	14	5	0	430	90.34
0.1	10	1	0	2	2	15	3.15
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

RSA 17

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	28	3	2	6	1	40	
At 48 hours:							
Control	238	19	4	24	1	286	98.62
0.003	239	14	0	12	0	265	91.38
0.01	263	20	3	4	0	290	100.00
0.03	40	7	1	9	0	57	19.66
0.1	0	2	1	28	0	31	10.69
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	50	20	16	0	0	88	
At 48 hours:							
Control	835	20	17	76	1	949	100.00
0.003	776	21	10	62	1	870	91.78
0.01	699	25	7	54	2	787	83.03
0.03	318	9	0	20	3	350	38.04
0.1	2	0	1	17	2	22	2.21
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	12	3	0	2	0	17	
At 48 hours:							
Control	210	34	0	1	0	245	98.39
0.003	192	38	1	1	0	232	93.17
0.01	209	39	0	1	0	249	100.00
0.03	139	49	1	2	0	191	76.71
0.1	0	0	1	3	0	4	1.61
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

MW 1

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	11	26	3	4	0	44	
At 48 hours:							
Control	105	108	45	41	0	299	73.65
0.003	207	84	70	45	0	406	100.00
0.01	163	79	56	35	0	333	82.02
0.03	97	99	38	26	0	260	64.04
0.1	3	3	1	13	0	20	4.93
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	9	7	0	0	0	16	
At 48 hours:							
Control	37	64	16	7	0	124	100.00
0.003	30	55	21	4	0	110	88.71
0.01	39	54	21	5	0	119	95.97
0.03	38	53	11	3	0	105	84.68
0.1	2	3	0	3	0	8	6.45
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	29	25	0	9	0	63	
At 48 hours:							
Control	498	5	7	31	3	544	81.19
0.003	611	14	6	35	4	670	100.00
0.01	564	13	4	40	2	623	92.99
0.03	402	12	4	33	3	454	67.76
0.1	3	1	1	12	2	19	2.84
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

MW 2

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	3	2	2	3	0	10	
At 48 hours:							
Control	20	8	9	9	3	49	96.08
0.003	25	5	10	6	5	51	100.00
0.01	18	5	8	9	4	44	86.27
0.03	6	12	7	9	10	44	86.27
0.1	13	7	4	6	8	38	74.51
0.3	7	7	6	8	6	34	66.67
1.0	6	3	7	5	8	29	0.00
3.0	2	6	6	8	8	30	0.00
Test 2							
Time 0	36	18	2	1	0	57	
At 48 hours:							
Control	130	183	67	78	1	459	95.62
0.003	89	185	70	135	1	480	100.00
0.01	73	198	76	127	1	475	98.96
0.03	87	203	70	57	0	417	86.88
0.1	7	5	2	5	0	19	3.96
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	7	3	0	5	0	15	
At 48 hours:							
Control	334	38	6	18	0	396	100.00
0.003	330	37	5	18	1	391	98.74
0.01	309	42	8	25	1	385	96.97
0.03	201	17	6	16	0	240	59.85
0.1	0	1	0	2	0	3	0.76
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
c Schizonts with 3 or more nuclei
d Where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

FCR-3

Pyrimethamine concentration (uM)	Parasitised erythrocytes/10^4 erythrocytes [a]						
	Rings	Tropho-zoites	Schizonts (=2N)[b]	Schizonts (>2N)[c]	Gameto-cytes	Total	Percentage control growth[d]
Test 1							
Time 0	15	4	0	1	0	20	
At 48 hours:							
Control	26	98	1	2	0	127	93.38
0.003	24	107	3	1	0	136	100.00
0.01	16	91	2	1	0	110	80.88
0.03	10	25	0	0	0	35	25.74
0.1	1	2	0	3	0	6	4.41
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 2							
Time 0	35	25	6	5	0	71	
At 48 hours:							
Control	190	161	72	106	0	529	85.46
0.003	202	179	59	140	0	580	93.70
0.01	207	198	74	140	0	619	100.00
0.03	91	154	46	73	0	364	58.80
0.1	2	1	1	7	0	11	1.78
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00
Test 3							
Time 0	8	5	0	0	0	13	
At 48 hours:							
Control	75	89	17	2	0	183	96.32
0.003	77	95	12	6	0	190	100.00
0.01	75	96	7	2	0	180	94.74
0.03	26	12	0	1	0	39	20.53
0.1	0	0	1	0	0	1	0.53
0.3	-	-	-	-	-	-	0.00
1.0	-	-	-	-	-	-	0.00
3.0	-	-	-	-	-	-	0.00

a Mean of 3 wells. Parasites were counted until the MIC was reached
b Schizonts with 2 nuclei
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In vitro response to halofantrine of 19 southern African reference isolates and 2 KwaZulu field isolates of *Plasmodium falciparum* in the radioisotope uptake assay

Reference isolates

RSA 1

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	7879.30	100.00
0.01	4114.42	52.22
0.02	2117.34	26.87
0.04	367.37	4.66
0.08	0.00	0.00
0.16	58.97	0.75
0.32	25.57	0.32
0.64	27.24	0.35

RSA 2

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	8585.69	100.00
0.01	7923.88	92.29
0.02	5175.05	60.28
0.04	1298.13	15.12
0.08	23.13	0.27
0.16	0.00	0.00
0.32	6.13	0.07
0.64	5.80	0.07

RSA 3

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	6901.30	100.00
0.01	6332.02	91.75
0.02	6356.90	92.11
0.04	6232.20	90.30
0.08	5687.95	82.42
0.16	2830.53	41.01
0.32	118.04	1.71
0.64	5.84	0.08

RSA 5

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	15634.84	100.00
0.01	10755.87	68.79
0.02	9055.97	57.92
0.04	3320.30	21.24
0.08	57.64	0.37
0.16	74.51	0.48
0.32	1.97	0.01
0.64	1.91	0.01

RSA 6

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	8968.82	100.00
0.01	7763.75	86.56
0.02	5300.67	59.10
0.04	2348.08	26.18
0.08	333.80	3.72
0.16	77.60	0.87
0.32	68.60	0.76
0.64	74.67	0.83

RSA 7

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	7252.59	100.00
0.01	7608.00	104.90
0.02	7406.71	102.13
0.04	5133.11	70.78
0.08	2312.91	31.89
0.16	55.53	0.77
0.32	0.00	0.00
0.64	0.00	0.00

RSA8

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	36149.44	100.00
0.01	30640.24	84.76
0.02	24907.17	68.90
0.04	10117.41	27.99
0.08	203.27	0.56
0.16	147.94	0.41
0.32	105.07	0.29
0.64	130.54	0.36

RSA 9

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	48855.67	100.00
0.01	42137.00	86.25
0.02	35610.56	72.89
0.04	20091.18	41.12
0.08	356.07	0.73
0.16	213.20	0.44
0.32	194.47	0.40
0.64	254.94	0.52

RSA 10

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	1505.32	100.00
0.01	2504.72	166.39
0.02	2594.00	172.32
0.04	2828.20	187.88
0.08	1722.80	114.45
0.16	2.00	0.13
0.32	0.00	0.00
0.64	0.00	0.00

RSA 11

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	14507.70	100.00
0.01	16168.42	111.45
0.02	16295.86	112.33
0.04	14386.71	99.17
0.08	387.00	2.67
0.16	53.74	0.37
0.32	66.07	0.46
0.64	109.87	0.76

RSA 12

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	17373.20	100.00
0.01	12739.62	73.33
0.02	7448.87	42.88
0.04	3122.70	17.97
0.08	91.60	0.53
0.16	0.00	0.00
0.32	0.00	0.00
0.64	0.00	0.00

RSA 13

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	6455.35	100.00
0.01	6264.22	97.04
0.02	6533.78	101.21
0.04	5875.83	91.02
0.08	3862.27	59.83
0.16	52.07	0.81
0.32	20.40	0.32
0.64	36.27	0.56

RSA 14

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	9472.30	100.00
0.01	3974.28	41.96
0.02	3095.07	32.67
0.04	1345.30	14.20
0.08	312.97	3.30
0.16	109.70	1.16
0.32	115.90	1.22
0.64	112.10	1.18

RSA 15

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	14857.90	107.12
0.01	13870.27	100.00
0.02	13140.46	94.74
0.04	8201.19	59.13
0.08	886.90	6.39
0.16	184.90	1.33
0.32	175.17	1.26
0.64	180.97	1.30

RSA 16

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	3416.21	100.00
0.01	3995.77	116.96
0.02	3667.40	107.35
0.04	3748.76	109.73
0.08	2225.76	65.15
0.16	270.06	7.91
0.32	13.93	0.41
0.64	0.00	0.00

RSA 17

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	4899.51	100.00
0.01	4757.83	97.11
0.02	4620.87	94.31
0.04	5231.99	106.79
0.08	5134.32	104.79
0.16	209.40	4.27
0.32	12.00	0.24
0.64	11.26	0.23

MW 1

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	10507.11	100.00
0.01	7649.79	72.81
0.02	3872.88	36.86
0.04	1229.33	11.70
0.08	82.93	0.79
0.16	0.00	0.00
0.32	0.00	0.00
0.64	0.00	0.00

MW 2

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	2585.91	100.00
0.01	1363.60	52.73
0.02	456.47	17.65
0.04	97.33	3.76
0.08	9.47	0.37
0.16	0.00	0.00
0.32	0.00	0.00
0.64	0.00	0.00

FCR-3

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	5010.46	100.00
0.01	4968.81	99.17
0.02	4443.03	88.68
0.04	3357.33	67.01
0.08	795.53	15.88
0.16	17.73	0.35
0.32	23.87	0.48
0.64	61.13	1.22

Field isolates

N35

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	4169.53	100.00
0.01	32.00	0.77
0.02	0.00	0.00
0.04	0.00	0.00
0.08	0.00	0.00
0.16	0.00	0.00
0.32	0.00	0.00
0.64	0.00	0.00

N42

Halofantrine concentration (μM)	Mean counts per minute	Percentage control growth
Control	15686.10	100.00
0.01	234.80	1.49
0.02	0.00	0.00
0.04	0.00	0.00
0.08	0.00	0.00
0.16	0.00	0.00
0.32	0.00	0.00
0.64	0.00	0.00

In vitro response to amodiaquine of 19 southern African reference isolates of *Plasmodium falciparum* in the radioisotope assay

RSA 1

Amodiaquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	4730.15	100.00
0.0025	4961.09	104.88
0.005	6282.63	132.82
0.01	3924.05	82.96
0.02	77.37	1.64
0.04	66.37	1.40
0.08	61.11	1.29
0.16	141.51	2.99

RSA 2

Amodiaquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	9382.86	100.00
0.0025	9433.98	100.54
0.005	9186.61	97.91
0.01	7506.54	80.00
0.02	233.80	2.49
0.04	0.00	0.00
0.08	0.00	0.00
0.16	0.00	0.00

RSA 3

Amodiaquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	5539.72	100.00
0.0025	6062.42	109.44
0.005	6158.45	111.17
0.01	2881.86	52.02
0.02	54.44	0.98
0.04	46.91	0.85
0.08	36.57	0.66
0.16	90.57	1.63

RSA 5

Amodiaquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	6623.80	100.00
0.0025	7394.29	111.63
0.005	7635.28	115.27
0.01	2430.52	36.69
0.02	98.57	1.49
0.04	64.77	0.98
0.08	51.44	0.78
0.16	103.44	1.56

RSA 6

Amodiaquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	2247.40	100.00
0.0025	2324.92	103.45
0.005	2126.06	94.60
0.01	1856.20	82.59
0.02	25.93	1.15
0.04	4.13	0.18
0.08	0.00	0.00
0.16	0.00	0.00

RSA 7

Amodiaquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	4304.29	100.00
0.0025	5116.44	118.87
0.005	2948.63	68.50
0.01	56.93	1.32
0.02	24.13	0.56
0.04	0.00	0.00
0.08	0.00	0.00
0.16	0.00	0.00

RSA 8

Amodiaquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	2721.42	100.00
0.0025	3028.09	111.27
0.005	2619.51	96.26
0.01	1762.49	64.76
0.02	7.00	0.26
0.04	0.00	0.00
0.08	0.00	0.00
0.16	0.00	0.00

RSA 9

Amodiaquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	5104.77	100.00
0.0025	6177.35	121.01
0.005	6041.52	118.35
0.01	4940.99	96.79
0.02	79.53	1.56
0.04	51.13	1.00
0.08	65.33	1.28
0.16	68.07	1.33

RSA 10

Amodiaquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	6475.46	100.00
0.0025	7417.36	114.55
0.005	7525.82	116.22
0.01	6278.56	96.96
0.02	175.53	2.71
0.04	57.47	0.89
0.08	100.20	1.55
0.16	92.93	1.44

RSA 11

Amodiaquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	3706.15	100.00
0.0025	3781.84	102.04
0.005	3450.96	93.11
0.01	3242.26	87.48
0.02	39.20	1.06
0.04	0.00	0.00
0.08	0.00	0.00
0.16	0.00	0.00

RSA 12

Amodiaquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	21286.73	100.00
0.0025	20674.13	97.12
0.005	20115.70	94.50
0.01	16073.21	75.51
0.02	246.40	1.16
0.04	0.00	0.00
0.08	0.00	0.00
0.16	0.00	0.00

RSA 13

Amodiaquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	5591.05	100.00
0.0025	6225.33	111.34
0.005	5897.23	105.48
0.01	4868.28	87.07
0.02	0.00	0.00
0.04	0.00	0.00
0.08	0.00	0.00
0.16	0.00	0.00

RSA 14

Amodiaquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	9102.60	100.00
0.0025	11399.91	125.24
0.005	12067.54	132.57
0.01	12599.26	138.41
0.02	12507.27	137.40
0.04	11389.24	125.12
0.08	202.70	2.23
0.16	73.30	0.81

RSA 15

Amodiaquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	16310.91	100.00
0.0025	18531.23	113.61
0.005	17970.68	110.18
0.01	18070.68	110.79
0.02	18612.24	114.11
0.04	17070.14	104.65
0.08	15087.39	92.50
0.16	160.64	0.98

RSA 16

Amodiaquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	2276.32	100.00
0.0025	2150.78	94.48
0.005	2063.84	90.67
0.01	161.00	7.07
0.02	0.00	0.00
0.04	0.00	0.00
0.08	0.00	0.00
0.16	0.00	0.00

RSA 17

Amodiaquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	3207.68	100.00
0.0025	2927.22	91.26
0.005	2999.81	93.52
0.01	130.73	4.08
0.02	0.00	0.00
0.04	0.00	0.00
0.08	0.00	0.00
0.16	0.00	0.00

MW 1

Amodiaquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	18284.76	100.00
0.0025	17763.84	97.15
0.005	16717.22	91.43
0.01	13964.26	76.37
0.02	55.80	0.31
0.04	0.00	0.00
0.08	0.00	0.00
0.16	0.00	0.00

MW 2

Amodiaquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	3947.10	100.00
0.0025	4489.85	113.75
0.005	4397.23	111.40
0.01	3040.04	77.02
0.02	50.87	1.29
0.04	49.53	1.25
0.08	77.13	1.95
0.16	45.20	1.15

FCR-3

Amodiaquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	5702.92	100.00
0.0025	6191.77	108.57
0.005	5619.92	98.54
0.01	4406.92	77.27
0.02	65.40	1.15
0.04	48.87	0.86
0.08	73.73	1.29
0.16	46.53	0.82

In vitro response to chloroquine of 19 southern African reference isolates of *Plasmodium falciparum* in the radioisotope assay

RSA 1

Chloroquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	2655.97	100.00
0.01	2340.78	88.13
0.02	1776.53	66.89
0.04	1055.44	39.74
0.08	192.91	7.26
0.16	51.24	1.93
0.32	118.71	4.47
0.64	23.77	0.89

RSA 2

Chloroquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	10208.24	100.00
0.01	8178.82	80.12
0.02	3270.42	32.04
0.04	147.67	1.45
0.08	5.00	0.05
0.16	26.47	0.26
0.32	0.00	0.00
0.64	15.00	0.15

RSA 3

Chloroquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	4218.91	100.00
0.01	105.17	2.49
0.02	67.97	1.61
0.04	67.24	1.59
0.08	21.77	0.52
0.16	58.64	1.39
0.32	114.04	2.70
0.64	28.51	0.68

RSA 5

Chloroquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	5421.16	100.00
0.01	2598.83	47.94
0.02	89.71	1.65
0.04	106.44	1.96
0.08	30.77	0.57
0.16	40.37	0.74
0.32	79.37	1.46
0.64	68.57	1.26

RSA 6

Chloroquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	24185.00	100.00
0.01	22201.82	91.80
0.02	23833.89	98.55
0.04	22986.48	95.04
0.08	22533.08	93.17
0.16	11853.05	49.01
0.32	727.47	3.01
0.64	180.74	0.75

RSA 7

Chloroquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	11548.12	100.00
0.01	11742.33	101.68
0.02	11733.18	101.60
0.04	10777.65	93.33
0.08	2590.55	22.43
0.16	155.93	1.35
0.32	45.26	0.39
0.64	18.53	0.16

RSA 8

Chloroquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	47333.00	100.00
0.01	49379.00	104.32
0.02	51336.67	108.46
0.04	50420.00	106.52
0.08	50055.00	105.75
0.16	22483.98	47.50
0.32	304.80	0.64
0.64	106.27	0.22

RSA 9

Chloroquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	52356.67	100.00
0.01	51916.67	99.16
0.02	53786.67	102.73
0.04	54388.34	103.88
0.08	53995.00	103.13
0.16	43559.67	83.20
0.32	1322.40	2.53
0.64	451.80	0.86

RSA 10

Chloroquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	3718.27	100.00
0.01	3646.15	98.06
0.02	3524.86	94.80
0.04	3133.16	84.26
0.08	1633.80	43.94
0.16	101.00	2.72
0.32	14.93	0.40
0.64	3.46	0.09

RSA 11

Chloroquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	20465.37	100.00
0.01	17203.89	84.06
0.02	19330.46	94.45
0.04	12665.40	61.89
0.08	18144.40	88.66
0.16	14733.10	71.99
0.32	1686.47	8.24
0.64	197.34	0.96

RSA 12

Chloroquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	20822.46	100.00
0.01	18875.16	90.65
0.02	18597.58	89.31
0.04	13193.80	63.36
0.08	2874.61	13.81
0.16	196.20	0.94
0.32	43.07	0.21
0.64	94.40	0.45

RSA 13

Chloroquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	3686.41	100.00
0.01	3995.38	108.38
0.02	3980.93	107.99
0.04	3270.93	88.73
0.08	1456.73	39.52
0.16	0.00	0.00
0.32	0.00	0.00
0.64	0.00	0.00

RSA 14

Chloroquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	14400.18	100.00
0.01	14971.38	103.97
0.02	14177.37	98.45
0.04	12018.39	83.46
0.08	5128.78	35.62
0.16	410.10	2.85
0.32	160.90	1.12
0.64	166.10	1.15

RSA 15

Chloroquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	16244.55	100.00
0.01	19751.64	121.59
0.02	18354.66	112.99
0.04	16014.58	98.58
0.08	17245.93	106.16
0.16	3842.82	23.66
0.32	714.64	4.40
0.64	252.84	1.56

RSA 16

Chloroquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	5468.18	100.00
0.01	4822.90	88.20
0.02	4909.04	89.77
0.04	4190.61	76.64
0.08	1766.99	32.31
0.16	398.20	7.28
0.32	30.53	0.56
0.64	0.00	0.00

RSA 17

Chloroquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	4500.96	100.00
0.01	4303.30	95.61
0.02	4063.24	90.27
0.04	3842.75	85.38
0.08	2511.80	55.81
0.16	822.00	18.26
0.32	47.86	1.06
0.64	13.00	0.29

MW 1

Chloroquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	18458.34	100.00
0.01	17039.96	92.32
0.02	15680.94	84.95
0.04	8475.54	45.92
0.08	807.40	4.37
0.16	125.27	0.68
0.32	2.47	0.01
0.64	52.47	0.28

MW 2

Chloroquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	2349.75	100.00
0.01	2111.60	89.86
0.02	1941.74	82.64
0.04	1084.33	46.15
0.08	143.40	6.10
0.16	36.87	1.57
0.32	12.60	0.54
0.64	17.00	0.72

FCR-3

Chloroquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	6834.41	100.00
0.01	6908.04	101.08
0.02	6892.59	100.85
0.04	5075.52	74.26
0.08	975.60	14.27
0.16	0.00	0.00
0.32	0.00	0.00
0.64	0.00	0.00

APPENDIX F

In vitro response to mefloquine of 19 southern African reference isolates of *Plasmodium falciparum* in the radioisotope assay

RSA 1

Mefloquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	4373.69	100.00
0.02	730.37	16.70
0.04	171.17	3.91
0.08	62.97	1.44
0.16	72.44	1.66
0.32	0.00	0.00
0.64	121.84	2.79
1.28	49.64	1.13

RSA 2

Mefloquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	8678.21	100.00
0.02	1823.15	21.01
0.04	453.73	5.23
0.08	58.20	0.67
0.16	9.13	0.11
0.32	2.13	0.02
0.64	6.73	0.08
1.28	0.00	0.00

RSA 3

Mefloquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	5327.72	100.00
0.02	5544.04	104.06
0.04	5383.27	101.04
0.08	4283.45	80.40
0.16	1291.71	24.25
0.32	0.00	0.00
0.64	91.37	1.71
1.28	72.97	1.37

RSA 5

Mefloquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	6507.84	100.00
0.02	2694.17	41.40
0.04	913.91	14.04
0.08	55.77	0.86
0.16	55.17	0.85
0.32	0.00	0.00
0.64	123.17	1.89
1.28	61.11	0.94

RSA 6

Mefloquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	20597.74	100.00
0.02	9894.75	48.04
0.04	75432.17	36.57
0.08	3263.26	15.84
0.16	391.74	1.90
0.32	173.60	0.84
0.64	176.87	0.86
1.28	122.00	0.59

RSA 7

Mefloquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	11065.38	100.00
0.02	5681.64	51.35
0.04	2187.94	19.77
0.08	716.20	6.47
0.16	55.06	0.50
0.32	37.46	0.34
0.64	57.86	0.52
1.28	65.06	0.59

RSA 8

Mefloquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	47413.34	100.00
0.02	29405.72	62.02
0.04	22501.45	47.46
0.08	5402.85	11.40
0.16	209.94	0.44
0.32	201.74	0.43
0.64	237.60	0.50
1.28	251.87	0.53

RSA 9

Mefloquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	48023.00	100.00
0.02	36979.89	77.00
0.04	31548.81	65.70
0.08	8174.96	17.02
0.16	314.67	0.66
0.32	213.87	0.45
0.64	280.07	0.58
1.28	320.27	0.67

RSA 10

Mefloquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	3361.48	100.00
0.02	2851.59	84.83
0.04	2454.34	73.01
0.08	559.53	16.65
0.16	26.00	0.77
0.32	44.40	1.32
0.64	50.73	1.51
1.28	47.93	1.43

RSA 11

Mefloquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	19923.73	100.00
0.02	14593.58	73.25
0.04	16197.82	81.30
0.08	10351.49	51.96
0.16	856.60	4.30
0.32	179.74	0.90
0.64	194.14	0.97
1.28	181.27	0.91

RSA 12

Mefloquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	19873.64	100.00
0.02	6456.38	32.49
0.04	3397.59	17.10
0.08	1281.40	6.45
0.16	44.07	0.22
0.32	0.47	0.00
0.64	23.40	0.12
1.28	12.47	0.06

RSA 13

Mefloquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	3422.89	100.00
0.02	2798.52	81.76
0.04	1868.45	54.59
0.08	676.07	19.75
0.16	0.00	0.00
0.32	0.00	0.00
0.64	0.00	0.00
1.28	0.00	0.00

RSA 14

Mefloquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	11164.64	100.00
0.02	5197.71	46.56
0.04	1827.40	16.37
0.08	1525.01	13.66
0.16	203.97	1.83
0.32	124.10	1.11
0.64	125.30	1.12
1.28	118.10	1.06

RSA 15

Mefloquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	16844.24	100.00
0.02	11724.59	69.61
0.04	7394.16	43.90
0.08	2302.35	13.67
0.16	369.70	2.19
0.32	203.70	1.21
0.64	181.24	1.08
1.28	224.50	1.33

RSA 16

Mefloquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	4676.90	100.00
0.02	2732.66	58.43
0.04	1984.99	42.44
0.08	1108.13	23.69
0.16	140.93	3.01
0.32	43.00	0.92
0.64	62.33	1.33
1.28	95.86	2.05

RSA 17

Mefloquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	4453.11	100.00
0.02	4049.39	90.93
0.04	4292.30	96.39
0.08	1957.84	43.97
0.16	95.00	2.13
0.32	51.66	1.16
0.64	94.73	2.13
1.28	77.40	1.74

MW1

Mefloquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	17369.95	100.00
0.02	4358.46	25.09
0.04	686.00	3.95
0.08	469.07	2.70
0.16	7.07	0.04
0.32	0.00	0.00
0.64	0.00	0.00
1.28	0.07	0.00

MW2

Mefloquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	2249.82	100.00
0.02	365.27	16.24
0.04	157.93	7.02
0.08	86.20	3.83
0.16	0.00	0.00
0.32	0.00	0.00
0.64	7.00	0.31
1.28	0.00	0.00

FCR-3

Mefloquine concentration (μM)	Mean counts per minute	Percentage control growth
Control	5955.76	100.00
0.02	3996.33	67.10
0.04	2221.86	37.31
0.08	683.80	11.48
0.16	0.00	0.00
0.32	0.00	0.00
0.64	0.00	0.00
1.28	0.00	0.00

APPENDIX G

In vitro response to quinine of 19 southern African reference isolates of *Plasmodium falciparum* in the radioisotope assay

RSA 1

Quinine concentration (μM)	Mean counts per minute	Percentage control growth
Control	3609.70	100.00
0.04	3994.14	110.65
0.08	3245.30	89.90
0.16	1633.71	45.26
0.32	594.91	16.48
0.64	147.77	4.09
1.28	203.24	5.63
2.56	70.77	1.96

RSA 2

Quinine concentration (μM)	Mean counts per minute	Percentage control growth
Control	2123.76	100.00
0.04	2926.56	137.80
0.08	2283.53	107.52
0.16	1053.13	49.59
0.32	380.07	17.90
0.64	21.93	1.03
1.28	36.27	1.71
2.56	0.00	0.00

RSA 3

Quinine concentration (μM)	Mean counts per minute	Percentage control growth
Control	6667.38	100.00
0.04	5738.33	86.07
0.08	5694.23	85.40
0.16	2418.34	36.27
0.32	1073.34	16.10
0.64	488.17	7.32
1.28	183.97	2.76
2.56	44.44	0.67

RSA 5

Quinine concentration (μM)	Mean counts per minute	Percentage control growth
Control	6523.00	100.00
0.04	5790.78	88.77
0.08	3485.00	53.43
0.16	1399.64	21.46
0.32	604.04	9.26
0.64	482.84	7.40
1.28	174.71	2.68
2.56	121.24	1.86

RSA 6

Quinine concentration (μM)	Mean counts per minute	Percentage control growth
Control	23611.67	100.00
0.04	20487.00	86.77
0.08	20689.00	87.62
0.16	17917.02	75.88
0.32	14579.05	61.75
0.64	7794.16	33.01
1.28	1959.58	8.30
2.56	358.27	1.52

RSA 7

Quinine concentration (μM)	Mean counts per minute	Percentage control growth
Control	12489.56	100.00
0.04	12912.27	103.38
0.08	12547.80	100.47
0.16	8872.00	71.04
0.32	5970.57	47.80
0.64	1849.60	14.81
1.28	326.86	2.62
2.56	76.93	0.62

RSA 8

Quinine concentration (μM)	Mean counts per minute	Percentage control growth
Control	51009.00	100.00
0.04	48804.34	95.68
0.08	49124.34	96.31
0.16	46641.00	91.44
0.32	40934.56	80.25
0.64	26115.65	51.20
1.28	4246.74	8.33
2.56	448.67	0.88

RSA 9

Quinine concentration (μM)	Mean counts per minute	Percentage control growth
Control	56557.34	100.00
0.04	51750.00	91.50
0.08	52260.00	92.40
0.16	49103.67	86.82
0.32	46323.67	81.91
0.64	31887.50	56.38
1.28	6463.32	11.43
2.56	788.20	1.39

RSA 10

Quinine concentration (μM)	Mean counts per minute	Percentage control growth
Control	3883.37	100.00
0.04	3959.55	101.96
0.08	4396.06	113.20
0.16	3388.54	87.26
0.32	2756.75	70.99
0.64	1523.26	39.23
1.28	283.20	7.29
2.56	21.33	0.55

RSA 11

Quinine concentration (μM)	Mean counts per minute	Percentage control growth
Control	18377.13	100.00
0.04	15976.28	86.94
0.08	16947.22	92.22
0.16	16558.51	90.10
0.32	16743.34	91.11
0.64	14994.45	81.59
1.28	5034.17	27.39
2.56	1064.40	5.79

RSA 12

Quinine concentration (μM)	Mean counts per minute	Percentage control growth
Control	32848.60	100.00
0.04	9817.85	29.89
0.08	9225.21	28.08
0.16	6349.28	19.33
0.32	6404.47	19.50
0.64	3242.73	9.87
1.28	883.37	2.69
2.56	392.63	1.20

RSA 13

Quinine concentration (μM)	Mean counts per minute	Percentage control growth
Control	2237.04	100.00
0.04	4135.83	184.88
0.08	4248.33	189.91
0.16	3947.08	176.44
0.32	3012.19	134.65
0.64	2284.36	102.12
1.28	650.67	29.09
2.56	0.00	0.00

RSA 14

Quinine concentration (μM)	Mean counts per minute	Percentage control growth
Control	19946.36	100.00
0.04	23416.32	117.40
0.08	20160.22	101.07
0.16	25092.47	125.80
0.32	14419.57	72.29
0.64	6537.61	32.78
1.28	1798.71	9.02
2.56	922.24	4.62

RSA 15

Quinine concentration (μM)	Mean counts per minute	Percentage control growth
Control	34409.41	100.00
0.04	37830.52	109.94
0.08	33145.44	96.33
0.16	37998.30	110.43
0.32	28252.83	82.11
0.64	15405.89	44.77
1.28	4768.01	13.86
2.56	1812.58	5.27

RSA 16

Quinine concentration (μM)	Mean counts per minute	Percentage control growth
Control	3092.71	100.00
0.04	4557.10	147.35
0.08	4422.89	143.01
0.16	3707.31	119.87
0.32	2840.70	91.85
0.64	1510.80	48.85
1.28	447.66	14.47
2.56	164.06	5.30

RSA 17

Quinine concentration (μM)	Mean counts per minute	Percentage control growth
Control	4384.02	100.00
0.04	4208.44	96.00
0.08	4152.61	94.72
0.16	3806.15	86.82
0.32	3500.65	79.85
0.64	1964.28	44.81
1.28	677.80	15.46
2.56	128.80	2.94

MW1

Quinine concentration (μM)	Mean counts per minute	Percentage control growth
Control	4900.09	100.00
0.04	5836.12	119.10
0.08	5729.59	116.93
0.16	5494.93	112.14
0.32	4822.87	98.42
0.64	3897.93	79.55
1.28	1497.44	30.56
2.56	293.57	5.99

MW2

Quinine concentration (μM)	Mean counts per minute	Percentage control growth
Control	5526.55	100.00
0.04	6017.08	108.88
0.08	7380.64	133.55
0.16	5584.20	101.04
0.32	5503.33	99.58
0.64	4491.36	81.27
1.28	2034.87	36.82
2.56	238.57	4.32

FCR-3

Quinine concentration (μM)	Mean counts per minute	Percentage control growth
Control	8036.43	100.00
0.04	7025.35	87.42
0.08	6942.72	86.39
0.16	6542.53	81.41
0.32	4982.12	61.99
0.64	2898.27	36.06
1.28	445.47	5.54
2.56	0.00	0.00

In vitro response to sulphadoxine/pyrimethamine of 19 southern African reference isolates of *Plasmodium falciparum* in the radioisotope assay

RSA 1

Sulphadoxine concentration (μM)*	Mean counts per minute	Percentage control growth
Control	2484.05	100.00
0.1	3531.11	142.15
0.3	4082.44	164.35
1.00	3646.54	146.80
3.00	3482.42	140.19
10.0	2663.59	107.23
30.0	2327.50	93.70
100.0	1198.84	48.26

RSA 2

Sulphadoxine concentration (μM)*	Mean counts per minute	Percentage control growth
Control	5078.15	100.00
0.1	5092.92	100.29
0.3	5131.33	101.05
1.0	5113.44	100.69
3.0	3273.26	64.46
10.0	994.00	19.57
30.0	144.20	2.84
100.0	101.40	2.00

RSA 3

Sulphadoxine concentration (μM)*	Mean counts per minute	Percentage control growth
Control	6627.18	100.00
0.1	7265.21	109.63
0.3	7774.24	117.31
1.00	3692.82	55.72
3.00	584.97	8.83
10.0	343.24	5.18
30.0	217.04	3.27
100.0	322.77	4.87

* Ratio of sulphadoxine:pyrimethamine = 80:1

RSA 5

Sulphadoxine concentration (μM)*	Mean counts per minute	Percentage control growth
Control	23592.63	100.00
0.1	12557.40	53.23
0.3	13364.36	56.65
1.0	12937.35	54.84
3.0	13034.86	55.25
10.0	12915.35	54.74
30.0	8537.24	36.19
100.0	2575.70	10.92

RSA 6

Sulphadoxine concentration (μM)*	Mean counts per minute	Percentage control growth
Control	18925.00	100.00
0.1	18305.56	96.73
0.3	18110.81	95.70
1.00	18545.61	98.00
3.00	10149.60	53.63
10.0	1127.14	5.96
30.0	587.40	3.10
100.0	591.00	3.12

RSA 7

Sulphadoxine concentration (μM)*	Mean counts per minute	Percentage control growth
Control	11315.49	100.00
0.1	11652.37	102.98
0.3	12084.31	106.79
1.0	12226.55	108.05
3.0	5283.51	46.69
10.0	717.66	6.34
30.0	311.20	2.75
100.0	259.00	2.29

* Ratio of sulphadoxine:pyrimethamine = 80:1

RSA 8

Sulphadoxine concentration (μM)*	Mean counts per minute	Percentage control growth
Control	29083.10	100.00
0.1	30265.96	104.07
0.3	31268.81	107.52
1.00	31099.29	106.93
3.00	15214.35	52.31
10.0	1970.33	6.77
30.0	1103.07	3.79
100.0	1107.94	3.81

RSA 9

Sulphadoxine concentration (μM)*	Mean counts per minute	Percentage control growth
Control	52868.34	100.00
0.1	50853.34	96.19
0.3	56555.00	106.97
1.0	54505.00	103.10
3.0	43391.67	82.07
10.0	8895.20	16.83
30.0	4596.29	8.69
100.0	3954.35	7.48

RSA 10

Sulphadoxine concentration (μM)*	Mean counts per minute	Percentage control growth
Control	2785.21	100.00
0.1	2921.23	104.88
0.3	2878.77	103.36
1.00	2914.53	104.64
3.00	1081.13	38.82
10.0	232.40	8.34
30.0	79.66	2.86
100.0	92.06	3.31

* Ratio of sulphadoxine:pyrimethamine = 80:1

RSA 11

Sulphadoxine concentration (μM)*	Mean counts per minute	Percentage control growth
Control	13294.78	100.00
0.1	14169.16	106.58
0.3	15627.38	117.55
1.0	15076.61	113.40
3.0	3329.68	25.05
10.0	1005.67	7.56
30.0	634.94	4.78
100.0	674.74	5.08

RSA 12

Sulphadoxine concentration (μM)*	Mean counts per minute	Percentage control growth
Control	12688.38	100.00
0.1	15639.46	123.26
0.3	16042.40	126.43
1.00	17132.41	135.02
3.00	6413.76	50.55
10.0	680.33	5.36
30.0	236.53	1.86
100.0	202.80	1.60

RSA 13

Sulphadoxine concentration (μM)*	Mean counts per minute	Percentage control growth
Control	3403.21	100.00
0.1	3795.99	111.54
0.3	3749.13	110.16
1.0	3490.38	102.56
3.0	783.00	23.01
10.0	19.27	0.57
30.0	25.20	0.74
100.0	0.00	0.00

* Ratio of sulphadoxine:pyrimethamine = 80:1

RSA 14

Sulphadoxine concentration (μM)*	Mean counts per minute	Percentage control growth
Control	11465.54	100.00
0.1	10572.08	92.21
0.3	12780.73	111.47
1.00	11836.05	103.23
3.00	2683.61	23.41
10.0	610.90	5.33
30.0	369.04	3.22
100.0	293.30	2.56

RSA 15

Sulphadoxine concentration (μM)*	Mean counts per minute	Percentage control growth
Control	17316.64	100.00
0.1	14816.41	85.56
0.3	15829.60	91.41
1.0	16544.97	95.54
3.0	6057.03	34.98
10.0	1151.24	6.65
30.0	687.84	3.97
100.0	561.37	3.24

RSA 16

Sulphadoxine concentration (μM)*	Mean counts per minute	Percentage control growth
Control	5767.24	100.00
0.1	5364.49	93.02
0.3	5772.99	100.10
1.00	5606.55	97.21
3.00	1031.46	17.88
10.0	81.93	1.42
30.0	107.20	1.86
100.0	102.80	1.78

* Ratio of sulphadoxine:pyrimethamine = 80:1

RSA 17

Sulphadoxine concentration (μM)*	Mean counts per minute	Percentage control growth
Control	5529.62	100.00
0.1	5294.80	95.75
0.3	5491.10	99.30
1.0	5441.73	98.41
3.0	1406.53	25.44
10.0	328.20	5.94
30.0	214.46	3.88
100.0	230.00	4.16

MW1

Sulphadoxine concentration (μM)*	Mean counts per minute	Percentage control growth
Control	7242.73	100.00
0.1	7965.28	109.98
0.3	8218.99	113.48
1.00	8239.79	113.77
3.00	1740.33	24.03
10.0	216.87	2.99
30.0	89.33	1.23
100.0	104.73	1.45

MW2

Sulphadoxine concentration (μM)*	Mean counts per minute	Percentage control growth
Control	2143.20	100.00
0.1	2169.45	101.22
0.3	2242.35	104.63
1.0	2209.94	103.11
3.0	1060.27	49.47
10.0	1556.40	72.62
30.0	1513.73	70.63
100.0	1101.87	51.41

* Ratio of sulphadoxine:pyrimethamine = 80:1

FCR-3

Sulphadoxine concentration (μM)*	Mean counts per minute	Percentage control growth
Control	12893.89	100.00
0.1	13212.78	102.47
0.3	13455.64	104.36
1.00	13174.89	102.18
3.00	4357.04	33.79
10.0	512.13	3.97
30.0	571.33	4.43
100.0	299.67	2.32

* Ratio of sulphadoxine:pyrimethamine = 80:1

In vitro response to chloroquine of field isolates of *Plasmodium falciparum* collected in KwaZulu in May 1987 from active cases.

A4

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	184.0	98.40
1	183.0	97.86
2	183.0	97.86
4	184.0	98.40
5.7	187.0	100.00
8	183.0	97.86
16	132.0	70.59
32	0.0	0.00

A6

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	43.5	58.00
1	43.0	57.33
2	30.0	40.00
4	75.0	100.00
5.7	3.5	4.67
8	0.0	0.00
16	0.0	0.00
32	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

A8

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	20.5	91.11
1	15.0	66.67
2	22.5	100.00
4	21.0	93.33
5.7	8.0	35.56
8	2.0	8.89
16	0.0	0.00
32	0.0	0.00

A51

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	54.0	100.00
1	19.0	35.19
2	-[c]	-
4	-	-
5.7	49.0	90.74
8	3.0	5.56
16	0.0	0.00
32	0.0	0.00

A53

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	70.0	100.00
1	58.0	82.86
2	55.0	78.57
4	60.0	85.71
5.7	62.0	88.57
8	66.0	94.29
16	51.0	72.86
32	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth
[c] Not counted

A56

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	34.0	100.00
1	20.0	58.82
2	6.0	17.65
4	4.0	11.76
5.7	0.0	0.00
8	0.0	0.00
16	0.0	0.00
32	0.0	0.00

A57

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	138.0	89.32
1	118.5	76.70
2	148.0	95.79
4	136.0	88.03
5.7	142.5	92.23
8	154.5	100.00
16	110.0	71.20
32	14.0	9.06

A58

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	98.0	94.69
1	103.5	100.00
2	83.0	80.19
4	79.0	76.33
5.7	77.0	74.40
8	75.0	72.46
16	51.0	49.28
32	35.0	33.82

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

A59

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	133.0	100.00
1	130.0	97.74
2	113.0	84.96
4	13.0	97.74
5.7	2.0	1.50
8	0.0	0.00
16	0.0	0.00
32	0.0	0.00

A61

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	27.0	80.60
1	33.5	100.00
2	15.0	44.78
4	16.0	47.76
5.7	0.0	0.00
8	0.0	0.00
16	0.0	0.00
32	0.0	0.00

A65

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	38.0	100.00
1	37.0	97.37
2	37.0	97.37
4	36.0	94.74
5.7	11.0	28.95
8	0.0	0.00
16	0.0	0.00
32	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

A67

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	121.5	100.00
1	98.5	81.07
2	99.0	81.48
4	96.0	79.01
5.7	11.0	9.05
8	0.0	0.00
16	0.0	0.00
32	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

The following isolates were obtained from patients who had ingested chloroquine in the 2 weeks prior to collection of the blood sample:

A4, A51, A53, A59.

In vitro response to chloroquine of field isolates of *Plasmodium falciparum* collected in KwaZulu in May 1987 from passive cases.

P2

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	36.0	100.00
1	22.0	61.11
2	16.5	45.83
4	13.5	37.50
5.7	12.5	34.72
8	15.5	43.06
16	11.5	31.94
32	0.0	0.00

P3

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	73.0	100.00
1	64.0	87.67
2	63.0	86.30
4	61.0	83.56
5.7	71.0	97.26
8	58.0	79.45
16	62.0	84.93
32	69.0	94.52

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

P11

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	12.5	89.29
1	13.0	92.86
2	12.5	89.29
4	14.0	100.00
5.7	12.5	89.29
8	3.5	25.00
16	0.0	0.00
32	0.0	0.00

P17

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	15.5	70.45
1	22.0	100.00
2	14.5	65.91
4	18.0	81.82
5.7	18.5	84.09
8	11.5	52.27
16	2.0	9.09
32	0.0	0.00

P18

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	55.5	48.26
1	71.0	61.74
2	92.5	80.43
4	96.0	83.48
5.7	115.0	100.00
8	94.0	81.74
16	13.0	11.30
32	0.0	0.00

[a] Mean of 2 counts

[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

P21

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	41.0	100.00
1	36.5	89.02
2	32.5	79.27
4	32.5	79.27
5.7	36.5	89.02
8	36.0	87.80
16	26.0	63.41
32	1.0	2.44

P22

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth
Control	118.5	89.10
1	114.5	86.09
2	106.0	79.70
4	119.0	89.47
5.7	133.0	100.00
8	129.5	97.37
16	108.0	81.20
32	26.0	19.55

P23

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	165.0	100.00
1	155.0	93.94
2	148.0	89.70
4	155.0	93.94
5.7	153.0	92.73
8	142.0	86.06
16	56.0	33.94
32	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

P24

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	119.0	96.75
1	121.0	98.37
2	123.0	100.00
4	100.0	81.30
5.7	51.0	41.46
8	34.5	28.05
16	14.0	11.38
32	0.5	0.41

P25

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	123.0	100.00
1	110.0	89.43
2	116.5	94.72
4	118.0	95.93
5.7	103.0	83.74
8	2.5	2.03
16	2.0	1.63
32	0.0	0.00

P26

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	72.5	100.00
1	72.5	100.00
2	63.0	86.90
4	57.0	78.62
5.7	37.0	51.03
8	15.0	20.69
16	5.5	7.59
32	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

P3 contained > 100 100 trophozoites/μl blood.

P23 was obtained from a patient who had ingested chloroquine in the 2 weeks prior to collection of the blood sample.

In vitro response to chloroquine of field isolates of *Plasmodium falciparum* collected in KwaZulu in January 1988 from passive cases.

J3

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	97.0	100.00
2	61.0	62.89
4	52.0	53.61
5.7	49.0	50.52
8	40.0	41.24
16	20.0	20.62
32	1.0	1.03
64	0.0	0.00

J4

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	58.5	60.31
2	59.5	61.34
4	77.0	79.38
5.7	97.0	100.00
8	44.0	45.36
16	2.5	2.58
32	0.0	0.00
64	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

J8

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	157.0	81.14
2	189.0	97.67
4	193.5	100.00
5.7	182.0	94.06
8	151.0	78.04
16	18.5	9.56
32	2.5	1.29
64	0.5	0.26

J10

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	41.0	74.55
2	33.5	60.91
4	-[c]	-
5.7	55.0	100.00
8	52.0	94.55
16	22.0	40.00
32	0.0	0.00
64	0.0	0.00

J11

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	128.0	75.74
2	69.0	40.83
4	169.0	100.00
5.7	113.5	67.16
8	122.5	72.49
16	55.5	32.84
32	0.0	0.00
64	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth
[c] Not counted

J12

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	102.0	100.00
2	61.0	59.80
4	75.0	73.53
5.7	49.0	48.04
8	25.0	24.51
16	39.0	38.24
32	0.0	0.00
64	0.0	0.00

J13

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	70.0	100.00
2	11.0	15.71
4	13.0	18.57
5.7	24.0	34.29
8	38.5	55.00
16	0.0	0.00
32	0.0	0.00
64	0.0	0.00

J14

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	83.0	100.00
2	56.0	67.47
4	55.0	66.27
5.7	81.0	97.59
8	45.0	54.22
16	18.0	21.69
32	0.0	0.00
64	0.0	0.00

[a] Mean of 2 counts

[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

J19

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	104.0	100.00
2	89.0	85.58
4	64.0	61.54
5.7	86.0	82.69
8	67.0	64.42
16	64.0	61.54
32	17.0	16.35
64	1.0	0.96

J20

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	55.0	100.00
2	3.0	5.45
4	2.0	3.64
5.7	28.0	50.91
8	6.0	10.91
16	8.0	14.55
32	0.0	0.00
64	0.0	0.00

J27

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth
Control	49.0	100.00
2	25.0	51.02
4	4.0	8.16
5.7	10.0	20.41
8	12.0	24.49
16	11.0	22.45
32	0.0	0.00
64	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

J34

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	74.0	92.50
2	74.0	92.50
4	80.0	100.00
5.7	78.0	97.50
8	44.0	55.00
16	24.0	30.00
32	0.0	0.00
64	0.0	0.00

J36

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	46.0	35.80
2	123.5	96.11
4	128.5	100.00
5.7	116.0	90.27
8	119.0	92.61
16	49.5	38.52
32	0.0	0.00
64	0.0	0.00

J37

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	50.5	100.00
2	49.0	97.03
4	1.0	1.98
5.7	39.0	77.23
8	48.0	95.05
16	41.0	81.19
32	3.0	5.94
64	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

J38

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	60.0	52.63
2	114.0	100.00
4	106.0	92.98
5.7	99.0	86.84
8	103.0	90.35
16	72.0	63.16
32	0.0	0.00
64	0.0	0.00

J40

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	12.0	12.90
2	65.0	69.89
4	93.0	100.00
5.7	88.0	94.62
8	88.0	94.62
16	86.0	92.47
32	31.0	33.33
64	0.0	0.00

J44

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	20.0	32.26
2	47.0	75.81
4	54.0	87.10
5.7	62.0	100.00
8	49.0	79.03
16	27.0	43.55
32	0.0	0.00
64	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

J45

Chloroquine concentration (pmols/well)	Schizonts per 200 asexual parasites[a]	Percentage control growth[b]
Control	134.0	100.00
2	116.0	86.57
4	108.0	80.60
5.7	133.0	99.25
8	16.0	11.94
16	25.0	18.66
32	0.0	0.00
64	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

The following isolates were obtained from patients who had ingested chloroquine in the 2 weeks prior to collection of blood samples:

J3, J44, J45.

J34 had < 500 trophozoites/µl blood.

In vitro response to amodiaquine of field isolates of *Plasmodium falciparum* collected in KwaZulu in April 1989, in the 24-hour microtechnique

P7

Amodiaquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	95.5	81.28
0.005	98.0	83.40
0.01	95.5	81.28
0.02	81.5	69.36
0.04	117.5	100.00
0.08	0.0	0.00
0.16	0.0	0.00
0.32	0.0	0.00

P9

Amodiaquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	171.1	93.70
0.005	172.5	94.52
0.01	147.5	80.82
0.02	145.0	79.45
0.04	182.5	100.00
0.08	178.0	97.53
0.16	46.0	25.21
0.32	1.5	0.82

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

P10

Amodiaquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	95.5	100.00
0.005	93.0	97.38
0.01	84.0	87.96
0.02	90.0	94.24
0.04	18.0	18.85
0.08	0.5	0.52
0.16	0.0	0.00
0.32	0.0	0.00

P12

Amodiaquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	71.5	100.00
0.005	56.0	78.32
0.01	43.0	60.14
0.02	42.0	58.74
0.04	10.0	13.99
0.08	1.0	1.40
0.16	0.0	0.00
0.32	0.0	0.00
0.64	0.0	0.00

P13

Amodiaquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	152.0	94.70
0.005	158.0	98.44
0.01	160.5	100.00
0.02	152.0	94.70
0.04	147.0	91.59
0.08	0.0	0.00
0.16	0.0	0.00
0.32	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

P14

Amodiaquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	46.0	82.14
0.005	56.0	100.00
0.01	42.0	75.00
0.02	42.5	75.89
0.04	26.5	47.32
0.08	0.5	0.89
0.16	0.0	0.00
0.32	0.0	0.00

P15

Amodiaquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	27.0	72.97
0.005	26.0	70.27
0.01	24.0	64.86
0.02	37.0	100.00
0.04	2.0	5.41
0.08	0.5	1.35
0.16	0.0	0.00
0.32	0.0	0.00

P16

Amodiaquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	83.5	78.04
0.005	80.5	75.23
0.01	84.5	78.97
0.02	107.0	100.00
0.04	89.5	83.64
0.08	95.0	88.79
0.16	3.0	2.80
0.32	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

P18

Amodiaquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	191.0	100.00
0.005	187.5	98.17
0.01	186.5	97.64
0.02	186.5	97.64
0.04	189.0	98.95
0.08	180.0	94.24
0.16	0.0	0.00
0.32	0.0	0.00

P22

Amodiaquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	32.5	98.48
0.005	31.0	93.94
0.01	33.0	100.00
0.02	18.5	56.06
0.04	0.0	0.00
0.08	0.0	0.00
0.16	0.0	0.00
0.32	0.0	0.00

P32

Amodiaquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	69.0	100.00
0.005	44.0	63.77
0.01	30.0	43.48
0.02	37.5	54.35
0.04	27.0	39.13
0.08	32.0	46.38
0.16	0.0	0.00
0.32	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

P33

Amodiaquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	75.0	100.00
0.005	65.0	94.20
0.01	60.5	80.67
0.02	57.0	76.00
0.04	73.0	97.33
0.08	50.5	67.33
0.16	0.0	0.00
0.32	0.0	0.00

P35

Amodiaquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	13.5	75.00
0.005	8.0	44.44
0.01	18.0	100.00
0.02	10.5	58.33
0.04	3.0	16.67
0.08	0.0	0.00
0.16	0.0	0.00
0.32	0.0	0.00

[a] Mean of 2 count

[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

In vitro response to chloroquine of field isolates of *Plasmodium falciparum* collected in KwaZulu in April 1989, in the 24-hour microtechnique

P7

Chloroquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	45.0	100.00
0.02	5.0	11.11
0.04	0.0	0.00
0.08	0.0	0.00
0.16	0.0	0.00
0.32	0.0	0.00
0.64	0.0	0.00
1.28	0.0	0.00

P9

Chloroquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	161.5	87.06
0.02	185.5	100.00
0.04	163.5	88.14
0.08	164.0	88.41
0.16	135.0	72.78
0.32	129.0	69.54
0.64	31.5	16.98
1.28	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

P10

Chloroquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	24.5	56.32
0.02	43.5	100.00
0.04	18.0	41.38
0.08	4.0	9.20
0.16	0.0	0.00
0.32	0.0	0.00
0.64	0.0	0.00
1.28	0.0	0.00

P12

Chloroquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	23.0	100.00
0.02	18.0	78.26
0.04	11.5	50.00
0.08	3.5	15.22
0.16	2.5	10.87
0.32	0.0	0.00
0.64	0.0	0.00
1.28	0.0	0.00

P13

Chloroquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	37.0	43.53
0.02	85.0	100.00
0.04	0.0	0.00
0.08	0.0	0.00
0.16	0.0	0.00
0.32	0.0	0.00
0.64	0.0	0.00
1.28	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

P14

Chloroquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	17.0	79.07
0.02	18.5	86.05
0.04	21.5	100.00
0.08	10.5	48.84
0.16	7.5	34.88
0.32	3.5	16.28
0.64	0.0	0.00
1.28	0.0	0.00

P15

Chloroquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	12.0	61.54
0.02	19.5	100.00
0.04	10.0	51.28
0.08	9.5	48.72
0.16	12.0	61.54
0.32	6.0	30.77
0.64	2.5	12.82
1.28	0.0	0.00

P16

Chloroquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	59.0	97.52
0.02	59.0	97.52
0.04	60.5	100.00
0.08	53.0	87.60
0.16	42.5	70.25
0.32	24.0	39.67
0.64	3.0	4.96
1.28	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

P18

Chloroquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	72.5	71.78
0.02	79.0	78.22
0.04	85.5	84.65
0.08	100.0	99.01
0.16	101.0	100.00
0.32	59.5	58.91
0.64	4.5	4.46
1.28	0.0	0.00

P22

Chloroquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	5.5	25.19
0.02	21.0	100.00
0.04	13.5	64.29
0.08	11.0	52.38
0.16	9.0	42.86
0.32	3.0	14.29
0.64	0.0	0.00
1.28	0.0	0.00

P32

Chloroquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	19.0	100.00
0.02	11.5	60.53
0.04	18.5	97.37
0.08	10.0	52.63
0.16	13.0	68.42
0.32	9.5	50.00
0.64	0.5	2.63
1.28	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth

P33

Chloroquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	37.5	80.65
0.02	39.5	84.95
0.04	32.5	69.89
0.08	46.5	100.00
0.16	35.0	75.27
0.32	27.0	58.06
0.64	14.0	30.11
1.28	0.0	0.00

P45

Chloroquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	47.0	100.00
0.02	37.0	78.72
0.04	36.0	76.60
0.08	45.0	95.74
0.16	41.5	88.30
0.32	2.0	4.26
0.64	0.0	0.00
1.28	0.0	0.00

P46

Chloroquine concentration (μM)	Schizonts per 200 parasites[a]	Percentage control growth[b]
Control	29.5	78.67
0.02	37.5	100.00
0.04	24.5	65.33
0.08	21.5	57.33
0.16	5.0	13.33
0.32	0.0	0.00
0.64	0.0	0.00
1.28	0.0	0.00

[a] Mean of 2 counts
[b] For the calculation of the IC_{50} value, where the mean schizont count in the control wells was less than that in some of the test wells, the highest count was taken as 100% control growth