Linkage Group Selection to Investigate Genetic Determinants of Complex Traits of Malaria Parasites

Sittiporn Pattaradilokrat

Thesis submitted for the degree of Doctor of Philosophy
to The University of Edinburgh

June 2008

Abstract

Malaria parasites of the species infecting humans and animal hosts exhibit genetic and phenotypic diversity. Some of this diversity, including the responses to anti-malarial drugs, growth rate and virulence and antigenic variability, is medically significant. This is because these phenotypes may determine the existence and survival of the parasites in the host and, in turn, contribute to the clinical outcome of infection. Understanding of the biological characteristics and the genetic basis underlying these complex phenotypes can thus lead to the development of effective control strategies against the disease, such as anti-malarial drugs and vaccines. Genetic studies in rodent malaria parasites have proved useful in providing insights into the genetic determinants of these complex traits and thus can be used to complement the study of human malaria.

The present studies aim to investigate genetic determinants underlying two major medically important phenotypes, Strain Specific Protective Immunity (SSPI) and Growth rate, using the newly devised genetic method of Linkage Group Selection (LGS). The results presented here relate to the accomplishment of these aims.

LGS analysis of SSPI using a genetic cross between clones AJ and CB-pyr10 of *Plasmodium chabaudi chabaudi* has identified a single region on chromosome 8 containing the gene for the Merozoite Surface Protein-1 as encoding a major target of SSPI. A similar finding was also obtained in a previous LGS study using a different genetic cross between clones AS-pyr1 and CB of *P. c. chabaudi* (Martinelli *et al.*, 2005). Hence, the results of two independent studies strongly indicate that a single locus within the parasite genome contains a major target antigen, or antigens, of SSPI against *P. c. chabaudi* malaria. These results have particular relevance for research on SSPI in human malaria and the choice of candidate antigens for malaria vaccine development.

LGS analysis of growth rate conducted upon a genetic cross between a fast-growing line, 17XYM, and a slow-growing line, 33XC, of *Plasmodium yoelii yoelii* has identified a ~ 1 megabase pair region on *P. y. yoelii* chromosome 13 as containing a major genetic determinant(s) of growth rate in these malaria parasites. This is consistent with the finding of the classical linkage analysis by Walliker *et al.*, (1976), that growth rate in *P. y. yoelii* is mainly determined at a single genetic locus. Because the fast-growing line 17XYM arose spontaneously during infection with a mild strain of *P. y. yoelii* 17X, identification of parasites with a slow growth rate phenotype derived from the same genetic stock as 17XYM can be useful in determining genes underlying growth rate in these malaria parasites. It has been shown here that parasites of the *P. y. yoelii* lines 17X consist of two completely distinct genotypes. One is represented by the fast-growing line, 17XYM, and a slow-growing line of *P. y. yoelii*, 17XNIMR. The other is represented by another slow-growing line 17XA. Comparing the region of *P. y. yoelii* chromosome 13 under strong growth selection between the two congenic lines, 17XYM and 17XNIMR, could lead to the identification of the gene(s) controlling growth rate differences in these two parasite lines. Such findings could be relevant to the location of genetic determinants of growth rate in human malaria.

Declaration of Contributions

I hereby declare that I alone have composed this thesis, and that, with the contribution mentioned below, the work and the opinions expressed herein are my own.

Before I began my PhD studies, experiments concerning with Linkage Group Selection analysis of strain specific protective immunity and growth rate were already designed and conceived by my principle supervisor, Professor Richard Carter.

Mice were made immune to strains of a rodent malaria parasite, *Plasmodium chabaudi chabaudi*, by Professor Richard Carter and Mr Les Steven.

Allele-specific quantitative real time quantitative-polymerase chain reaction and Pyrosequencing™ assays used throughout this study were made available by my co-supervisor, Dr Sandra J Cheesman.

Nucleotide sequences of the genes encoding Merozoite Surface Protein-1 (MSP-1) and Apical Membrane-1 from lines of the rodent malaria parasites, *P. chabaudi* and *Plasmodium yoelii* (except *P. y. yoelii* line 17XNIMR) were supplied by Dr Sandra J Cheesman and Mrs Kathryn Degnan.

Nucleotide sequences of genes encoding Merozoite Surface Protein-1 of *P. yoelii* line 17XNIMR were kindly provided by Dr Anthony A Holder (The National Institute for Medical Research, London, The United Kingdom).

This work has not been submitted for any other degrees or professional qualification.

Sittiporn Pattaradilokrat

June 2008

Acknowledgments

I see myself, first and foremost, very fortunate to be supervised by Professor Richard Carter. He was always generous with his endless support, constructive advice and friendly encouragement in all aspects of my work. I am also grateful to my co supervisors, Dr Sandra Cheesman and Dr Jeff Bond, whose scientific input was deeply appreciated.

I acknowledge the help of Dr Axel Martinelli and Dr Richard Culleton in passing on their expertise in AFLP and for their helpful suggestion and unfailing interest. A special thank goes to Naomi J Gadsby for having offered constructive critique during the writing of this thesis.

I offer my sincere thanks to Dr Paul Hunt, Dr Elaine M O' Mahony, Dr Alison M Creasey and Katarzyna Modrzynska for their personal kindness. Dr Paul Hunt also helped with advice on specific aspects of the work. I am also thankful to Bette Boyle, Les Steven, Kathryn Degnan, Ronnie Mooney, Richard Fawcett, Sheena Boath and Ray Mclnnes, who provided their excellent technical assistance at all times.

I wish to extend my gratitude to the following people: Emeritus Professor Sodsri Thaithong, Dr Pongchai Harnyuttanakorn, Dr Naowarat Kanchanakhan, Aree Seugorn, Napaporn Siripoon and Tepanata Pumpaiboon (WHO/CC for the Reference and Research on Biological Characterization of Malaria Parasites, Institute of Health Research, Chulalongkorn University, Thailand) in passing their expertise in molecular biology skills used in the present studies and for their continuing support.

My sincere gratitude goes to Dr Worrawan Marungsith and Dr Itsara Pracharoenwattana whom I am delighted to count as my 'special' sister and brother, respectively. They have been incredible by believing in me that I can do this and supporting me. I extend my sincere gratitude to Thadsin Khamkanya, Wimolpat Bumbudsanpharoke, Apirat Chaikuad and the staff at Thai restaurants. Their friendship is greatly valued. I would also like to thank Kelwalin Dhanasarnsombut for always keeping a smile on my face.

The Development and Promotion of Science and Technology Talents Project, the Royal Thai Government (Thailand) provided financial support for my post-graduate study. The staff at the Office of Educational Affairs, the Royal Thai Embassy (London), have all been great in sorting out the administrative parts of my PhD studies. The Wellcome Trust of the United Kingdom generously funded this research.

Finally, I wish to express my deep appreciation to my family for their unconditional love, understanding and being so wonderfully patient with me over the years. I dedicated this thesis to my grandma, my auntie and my mum, whom I love most. Without their wholehearted support throughout all my studies, this thesis would have never been achieved.

Table of Contents

Page number

Page number

List of Figures

Page number

Chapter 1

Chapter 2

Page number

Chapter 3

Page number

Chapter 4

List of Tables

Page number

Chapter 1

Chapter 2

Chapter 3

Page number

Chapter 4

Appendix 1

Appendix 2

Page number

Appendix 3

Abbreviations

ACTs	artemisinin combination therapies
AFLP	amplified fragment length polymorphism
ama-1	apical membrane antigen-1 (gene)
AMA-1	apical membrane antigen-1 (protein)
ANOVA	analysis of variance
AT content	Adenine and Thymine content
BLAST	Basic Local Alignment Search Tool
˚C	the degree Celsius
CI	comparative intensity
CHEF	contour-clamped homogeneous electric field
cM	centiMorgan
cm^3	cubic centimetre
CSP	circumsporozoite protein (protein)
DHFR	dihydrofolate dehydrogenase
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
g	g-force (for centrifugation)
GDH	NADP-dependent glutamate dehydrogenase
GPI	glucose phosphate isomerase
HbsAg	Hepatitis B surface antigen
HCl	hydrochloric acid
hr	hour
i.p.	intra-peritoneal
L	liter
LDH	lactate dehydrogenase
LGS	Linkage Group Selection
LSHTM	London School of Hygiene and Tropical Medicine (UK)
M	Molar
Mb	megabase pair
mg	milligram
min	minute
mM	milliMolar
mm^3	cubic millimetre
ml	millilitre
msp	merozoite surface protein (gene)
MSP	merozoite surface protein (protein)
MVA-CS	modified vaccinia virus Ankara
NIH	National Institutes of Health (USA)
NIMR	National Institute for Medical Research (UK)
PABA	*para*-aminobenzoic acid
PCR	polymerase chain reaction
PfEMP-1	*P. falciparum* erythrocyte membrane protein-1
PFGE	pulsed field gel electrophoresis

pir	*Plasmodium* interspersed repeats (gene)
PNG	Papua New Guinea
pRBC	parasitised red blood cells
PSQ	Pyrosequencing™
6PGD	6-phospho gluconate dehydrogenase
RESA	ring-infected erythrocyte surface antigen (protein)
RIFIN	repetitive interspersed family
RII	relative intensity index
RFLP	restriction fragment length polymorphism
pyr	pyrimethamine
RMP	rodent malaria parasites
RNA	ribonucleic acid
RTQ-PCR	real time quantitative-polymerase chain reaction
SEM	standard error of mean
sec	second
SNP	single nucleotide polymorphism
SSPI	Strain Specific Protective Immunity
STEVOR	sub-telomeric variable open reading frame
TBE	Tris Borate EDTA
TE	Tris EDTA
TERT	telomerase reverse transcriptase (protein)
TIGR	The Institute for Genomic Research (USA)
µg	microgram
µl	microlitre
µM	microMolar
v/v	volume for volume
w/v	weight for volume
WRIAR	Walter Reed Army Institute of Research (USA)
WHO	World Health Organisation
YIR	yoelii interspersed repeats (protein)
%	percent

CHAPTER 1: Introduction

1.1 General Overview

The purpose of this chapter is firstly to provide a short introduction to the life cycle of a malaria parasite. This is followed by a brief account of the current situation of the human malaria disease, including the global distribution of the disease, the socio-economic impact of malaria and its consequences, and the problems related to malaria control. Then, the genetics and genomics of the human and rodent malaria parasites is reviewed. Finally, there is an introduction to the principle of Linkage Group Selection (LGS), a method that enables the identification of regions in the *Plasmodium* genomes containing genes controlling medically important phenotypes, including antigens controlling Strain Specific Protective Immunity (SSPI) and blood stage growth Rate.

1.2 Life Cycle of Malaria

Malaria is a disease that is caused by parasitic protozoa that belong to the genus *Plasmodium*, members of a large phylum *Apicomplexa*. There are at least 172 described species of *Plasmodium* infecting a range of vertebrate host species, including 89 species infecting reptiles (Telford, 1994), 32 species infecting birds (van Riper *et al.*, 1994) and 51 species infecting mammals. The latter group includes 11 species infecting rodents (Cox, 1988; Cox, 1993), 25 species infecting non-human primates (Collins *et al.*, 1993), and 4 species infecting humans. The four species of human malaria parasite are *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium vivax*. Their only reservoir is humans, except for *P. malariae*, which is common to man, the African apes and probably some South-American monkeys (Fandeur *et al.*, 2000). It is also known that non-human primate malaria parasite species, such as *Plasmodium knowlesi*, do occasionally infect humans under natural conditions (Jongwutiwes *et al.*, 2004; Singh *et al.*, 2004).

All species of *Plasmodium* infecting mammals have a complex life cycle that involves both an invertebrate vector and a mammalian host. A typical life cycle of a malaria parasite is illustrated in Figure 1.1.

Malaria transmission from a mammalian host to a mosquito can only be initiated by intra-erythrocytic sexual blood stages of the life cycle – the gametocytes. During a mosquito blood meal the mosquito ingests male and female gametocytes from an individual who is infected with malaria. Inside the mosquito's midgut, the gametocytes of both sexes undergo gametocytogenesis. The female gametocyte escapes from the erythrocyte membrane and matures into a single macrogamete. Male gametocytes undergo three rounds of DNA replication resulting in eight motile microgametes, and they are released from the erythrocyte by a process called exflagellation. The macrogamete and microgamete combine to form a zygote. By 12 to 24 hours after the blood meal, the zygote has transformed into a motile ookinete that penetrates the midgut wall of the vector and becomes an oocyst. Then, the oocyst matures, with the formation of thousands of sporozoites. Sporozoite

development in the oocysts takes between 10 and 25 days depending on the species of *Plasmodium* and environmental factors such as temperature. When the mature oocyst ruptures, the sporozoites are released into the haemocoel of the mosquito. Sporozoites that make their way into the salivary glands are in position to be injected into a new host when the mosquito takes a blood meal [reviewed by Vlachou *et al.*, 2006].

Following the bite of an infected female mosquito, the phase of development of the malaria parasite in a mammalian host starts. During the blood meal, sporozoites from the salivary glands of the mosquito are injected into the skin or directly into the bloodstream (Amino *et al.*, 2006). Despite the fact that the salivary glands of an infected mosquito may contain thousands of sporozoites (Sinden, 1997), less than 100 of these are believed to be transmitted in any one bite (Rosenberg *et al.*, 1990). The sporozoites that enter the bloodstream localise in liver sinusoid and there they invade hepatocytes, where they develop into liver schizonts and replicate asexually, thus producing 30,000 to 50,000 merozoites per sporozoite (Sinden *et al.*, 2002). This period is known as the pre-erythryocytic stage [reviewed by Prudêncio *et al.*, 2006]. It normally takes between 1 to 2 weeks in human malaria parasites (Garnham, 1966). In *P. vivax* and *P. ovale*, dormant stages (hypnozoites) can persist in the liver and cause relapses by invading erythrocytes months, or even years later (Mangoni *et al.*, 2003).

Then, the liver-stage schizonts rupture and release the merozoites into bloodstream. The merozoites continue to invade circulating erythrocytes where they mature and multiply by shizogony over a period of a few days. The merozoite then differentiates into a ring-form, then a trophozoite and then a schizont. The schizont eventually bursts, lysing the erythrocyte and releasing newly matured merozoites that immediately proceed to invade new erythrocytes to repeat the cycle. This is the erythrocytic stage of the parasite's life cycle. The molecular basis of the malaria parasite's maturation and muliplication in the erythrocytes has been described elsewhere (Galinski *et al*., 2005; Topolska *et al.*, 2005).

Some of the merozoites do not form the erythrocytic-stage schizonts, but eventually differentiate into gametocytes. The cellular events of gametocytogenesis have been described in detail previously (Khan *et al.*, 2004; Janse *et al.*, 2005; Kooij *et al.*, 2007). After being taken up by an anopheline mosquito during a blood meal, the gametocytes of both sexes mature and sexually combine in the insect host to generate a zygote. The latter develops into an oocyst, from which new sporozoites are generated and migrate into the salivary glands of the mosquito, ready to reinitiate the cycle.

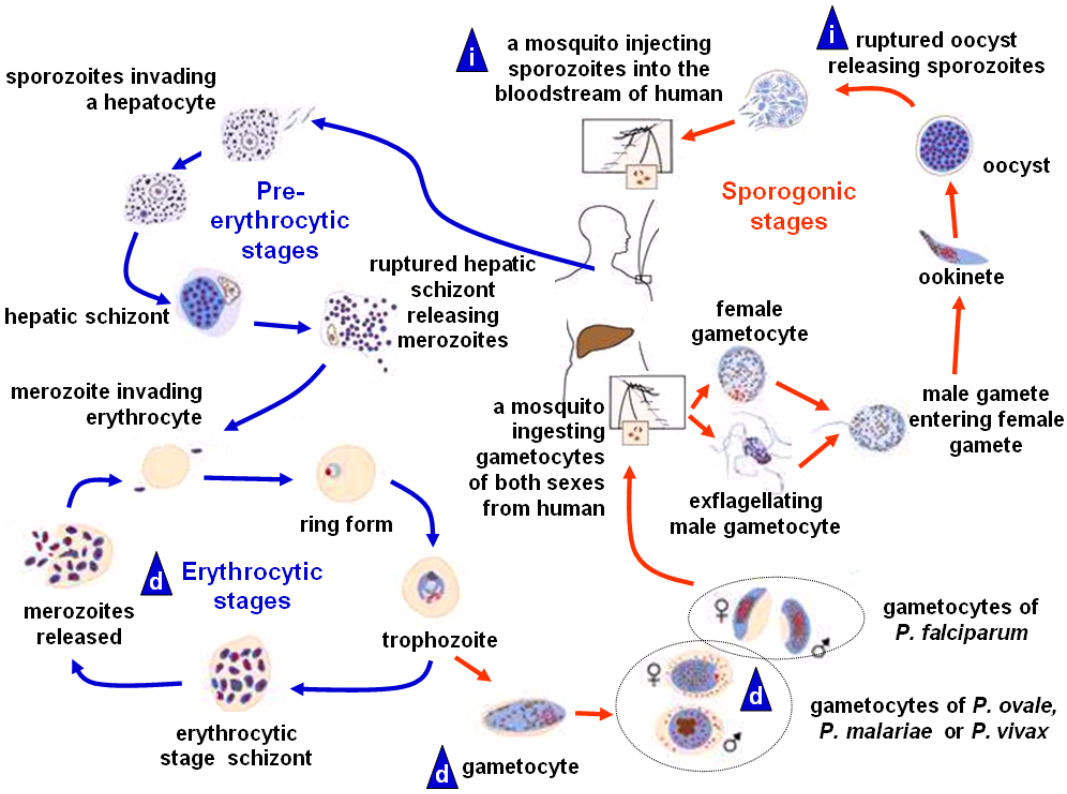

Figure 1.1. A schematic representation of the life cycle of human malaria. The malaria parasite life cycle involves two hosts: a man (blue arrows) and a female Anopheline mosquito (red arrows). The letter'i' in the blue triangle represents the infective phase (sporozoite stage) of the parasite. The letter'd' in the blue triangle represents the diagnostic phase (asexual erythrocytic stages) of the parasite in human. This figure was prepared with the help of artwork from the website of Centres for Disease Control and Prevention, Division of Parasitic Disease, National Centre for Infection Disease. http://www.cdc.gov/malaria/biology/life_cycle.htm.

All the clinical symptoms associated with malaria are commonly attributed to the erythrocytic stages of the parasite's life cycle. In human malaria infections, symptoms associated with malaria include chills and high fever (paroxysms) that recur every 48 hours in tertian malaria (*P. falciparum*, *P. ovale* and *P. vivax*) or every 72 hours in quartian malaria (*P. malariae*) (Garnham, 1966; Warrell, 2002), in synchrony with the release of malaria parasites (merozoites) from ruptured erythrocytes. A paroxysm lasts for 4 to 12 hours, after which time there is typically a cessation of symptoms for a period, before another wave of paroxysm.

Without correct diagnosis and treatment with effective anti-malarial drugs, malaria can cause severe disease, which occurs most frequently with *P. falciparum*. Severe malaria is characterised by symptoms, such as severe anaemia, organ dysfunction and cerebral malaria, all of which are frequently fatal. These are most commonly found in individuals who have not acquired immunity to malaria, such as young children, pregnant women and travellers, and generally in areas of low endemicity (Chen *et al.*, 2000; Casals-Pascual *et al.*, 2006). The vast majority of malaria morbidity and mortality is caused by *P. falciparum*. *P. vivax* almost always causes mild disease, although it can cause severe clinical symptoms and mortality in humans (Choi *et al.*, 2004). *P. malariae* and *P. ovale* are relatively infrequent causes of morbidity and are responsible for a small percentage of the total cases worldwide.

1.3 Malaria Burden: The Current Situation

1.3.1 Geographical Distribution and Burden of Malaria

As recently as the early 1900s, human malaria was endemic on every continent except Antarctica. In the 1950s, the World Health Organisation (WHO) launched a global malaria control/eradication programme based on the use of insecticides. Despite the success of the eradication of malaria from North America, Europe and Australia, malaria remains endemic in a number of tropical and subtropical countries worldwide (Figure 1.2). The numbers of countries and territories with malaria transmission for 2004 is 107 (WHO, 2005). Nowadays, around three billion people living in these countries, which are about half of the world's population, are at risk of exposure to malarial infections (Guerra *et al.*, 2006).

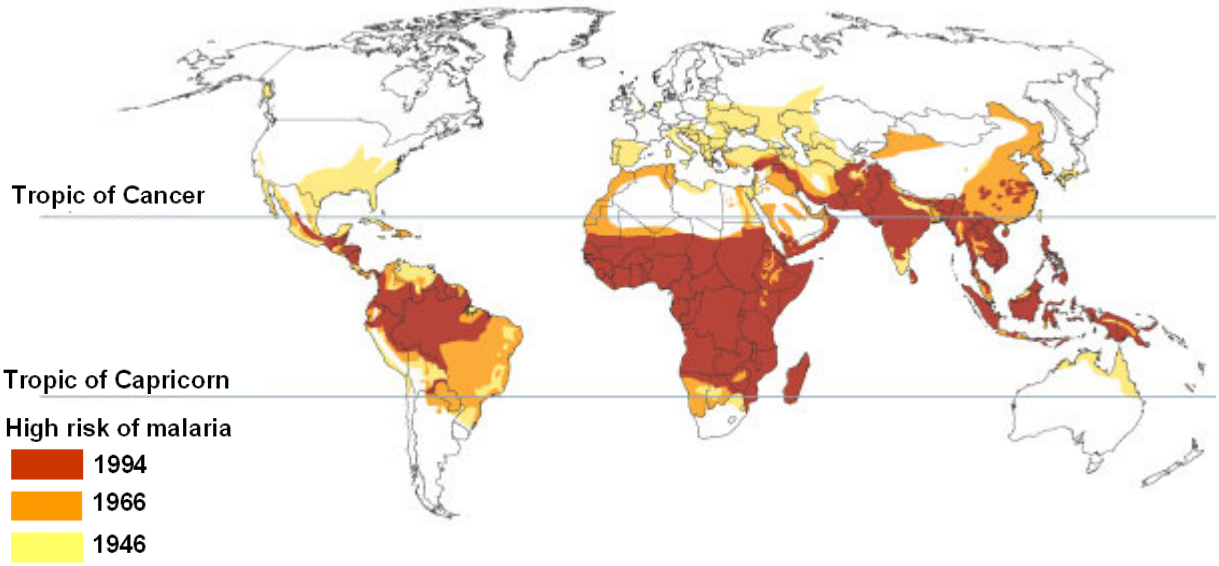

Figure 1.2. The global distribution of malaria transmission from 1946 to 1994. The changing global distribution of malaria risk shows a disease burden that is being limited to tropical and subtropical countries in five continents. (Figure reproduced from Sachs *et al.*, 2002).

Malaria causes an estimated annual death toll of over 1 million (WHO, 2005), mainly caused by infections with *P. falciparum*. Approximately 800,000 deaths occur among

children younger than 5 years of age, especially in remote rural areas in Africa with poor access to health care. Globally, 8% of all deaths in children are attributed to malaria, this proportion increasing to 18% in sub-Saharan African countries (Black *et al.*, 2003; Bryce *et al.*, 2005; Rowe *et al.*, 2006). This is due in part to the fact that severe anaemia and cerebral malaria are most frequent in African children and are responsible for 4 and 1 million cases annually, respectively (Greenwood *et al.*, 2005).

The WHO estimates for 2004 that there are 350 to 500 million actual illnesses from malaria every year worldwide (WHO, 2005). 70 to 80 million cases are caused by *P. vivax* (Mendis *et al.*, 2001) and 270 to 400 million are attributed to *P. falciparum* (Nahlen *et al.*, 2005). However, a recent publication increased the estimate of global *P. falciparum* malaria episodes for 2002 to 515 million (range 300 to 600 million) (Snow *et al.*, 2005). Around 70% of the cases of clinical malaria caused by *P. falciparum* occur in Africa and most of the other 30% occur in Asia (Snow *et al.*, 2005). By contrast, the small number of malaria cases still found in Europe (about 50,000 cases in 1999; Sabatinelli *et al.*, 2001) and an even smaller number in the USA (about 1,300 cases in 2004; Skarbinski *et al.*, 2006) are essentially all imported cases in travellers or military personnel. With a rapidly growing human population in regions with high malaria transmission, it has been estimated that, in the absence of effective intervention strategies, the number of malaria cases will double by 2021 (Bremen *et al.*, 2001).

1.3.2 Social and Economic Impacts of Malaria

Malaria impairs the activity of infected individuals and the social development of the endemic community (Gallup *et al.*, 2001; Sachs *et al.*, 2002). Malaria causes sickness in adults, resulting in loss of working days and lost incomes. Malaria not only causes morbidity in pre-school children (i.e. those less than 5 years of age) but also affects a large number of school-aged children, resulting in school-absenteeism. A study in Kenya attributed 13 to 50% of medically related school absences to malaria (Brooker

et al., 2000). Consistent with the above, a study in Sri Lanka showed that malaria had an adverse effect on the school performance of children (Fernando *et al.*, 2003). They found a strong negative correlation between the number of uncomplicated malaria attacks and both language and mathematical scores (Fernando *et al.*, 2003). Thus, the burden of malaria extends beyond affecting human health and activity to impairing the economic development of countries.

High levels of the malaria burden are also commonly considered a cause of poverty and depressed economy in many countries where malaria is endemic (Gallup *et al.*, 2001; Sachs *et al.*, 2002). This is partly because malaria incidence appears to coincide with the world's poorest countries, mainly in Africa south of the Sahara-desert (Figure 1.2). Malaria has a deleterious effect on the wealth of individuals and the economic development of nations. Microeconomic costs include the price of household-level malaria prevention, such as insecticides and bed nets (Gallup *et al.*, 2001; Sachs *et al.*, 2002). It also includes individual spending on diagnosis and treatment (Gallup *et al.*, 2001; Sachs *et al.*, 2002). The disease also reduces productivity and economic effectiveness of affected populations, which can represent about a 20% of an annual household income (Malaney *et al.*, 2004). Macroeconomic costs include the large proportion of government public expenditure for maintaining public health facilities as well as continuing malaria vector control programs. The estimated costs of malaria, in terms of the strain on health services, are enormous. In malaria endemic countries, as many as 3 out of 10 hospital beds are occupied by malaria infected patients (WHO, 2005). Apart from the above examples, foreign trade and investment, tourism, job-seeking migrations and agricultural practices are all transformed by the existence of malaria, having a deleterious effect on the macroeconomy (Guinovart *et al.*, 2006). The combined micro and macroeconomic cost of malaria in sub-Saharan Africa is estimated to be 1 to 5% pre-capita gross domestic product, a cost of US$12 billion a year (Sachs *et al.*, 2002).

There is no doubt that effectively reducing the burden of malaria will improve the quality of life for people and, therefore, increase the economic development of the countries affected.

1.4 Malaria control: Strategies and Problems

Traditional strategies for the control of malaria depend upon the reduction of vector breeding sites and vector density, reducing human-mosquito contact, the effective use of the anti-malarial drugs delivered to malaria-infected individuals, or vaccines.

1.4.1 The Vector Control

Malaria is primarily a rural disease and is transmitted by the female infected anopheline mosquito. The use of mosquito nets and repellent as a protection from mosquito bites during the night has been practised from very early times. Environmental modification and manipulation, such as drainage of swamps and the use of larvalicides and insecticides, have also been among the main approaches to reducing the number of vectors. However, in many endemic areas drainage and general environmental planning were not feasible (Beales *et al.*, 2002). It was also discovered that mosquitoes were able to become resistant to the cheap insecticides directed against them such as DDT, and thus DDT spraying has now been abandoned in many countries (Najera, 1989; Georghiou, 1990).

Currently, insecticide-treated bed nets (ITN) have been increasingly used in many malaria endemic countries to replace indoor house spraying with insecticides (WHO, 2005). It has recently shown the efficacy of 17 and 43% in reducing all causes of malaria mortality and morbidity in children younger than 5 years and also provides protection to pregnant women (Binka *et al.*, 2006). However, the ITN in malaria endemic areas, particularly in Africa, is currently in a limited supply. It has been estimated that between 130 to 264 million ITNs are required in 2007 (Miller *et al.*, 2007). These numbers will reach the 80% coverage target for about 133 million children younger than 5 years and pregnant women living in 123 million households at high risk from malaria (Miller *et al.*, 2007).

In addition, where the uses of ITNs are in practice, a sufficient coverage of ITN in malaria-transmission areas and good community participation at a sustained level are

needed. Furthermore, malaria surveillance must be conducted to identify episodes of local transmission and to monitor emergence of vectors resistant to the particular insecticides and densities of malaria-infected mosquitoes throughout areas. Without such data, the long-term success of the ITN-based malaria control programs would not be possible.

1.4.2 Chemotherapy

A malarial infection in humans is currently treated with a variety of anti-malarial drugs. Many anti-malarial drugs, including quinine, chloroquine, sulfadoxine-pyrimethamine, mefloquine and atovaquone, interfere with the replicating cycles of the erythrocytic stage malaria parasites (see Figure 1.1, section 1.2), thus reducing the parasite numbers in the blood and terminating the clinical attack (Wernsdorfer *et al.*, 1991; White, 1999; Wongsrichanalai *et al.*, 2002). Some are also gametocytocial drugs, such as tafenoquine, artemisinine and artesunate (Colman *et al.*, 1992; Kumar *et al.*, 1990; Chen *et al.*, 1994, Chotivanich *et al.*, 2006), which are active against the sexual forms of the parasite in the blood, thus having the potential to reduce transmission of malaria.

However, emergence of parasites that are resistant to antimalarial-drugs, however, commonly develops within 1 year to 15 years after the introduction of a drug (Table 1.1). The high prevalence of strains of *P. falciparum* and *P. vivax* resistant to chloroquine and sulfadoxine-pyrimethamine has been rising in Africa, South-East Asia and South-America over the past decades, and it has now become the major challenge that most malaria control programmes are facing.

Nevertheless, so far there has not been any solid evidence of resistance to artemisininand its derivatives (e.g. artemether, artesunate, dihydroartemisinin) in human malaria. These compounds produce a very rapid therapeutic response (i.e. reduction of the parasite number and resolution of clinical symptoms) against multidrug resistant *P. falciparum*, and they reduce gametocyte carriage (Kumar *et al.*, 1990; Chen *et al.*, 1994; Chotivanich *et al.*, 2006). Consequently, many countries

have now adopted artemisinine derivatives as their first line therapies, in combination with other drugs (such as benflumetol plus artemether, amodiaquine plus artesunate, and sulfadoxine-pyrimethamine plus artesunate) (WHO, 2006). These artemisinin combination therapies (ACTs) present favourable pharmacokinetics (i.e. the process by which a drug is absorbed, distributed, metabolized, and eliminated by the body) and are thought to reduce the probability of mutations that underlie resistance and treatment failure emerging in parasite populations (Yeung *et al.*, 2004; WHO, 2006). However, the main problem with these drugs is that they are still in limited supply and are often too expensive to be afforded by poor countries (Enserink, 2005). Therefore, a safe, effective and affordable malaria vaccine is an urgently needed tool for reducing the global burden of the disease and is considered as a critical global public health priority (Malkin *et al.*, 2006).

Anti-malarial Drugs	Year of Introduction	Year of the First Reported Resistance	References
Chloroquine	mid 1940s	1959-1960	Maberti (1960) cited by Wernsdorfer *et al.*, (1991)
Proguanil	1948	1949	Edeson (1950)
Sulfaxodine-pyrimethamine	1967	1967	Verdrager *et al.*, (1967) cited by Bjorkman *et al.*, (1990)
Mefloquine	1977	1982	Boudreau *et al.*, (1982)
Atovaquone	1996	1996	Looareesuwan *et al.*, (1996)

Table 1.1 Year of introduction and first reports of anti-malarial resistance in *Plasmodium falciparum* malaria (Table adapted from Wongsrichanalai *et al.*, 2002)

1.5 Human malaria vaccines

Human malaria vaccine-based intervention could be used to supplement strategies of vector control and medical treatment for reducing rates of morbidity and mortality. The complexity of the life cycle of the malaria parasite leads to the development of an array of diverse strategies for malaria vaccines. The approaches to the generation of malaria vaccines have focused on the targeting of one of the three main stages of the life cycle of malaria parasites (see Figure 1.1, section 1.2): the sexual stage, the pre-erythrocytic stage and the erythrocytic stage.

Sexual stage vaccine strategies aim to raise antibodies that target the sexual stages of malaria parasites in the anopheline mosquito gut [reviewed by Saul, 2007]. During a blood meal such antibodies are taken up by the mosquitoes and can block further parasite development. Although this type of vaccine would not directly prevent infection in the vaccinated individuals (vaccinees), the community where the vaccinees live would experience a decrease in transmission of the parasite to new hosts. Hence, this type of vaccine is known as a transmission-blocking vaccine.

Pre-erythrocytic stage vaccine strategies aim to generate protective immunity directed against sporozoites via antibody responses and to destroy intra-hepatic parasitic stages via cell-mediated immunity [reviewed by Mikolajczak *et al.*, 2007]. This would stop the parasites from reaching the erythrocytic stage and thus prevent any clinical manifestation. This type of vaccine would benefit individuals from areas with no exposure to malaria who travel to a region of high malaria endemicity. Among key target antigens at this stage are the circumsporozoite protein (CSP) and the thrombospondin-related adhesive protein (TRAP), which are expressed abundantly on the surface of sporozoites, and the liver stage antigen-1 (LSA-1), which are expressed on the surface of infected hepatocytes.

Erythrocytic stage vaccine strategies aim to elicit protective immunity directed against the blood stages of the parasites, mainly the merozoite [reviewed by Genton *et al.*, 2007]. They should, therefore, prevent the invasion of the merozoites into

erythrocytes, inhibiting the development of the parasites in erythrocytes and speeding the clearance of parasitised erythrocytes. This type of vaccine should reduce the blood parasite density, thereby reducing the incidence of severe malaria and malaria-associated mortality in infants and young children with heavy exposure to malaria infections, such as those living in sub-Saharan Africa. A number of malarial surface antigens, including the merozoite surface proteins (MSP-1, MSP-2 and MSP-3) and the apical membrane antigen-1 (AMA-1), have been widely used as components of this type of vaccine.

In addition to the above-mentioned conventional approach, the multistage or multivalent vaccine strategy has been developed. Although the incorporation of combined malarial antigens from different stages (multistage), or different antigens from the same stages (multivalent) may help trigger an intense and sequential immune response and increase vaccine efficacy, it should be noted that the inclusion of non-protective vaccine components could yield the unnecessarily high cost and may lead to any undesired effects. The most well-known multiple component vaccine is Combination B, comprising three blood-stage antigens MSP-1, MSP-2 and the ring-stage infected-erythrocyte surface antigen (RESA) (Lawrence *et al.*, 2000; Genton *et al.*, 2002) (see below for details).

Currently, 75 candidate vaccines are being developed, most of which are still being assessed in humans in pre-clinical phases (Aide *et al.*, 2007; Girard *et al.*, 2007) [see also the portfolio of candidate malaria vaccines at the WHO website http://www.who.int/vaccine_research/documents/en/malaria_table.pdf]. The majority of the vaccines in active development today are the pre-erythrocytic stage and the erythrocytic stage vaccines. Since *P. falciparum* is responsible for most malaria mortality among the four species of human malaria parasites, this malaria species receives most attention in vaccine research. In the following section, I will describe progress in the vaccine development against the pre-erythrocytic stages and the erythrocytic stages of the human malaria parasite *P. falciparum*.

1.5.1 *Pre-erythrocytic vaccines*

Three main approaches are currently being undertaken to produce *P. falciparum* pre-erythrocytic vaccines [reviewed by Mikolajczak *et al.*, 2007]: (1) whole parasite vaccine approach, (2) heterologous prime-boost approach and (3) subunit protein vaccine approach.

The whole parasite vaccine approach involves immunisation of individuals with large numbers of live irradiation-attenuated sporozoites or genetically attenuated sporozoites. It had already been shown in experimental animals (Nussenzweig *et al.*, 1976) and in humans that immunisation with irradiated sporozoites could confer a solid and sterile protection from laboratory-induced challenge infection (Hoffman *et al.*, 2002). However, there are several obstacles associated with the whole parasite vaccine approach (Todryk *et al.*, 2007). For instance, the irradiated sporozoites must be delivered though irradiated, infected mosquitoes. In addition, there are problems associated with mass production and cryopreservation of sufficient sporozoites. Nevertheless, the applications of reverse genetics and bioengineering in production process development of live attenuated vaccines are in progress and this would overcome problems that currently prevent their large-scale application (Kanoi *et al.*, 2007).

The heterologous prime-boost approach is another vaccination approach that uses a regime of priming the immune response with antigens expressed as plasmid DNA or in T-cell inducing attenuated viral vectors, such as the fowlpox strain FP9 (FP9) or adenovirus, followed by boosting with another antigen-coding viral vector, such as the modified vaccinia virus Ankara (MVA) (Dunachie *et al.*, 2003; Hill, 2006). This approach has involved the use of complex antigens. For example, the sporozoite surface antigen TRAP has been fused to a string of liver stage antigen T-cell epitopes to enhance the breadth of the T-cell response. Although immunisation of malaria-naïve volunteers with TRAP-MVA vaccination has provided significant T-cell responses and protection from sporozoite challenge infection (Webster *et al.*, 2005), trials in the Gambia following the same regimens showed no protection against occurrence of the disease in human volunteers (Dunachie *et al.*, 2006) and showed

much lower immunogenicity in adults and children in Kenya (Bejon *et al.*, 2006). Nevertheless, a new efficacy trail with FP9-TRAP vaccination is currently ongoing in Kenya [quoted from Girarad *et al.*, 2007].

The third approach to the development of pre-erythrocytic vaccines is based on the use of recombinant proteins or peptides derived from the surface antigen(s) of the sporozoites. The subunit vaccine development aims to reproduce or enhance the level of immunity achieved with the whole attenuated parasite. The vaccine of this type that is currently most advanced in development is the RTS,S vaccine in a potent proprietary AS02 adjuvant, developed by GlaxoSimthKine Biologicals (GSK). The RTS,S/AS02A vaccine is a fusion protein of the central repeat sequence (R) and major T-cell epitopes (T) of the CSP of *P. falciparum* (strain NF54, laboratory clone 3D7), fused to the entire surface antigen (S) of the hepatitis B virus, and expressed in the form of virus-like particles with nonrecombinant S antigen in yeast *Saccharomyces cerevisiae* (Bojang, 2006). Initial Phase I clinical trials of RTS,S formulated with the AS02 adjuvant, containing an oil-in water emulsion plus the immune stimulant monophosphoryl lipid A and QS21, showed protection against a laboratory-based homologous sporozoite challenge in six out of seven volunteers, although the duration of protection appeared to be short-lived (Stoute *et al.*, 1997; Stoute *et al.*, 1998). Subsequent evaluations of RTS,S/AS02A have confirmed tolerability and immunogenicity in malaria-naïve and malaria-experienced adult populations. A dose-range Phase I/II study has demonstrated levels of efficacy from 30% (single-dose vaccination schedules) to 55% (three-dose vaccination schedules), with an overall protective efficacy of 40% in non-immune vaccines against sporozoite challenge (Kester *et al.*, 2001).

The first field trials of the RTS,S/AS02A vaccine were conducted in an area of low seasonal malaria transmission in the coastal region of The Gambia and in a hyperendemic region of western Kenya. The trials in The Gambia demonstrated a protective efficacy of 71% against naturally acquired malarial infection for the first 9 weeks of follow-up (Bojang *et al.*, 2001). However, protection appeared to wane, declining to 0% in the last 6 weeks of follow-up (Bojang *et al.*, 2001). In a phase IIb

clinical carried out in children 1 to 4 years of age in Mozambique, this vaccine reduced the risk of *P. falciparum* infection, uncomplicated malaria and severe disease, and this protection lasted for at least 18 months (Alonso *et al.*, 2004; Alonso *et al.*, 2005). More recently, the safety, immunogenicity and initial efficacy of the RTS,S vaccine has been assessed in Mozambican young infants (e.g. children younger than one year). This vaccine was found to be safe, well-tolerated and immunogenic in young infants, with an overall protective efficacy of 66% (Aponte *et al.*, 2007) against natural malaria infections. The results obtained from these trails are encouraging and these findings set the stage for large-scale, multi-centre phase III efficacy studies.

1.5.2 *Erythrocytic vaccines*

The goal of the asexual erythrocytic malaria vaccines is to induce immune responses against merozoites in order to reduce parasite burden and, hence, protect against pathological severity of disease. Currently, eighteen clinical trails with erythrocytic vaccines have been registered in the main clinical trials registries [quoted from Genton *et al.*, 2007; see also the World Health Organisation website at http://www.who.int/trialresearch]. The majority of erythrocytic vaccines are based on the use of the proteins expressed at the merozoite surface, e.g. MSP-1, MSP-2 and MSP-3 and the proteins present at the apical organelles of the malaria parasites, e.g. AMA-1. A number of these vaccines being developed are based on the use of single malaria antigens and, hence, registered as single-type antigen vaccines. There are only a small number of erythrocytic vaccines being developed through a multicomponent approach by combining more than one blood-stage antigen in a vaccine formulation.

In the following section, I will give a brief account of candidates of erythrocytic stage vaccines and summarise the progress towards *P. falciparum* erythrocytic vaccine development.

1.5.2.1 *MSP-1 based vaccines*

MSP-1 is the most abundant complex protein on the merozoite surface. *P falciparum* MSP-1 is initially synthesized as a polypeptide of ~200 kDa which undergoes proteolytic cleavage before schizont rupture into fragments of 83kDa (N-terminus), 28kDa, 38kDa (Central portions) and 42kDa (C-terminus) (Holder, 1988; Stafford *et al.*, 1994; Stafford *et al.*, 1996). The latter fragment called MSP-1_{42} remains membrane-bound by means of a GPI anchor and undergoes secondary processing into 33kDa and 19kDa fragments during red cell invasion. While the other fragments are shed from the parasite surface, forming a complex by non-covalent bonds, the 19 kDa fragment, known as MSP-1_{19}, composed of two epidermal growth factor-like domains, remains membrane-bound and is carried inside the erythrocyte.

The gene for MSP-1 is located on chromosome 9 in *P. falciparum*. According to the nucleotide sequence comparison from a number of *P. falciparum* isolates, the gene is divided into 17 blocks of variable and conserved regions (Tanabe *et al.*, 1987; Miller *et al*., 1993). With the exception of the highly polymorphic block 2, the genes are dimorphic and can be classified into the MAD-20 and Wellcome families with additional variance being produced by limited intragenic recombination between the families. Block 2 of MSP-1 can be classified into three families, MAD-20, Wellcome and RO33 (Miller *et al.*, 1993). Block 17 at the extreme C-terminus, which corresponds to MSP-1_{19}, is highly conserved, although there are limited polymorphisms identified at specific residues in this region (Kang *et al.*, 1995; Tolle *et al.*, 1995).

The protective role of the C-terminal regions of MSP-1 has been assessed in a number of studies that use recombinant MSP-1_{42} and MSP-1_{19} (reviewed by Holder *et al.*, 1996). It has been shown that the presence of antibodies against the MSP-1_{42} (Riley *et al.*, 1992) and MSP-1_{19} (Egan *et al.*, 1996; Branch *et al.*, 1998; Perraut *et al.*, 2005) are associated with protection from clinical *P. falciparum* infection. In addition, a longitudinal survey conducted with infants in Liberia indicated that the high levels of maternally derived antibodies against MSP-1_{19} in the first year of life

were associated with protection from clinical malaria (Hogh *et al.*, 1995). Another study conducted with children and adults in the Gambia demonstrated a correlation between protection from clinical malaria and the ability of sera to block binding of two monoclonal antibodies against different epitopes on MSP-1_{19} by ELISA (Shai *et al.*, 1995). Moreover, immunisation with a recombinant baculovirus *P. falciparum* MSP-1_{42} (strain FUP) in complete Freund's adjuvant provides protection in Aotus monkeys against lethal parasite challenge (Chang *et al.*, 1996). All vaccinated, protected Aotus monkeys produced antibodies which inhibited *in vitro* parasite growth, indicating that the immunity induced by MSP-1_{42} immunization is mediated, at least in part, by a direct effect of antibodies against the MSP-1 C-terminal region. Likewise, vaccination with a recombinant MSP1_{42} from *P. falciparum* strain FVO expressed in *Escherichia coli* protected monkey against the blood challenge infection with homologous strain (Darko *et al.*, 2005). Protective immunity in these monkeys was found to be correlated with antibodies against the epidermal growth factor (EGF)-like domain 2 fragment of MSP1_{42} (Darko *et al.*, 2005). Therefore, these lines of evidence support further development of MSP-1_{42} and MSP-1_{19} as a candidate malaria vaccine.

The most advanced MSP-1 based vaccine is FMP1/AS02. This vaccine consists of a MSP-1_{42} fragment from *P. falciparum* clone 3D7 expressed in *E. coli*, and formulated in the AS02 adjuvant. Preclinical evaluation of FMP1 in the rhesus monkey safety and immunogenicity model identified AS02A as a safe, well-tolerated, and highly immunogenic adjuvant (Pichyangkul *et al.*, 2004). FMP1/AS02 have been subsequently assessed in clinical trials in human volunteers in the USA, Kenya and Mali (Ockenhouse *et al.*, 2006; Stoute *et al.*, 2007) and found to be safe and immunogenic. However, in a subsequent proof of concept efficacy phase IIb trail in Kenyan children, the vaccine showed no evidence of protection from natural malaria infections and no efficacy to reduce the number of episodes of clinical malaria [quoted from Genton *et al*., 2007].

Another leading MSP-1 based erythrocytic vaccine candidate is MSP-1 C1, based on the use of the 42kDa fragment from *P. falciparum* clones 3D7 and FVO, formulated in Alhydrogel. The *msp*-1 alleles of the 3D7 and FVO parasites lines represent the two major allelelic types, MAD20 and K1, of this molecule. In a phase I trial in malaria-naive volunteers in the USA, MSP1(42)-FVO/Alhydrogel and MSP1(42)-3D7/Alhydrogel was found to be safe, but not sufficiently immunogenic (Malkin *et al.*, 2007). Vaccine development is currently in progress to improve immunogenicity of the Alhydrogel formulation in humans. A vaccine candidate based upon the 19 kDa C-terminal fragment of MSP-1 has also been produced at Baylor University, but a Phase I trial carried out demonstrated that this vaccine had unacceptable side effects and was poorly immunogenic (Girard *et al.*, 2007).

Though the conserved MSP-1_{42} and MSP-1_{19} fragments have been the focus of vaccine development efforts, there is evidence from epidemiological and immunological studies that the polymorphic N-terminal block 2 region of MSP-1 may be a target of protective immunity and of stain-specific protective immunity against *P. falciparum* infection (Conway *et al.*, 2000; Polley *et al.*, 2003a; see section 1.6.1 for discussion). Currently, block 2 of *P. falciparum* MSP-1 has been selected for inclusion in a subunit vaccine against malaria that is under development at Edinburgh University (D. Cavanagh, personal communication).

1.5.2.2 MSP-2 based vaccines

P. falciparum MSP-2 is a protein on the merozoite surface, encoded by a single-copy gene on chromosome 2, with a predict molecular weight between 46 and 53 kDa (Smythe *et al.*, 1988). The gene for MSP-2 is located on *P. falciparum* chromosome 2 (Marshall *et al.*, 1998) and is divided into five blocks. Blocks 1 and 5 encode highly conserved N- and C-termini, respectively. The central variable region, block 3, consists of centrally located repeats, which are flanked by non-repetitive sequences. MSP-2 sequences are assigned into one of the two families, FC27 and IC-

1/3D7, on the basis of the non-repetitive sequences (Smythe *et al.*, 1990; Smythe *et al.*, 1991; Marshall *et al.*, 1992; Felger *et al.*, 1997).

Evidence for involvement of MSP-2 in protective immunity derives from several studies. The presence of IgG antibodies to the 3D7 allele of MSP-2 has been found to be associated with protection from clinical malaria in Papua New Guinea (Al-Yeman *et al.*, 1994), in the Gambia (Taylor *et al.*, 1998; Metzger *et al.*, 2003) and, more recently, in Kenya (Polley *et al.*, 2006). The extent of antibody reactivity to MSP-2 is sequence dependent, such that antibodies are inhibitory to parasites expressing on the same allelic type of MSP-2 but not parasites expressing a different allelic type (Ranford-Cartwright *et al.*, 1996).

A recombinant MSP-2 protein from *P. falciparum* clone 3D7 was included in the multivariant erythrocytic vaccine Combination B, which also contained the conserved blocks 3-4 of MSP-1 from *P. falciparum* strain K1 and part of the ring-infected erythrocytic surface antigen (RESA) in a Montaside 720 adjuvant formulation (Lawrence *et al.*, 2000). Though vaccination of naïve volunteers with Combination B vaccines followed by blood-stage challenge failed to provide any protection (Lawrence *et al.*, 2000), the vaccine demonstrated a 62% reduction in parasite densities in the vaccine group in those not pretreated with sulfadoxine-pyrimethamine when compared to those in the placebo group (Genton *et al.*, 2002) in a phase IIb trial conducted in children, aged 5 to 9 years, in Papua New Guinea. Furthermore, it was found that the prevalence of parasites carrying alleles belong to the 3D7 family appeared to be lower in vaccinated children when compared to the placebo group. However, the prevalence of parasites carrying alleles of the other allelic type of MSP-2, FC-27, was not different between the vaccinee and placebo groups. These findings demonstrated that the activity of the Combination B vaccine is due, at least in part, to the MSP-2 component (Genton *et al.*, 2002), which seems to confer protection in the vaccinees against blood stage infections with malaria parasites containing the homologous allele of MSP-2.

Following the promising results of the phase IIb trial in Papua New Guinea (Genton *et al.*, 2002), an MSP-2 based vaccine that comprise two major variants of MSP-2 from *P. falciparum* clones 3D7 and FC27 is now developed. A phase I trial in malaria-naïve adults in Australia is underway with a Montanide ISA720 formulation (R. Anders, unpublished; quoted from Genton *et al.*, 2007).

1.5.2.3 MSP-3 based vaccines

MSP-3 is a ~45-76 kDa polypeptide that is synthesised by mature stage parasites and secreted into the parasitophorous vacuole (McColl *et al*., 1994; Oeuvray et al., 1994a). The MSP-3 protein was first detected using human hyperimmune serum and with antibodies that inhibit *P. falciparum* asexual blood stage growth in vitro by incorporation with monocytes in an antibody-dependent cellular inhibition (ACDI) assay (Oeuvray *et al*., 1994b). There is evidence to implicate MSP-3 as a potential target of protective immune responses against malaria infection. In a primate model, vaccination with recombinant *P. falciparum* MSP-3 can protect Aotus monkeys from a lethal blood stage challenge infection (Hisaeda *et al.*, 2002). More recently, evidence from epidemiological and immunological studies showed that the levels of IgG3 antibodies against the allele-specific and conserved epitopes in MSP-3 were strongly associated with protection from clinical malaria (Osier *et al.*, 2007; Polley *et al.*, 2007; Roussolhon *et al.*, 2007; Soe *et al.*, 2007), suggesting the involvement of MSP-3 in protective immunity against malaria in humans. These observations support the further development of MSP-3 based vaccine formulations.

Thus, the Pasteur institute and the Europian Malaria Vaccine Initiative have developed an erythrocytic vaccine based on MSP-3. It has been developed as a long synthetic peptide incorporating regions of MSP-3 and containing three human B cell epitopes and T cell epitopes. These epitopes were selected on the basis of the finding that they were targeted by cytophilic antibodies that participate in ACDI (Oeuvray *et al.*, 1994b). In a phase Ia study in Burkina Faso, the MSP-3 vaccine, formulated in either alum or Montanide ISA720, induced sustained high levels of antibody

responses displaying ACDI activity (Audran *et al*., 2005). When passively transferred into *P. falciparum*–infected humanized SCID mice, the antibodies from the vaccinated volunteers could abrogate parasitaemia in mice (Druilhe *et al.*, 2005). The encouraging safety and immunogenicity of this vaccine in adults allows its entry into a phase Ib trial in children. Such vaccine trials are currently underway in Burkina Faso and Tanzania (quoted from Genton *et al.*, 2007).

1.5.2.4 *Erythrocytic vaccines based on other antigens*

Additional blood stage antigens of *P. falciparum* under development as vaccine candidates include AMA-1, the serine repeat antigen (SERA), the Glutamine-rich protein and other merozoite surface proteins (MSP-4 and MSP-5). Progress towards the development of erythrocytic vaccines based these antigens is described elsewhere (Genton *et al.*, 2007; Girard *et al.*, 2007).

1.5.3 Challenges

Results obtained from clinical trials of human malaria vaccines have shown only moderate success. One of the major obstacles to the development of malaria vaccines is polymorphism of the vaccine candidates. For instance, following the clinical trials of the Combination B (Genton *et al.*, 2002), *P. falciparum* identified in breakthrough infections from the vaccinated volunteers were found to carry alleles of MSP-2 that were not present in the vaccine formulation (Felger *et al.*, 2003; Flück *et al.*, 2007). This suggests that all allelic variants of MSP-2 need to be included in the vaccine in order to counteract the effect of polymorphism of the candidate antigen. Since merozoite surface antigens, such as MSP-1 and AMA-1, also have a substantial polymorphism, this may have an impact on immunogenicity and protective effectiveness (Tanabe *et al.*, 1987; Miller *et al.*, 1993; Marshall *et al.*, 1996). Thus, there is an urgent need to obtain molecular epidemiological data of all the possible variants of the antigens present in a vaccine testing site prior to conducting clinical

vaccine trials. This information would enable measurement and interpretation of the host and parasite responses to vaccines, both during efficacy trials and after introduction of vaccines into the population (Genton *et al.*, 2007; Sutherland, 2007). This data may provide invaluable insights into vaccine-induced selection pressure acting on antigen genes and the potential implications of allele-specific immunity. Such an investigation was already performed in a vaccine-testing site in Mali in which the population-level dynamics of 14 different haplotypes encoding MSP-1_{19} were analysed prior to conducting clinical trials (Takala *et al.*, 2007).

To improve malaria vaccine research and development we must also gain the definitive knowledge on the nature of host protective immunity to malaria and understand the mechanisms by which parasites escape immunity. This information would allow us to identify and prioritise appropriate antigens as vaccine candidates and, therefore, develop formulations that could elicit desired protective immune responses (Girard *et al.*, 2007).

1.6 Rodent malaria parasites

There are eleven species of malaria parasite that occur naturally in African rodents (Cox, 1988; Cox, 1993). Of these species, four from murine rodents have been grown in laboratory mice: *Plasmodium berghei* (Vincke and Lips, 1948), *Plasmodium vinckei* (Rodhain, 1952), *Plasmodium chabaudi* (Landau, 1965) and *Plasmodium yoelii* (Landau and Killick-Kendrick, 1966; Killick-Kendrick, 1974). The main features of the four rodent malaria species are described elsewhere (Garnham, 1966; Carter *et al.*, 1977; Landau *et al.*, 1978; Cox, 1988; Cox, 1993; Landau *et al.*, 1994; Landau *et al.*, 1998). The locations from which the four species of most commonly used rodent malaria parasites were isolated are illustrated in Figure 1.3.

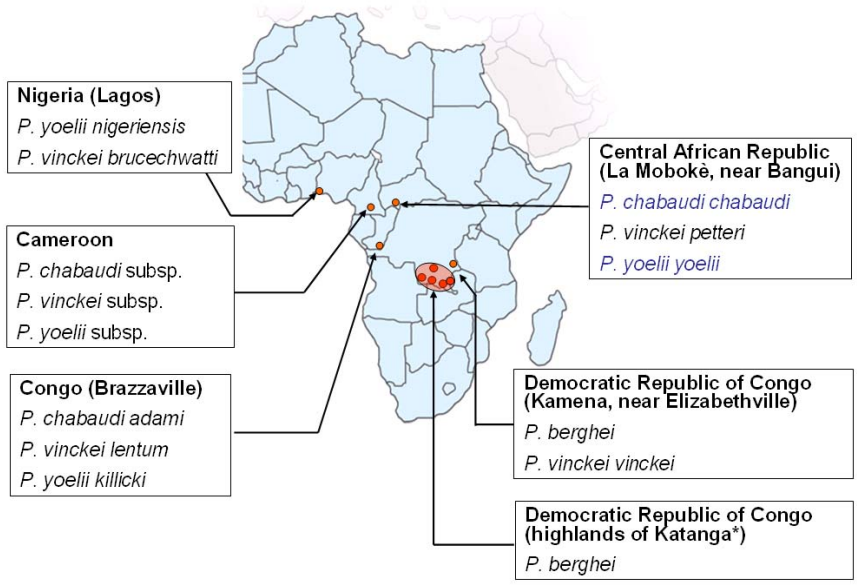

Figure 1.3. The origin of the four species of rodent malaria parasites in Africa. Two species of rodent malaria, *Plasmodium yoelii yoelii* and *Plasmodium chabaudi* used in the present studies (blue letters) originated from thicket rats, captured in the Central African Republic (see text). Red dots represent foci from which murine hosts were collected. The distribution of the rodent malaria parasites in Africa was previously described by Carter *et al.*, (1977); Killick-Kendrick (1978) and Landau *et al.*, (1994).
* Foci in the highlands of Katangain Democratic republic of Congo from which *Plasmodium berghei* was isolated included Kisanga and Kasapa near Elizabethville; Kanzenzi near Kolwezi; near Albertsville; Plateau of Kundelungu; Kamina; Sandoa, represented by red dots in a transparent oval, (Garnham, 1966).

The experiments described herein were carried out using two species of rodent malaria parasites: *Plasmodium chabaudi* and *Plasmodium yoelii*.

Plasmodium chabaudi

P. chabaudi was originally isolated from blood of wild thicket rats, *Thamnomys rutilans*, captured in the Central African Republic (La Mobokè, near Bangui) in 1965 (Landau, 1965) and in the Congo (Brazzaville) between 1970 and 1972 (Carter *et al.*, 1976) (see Figure 1.3). The parasites in each region were accorded subspecies status as *Plasmodium chabaudi chabaudi* (Landau, 1965) and *Plasmodium chabaudi adami* (Carter and Walliker, 1976), respectively. Many strains of *P. c. chabaudi* have been derived and used in many laboratories (Beale *et al.*, 1978; Walliker, 1983). Two cloned strains denoted AJ and CB of *P. c. chabaudi* used in the present study were derived from isolates AJ and CB (Carter, 1978) (see Chapter 2).

Parasites belonging to the *P. chabaudi* group show a predilection for mature erythrocytes (normocytes), which more closely resembles *P. vinckei* than *P. berghei* or *P. yoelii*. Blood infections of *P. chabaudi* are synchronous with a periodicity of 24 hours. The main distinguishing features are the virtual absence of malaria pigment in the growing trophozoite stage, although the pigment is apparent in blood stage schizonts as a single large granule of black pigment at the centre or edge of the dividing parasite (Carter *et al.*, 1975a; Carter *et al.*, 1977). The number of merozoites per schizont is between 6 and 8 (Carter *et al.*, 1975a). The two subspecies of *P. c. chabaudi* are identical morphologically; they are distinguishable only by the electrophoretic enzyme characteristics of the blood stage parasites (see Table 1.2).

Plasmodium yoelii

P. yoelii was isolated from blood of adult thicket rats, *T. rutilans*, captured in three geographically isolated localities: the Central African Republic (La Mobokè, near Bangui) in 1963, 1965, 1969 and 1970, Congo (Brazzaville) in 1966 and 1968, and Nigeria (Lagos) in 1970 (Carter, 1978; Beale *et al.*, 1978) (see Figure 1.3). The

parasites in each region were given subspecies status: *Plasmodium yoelii yoelii* (Landau and Killick-Kendrick 1966), *Plasmodium yoelii killicki* (Landau, Michel and Adam, 1968), and *Plasmodium yoelii nigeriensis* (Killick-Kendrick, 1973). *P. yoelii* was initially classified as a subspecies of *P. berghei* under the name of *P. berghei yoelii* (Landau *et al.*, 1966). This parasite was renamed *P. y. yoelii* by Killick-Kendrick in 1974. Numerous strains of *P. y. yoelii* have been derived (Beale *et al.*, 1978). One of the most commonly studied strains of this parasite is denoted 17X (see Chapter 3).

In the morphology of their blood stages, the three subspecies of *P. yoelii* and, indeed, *P. berghei* are indistinguishable from each other. Blood infections of *P. yoelii* are asynchronous (Carter *et al.*, 1977). The blood stages of both species preferentially invade immature erythrocytes (reticulocytes), although some strains of *P. y. yoelii* are able to invade normocytes extensively during the late phase of an infection (Yoeli *et al.*, 1975; Finerty *et al.*, 1976; Finerty *et al.*, 1977; Burns *et al.*, 1989) (see also Chapter 3 and 4). The number of merozoites per schizont is between 12 and 18 (Landau *et al.*, 1978). The main distinction between *P. yoelii* and *P. berghei* lies in the characteristics of the sporogonic stages and in the enzyme character of the blood stages. The sporogonic development of *P. yoelii* is completed between 24˚C and 26˚C, but at this temperature *P. berghei* fails to develop. The optimum temperature for the sporogonic development of *P. berghei* is between 19˚C and 21˚C. All lines of *P. yoelii* so tested are resistant to chloroquine in the presence of *para*-aminobenzoic acid (PABA) in the host diet (Carter, 1972), whereas strains of *P. berghei* are normally susceptible to this drug with or without the presence of PABA supplemented in the diet. The distinctive enzyme characteristics of the three subspecies of *P. yoelii* are shown in Table 1.2.

Species and Subspecies of rodent malarias	Characteristic enzyme forms			
	GPI	GDH	LDH	6PGD
Plasmodium berghei	3	3	1	1
Plasmodium yoelii yoelii	1,2,10	4	1	4
Plasmodium yoelii killicki	1	1	1	4
Plasmodium yoelii nigeriensis	2	2	1	4
Plasmodium vinckei	5,6,7,9,11	6	6,7,9	5,6
Plasmodium chabaudi chabaudi	4	5	2,3,4,5	2,3,7
Plasmodium chabaudi adami	8	5	8,10	2

Table 1.2. Characteristic enzyme forms among species and subspecies of African rodent malaria parasites (Carter, 1978).
GPI, glucose phosphate isomerase;
GDH, NADP-dependent glutamate dehydrogenase;
LDH, Lactate dehydrogenase;
6PGD, 6-phospho gluconate dehydrogenase.

1.7 Medically important phenotypes of malaria parasites

Malaria parasites exhibit wide variation in their phenotypes between species and between strains of the same species. Examples of phenotypes are antigenic polymorphisms (i.e. the presence of allelic forms) in genes encoding surface antigens of malaria parasites) and blood stage growth rate. This diversity is of medical importance since (1) the antigenic polymorphism may contribute to evasion of host immune response, probably due to allele specificity of host protective immunity (Mendis *et al.*, 1991), and (2) growth rate can be positively correlated with pathological severity of an infection (Dondrop *et al.*, 2005). Thus, these phenotypes can not only determine the survival of the parasites in the host but also influence the outcome of an infection. The genetic determinants that control some of the medically important phenotypes of malaria parasite, such as antigens determining strain specific protective immunity and proteins involved in blood stage growth rate, are, however, poorly understood. These two phenotypes are, therefore, the main subjects of the present studies.

1.7.1 Strain Specific Protective Immunity

Protective, but not sterilising, immunity against blood stages of malaria parasites, particularly human malaria parasites, can be induced in individuals after several years of exposure to endemic malaria (McGregor *et al.*, 1956; McGregor, 1974; Day *et al.*, 1991). This reflects the need to be infected with a large number of antigenically diverse parasite populations in order to become effectively immune (Mendis *et al.*, 1991). By contrast, some studies showed that acquired protective immunity against the blood stages of human malaria parasites can be readily achieved after only one or a few infections (Ciuca *et al.*, 1934; Boyd *et al.*, 1936). A study reported by Jeffrey (1966) showed that immunity was most effective in individuals who had been previously infected with the homologous genotype (strain) of human malaria parasites, while it became less effective against a heterologous (genetically distinct) strain. This effect has led to the concept of ‘Strain Specific Protective Immunity’ (SSPI) in malaria.

The first study on SSPI in rodent malaria was conducted using *P. c. chabaudi* lines AS and CB by Jarra and Brown (1985) in a protocol in which parasites of one or the other strain were used to induce an immunity in laboratory mice and subsequently used for challenge infection. The studies showed that mice infected with strain AS developed strong immunity against the challenge infection with blood stages of the homologous strain (AS), but developed much weaker immunity against those of a heterologous strain (CB). In a reciprocal experiment, mice infected with strain CB were protected from challenge infection with CB, but not AS. This experiment was recently repeated and extended in our laboratory, using other genetically distinct strains of *P. c. chabaudi*, including AJ, AS, AQ and CB (Martinelli *et al.*, 2005a; Cheesman *et al.*, 2006). These studies again confirmed the existence of SSPI of one degree or another to all strain combinations of *P. c. chabaudi* in laboratory mice (Martinelli *et al.*, 2005a; Cheesman *et al.*, 2006) as was also found in the previous study (Jarra *et al.*, 1985).

Several antigens of the blood stages of malaria parasites might be targets of SSPI. It would, however, be necessary that the antigens targeted by SSPI are antigenically and genetically different among different strains, or genetically distinct lines, of malaria parasite. There is a high degree of allelic polymorphism in genes encoding parasite surface-located antigens. This antigenic diversity means that among genetically distinct strains of the same species of malaria parasite, each candidate can be represented in more than one allelic form, each of which may be a target of host protective immunity. Prominent candidate antigens of SSPI are the merozoite surface protein-1 (MSP-1) and apical membrane antigen-1 (AMA-1). In the following section I will describe literature that demonstrates the possible involvement of MSP-1 and AMA-1 in SSPI in rodents and humans.

MSP-1 is considered a prime candidate target of protective immunity and of SSPI in humans (see section 1.5.2.1) and rodents, and is also the most extensively studied surface antigen of the merozoites. Data from several experimental studies in rodent malarias have demonstrated that MSP-1 exhibit extensive sequence polymorphism

among laboratory strains of the rodent malaria parasite *P. c. chabaudi* and *P. yoelii* (Cheesman S., unpublished data). Immunisation with recombinant MSP-1 could induce antibody-mediated immune responses, which are highly effective in eliminating parasites and protecting hosts against a malarial infection in a strain specific manner. For instance, immunisation with the recombinant C-terminus MSP-1 of *P. y. yoelii* 17XYM completely protected mice against sporozoite-induced infections with the same strain, but was not protective against challenge with sporozoites of a non-homologous strain, *P. y. yoelii* 265BY (Renia *et al.*, 1997). In addition, passive transfer of a monoclonal antibody raised against the C-terminus of MSP-1 of *P. c. chabaudi* AS protected mice specifically against challenge with the homologous strain (AS) (Boyle *et al.*, 1982), but had no effect on challenge infection with a heterologous strain CB (Brown *et al.*, 1985).

Furthermore, there is evidence from epidemiological studies in human malarias to suggest the possible involvement of MSP-1 in SSPI in humans. Field studies on malaria in endemic human populations have used molecular population genetic approaches to test the involvement of antigens in protective immunity and to test the "strain" or allelic-type specificity of this immunity. The underlying hypothesis of these studies is that target loci encode products that induce allele specific protective immune responses. Conway and colleagues (2000) proposed that there should be frequency-dependent selection on a polymorphic target antigen of anti-parasitic immunity, so that a parasite with a more frequent allelic type is more likely to be eliminated by protective immunity than is a parasite with a rare allelic type (Conway *et al.*, 2000). Thus, the high prevalence of a frequent antigenic type should lead to its reduction in prevalence, while prevalence of a rare antigenic type is increased under anti-parasitic allele specific immune selection. This leads to a concept of 'balancing selection' which perpetuates both polymorphisms in the population at stable equilibrium frequencies.

Following this approach, Conway and colleagues measured the allele-frequencies on different blocks of the *P. falciparum msp*-1 gene in parasite populations in different geographical regions in Africa and Asia (Conway *et al.*, 2000). Their results showed

that block 2 of MSP-1 had relatively conserved allele frequencies in parasite populations from different endemic regions, while the other blocks of MSP-1 exhibited more divergent allele frequencies in populations from different geographical regions. These findings are consistent with block 2 of MSP-1 evolving under allele specific balancing selection as would be expected of a target of SSPI against a malarial infection.

To test whether MSP-1 block 2 is the target of protective immunity, Conway and colleagues collected serum prior to malaria transmission season from a cohort of children (3 to 7 years old) in The Gambia and tested for antibody reactivity to recombinant proteins representing different allelic forms of full-length block 2 of MSP-1. They determined the ratios of individuals who experienced clinical malaria at any time during the transmission season, among those with or without detectable serum IgG levels against recombinant MSP-1 antigens at the start of the study. The results indicated that antibodies against either of the two most frequent allelic types of MSP-1 block 2 were strongly associated with a lower prospective risk of clinical malaria, whereas antibodies to the rarest block 2 type were not significantly associated with protection from *P. falciparum* malaria (Conway *et al.*, 2000). Consistent with the above, Cavanagh and his co-workers (2004) found that antibodies to full-length antigens from the two common MSP-1 block 2 allelic types were strongly associated with protection against malaria in a cohort of children (3 to 15 years old) in Ghana. The study also showed that while there were no significant association between antibodies to the MSP-1 block 2 flanking sequence antigens of either the K1 or the MAD20 block 2 types and a reduced risk of clinical malaria, antibodies to the MSP-1 block 2 repeat sequence antigens are strongly associated with protection from clinical malaria (Cavanagh *et al.*, 2004). The above finding is consistent with results from another cohort from The Gambia (Polley *et al.*, 2003a). Together, these studies provide evidence for the involvement of MSP-1 block 2 as a target of protective immunity against *P. falciparum* malaria in humans, and that this immunity acts specifically against the products of allelic types of the *msp*-1 gene.

AMA-1 is also considered to be a possible target of SSPI against human and rodent malaria parasites. Mice immunised with the refolded ectodomain of the *P. c. adami* DS allele of AMA-1 were protected against challenge with blood stages of the homologous strain, DS, but were not protected against challenge with a non-homologous strain, *P. c. adami* 556KA (Crewther *et al.*, 1996). In a reciprocal experiment, immunisation of mice with the refolded ectodomain of the *P. c. adami* DK allele of AMA-1 induced protective immunity against challenge with DK strain but not DS strain (Barclay V. and Read A.F., personal communication). Furthermore, on passive transfer into non-immune mice, the IgG antibodies which had been raised against the refolded recombinant DS AMA-1, were able to confer protection against challenge with the homologous strain, but had no protective effects on challenge with a heterologous strain (Crewther *et al.*, 1996).

Furthermore, data from epidemiological studies in human malaria have indicated the possible association of AMA-1 with SSPI in humans. Using the molecular population genetic approaches of Conway *et al.* (2000), Polley and others analysed sequences of the gene for AMA-1 from natural populations of *P. falciparum* from Nigeria (Polley *et al.*, 2001) and Thailand (Polley *et al.*, 2003b). In both of these cases, Domains I and III of the ectodomain region (N-terminus) of AMA-1 were considered to be evolving under strong balancing selection. It was, therefore, predicted that naturally acquired protective immune responses exist against allele-specific epitopes in the Domains I and III of AMA-1 protein (Polley *et al.*, 2001; Polley *et al.*, 2003b). Thus, Polley and colleagues (2004) collected serum from two cohorts (e.g. two villages) of children and adults (0 to 85 years old) in Kenya prior to a malaria transmission season, to determine the relationship between antibodies to the recombinant AMA-1 proteins representing the *P. falciparum* FVO or 3D7 full-length ectodomain and subsequent incidence of malaria. The results showed that the presence of the antibodies to the recombinant proteins representing either the full-length ectodomain of FVO or 3D7 AMA-1 reduced the risk of having a subsequent episode of malaria (Polley *et al.*, 2004). These findings have indicated, therefore, that AMA-1 appears to be a target of allele-specific protective immunity against *P. falciparum* malaria in human populations.

1.7.2 Growth rate

In the context of the present subject, the following definitions of terms are used.

'*Growth rate*' refers to the replication rate of malaria parasites in the blood. Lines of malaria parasites that are considered to have a 'fast' growth rate phenotype refers to those developing a high parasite density (high levels of parasitaemia), and causing a fulminating and lethal infection in their host. Lines of malaria parasites that are considered to have a 'slow' growth rate phenotype refers to those producing a low parasite density (low levels of parasitaemia) throughout infection, and normally causing a mild, self-limiting disease. The term 'growth rate' described herein has a different meaning from 'parasite multiplication rate', which is an indication of how many erythrocytes are newly infected when a schizont bursts (Chotivanich *et al.*, 2000; Deans *et al.*, 2006).

'*Virulence*' refers to the ability of malaria parasites to do harm to their host following infection, where harm is defined as disease severity (morbidity) and/or mortality (Mackinnon *et al.*, 2002; Mackinnon *et al.*, 2004).

All *Plasmodium* species have an asexual haploid life stage that replicates in the blood of their vertebrate hosts, thereby causing destruction of infected erythrocytes leading to general pathology. The clinical outcome of a malarial infection can be highly variable and dependent upon the interactions of multiple factors from the hosts and parasites themselves (Miller *et al.*, 2002). Environmental changes, such as the effects of PABA in the growth of *P. berghei* (Jacobs, 1964; Carter, 1972), or concurrent infections with other organisms (Cox, 1978; Cox, 2001), are well-known causes for alternation in the natural course of infection of *Plasmodium*. Host genetic factors determine the ability of parasites to grow in the host. For instance, while individuals that are homozygous for the allele of the β-globin gene for haemoglobin variant S (HbS) develop sickle cell anaemia, heterozygotes are healthy and strongly protected against severe *P. falciparum* malaria (Beet, 1946; Allison, 1954; Aidoo *et al.*, 2002). HbS is caused by a single nucleotide substitution (A to T) of codon GAG, which results in an amino acid substitution from glutamic acid to valine at position 6 in the

β-globin. The Duffy-negative ($FY^{null}FY^{null}$) erythrocyte genotype is also involved in resistance to *P. vivax* infection (Miller *et al.*, 1976). Furthermore, different strains, or genotypes, of laboratory mice can influence the growth rate of a rodent malaria parasite. Some strains of mice, e.g. DBA/2, are resistant to a slow-growing strain of *P. y. yoelii* 17X, while being highly susceptible to a lethal strain of *P. y. yoelii* 17XYM. In contrast, it has been reported that some mouse strains, e.g. C57BL/6, are resistant to *P. y. yoelii* strain 17XYM, but are sensitive to strain 17X (Sayles *et al.*, 1988). However, when C57BL/6 mice were infected with sporozoites, or inoculated intra-peritoneally with blood stages, of *P. y. yoelii* strain 17XYM in our laboratory, we found that no mice of this strain, C57BL/6, survived (Pattaradilokrat S. and Carter R., unpublished observation). Our observation has been made several times and is in absolute contrast to that reported by Sayles and Wassom (1988). The reason for this discrepancy is not known, and this subject needs further investigation. However, mixing of the two parasite strains of 17X must be considered to be a possibility.

In addition to environment and host factors, parasite-associated factors, as already mentioned above, appear to contribute to differences in the clinical outcome of a malarial infection (see a review by Miller *et al.*, 2002). One factor positively associated with severe pathology is the rapid multiplication of the parasites in the host, or blood stage growth rate (Yoeli *et al.*, 1975; Dondrop *et al.*, 2005). Differences in growth rate of malaria parasites of humans and rodents are likely to be due to variation in multiplication ability of the blood stages and their selectivity for different classes, e.g. age, of erythrocyte. It is known that different strains of malaria parasites that vary in growth rate have different multiplication ability (Simpson *et al.,* 2002). In a clinical study of *P. falciparum* malaria in Thailand, isolates from patients with severe clinical symptoms were found to multiply three times as fast and to be less selective in their invasion of erythrocytes, than those from mild cases (Chotivanich *et al.,* 2000). By contrast, this was not found to be the case with *P. falciparum* isolates from Mali and Kenya, in which there were no significant differences in selectivity or multiplication ability between parasites from severe or mild malaria cases (Deans *et al.*, 2006).

Differences in growth rate can be related to the ability of the blood stages of the parasite to invade erythrocytes of different ages. It has long been known that merozoites of certain malaria species infecting humans or other animals have a predilection for a minor sub-population of erythrocytes, the reticulocytes. These include the human malaria parasites, *P. vivax* (Craik, 1920) and *P. ovale* (Garnham, 1966), a simian malaria parasite, *P. cynomolgi* (Garnham, 1966), and a rodent malaria parasite, *P. berghei* (Garnham, 1966) and *P. yoelii* (Carter *et al.*, 1977; Landau *et al.*, 1978). Since reticulocytes only make up a small proportion of the total erythrocyte content, usually below 2% in humans (Bessman, 1990), reticulocyte-preferring *Plasmodium* species tend to have slow growth rate and low parasite density, with rare severe pathology and mortality. By contrast, the human malaria parasite, *P. falciparum* (Bruce-Chwatt, 1948), and the rodent malaria parasite, *P. chabaudi* (Carter *et al.*, 1975a; Carter *et al.*, 1976), generally invade erythrocytes of all ages, although they are mostly seen in mature erythrocytes (normocytes). *Plasmodium* species of this phenotype could lead to a much higher parasite density, a condition that frequently causes severe anaemia and other potentially life-threatening disease symptoms, compared to the *vivax*-type malaria parasites.

Such variation in growth rate is also observed between genotypes, or 'clones', of the same species of the malaria parasite infecting rodents (see Chapter 3). Many *P. y. yoelii* lines derived from isolate 17X, such as 17XA and 17XNIMR, have a slow growth rate phenotype. Parasites of this phenotype show marked preference for reticulocytes (Figure 1.4, panel B), and undergo a mild course of infection in mice. In contrast, there are a few *P. y. yoelii* lines with fast growth rate, designated 17XYM (Yoeli *et al.*, 1975) and 17XL (Finerty *et al.*, 1976; Finerty *et al.*, 1977). These strains emerged spontaneously during passage of the normally slow-growing *P. y. yoelii* strain 17X, and developed a dramatic increase in growth rate and virulence, compared to their original stock. Although retaining reticulocyte invasion preference in early infection, after four or five days these parasites switch to invade mainly normocytes (Figure 1.4, panel A). Parasites of this group are able to produce a high parasite density. As a result, infected animals normally die on the six or seventh day

of infection, with mean parasitaemia of 60 to 80% (Yoeli *et al.,* 1975; Burns *et al.,* 1989).

The first genetic study of growth rate in a rodent malaria parasite was conducted by Walliker and his co-workers (1976), by crossing two lines that differed in growth rate, isozyme type and pyrimethamine-sensitivity. The fast-growing line was *P. y. yoelii* 17XYM, and the slow-growing line was *P. y. yoelii* A/C. Analysis of the cloned progeny of the 17XYM x A/C genetic cross showed that the fast growth rate and slow growth rate characters segregated in the cloned progeny, implicating a single locus for genes conferring these growth characters. However, the genetic determinants of growth rate in this parasite are not known. This subject will be investigated in the present study.

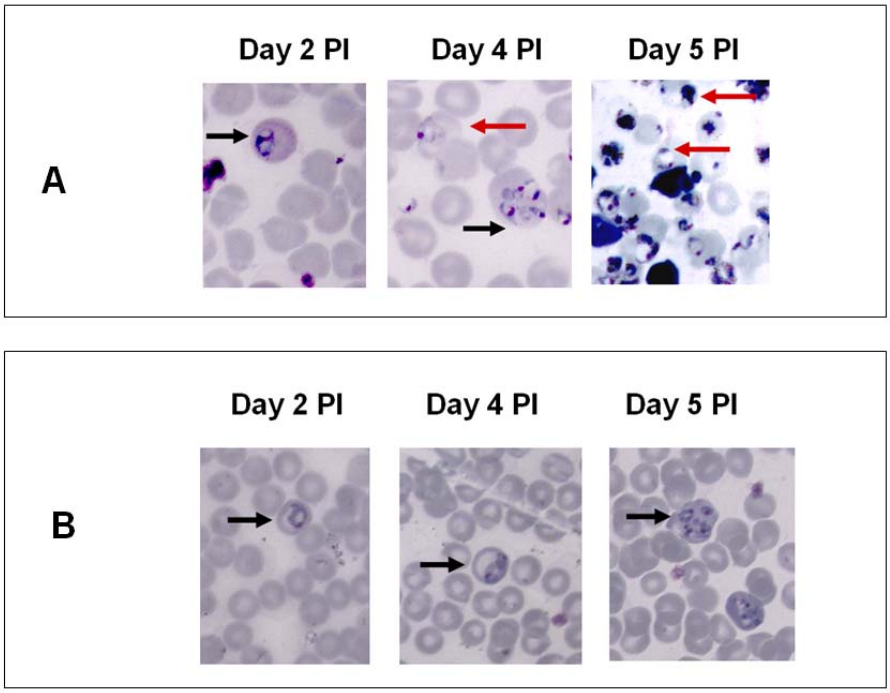

Figure 1.4 Microscopic examination of blood stage-induced infections with (panel A) a fast- and (panel B) slow-growing line of the rodent malaria parasite *Plasmodium yoelii yoelii*. Parasites with fast growth rate were 17XYM. Parasites with slow growth rate were 17XA. Laboratory mice were inoculated intra-peritoneally with 1 x 10^6 parasitised erythrocytes of either 17XYM or 17XA line. Infections were monitored by light microscopy of thin blood smears stained with Giemsa's stain on day 2, 4 and 5 post-inoculation. All photos were taken at 1000 x magnification. Parasitised reticulocytes and normocytes are indicated by black and red arrows, respectively. Photos were kindly supplied by Dr Richard Culleton (Nagasaki University).

1.8 Genetics of malaria parasites

1.8.1 Glossary

In the context of this thesis, the following definitions of terms are used (Carter, 1973; Carter, 1978; Walliker, 2005).

An '*isolate*' refers to a single sample of parasite material that has been collected from a naturally infected host specimen on a single occasion. The parasite cells are not necessarily genetically identical and may contain representatives of several distinct species.

A '*line*' refers to the progeny of parasites derived by laboratory passage on a unique occasion. Like an isolate, a line of parasites may not be genetically homogeneous.

A '*strain*' may be used to refer to lines of parasites presumed to represent a single species and having derived from the same isolate. For example, lines derived from isolate 17X and apparently containing only *P. y. yoelii* may be referred to collectively as *P. y. yoelii* strain 17X.

A '*clone*' refers to the asexually dividing progeny of a single cell. Assuming that no spontaneous mutations or mitotic recombination had taken place during parasite growth, all parasite cells of a single clone should have identical genetic constitutions.

1.8.2 Genetic variation in malaria parasites

Considerable phenotypic variation occurs in natural populations of human and rodent malaria parasites of the same species. Many studies in the past reported that some variation did occur between 'strains' of the human malaria parasites, *P. falciparum* and *P. vivax*, isolated from infected individuals from various geographical regions. Some of the variation is manifest as differences in virulence (see section 1.7.2 for definition) and in ability to escape the "strain specific" components of the host's protective immune response. One of the earliest examples was found nearly 80 years

ago following the use of induced malaria for the therapy of neurosyphilis. It was found that "A striking difference can be observed between the clinical virulence of different geographical races or strains of the same morphological species of the parasite; the difference is particularly apparent between various races of *P. falciparum*" (quoted from James *et al.*, 1932; page 1176). In *P. vivax* malaria, there is also natural variation that appears to exist between a number of different 'strains', as shown by the absence of cross protective immunity between them. Evidence to support this view came from a study by Boyd and co-workers that described, "an immunity derived from the local Floridan strain (McCoy) of *P. vivax* [from Florida, USA] was unable to protect against infection with four other strains (two of which were local and two exotic). There is an absence of cross immunity between the American St Elizabeth [from Washington, USA] and the New Guinea Chesson [from Papua New Guinea] strains; also between the Dutch [from Holland] and the Madagascar strains" (quoted from Garnham, 1966; page 143). Such absence of cross protective immunity in the host is considered a reflection of variant forms of antigens that induce protective immunity in the host.

Over the past three decades the 'true' extent of genetic variation within natural populations of malaria parasites of the same species has become clear. The detection of variation depended initially upon the observation of protein variation, usually using starch gel electrophoresis (Carter, 1970). Since enzymes are proteins, which are the products of genes, protein variation is thus a direct reflection of genetic variation. The two species of rodent malaria parasite in which protein variation was most studied are *P. yoelii* and *P. chabaudi*. A large number of electrophoretic enzyme variants have been observed among natural isolates and clones of these organisms (Carter, 1973; Carter, 1978; Beale *et al.*, 1978; Walliker, 1983). Protein variation was also detected in natural populations (isolates) of *P. falciparum* (Carter *et al.*, 1973; Carter *et al.*, 1975b). Subsequently, the extent of protein variation could be studied by 2-dimensional protein gel electrophoresis, in which variant forms of proteins among clones of *P. falciparum* were differentiated by iso-electric point and molecular size (Fenton *et al.*, 1985), since blood stage malaria parasites were readily

cloned out from clinical isolates of the human malaria parasite, *P. falciparum*, (Rosario, 1981) using methods such as limiting dilution.

In more recent times, genetic variation in natural populations of human and rodent malaria parasites has been detected with DNA-based methodology, such as the polymerase chain reaction (PCR). The identification and sequencing of *Plasmodium* genes can be used to detect polymorphism at the molecular level (nucleotide sequence level). By contrast, a range of advance molecular techniques, including Restriction Fragment Length Polymorphism (RFLP) (Walker-Jonah *et al.*, 1992; Carlton *et al.*, 1995) and microsatellites (Su *et al.*, 1996; Li *et al.*, 2007), has facilitated the assessment of genetic polymorphisms in malaria parasites at the genome scale. An additional technique - amplified fragment length polymorphism (AFLP) - is able to detect restriction site-associated polymorphisms without prior sequence knowledge using PCR (Vos *et al.*, 1995; Rubio *et al.*, 2001; Grech *et al.*, 2002). These molecular techniques have a number of advantages over isozyme electrophoresis, particularly in their requiring much smaller quantities of material to work with. The AFLP method has been used throughout the present work to detect genetic variation between strains of the rodent malaria parasites, *P. c. chabaudi* (Chapter 2) and *P. y. yoelii* (Chapter 3 and 4).

1.8.3 Genetic recombination of malaria parasites

All genetic variation arises by genetic mutation, while novel genotypes arise through genetic recombination (Walliker, 1983; Walliker *et al.*, 1998). The existence of a sexual phase during the parasite's life cycle in the Anopheline mosquito presents an opportunity for crossing between genetically variant clones of malaria parasites, with the subsequent generation of new genotypes through genetic recombination.

How does genetic recombination occur in malaria parasites? Genetic recombination in eukaryotes occurs principally during chromosome pairing at meiosis. The process takes place during the early phase of development of the malaria parasite in the mosquito. Following the release of 'haploid' male and female gametes in the mid-gut of the mosquito, fertilisation takes immediately and a 'diploid' zygote is formed. The

fertilised zygote develops into a motile ookinete which traverses the wall of the mosquito stomach and emerges to be beneath the basal lamina of the midgut. There, it comes to rest and begins differentiation into an oocyst. Meiosis appears to occur in the zygote/ookinete, with the appearance of a synaptonemal complex within the nucleus (based on *P. berghei*) as described by Sinden *et al.*, (1985). During meiotic cell division, the homologous chromosomes pair (synapsis) at the time when each chromosome already consists of two chromatids. If the zygote is derived through cross-fertilisation between gametes of the two genetically distinct parasites, genetic exchange can occur by means of 'crossing-over' between the non-sister chromatids of a pair of homologous chromosomes resulting in the recombination of genetic material at homologous loci. In addition to inter-chromosomal crossing events, genetic recombination during meiosis of heterozygotes can be produced by simple reassorment between members of chromosome pairs. Each zygote undergoes two successive nuclear divisions to obtain four 'haploid' nuclei. As a result, the maximum number of genetically distinct types of haploid daughter cells (progeny) produced by a single heterozygote is four. This event is all-important in determining the genetic fate of the progeny of the zygote. The production of recombinant forms at meiosis is summarised in Figure 1.5.

The earliest attempt to carry out a genetic cross on malaria parasites made use of the avian species, *Plasmodium gallinaceum* (Greenberg *et al.*, 1954). The approach involved (1) mixing blood forms of *P. gallinaceum* strains M and BI that differed in patterns of pre-erythrocytic schizogony and responses to pyrimethamine, (2) transmitting the mixture through mosquitoes to allow cross-fertilisation between gametes of the two parental genotypes and (3) examining for evidence of recombination between characters of the parental strains in the progeny of a genetic cross. It was found that the uncloned progeny of a genetic cross were able to resist to a high degree of pyrimethamine [behaving as the pyrimethamine-resistant strain] while retaining a pattern of pre-erythrocytic schizogony of the pyrimethamine-sensitive parental strain. Although it was claimed to be evidence of hybridisation in the genetic cross by the authors, this work was inconclusive because evidence of recombination was not demonstrated in the 'cloned' progeny of a genetic cross. Since then clear demonstrations of genetic recombination following crossing were

made with rodent malaria species (Walliker *et al.*, 1973; Walliker *et al.*, 1975). This is due to the availability of cloning methods and molecular genotyping methods (e.g. starch gel electrophoresis of isozymes) that allowed the precise genetic characterisation of genetically distinct clones. A genetic cross of rodent malaria was first conducted between two cloned lines, 17XA and 33XC, of *P. y. yoelii* that differed in pyrimethamine sensitivity and in electrophoretic motility of the enzyme protein glucose phosphate isomerase (GPI) (Walliker *et al.*, 1973). Following this work, a second genetic cross was conducted between two cloned lines, AS and AJ, of *P. c. chabaudi*, which differed in electrophoretic forms of lactate dehydrogenase (LDH) and 6-phospho gluconate dehydrogenase (6PGD) as well as in their responses to pyrimethamine (Walliker *et al.*, 1975). In both studies, products of the genetic crosses were cloned by limiting dilution and examined for enzyme forms and drug responses. It was found that only one form of any character was present in any of the cloned progeny of a genetic cross, and that the frequency of recombination between variants of these characters corresponded to random reassortment. Such recombination between, and segregation of, allelic pairs representing different inherited traits conforms to that pattern of inheritance originally observed and described by Mendel.

The production of heterozygous oocysts following mixed feeding to mosquitoes, of two genetically distinct malaria parasites has been shown to occur experimentally with *P. falciparum* (Ranford-Cartwright *et al.*, 1993). This study examined oocysts in mosquitoes fed on mixed clones of *P. falciparum* designated 3D7 and HB3, in which genes coding for merozoite surface proteins MSP-1 and MSP-2 are polymorphic. Three types of oocysts were found: some oocysts contained alleles exclusively from HB3 (homozygotes), some contained alleles only from 3D7 (homozygotes), and the remainder contained alleles from both parents at both loci. Oocysts of the latter group were, therefore, hybrids (heterozygotes) which are the products of cross-fertilisation between gametes of the two different parental genotypes. The proportions of homozygous and heterozygous forms were consistent with those predicted by Hardy-Weinberg expectations for random fertilisation between gametes of the two parental genotypes (Ranford-Cartwright *et al.*, 1993).

In addition to meiosis, genetic recombination during asexual replication of malaria parasites in the blood has been documented in *P. falciparum* (Freitas-Junior *et al.*, 2000), albeit of at a frequency much lower than in meiosis. The mitotic recombination events can occur between non-homologous chromosomes at sub-telomeric regions within the same genome and appeared to be facilitated through the clustering of telomeres at the nuclear periphery (Freitas-Junior *et al.*, 2000). Because these regions of the chromosomes usually comprise a number of genes coding for variable parasite proteins, such as *var* genes (Smith *et al.*, 1995; Su *et al.*, 1995), this mechanism appears to facilitate recombination of alleles of these genes to promote the diversity of antigenic phenotypes (Freitas-Junior *et al.*, 2000).

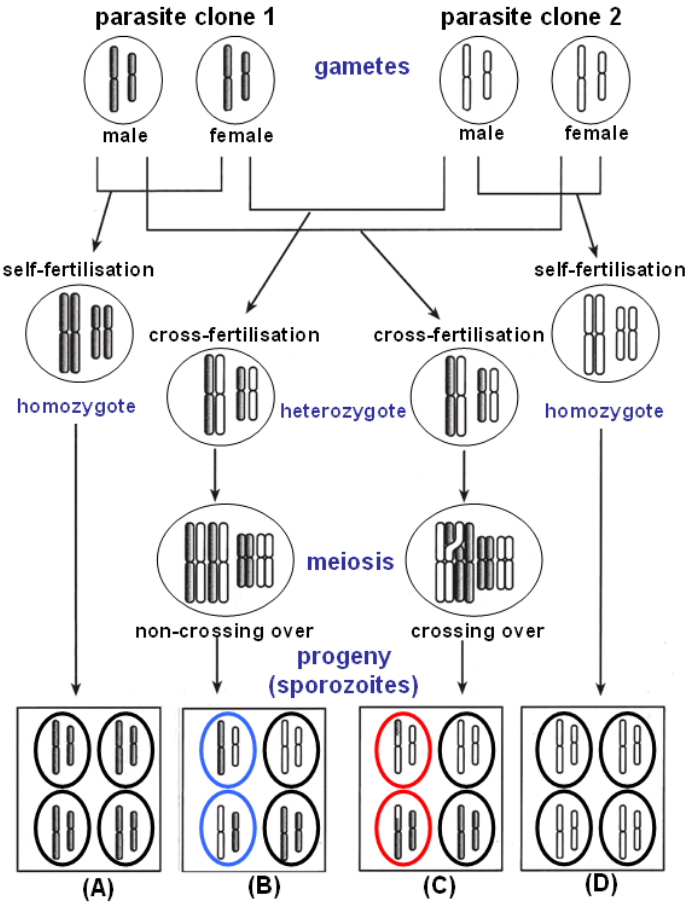

Figure 1.5. Inheritance of nuclear genes in malaria parasites and the production of recombinant progeny at meiosis. Note that genetic recombination can be generated by chromosomal reassortment or by crossing-over events. (A) or (D) Homozygous progeny produced by selfing between gametes of the same parental clone 1 or 2, respectively. (B) Heterozygous progeny produced by crossing between gametes of the two different parental clones, recombinant nuclei (blue ovals) being formed by chromosomal reassortment alone. (C) Heterozygous progeny produced from cross-fertilisation between gametes of the two different parent clones, recombinant nuclei (red ovals) being formed by crossing over between the non-sister chromatids of the homologous chromosomes. (Figure adapted from Walliker *et al.*, 1998)

1.8.4 Genetic linkage maps of malaria parasites

Linkage of genetic markers on a chromosome of an organism can be represented in the form of a genetic map. This shows the linear order of the genetic markers along the chromosome with distances between adjacent markers being proportional to the frequency of recombination between them. Genetic linkage maps have provided a powerful means to locate genes controlling heritable phenotypes in organisms that undergo sexual recombination. The construction of a genetic linkage map for malaria parasites involves (1) crossing two genetically distinct clones of the same malaria parasite species and (2) cloning out products of the genetic crosses. Individual parasite clones from the crosses can be typed with parasite clone-specific, polymorphic, DNA markers at hundreds of loci which represent one or the other allele of the parental clones at each locus. A pattern of inherited parental alleles (haplotype) is defined for each parasite clone. The markers of each parent are expected to reassort in the progeny of a genetic cross. However, markers that appear to be inherited more often than expected from random reassortment are considered to be 'genetically linked' in malaria parasites. Genetic markers that represent each such linkage group can be hybridised to chromosomes, separated by pulsed field gel electrophoresis (PFGE), in order to determine the physical location of each linkage group onto a specific chromosome. Together, these processes can yield a genetic linkage map across all of the chromosomes of a malaria parasite.

Three genetic crosses in *P. falciparum* have been generated and published so far. The three crosses are: HB3 x 3D7 (Walliker *et al.*, 1987), HB3 x Dd2 (Wellems *et al.*, 1990; Wellems *et al.*, 1991) and 7G8 ×GB4 (K. Hayton, unpublished; cited from Su *et al.*, 2007), from which 21, 35 and 33 independent recombinant progeny clones were established, [the number of clones from these crosses quoted from Su *et al.*, (2004) and Su *et al.*, (2007)]. The first genetic linkage map of *P. falciparum* was constructed after the characterisation of 16 independent recombinant progeny of the *P. falciparum* HB3 x Dd2 genetic cross (Walker-Jonah *et al.*, 1992). The map was established with 90 parasite clone-specific RFLP markers that were allocated to 14 inferred linkage groups corresponding to the 14 nuclear chromosomes (Walker-Jonah

et al., 1992). Crossover rates obtained from parasite clone-specific RFLP markers mapped on *P. falciparum* chromosomes allowed calculation of the map unit for this malaria parasite. A map unit (one centiMorgan) is a unit of distance in a linkage group that corresponds to a recombination frequency of 1%. An average map unit in the RFLP-based linkage map of *P. falciparum* was 15 to 30 kilobase pairs per centiMorgan of genetic distance (kb cM^{-1}) (Walker-Jonah *et al.*, 1992).

The association between the inheritance of a phenotype (e.g. drug resistance) and the inheritance of genetic markers in the cloned progeny of the genetic crosses is used to identify chromosomal regions that harbour genes underlying such phenotypes. For example, the availability of RFLP inheritance patterns from the HB3 x Dd2 cross allowed the location of a locus underlying chloroquine resistance to a 400-kb segment of *P. falciparum* chromosome 7 (Wellems *et al.*, 1991). Likewise, the genetic determinants of a male gametocytogenesis defect were mapped to a location on *P. falciparum* chromosome 12 (Vaidya *et al.*, 1995; Guinet *et al.*, 1996) and genes conferring sulfadoxine resistance to a locus on *P. falciparum* chromosome 4 (Wang *et al.*, 1997).

Following this work, about 900 microsatellite markers were used to construct a genetic linkage map of *P. falciparum* (Su *et al.*, 1999) using 35 independent progeny of the *P. falciparum* HB3 x Dd2 genetic cross. The markers were then assigned to 14 inferred linkage groups, using the locations of RFLP markers from the previous genetic map (Walker-Jonah *et al.*, 1992) to anchor the 14 linkage groups of microsatellite markers to the 14 nuclear chromosomes of *P. falciparum*. Consistent with the previous estimation based on the earlier RFLP-based linkage map for *P. falciparum* (Walker-Jonah *et al.*, 1992), the size of an average genetic map unit derived from the microsatellite-based linkage map was 17 kb cM^{-1} (Su *et al.*, 1999). This high-resolution genetic map enabled the location of the *pfcrt* gene conferring chloroquine resistance in this parasite (*pfcrt* encodes the "chloroquine resistant transporter" (Fidock *et al.*, 2000) to a 36-kb region in *P. falciparum* chromosome 7 (Su *et al.*, 1997)

A genetic linkage map of *P. c. chabaudi* has also been constructed, using the inheritance patterns of 45 RFLP markers in 20 independent recombinant progeny clones of a genetic cross between the chloroquine-resistant, AS, and chloroquine-sensitive clone, AJ (Carlton *et al.*, 1998a). This map comprises 14 linkage groups that correspond to the 14 chromosomes. The RFLP inheritance patterns of the cloned progeny of the *P. c. chabaudi* AS x AJ cross indicated a locus responsible for chloroquine resistance in this malaria parasite on chromosome 11 (Carlton *et al.*, 1998a). Subsequently, AFLP has been developed in our laboratory for *P. c. chabaudi* (Grech *et al.*, 2002). It has proved useful in generating a large number of parasite clone specific genetic markers in this malaria parasite (Grech *et al.*, 2002). The inheritance of 672 AFLP markers from the two parental clones of *P. c. chabaudi* AS and AJ was determined in 28 independent recombinant cloned progeny from the two AS x AJ genetic crosses (Martinelli *et al.*, 2005b). These AFLP markers could be organised into 22 linkage groups together with a previous set of RFLP markers used as an anchor for chromosome identification (Carlton *et al.*, 1998a). Ten of these linkage groups correspond to *P. c. chabaudi* chromosomes 1, and 5 to 13. Twelve other groups of AFLP markers could not be assigned to specific chromosomes and are called *P. c. chabaudi* unassigned linkage groups (Martinelli *et al.*, 2005b). The overall estimated size of map unit for *P. c. chabaudi* is 15.1 kb cM^{-1} (Martinelli *et al.*, 2005b). The AFLP-based genetic linkage map for *P. c. chabaudi* has become a tool for identifying the genetic location of AFLP markers that are genetically linked to loci conferring important phenotypes. This map was used to successfully locate a 250-kb region on *P. c. chabaudi* chromosome 11 which contains a locus underlying chloroquine resistance in this parasite (Hunt *et al.*, 2004).

In addition, the AFLP-based genetic linkage map for *P. c. chabaudi* has already enabled us to identify loci linked to genes underlying other medically important phenotypes, such as the artemisinine sensitive/resistant traits (Hunt *et al.*, 2007), using the Linkage Group Selection approach (LGS) (Culleton *et al.*, 2005; Carter *et al.*, 2007) (see section 1.8 for detailed description of LGS). LGS is a genetic method that involves the mass screening with quantitative genetic markers of the 'uncloned' progeny of a genetic cross following its growth under a selection pressure

representing the phenotype of interest. In the present work, the AFLP-based genetic linkage map is now utilised to identify regions in the *P. c. chabaudi* genome containing genes encoding targets of SSPI in *P. c. chabaudi* (Chapter 2).

1.9 Genomes of malaria parasites

The genome of the malaria parasite comprises three components: (1) a mitochrondrial genome, (2) a genome associated with an organelle known as an apicoplast and (3) the nuclear genome.

1.9.1 Mitochrondrial and apicoplast genomes

Each malaria parasite cell has two organellar genomes; the mitochondrial genome and the apicoplast genome. The mitochrondrial genome of malaria parasites was first identified in the rodent malaria parasite, *P. yoelii*, as a multiple-copy 6 kb linearly re-iterated molecule (Vaidya *et al.*, 1987). The mitochondrial genome comprises genes encoding two truncated ribosomal RNAs and three components of cellular respiration (cytochrome *c* oxidase subunits I and III, and cytochrome *b*) (Vaidya *et al.*, 1989; Feagin, 1992; Williamson, 1998). Its copy number varies over the species of malaria parasite, ranging from 20 copies per cell in *P. falciparum* (Preiser *et al.*, 1996) to 150 copies per cell in *P. y. yoelii* (Vaidya *et al.*, 1987). The apicoplast genome is a low-copy number, 35 kb circular molecule (Wilson *et al.*, 1996; Wilson *et al.*, 1997; Wilson, 1998). It is composed of genes coding for around 30 proteins, most of which are RNA polymerase, and other proteins involved in transcription processes (Wilson *et al.*, 1996; Wilson *et al.*, 1997; Wilson, 1998).

The mitochondrial and apicoplast genomes of the human malaria parasite, *P. falciparum*, were shown to be inherited uniparentally (Creasey *et al.*, 1993; Vaidya *et al.*, 1993). In the majority of eukaryotes, these genomes are inherited through one parent only, mostly the female. This was also proven to be the case in *P. gallinaceum*, since DNA probes specific to the mitochondrial or apicoplast genome hybridised with the DNA of female gametes, but not male gametocytes (Creasey *et al.*, 1994).

1.9.2 Nuclear genome of *P. falciparum*

The first malaria parasite nuclear genome sequence data was generated from *P. falciparum* clone 3D7, undertaken by an international consortium of genome sequencing centres (Hoffman *et al.*, 1997) including The Institute for Genomic Research (TIGR, USA), the Wellcome Trust Sanger Institute (UK) and Stanford Genome Technology Centre (USA). Each centre used the chromosome-by-chromosome shotgun strategy to generate sequences of each of the 14 chromosomes, which were separated by PFGE. The genome of *P. falciparum* 3D7 was sequenced to an average coverage of 14.5 fold (i.e. every base in the genome is represented in the shotgun sequence [raw nucleotide sequence] on average, 14.5 times) [quoted from Kooij *et al.*, 2006] (Table 1.3). The raw sequences were then assembled into contiguous DNA sequences termed 'contigs'. These contigs could be subsequently orientated and allocated to specific chromosomes in the *P. falciparum* genome with reference to previous *P. falciparum* map data, such as the microsatellite-based genetic linkage map from the HB3 x Dd2 cross (Su *et al.*, 1999). Because the genomes of malaria parasite are so Adenine (A) and Thymine (T) rich (McCutchan *et al.*, 1984), it is difficult to obtain sequences for some regions due to the instability of the fragment in cloning vectors. This results in 'gaps' in the genome. At the time of the publication of the *P. falciparum* genome sequence in 2002 (Gardner *et al.*, 2002), there were still 79 gaps in the whole genomic sequence. At present, just three chromosomes (7, 8 and 13) are still awaiting closure, with only six gaps remaining (M Berriman, unpublished, quoted from Kooij *et al.*, 2006)

The *P. falciparum* clone 3D7 nuclear genome consists of 22.8 megabase pairs (Mb) distributed among 14 chromosomes ranging in size from 0.643 Mb for chromosome 1 to 3.29 Mb for chromosome 14 (Gardner *et al.*, 2002). The genome contains a high proportion of AT content (~80.6%). This proportion, however, rises to ~ 90% in introns and intergenic regions, and to > 97% in putative centromeric regions (Gardner *et al.*, 2002). The nuclear genome of *P. falciparum* appears to contain 5,268 predicted genes encoding proteins (Gardner *et al.*, 2002). A summary of general *P. falciparum* genome characteristics is given in Table 1.3.

Comparison of the annotation of the *P. falciparum* nuclear genome with previously published genomes of other organisms, e.g. yeasts, *Saccharomyces cerevisiae* (Wood *et al.*, 2001) and *Schizosaccharomyces pombe* (Wood *et al.*, 2002), and a plant, *Arabidopsis thaliana* (Arabidopsis Genome Initiatives, 2000), showed that around 40% of all gene products are similar to those in these other organisms. These proteins are predicted to be involved in maintaining cellular functions, such as metabolism (8%) and cell-to-cell adhesion or invasion of host cells (1.3%). A further 10% of the nuclear-encoded proteins are predicted to be transported to the apicoplast (Gardner *et al.*, 2002). These are known to function in synthesis of isoprenoid, haeme and fatty acids (Foth *et al.*, 2003). The *P. falciparum* nuclear genome also contains multigene families clustered towards the sub-telomeric regions of chromosomes. Three such families, accounting for 5 to 10% of all genes, termed *var*, *rif* and *stevor*, code for highly variable proteins known as *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) (Smith *et al.*, 2005, Su *et al.*, 1995), repetitive interspersed family (RIFIN) (Cheng *et al.*, 1998; Kyes *et al.*, 1999) and sub-telomeric variable open reading frame (STEVOR) (Cheng *et al.*, 1998), respectively. PfEMP-1 and RIFIN are exported to the surface of infected erythrocytes and undergo antigenic variation (Kyes *et al.*, 2001, Bull *et al.*, 2005). While the function of RIFIN and STEVOR is not known, PfEMP-1 mediates adherence to host endothelial receptors, resulting in the sequestration of infected erythrocytes in various organs (Pain *et al.*, 2001) and thus contributing to the development of severe malaria (Miller *et al.*, 2002). The remaining part of the nuclear genome (~ 60%) contains predicted genes encoding proteins of unknown function; so-called hypothetical proteins. Nevertheless, among them are likely to be novel antigens for malaria vaccine choice as well as *de novo* targets for anti-malarial drugs.

1.9.3 Nuclear genomes of rodent malaria parasites

With the completion of the *P. falciparum* genome sequencing project (Gardner *et al.*, 2002), a series of initiatives has begun to generate more genome information from other *Plasmodium* species. The genome sequencing projects of three rodent malaria species were undertaken by the two sequencing centres: TIGR for the *P. y. yoelii* 17XNLclone1.1 genome and the Sanger Institute for the *P. c. chabaudi* clone AS and *P. berghei* clone ANKA genomes. A whole-genome shotgun sequencing approach (i.e. the whole genome subjected to the shotgun sequencing without prior separation of individual chromosomes by PFGE) was utilised to sequence and assemble the sequence data. The partial genomic sequences from these three clones have been now published (Carlton *et al.*, 2002; Hall *et al.*, 2005). The *P. y. yoelii* genome project has completed with average five fold average sequence coverage (Carlton *et al.*, 2002), while the *P. c. chabaudi* and *P. berghei* genome projects have finished with average eight fold average sequence coverage. The summary of the three rodent malaria genome projects is given in Table 1.3.

Examination of data from the rodent malaria genome projects shows that the genomes of rodent malaria parasites have a standard size of approximately 23 to 30 Mb distributed among 14 linear chromosomes. Like *P. falciparum* chromosomes, the estimated sizes of rodent malaria parasite chromosomes range from 0.5 to 3.0 Mb, using PFGE (Janse *et al.*, 1994). The genomes of rodent malaria parasites are high in AT content (70 to 80%). However, because the average sequence coverage of the rodent malaria parasite genomes (5 to 8X coverage, quoted from Kooij *et al.*, 2006) is lower than that of the *P. falciparum* genome, a large number of gaps are still present in their genomes. Also, the number of contigs is higher and the relative size of contigs is much smaller (~2 to 4 kb), compared to those generated by the *P. falciparum* genome (~ 200 kb) (Table 1.3).

	P. falciparum	*P. y. yoelii*	*P. c. chabaudi*	*P. berghei*
clone	3D7	17XNL1.1	AS	ANKA
Statistics				
Estimated genome size (Mb)	23.3	23.1	30 [a]	26 [b]
Genome sequence coverage [c]	14.5X	5X	8X [a]	8X [b]
No. of chromosomes	14	14	14	14
(A+T) % [d]	80.6	77.4	75.6	76.1
No. of contigs	less than 93[e]	5,687	10,679	7,497
Average contig size (kb)	213	4	2	2
No. gaps	6 [e]	5,812	ND	ND
No. of predicted genes coding proteins	5,268	5,878 [f]	5,698 [f]	5,864 [f]
Annotated full length genes[e]	5,403	4,034	4,100	4,617
References	Gardner *et al.* 2002	Carlton *et al.*, 2002	Hall *et al.*, 2005	Hall *et al.*, 2005

Table 1.3. A comparison of general genome characteristics between the human malaria parasite, *Plasmodium falciparum*, and three rodent malaria species, *Plasmodium yoelii yoelii*, *Plasmodium chabaudi chabaudi* and *Plasmodium berghei*. ND, not determined

(a) For the current status of *P. c. chabaudi* genome project (September 2007) see the websites: http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genomeprj&cmd=ShowDetailView&TermToSearch=9537 and http://www.sanger.ac.uk/Projects/P_chabaudi/

(b) For the current status of *P. berghei* genome project (September 2007) see the websites: http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genomeprj&cmd=ShowDetailView&TermToSearch=9536 and http://www.sanger.ac.uk/Projects/P_berghei/

(c) Genome sequence coverage indicates how many times the genome was sequenced

(d) (A+T) % is defined as the percentage of the total nuclear genomic DNA content (i.e. both coding and non-coding regions) that is Adenine(A)-Thymine(T) pairs.

(e) Updated information of the *Plasmodium* genome projects (March 2006) quoted from Kooij *et al.*, (2006)

(f) Likely to be an over-estimate due to inclusion of partial sequences (Carlton *et al.*, 2002; Hall *et al.*, 2005)

Each rodent malaria parasite species appears to have 5000 to 6000 predicted genes per genome (Table 1.3). Comparative genomic analyses based on bi-directional BLAST searches between the rodent malaria genomes and the annotated human malaria genome have inferred the annotation of these genes in the rodent malaria parasite genomes (Carlton *et al.*, 2002; Hall *et al.*, 2005). As shown in Figure 1.6, the first comparative analysis between the *P. falciparum* and *P. y. yoelii* genomes revealed that about 60 to 70% of the predicted *P. y. yoelii* proteins had orthologous gene pairs (i.e. genes related to each other by descent from a common ancestral DNA sequence and not necessarily the having same function) in *P. falciparum* (Carlton *et al.*, 2002). Similar figures for orthologous gene pairs were also found between *P. falciparum* and *P. berghei* and between *P. falciparum* and *P. c. chabaudi* (Hall *et al.*, 2005) (Figure 1.6). Comparative analyses were also made between the genomes of the three rodent malaria species (Carlton *et al.*, 2002; Janssen *et al.*, 2002; Gardner *et al.*, 2002; Janssen *et al.*, 2004; Hall *et al.*, 2005). The results showed that about 80 to 90% of genes encoding proteins were shared between the rodent malaria genomes. Almost all of the putative orthologues could be located in the central portions of the chromosomes. There are, however, a number of genes that have no orthologues between malaria species. Most of these genes are commonly found in the sub-telomeric regions of chromosomes and are also known to be a repertoire of highly variant, species-specific gene families. The three rodent malaria parasites share *Plasmodium* interspersed repeats (*pir*) superfamily (*bir* gene family in *P. berghei*, *yir* gene family in *P. y. yoelii*, *cir* gene family in *P. c. chabaudi*) (Janssen *et al.*, 2002). The *pir* superfamily is also present in the human malaria parasite *P. vivax* (*vir* gene family) (del Portillo *et al.*, 2001) and in the simian malaria parasite *P. knowlesi* (*kir* gene family) (Janssen *et al.*, 2004). However, *P. falciparum* parasites lack the *pir* superfamily, but the sub-telomeric regions of their chromosomes harbour the gene families, including *var*, *rif* and *stevor*.

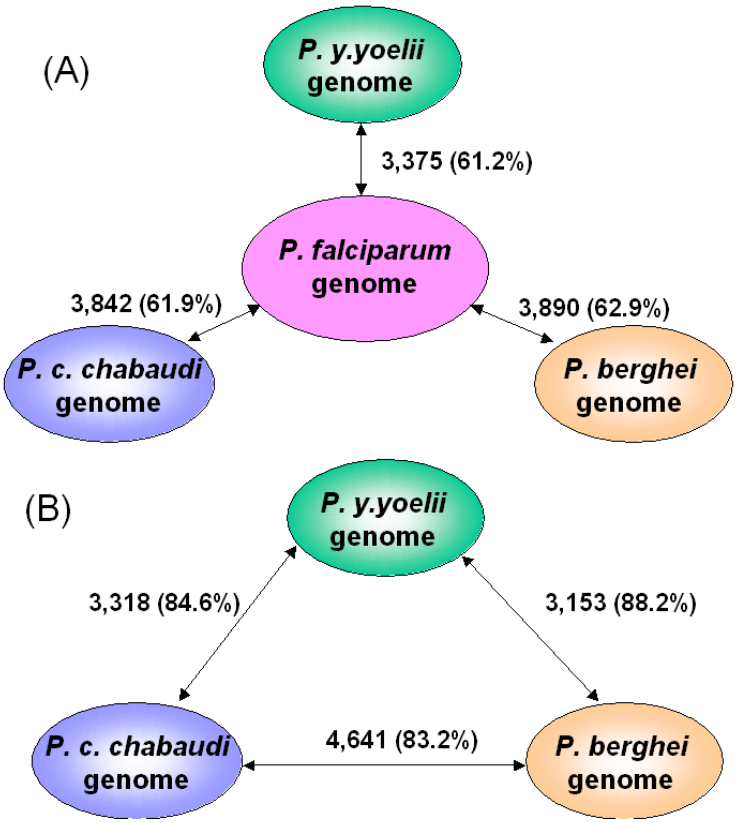

Figure 1.6. The number of orthologous gene pairs in the genome of four *Plasmodium* species. (A) shows a comparison between the human malaria parasite, *Plasmodium falciparum*, and three rodent malaria species, *Plasmodium yoelii yoelii*, *Plasmodium chabaudi chabaudi* and *Plasmodium berghei*. (B) shows a comparison between the three rodent malaria species. Numbers in brackets represent average protein identity. (Carlton *et al.*, 2002; Hall *et al.*, 2005)

1.9.4 Genetic synteny in *Plasmodium* species

Genetic synteny refers to the degree of conservation of gene order between two genomes. The original studies of gene synteny between *Plasmodium* species were conducted from gene-mapping on PFGE-separated chromosomes (Janse *et al.*, 1994; Carlton *et al.*, 1998b; Carlton *et al.*, 1999). The availability of the genome sequence data from *P. falciparum* and the three rodent malaria species allowed studies of gene synteny to be performed using bioinformatic techniques across the entire genomes. The first genome-wide synteny map was constructed between the *P. falciparum* and *P. y. yoelii* genome (Carlton *et al.*, 2002). Each genome sequence was translated in all 6 reading frames using the MUMer program (Delcher *et al.*, 2002) to identify all

exact amino sequence matches longer than 5 amino acids between species. Over 70% (~2,200 sequences) of the *P. y. yoelii* genome content were aligned to specific *P. falciparum* chromosomes, representing a cumulative length of 16.4 Mb of sequence. These *P. y. yoelii* contigs were subsequently joined up to generate "groups of contigs" by the use of mate-pair information, PCR amplification of products from the gaps between contigs, and identification of genes that spanned the gap (Carlton *et al.*, 2002). Accordingly, these groups of contigs represented regions of conserved gene synteny. Physical mapping of the representatives of these contigs to a partial physical map of *P. y. yoelii* (PFGE-separated chromosomes) (Janse *et al.*, 1994) provided a map of conserved gene synteny and identified potential syntenic break points. Subsequently, partial genome sequence data of the rodent malaria genome *P. berghei* and *P. c. chabaudi* were aligned to the *P. y. yoelii* genome to identify overlapping contigs and, therefore, to generate composite contigs of the rodent malaria parasite (Kooij *et al.*, 2005). This data was then used to construct a new synteny map of *P. falciparum* and the composite rodent malaria genome using the MUMer programme, as just described. The whole-genome synteny map between *P. falciparum* and the three rodent malaria parasites is illustrated in Figure 1.7.

Throughout the present study, the whole-genome synteny map between *P. falciparum* and the three rodent malaria parasites species has been used as a means to locate genetic markers in the *Plasmodium* genome. Genetic markers of rodent malaria parasites which are identified from genetic analysis can be sequenced. Orthologous loci of the markers can be found in the more-or-less completely annotated genome of the human malaria parasite, *P. falciparum*. The corresponding loci of *P. falciparum* orthologues of genetic markers in the rodent malaria parasite genome can, in turn, be identified through the conserved synteny (Figure 1.7). This data has enabled us to identify genomic regions containing genes underlying phenotype of interest and to determine whether possible candidate genes are present in such genomic regions (Chapter 2 and 4).

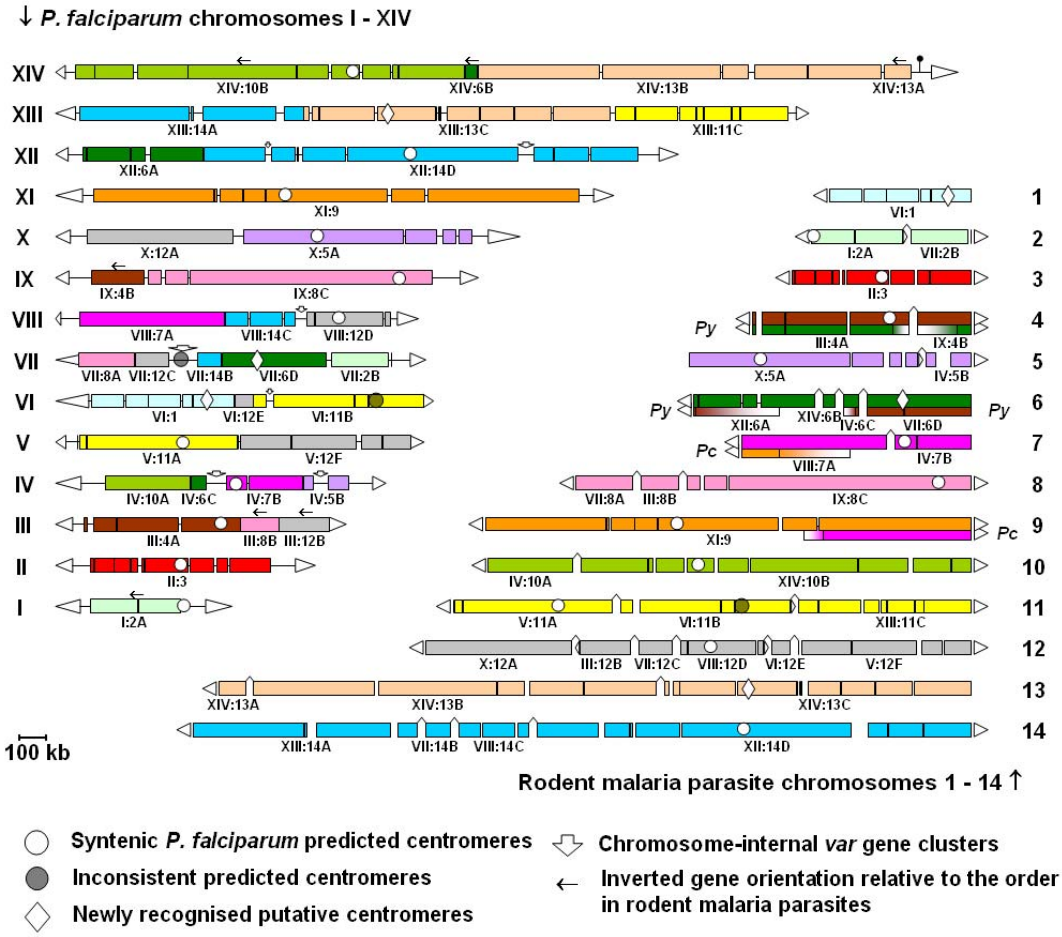

Figure 1.7. A whole-genome synteny map of the human malaria parasite *Plasmodium falciparum* and three rodent malaria parasites (RMP): *Plasmodium yoelii yoelii* (*Py*), *Plasmodium chabaudi chabaudi* (*Pc*) and *Plasmodium berghei*. A synteny map of the core regions of the 14 chromosomes of *P. falciparum* (left) and the rodent malaria parasites (right) show the 36 synteny blocks, 22 synteny breakpoints, 14 predicted centromeres, and *P. falciparum*-specific indel and translocations in the RMP chromosomes. The 36 synteny blocks, coloured according to their chromosomal location in the composite rodent malaria parasite (cRMP) genome are named with a Roman and an Arabic number referring to the corresponding chromosomal location in *P. falciparum* and the cRMP genomes, respectively. Letters give the order in which the synteny blocks are connected. Telomeres and sub-telomeric linked ends are shown as white arrows. The bars under the cRMP chromosomes represent the differences in the organisation of the synteny blocks of *Py* and *Pc* from *Pb* as a result of chromosomal translocations. Colours indicate the cRMP chromosome within which recombination has taken place and colour gradients represent the ill-defined regions of the translocation breakpoints. (Figure reproduced from Kooij *et al.*, (2005; 2006).

1.10 Linkage Group Selection

The identification of genes in malaria parasites which determine medically important traits, such as drug resistance, growth rate, transmissibility and SSPI can have great benefits for malaria control. The conventional approach of a genetic analysis of malaria parasites that attempts to identify genes underlying such traits involve crossing two genetically distinct lines of malaria parasites that differ in phenotypes of interest, cloning the products of a genetic cross, and characterising genotypes of individual clones with a large number of genetic markers (Walliker, 2005). Recently, an alternative approach to identifying genes in malaria parasite crosses has been developed and called "Linkage Group Selection" (LGS) (Carter *et al.*, 2007) (see Figure 1.8). In this method, the laborious procedure of cloning and characterizing the clones of genetic crosses is circumvented. Rather, the entire "uncloned" progeny of genetic crosses is placed under a selection pressure that represents a biological characteristic of interest, e.g. growth rate or SSPI. DNA of the selected progeny is screened *en masse* with quantitative, parental clone-specific genetic markers, such as AFLP, across the genome. It would be expected that alleles of genes not linked to the locus affected by the selection pressure would retain approximately the same frequency in the cross progeny before and after selection. By contrast, genetic markers of the parental clone sensitive to the selection pressure which are linked to a locus conferring susceptibility to the selection pressure will be removed or reduced in the cross progeny following selection. These 'selected' markers can be sequenced, and their positions located in the parasite genome in order to identify the linkage group that contains the locus sensitive to the selection pressure – hence the name "Linkage Group Selection". Within the linkage group of the selected markers, the closer the markers are genetically linked to the target locus, the greater the reduction in their representation in the selected population. Thus, a "selection valley" will be formed around the locus under selection with its walls sloping down towards the target of the selection force at its base (Carter *et al.*, 2007) (Figure 1.9).

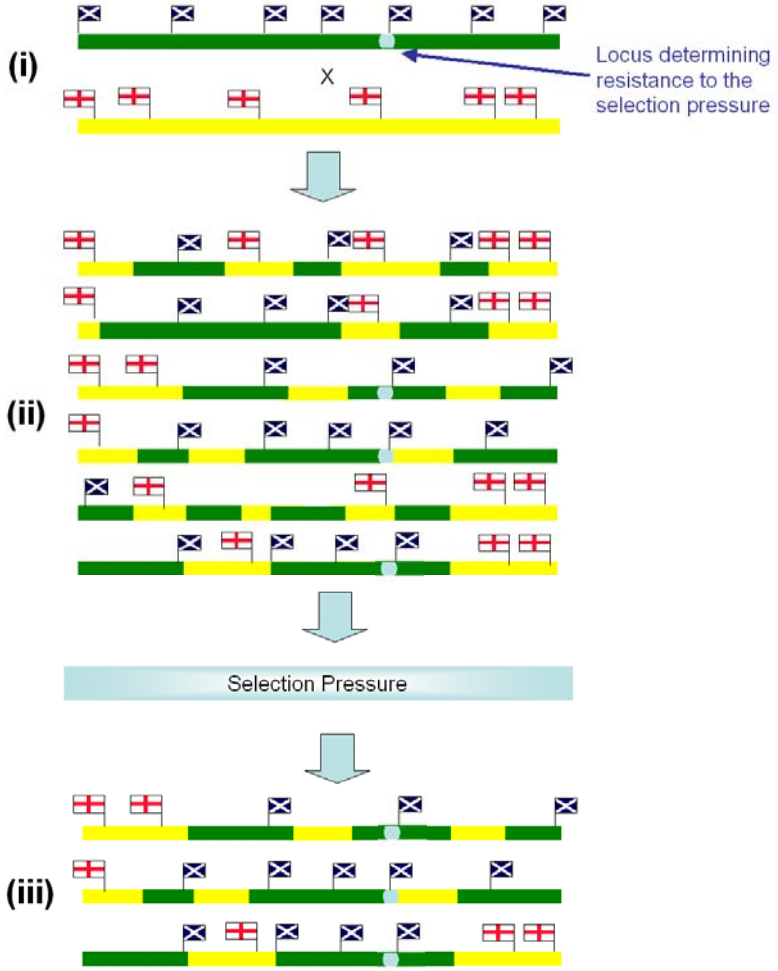

Figure 1.8. A schematic representation of the principles of Linkage Group Selection (LGS).

(i) LGS involves crossing two strains of malaria parasites that differ in their sensitivity to a particular selection pressure. Each bars represent a chromosome in the genome of parasite (resistant parent, green; sensitive parent, yellow). Resistance to the selection pressure is conferred by a single locus (blue oval), while the flags represent chromosome-wide markers between the two strains (resistant parent specific markers, Scottish flags; sensitive parent specific markers, English flags).

(ii) The resulting recombinant progeny population will contain thousands of parasites, each bearing a random assortment of parental alleles, and also a random assortment of parental markers (only 6 examples shown here). This uncloned population is then placed under the selection pressure representing the phenotype of interest. The process will select for parasites that possess the resistant allele of the gene that controls the phenotype of interest (blue oval), any markers linked to this locus will be mainly from the resistant parent, their proportion increasing as the distance from the locus under selection decreases.

(iii) In the resulting surviving population, we search for markers of the sensitive parent (English flags) that are removed or reduced by the selection pressure; the greater the degree of reduction of a sensitive marker, the closer it is linked to the allele removed by the selection pressure (see also Figure 1.9). (Figure adapted from Culleton *et al.*, 2005)

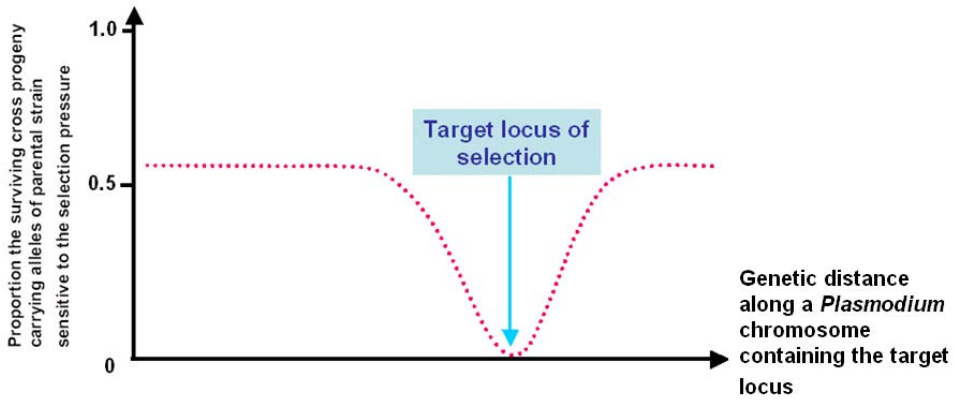

Figure 1.9. A simulation of the result of a Linkage Group Selection analysis of a simple monogenic trait in a malaria parasite. Following growth of uncloned progeny of a genetic cross between two lines of malaria parasites, one 'Resistant' and the other 'Sensitive' to a specific selection pressure, DNA of the selected progeny will be screened *en masse* for presence and intensity with the quantitative genetic markers specific to the sensitive parental strain (red dots). It is, theoretically, expected that any uncloned cross progeny carrying the allele of the gene (blue box) from the Sensitive parent conferring 'sensitivity' to the selection pressure will be removed from the uncloned progeny population. The target locus of selection directly affected by the selection pressure will be removed from the progeny, and so markers of the sensitive parent of other loci on the same linkage group would be reduced in proportion to their decreasing genetic distance from the target locus. Proportions of parasite DNA carrying the allele of genes at the genome-wide loci, represented by the relative intensities of quantitative markers of the Sensitive parent line, are plotted against the genetic distance of each marker in the *Plasmodium* chromosome containing the target under selection. Hence, a 'selection valley' will be formed. Markers of the Sensitive parent at the lowest point in the selection valley are expected to be closely linked to the locus determining 'Resistance' or 'Sensitivity' to the selection pressure representing the biological property of interest.

The application of LGS was validated by our research group using two genetically distinct lines, AS-pyr1 and AJ, of the rodent malaria parasite, *P. c. chabaudi*, that differ in responses to pyrimethamine (Culleton *et al.*, 2005), and for which the controlling genetic factors were already understood. The pyrimethamine-resistant phenotype is conferred by a single point mutation in codon 106 of dihydrofolate reductase coding gene in *P. c. chabaudi* (Cheng *et al.*, 1994a). The result showed that the base of the pyrimethamine-selected valley was closely linked to the position of the *dhfr* gene, already known to encode resistance or sensitivity to pyrimethamine, on a genetic linkage map of *P. c. chabaudi* on chromosome 7 (Culleton *et al.*, 2005). More recently, LGS analysis has enabled the location of regions in the *P. c. chabaudi* genome containing loci conferring resistance to anti-malarial drugs, artemesinin and artesunate (Hunt *et al.*, 2007). It has led to the location of a potential target of

artiminsinin resistance to a 200-kb region on *P. c. chabaudi* chromosome 2 which contains the gene encoding a deubiquitinating enzyme (Hunt *et al.*, 2007).

Thus, the practise of LGS analysis in malaria parasites is now well established and its principles have been validated (Culleton *et al.*, 2005). The method of LGS has many potential applications in the field of malaria parasite genetics. LGS can be used to investigate genes controlling other important phenotypes, such as SSPI and growth rate. In order to exploit LGS to investigate SSPI, the uncloned progeny of a genetic cross between antigenically distinct strains of malaria parasite need to be grown in individual hosts previously immunised with either one or the other of the two parental clones used to generate such a genetic cross. The first LGS analysis of SSPI was conducted in this laboratory using progeny of a genetic cross between two genetically distinct strains of *P. c. chabaudi* AS-pyr1 and CB. This study identified a selection valley on *P. c. chabaudi* chromosome 8 under SSPI selection. The base of which contains the gene encoding the highly variable merozoite surface antigen, Merozoite Surface Protein-1 (MSP-1) (Martinelli *et al.,* 2005a), a candidate target of SSPI to rodent malarias. However, whether the gene coding Apical Membrane Antigen-1 (AMA-1), another SSPI-target candidate, was involved in SSPI could not be tested because AS-pyr1 and CB possess identical amino acid sequences within the cell surface, ectodomain-coding region of AMA-1 (S. Cheesman and R. Carter, unpublished data). Therefore, the present study will be conducted using a new strain combination of *P. c. chabaudi* that inherit distinct allelic forms of the genes for both MSP-1 and AMA-1. The progeny of the cross will be subjected to growth in the strain-specific immunised mouse as well as being passaged in a non-immune mouse. Results of these experiments will be described in Chapter 2.

In the context of growth rate, the uncloned cross progeny between a faster-growing and a slower-growing strain of *P. y. yoelii* will be passaged in the vertebrate host to select those parasites that grow fast. This process is expected to select the allele(s) controlling growth rate in the faster growing parental strain. The use of LGS approach to determine genes controlling growth rate will be described in the *Results* in Chapter 4.

1.11 Aims of the project

The first part of the present study focuses on the identification of genes encoding target antigens of SSPI in malaria parasites. I conducted LGS analysis using two cloned lines of *P. c. chabaudi* AJ and CB-pyr10 in which both genes encoding two major targets of SSPI, MSP-1 and AMA-1, are polymorphic. This aimed to determine the involvement of both loci in SSPI. Experiments were designed in which the uncloned progeny of the AJ x CB genetic cross were exposed to the strain specific protective immune selection pressure (e.g. in mice which had been immunised with either strain AJ or CB) as well as being passaged in the absence of the relevant selection pressure (e.g. in a non-immune batch mate of the strain specific immunised mice). Proportions of parental alleles in the SSPI-selected and unselected cross progeny were measured using quantitative genome-wide molecular markers, such as AFLP. The regions of the *P. c. chabaudi* genome containing genes for target of SSPI were successfully identified. Results of LGS analysis of SSPI using these parasites are presented in **Chapter 2**.

The second part of the present study focuses on the genetic basis of growth rate in the rodent malaria parasite *P. y. yoelii*. Before reporting on the full LGS analysis of growth rate, I present the results of an investigation of the genetic relationship between different cloned lines of *P. y. yoelii* 17X that vary in growth rate. A comparative genetic analysis of these parasite lines was made using (i) nucleotide sequences for specific genes, which are expected to be polymorphic, and (ii) restriction site-associated polymorphism by AFLP. The purpose of this study was to determine from which slow-growing line of *P. y. yoelii* 17X the fast-growing line 17XYM, using in the LGS analysis of growth rate, had arisen. Results from this study are presented in **Chapter 3.** Finally, I report the results of LGS analysis of the genetic determinants of growth rate in *P. y. yoelii*. Experiments were conducted using a genetic cross between the fast-growing, 17XYM, and a slow-growing and genetically unrelated line, 33XC, of *P. y. yoelii*. The uncloned progeny was allowed to grow in laboratory mice. The growth rate selected cross progeny were subjected to a genome-wide scan by AFLP to determine the locus controlling growth rate in this parasite. These experiments are described in **Chapter 4**.

CHAPTER 2: Linkage Group Selection: Towards Identifying Genes Controlling Strain Specific Protective Immunity in Malaria

A version of this chapter has been published as: Pattaradilokrat S., Cheesman S. J., Carter R. (2007) Linkage Group Selection: Towards Identifying Genes Controlling Strain Specific Protective Immunity in Malaria. *PLoS One* **2**(9):e857.

2.1 Abstract

A part of anti-parasitic protective immunity against the pathogenic blood stages in malarial infections has been shown to be specific to the genotype, or strain, of the parasites in humans and laboratory animals. It is expected that the target antigens of Strain Specific Protective Immunity (SSPI) would, therefore, be antigenically and genetically different among different strains, or genetically distinct lines, of malaria parasite. Two highly polymorphic antigens, merozoite surface protein-1 (MSP-1) and apical membrane antigen-1 (AMA-1), have been implicated as targets of SSPI in human and rodent malarias. Here we describe the use of a genetic and genomic approach, Linkage Group Selection (LGS), to locate region(s) containing the target(s) of SSPI against the blood stages of the rodent malaria parasite *Plasmodium chabaudi chabaudi*. In a previous analysis using the progeny of a genetic cross between two genetically distinct cloned lines, AS-pyr1 and CB, of *P. c. chabaudi*, a region on *P. c. chabaudi* chromosome 8 that contains the gene encoding the *P. c. chabaudi* MSP-1 was found to be under strong SSPI selection. However, the possible involvement of *P. c. chabaudi* AMA-1 in SSPI could not have been evaluated in this cross as AS-pyr1 and CB have identical amino acid sequences within the cell surface, ectodomain-coding region of this protein. In the present study, LGS analysis of SSPI has been conducted with a different combination of genetically distinct lines, CB-pyr10 and AJ, of *P. c. chabaudi*, in which the genes for both MSP-1 and AMA-1 proteins are genetically distinct. This analysis has identified the region on *P. c. chabaudi* chromosome 8 containing the gene for MSP-1 as encoding a major target of SSPI as was also found in the previous study. The results of this study also revealed that two other regions, one on *P. c. chabaudi* chromosome 9 containing the

gene for AMA-1 and the other on chromosome 7, may be associated with SSPI but to a lesser degree than that for MSP-1. Together, the evidence from these two independent studies indicates that SSPI in *P. c. chabaudi* in mice is mainly determined by a single, narrow region of the *P. c. chabaudi* genome containing the gene for the *P. c. chabaudi* MSP-1 protein.

2.2 Introduction

In regions of the world where malaria is endemic, anti-parasitic protective immunity against infection with the pathogenic blood stages of the parasites is acquired gradually after repeated exposure to malaria (McGregor *et al.*, 1956; McGregor, 1974; Day *et al.*, 1991). This immunity is non-sterile, leading to a state of premunition in which low parasite densities are maintained in the host without causing disease symptoms. In contrast to the slow acquisition of protective immunity to malaria under natural conditions, such immunity can be achieved relatively quickly in humans under clinically controlled conditions and in laboratory animals after one, or a few, blood stage-induced infections with a single cloned strain (genotype) of malaria parasites followed by drug cure (Jeffrey, 1966; Cardigan *et al.*, 1969; Powell *et al.*, 1972; Jarra *et al.*, 1985; Jones *et al.*, 2000; Martinelli *et al.*, 2005a; Cheesman *et al.*, 2006). However, while this immunity can be very effective in protecting individuals against blood stage-induced infection with malaria parasites of the same genotype, it is often less effective against challenge infection with blood stage parasites of a different genotype. These observations imply the existence of Strain Specific Protective Immunity (SSPI) in malaria. Thus, the slow acquisition of immunity to naturally acquired malarial infection is likely to be at least partly due to the existence of polymorphism in the target antigens of protective immunity against the parasites (i.e. multiple allelic forms of an antigen-coding gene; Mendis *et al.*, 1991).

Natural populations of human malaria parasites (Carter *et al.*, 1973; McBride, *et al.*, 1982; Conway *et al.*, 1991a; Conway *et al.*, 1991b; Babiker *et al.*, 1999; Magesa *et al.*, 2002) and laboratory strains of rodent malaria parasites (Carter *et al.*, 1975b; Carter, 1978; Beale *et al.*, 1978) are, indeed, genetically highly polymorphic. Molecular characterisation of genes for protein antigens expressed on the surface of merozoites during blood stage malarial infection has revealed extensive sequence polymorphism in many of these antigen-coding genes. Prominent amongst them are genes encoding the merozoite surface protein-1 (MSP-1) (Tanabe *et al.*, 1987; Miller *et al.*, 1993; S. Cheesman and R. Carter, unpublished data) and the apical membrane antigen-1 (AMA-1) (Thomas *et al.*, 1990; Cheng *et al.*, 1994b; Kappe *et al.*, 1996;

Marshall *et al.*, 1996, S. Cheesman and R. Carter, unpublished data). Both MSP-1 and AMA-1 have been implicated as targets of SSPI in *Plasmodium falciparum* malaria in humans (Conway *et al.*, 2000; Polley *et al.*, 2001; Polley *et al.*, 2003a; Polley *et al.*, 2003b; Polley *et al.*, 2004; Cavanagh *et al.*, 2004) and in *Plasmodium chabaudi* malaria in rodents (Boyle *et al.*, 1982; Brown *et al.*, 1985; Crewther *et al.*, 1996; Renia *et al.*, 1997; Rotman, *et. al.*, 1999).

In a previous study using Linkage Group Selection (LGS) (Martinelli *et al.*, 2005a) to search for genes encoding targets of SSPI in the *P. chabaudi chabaudi* rodent malaria, we located a region of the genome on *P. c. chabaudi* chromosome 8 which contains the gene for MSP-1 (Martinelli *et al.*, 2005a) as encoding a major target of SSPI. However, the two *P. c. chabaudi* strains, AS-pyr1 and CB, used in that study are genetically identical for the cell-surface, ectodomain region of the gene for AMA-1 (S. Cheesman and R. Carter, unpublished data), another candidate for SSPI in rodent malaria (Crewther *et al.*, 1996). It is highly unlikely, therefore, that this LGS analysis could have detected AMA-1 as a target of SSPI (Martinelli *et al.*, 2005a). In the present study, we have conducted LGS analysis of SSPI on two *P. c. chabaudi* strains, AJ and CB-pyr10, which are genetically different for the genes for both MSP-1 and AMA-1 (S. Cheesman and R. Carter, unpublished data). The present analysis has again identified the region on *P. c. chabaudi* chromosome 8 containing the gene for MSP-1 to be under strong SSPI selection. A weaker SSPI selection was also found in the regions on *P. c. chabaudi* chromosome 9 containing the gene for AMA-1 and on chromosome 7 in which no candidates of SSPI have been identified. However, no region in the genome of *P. c. chabaudi* was identified under strength of SSPI selection comparable to that on the region containing the gene for MSP-1. The combined results from these two studies indicate, therefore, that the main force of SSPI is determined by the single region of the *P. c. chabaudi* genome containing the gene for the protein antigen MSP-1.

2.3 Materials and methods

2.3.1 Parasites, laboratory mice and mosquitoes

Two cloned strains of the rodent malaria species *P. c. chabaudi* denoted CB-pyr10 and AJ were used in these experiments. CB-pyr10 is a pyrimethamine resistant cloned line derived through pyrimethamine selection from CB (a pyrimethamine sensitive line), originally cloned from isolate CB (Walliker *et al.*, 1975). In this study, CB-pyr10 will be referred to as CB for simplicity. AJ is a pyrimethamine sensitive line, cloned from isolate AJ (Carter, 1978). Both cloned lines are known to be genetically distinct from each other (Carter, 1978). AJ and CB were isolated from wild thicket rats, *Thamnomys rutilans*, captured in the same locality in the Central African Republic in 1969 and 1970, respectively (Carter, 1978; Beale *et al.*, 1978). Following their cloning, both parasite lines were maintained in the laboratory by serial blood transfer in mice, with occasional transmission through mosquitoes *Anopheles stephensi* and occasional periods in liquid nitrogen as stabilates, according to the laboratory record of Richard Carter (The University of Edinburgh).

Females of inbred laboratory mouse strains, CBA/Ca and C57BL/6J, were used, aged 5 to 6 weeks old on first infection. These mouse strains will be referred here to as CBA and C57. Mosquitoes were from a laboratory-bred colony of *Anopheles stephensi*. Mice were housed in polypropylene cages with sawdust bedding at Animal Husbandry Unit (The University of Edinburgh). Cages were maintained at a constant temperature (25 ± 1°C), on 12:12 hours light:dark cycle). They were allowed to feed on 41B rat and mouse maintenance diet (Harlan-Tekld, England) and supplied with 0.05% *para*-aminobenzoic acid (PABA) in drinking water *ad libitum* to enhance parasite growth (Jacobs, 1964). A complete description of mouse maintenance is published elsewhere (de Roode *et al.*, 2004)

Mosquitoes were from a laboratory-bred colony of *A. stephensi*. They were cultured in nylon cages kept in a temperature and humidity controlled room (24 to 26°C, 70 to 85% humidity) on 12:12 hours light:dark cycle). Adult mosquitoes were supplied with 10% glucose and 2.00% PABA supplemented water solution. Mosquitoes were

maintained with slight modifications from the method previously described (Ferguson *et al.*, 2002). In order to obtain high quality adults, 500 larvae were grown in a low-density condition, in 1 L of distilled water in a 1,000-cm^3 open dish supplied with approximately 1 mg of sodium bicarbonate (Na_2CO_3). After hatching, the larvae were given Liquify (Interpet Ltd.) daily for 7 days, and then Tetrafin (Tetra GmbH) until they developed into the pupa stage and transferred to the adult mosquito cages for emerging. All animal work in this study was carried out in accordance with the Animals (Scientific Procedures) Act 1986 UK.

2.3.2 Induction and characterisation of SSPI between cloned strains CB and AJ of *P. c. chabaudi*

Groups of CBA mice were immunised by two consecutive rounds of blood stage infection with *P. c. chabaudi* followed by mefloquine cure with slight modifications from the method previously described (Cheesman *et al.*, 2006). In brief, each experimental mouse was inoculated intra-peritoneally (i.p.) with 5 x 10^5 parasitised red blood cells (pRBC) of either strain, CB or AJ, of *P. c. chabaudi*. Blood stage malarial infections were terminated by four consecutive daily doses of 20 mg/kg mouse body weight of mefloquine in 0.1 ml Dimethyl Sulfoxide, by oral gavage. Mefloquine treatment started before parasitaemias exceeded 50% but after they had passed 20% of infected red blood cells. The blood stage parasites were monitored daily by microscopic examination of thin blood smears stained with Giemsa's stain. Following each mefloquine treatment all mice remained blood smear negative. Twenty-six days after the last day of the first mefloquine treatment, mice were given a second i.p. infection with 5 x 10^6 pRBC of the *P. c. chabaudi* strain homologous to the first immunising one. Infections were grown for 5 days and drug cure treated as before.

To test for the presence of SSPI, two mice previously immunised by blood stage parasite infection with either CB or AJ from each group were randomly chosen sixteen weeks after the second mefloquine treatment. Each mouse was challenged with a mixture of 5 x 10^6 pRBC containing an equal proportion of blood stage parasites, CB and AJ. An equivalent inoculum from the same mixture was inoculated into a non-immune mouse, which was a batch mate of the immunised mice. The

resulting infections were examined daily by microscopic analysis. A 20 µl sample of tail blood in physiological citrate saline (0.9% (w/v) Sodium Chloride, 1.5% (w/v) Trisodium Citrate Dihydrate) was collected from each mouse 5 to 8 days post infection, so as to accurately measure proportions of parasites of each strain (CB and AJ) using strain specific RTQ-PCR for CB and AJ alleles of the *msp*-1 gene (Cheesman *et al.*, 2003; Cheesman *et al.*, 2006). Tail blood samples were centrifuged at 5,500g for 3 minutes. The red blood cell pellet was washed with 200 µl phosphate buffered saline (Sigma) and stored at –70°C prior to DNA extraction using a High Pure PCR template preparation kit (Roche Diagnostics). RTQ-PCR analysis was performed as previously describe by the method of Cheesman *et al.*, (2003; 2006).

2.3.3 Preparation of a genetic cross between cloned strains CB and AJ of *P. c. chabaudi*

Single strain infections with CB and AJ were individually grown in donor C57 mice. Parasites of the two strains were harvested from the donors and accurately mixed to produce three inocula in proportions of 1:1, 1:2 and 2:1 of CB and AJ, respectively. The mixtures were inoculated i.p. at 10^6 pRBC per mouse into three groups of C57 mice (four mice each). The parasitaemias from the mixed strain infections were followed microscopically on thin tail blood smears stained with Giemsa's stain. Six days post inoculation of the parasites, when parasitaemias were ~20% and the presence of gametocytes of both sexes was confirmed microscopically, mice from each mixed strain infection group were anaesthetised and placed on a mosquito cage, each containing ~ 250 female mosquitoes. Mosquitoes were aged 5 to 7 days post emergence from pupae. The mosquitoes were allowed to feed on the mice for 20 minutes without interruption. The mice were thereafter humanely killed before recovery from the anaesthesia. Eight days after the blood meal, samples of mosquitoes from each cage were dissected and examined for the presence of oocysts on their midguts. 11 mosquitoes from those fed on the 1:1 CB:AJ mixture had a mean of 9.5 oocysts per gut (Standard Error of Mean, SEM = 9.26); 16 mosquitoes from those fed on the 1:2 CB:AJ mixture had a mean of 4.06 oocysts per gut (SEM = 3.96); 18 mosquitoes from those fed on the 2:1 CB:AJ mixture had a mean of 3.13 oocysts per gut (SEM = 6.02) (Table 2.1).

Proportion of CB and AJ in mixtures Used to induced the blood infections	No. of mosquitoes dissected for oocysts (infected)	Average no. of oocysts per gut (SEM)	No. of mosquitoes dissected for sporozoites	Predicted no. of oocysts represented	Predicted no. of recombinant lines
1 : 1	11 (8)	9.5 (9.26)	**190**	1,312	**2,624**
1 : 2	16 (7)	4.06 (3.96)	**190**	337	**674**
2 : 1	18 (13)	3.13 (6.02)	**125**	283	**566**
	total 45 (28)		**total 505**	**total** 1,932	**total 3,864**

Table 2.1. The parameters used to predict the maximum number of recombinant lines present in the pooled progeny of the genetic cross between strains CB-pyr10 and AJ of *Plasmodium chabaudi chabaudi*. The predicted number of such recombinants is calculated as described (see *Materials and Methods*, section 2.3.3). SEM, standard error of mean.

Sixteen days after the blood meal, when sporozoites were present in the mosquito salivary glands, the mosquitoes were allowed to feed on 6 non-immune CBA female mice in order to naturally transmit sporozoites of the progeny of the AJ x CB genetic cross. All surviving mosquitoes of these three batches were then dissected for sporozoites from the salivary glands (190 mosquitoes from the 1:1 CB:AJ mixture, 190 mosquitoes from the 1:2 CB:AJ mixture and 125 mosquitoes from the 2:1 CB:AJ mixture). The glands were gently crushed in 0.2 to 0.4 ml volumes of 1:1 Foetal Bovine Serum (GIBCO BRL): Ringer's solution (420 mg L^{-1} Potassium Chloride, 250 mg L^{-1} Calcium Chloride, 9 g L^{-1} Sodium Chloride) and injected i.p. into 8 non-immune CBA mice. All 14 mice, naturally infected or inoculated with sporozoites dissected from salivary glands of the mosquitoes, became infected. The blood stage parasites representing the progeny of the genetic cross were harvested on day 9 post infection (mean parasitaemia = 15%, min = 0.6% and max 61%) and pooled prior to inoculation of mice previously immunised with parasites of either one or the other of the parental strains CB or AJ of *P. c. chabaudi* for SSPI selection, or else into a non-immune batch mate. The remaining pooled blood was frozen as stabilates and stored in liquid nitrogen.

From the data on the number of oocysts per mosquito and the number of mosquitoes dissected for inoculation of sporozoites (given above), it is possible to estimate the likely maximum numbers of recombinant lines that a genetic cross could have generated. On an assumption that gametes of the two parasite strains are generated in equal numbers in the mixed strain infection used to make a genetic cross, and that fertilisations occur randomly between them, half of all fertilisations will generate hybrid zygotes between the gametes of the two parental strains of parasite, while the second half will derive equally from the self fertilisations representing one or other of the two parental strains. Each hybrid zygote undergoes meiosis and produces four recombinant progeny lines. Thus, on average, for every four oocysts in such a genetic cross, there will be two parental and two hybrid oocysts, yielding a total of eight recombinants. Overall, therefore, there are, on average, two recombinant lines for every oocyst present. Using this logic we was able to estimate the likely maximum number of recombinant lines present in the pooled cross progeny (Table 2.1).

2.3.4 SSPI selection of uncloned cross progeny for Linkage Group Selection analysis

The blood stage parasites from a total of 14 non-immune CBA mice (six and eight mice from the natural transmission and the sporozoite-induced infection, respectively) were harvested in physiological saline and pooled to produce the blood inoculum containing the uncloned CB x AJ cross progeny. One mouse from the batches immunised with blood stage parasites of either CB or AJ and previously tested for SSPI to these strains was inoculated i.p. with 5 x 10^6 pRBC of the uncloned cross progeny. At time of infection with the cross progeny, the immunised mice were twenty weeks after the last day of the second mefloquine treatment (see above). The blood stage parasites from the cross progeny grown in these mice which had been immunised with CB or AJ were designated "CB-immune selected cross progeny" or "AJ-immune selected cross progeny", respectively. An equal number of the cross progeny designated "non-immune selected cross progeny" was passaged into a non-immune batch mate of the immunised mice. The resulting infections were followed by microscopic examination of thin blood smears stained with Giemsa's stain.

The uncloned cross progeny were grown in the CB- and AJ-immunised mice for 11 and 12 days, respectively. We harvested blood from these experimental mice at these timepoints because the differential effects of SSPI upon parasites of the immunising and non-immunising strains were expected to be greatest but before pan specific immunity had overwhelmed the infection. This was in order for there to be the greatest differences in relative intensity between markers under SSPI selection and those which were not.

In the non-immune batch mate, the cross progeny were allowed to grow for 9 days. We could expect in the uncloned cross progeny in the non-immune mouse that the proportions of parasites carrying genetic markers linked to targets of SSPI would remain stable. This is based on the observation that the proportions of the two strains in parasite mixtures of AJ and CB were remarkably constant during the course of infection (Cheesman *et al.* 2006).

To grow parasites in sufficient quantities to prepare DNA for subsequent analysis, 10^6 pRBC from each experimental mouse were inoculated into four non-immune mice. The parasites were grown in these mice to parasitaemias of ~ 35 to 50% before harvesting and preparing them for DNA extraction. We, therefore, aimed to harvest parasites of the uncloned cross progeny grown in the strain specific immunised mice on the day of infection

2.3.5 Extraction of genomic DNA from blood-stage parasites

DNA was extracted from parasites collected after the immune selection experiments. In brief, mice were anaesthetised and bled out. The blood were harvested in 5 ml of chilled physiological citrate saline and passed through two columns of fibrous cellulose CF11powder (Whatman) (Homewood *et al.*, 1976) to remove white blood cells. Blood was filtered through Plasmodipur™ filters (Euro-Diagnostics) twice Parasites were released by saponin lysis (Bowman *et al.*, 1960) and centrifuged at 4000g for 7 min at 4°C. The pellets were re-suspended in 400 µl of lysis solution (10 mM Tris-HCl, pH 8.0; 50 mM EDTA; 0.1% SDS; 1 mg ml^{-1}Proteinase K) and incubated at 42°C overnight. After phenol/chloroform extraction, DNA was precipitated by addition of chilled iso-propanol and 3 M sodium acetate pH 5.2 (Sigma) and treated by standard procedures (Sambrook *et al.*, 1989) prior to subsequent molecular analyses.

2.3.6 Quantitative measurement of strain specific Amplified Fragment Length Polymorphism (AFLP) Markers

To locate regions of the *P. c. chabaudi* genome affected by SSPI selection, we generated ^{33}P-radiolabelled AFLP markers from genomic DNA of the CB- and AJ-immune selected cross progeny, the non-immune selected cross progeny and the two parental cloned strains (CB and AJ) by the method of Grech *et al.*, (2002). AFLP products were resolved on 6% denaturing polyacrylamide gel electrophoresis and visualised using a Phosphoscreen (Amersham) and an X-ray film (Kodak) (see Figure 2.1). The intensities of AFLP bands were quantitatively measured using ImageQuant™ software version 1.2 Build 039 (Molecular Dynamics), as described by Martinelli *et al.*, (2004). The values were converted to a "*Relative Intensity Index*

(RII)", which is a measurement of the proportions of the parasite population carrying a specific allele for CB or AJ represented by individual AFLP markers in the immune and non-immune selected materials. This is defined as the intensity of the strain specific AFLP marker band in the mixture divided by the intensity of a designated non-polymorphic band in the same mixed parasite sample, divided by the equivalent ratio of the two relevant bands (strain specific AFLP and non-polymorphic band) measured in a sample of the pure parental strain (Martinelli *et al.*, 2004). The RII of parasites bearing specific AFLP markers for CB and AJ in the immune selected cross progeny (RII_i) were, thereafter, quantitatively compared to the RII of the same markers derived from the non-immune selected cross (RII_{ni}). These proportions were, thereafter, used to calculate the "*Comparative Intensity* (CI)" which is a measurement of proportional changes of strain specific AFLP markers between the immune and non- immune selected cross progeny. The CI is defined by the RII of a strain specific AFLP marker of the immune-selected cross progeny (RII_i) divided by the RII of the corresponding marker in the non-immune selected cross progeny (RII_{ni}), and expressed as a percentage, i.e., $CI = (RII_i/RII_{ni}) \times 100$ (Martinelli *et. al.*, 2005a). Specific AFLP markers of the immunising parasite strain whose CI values were less than 50% were considered to be under SSPI selection (Martinelli *et al.*, 2005a).

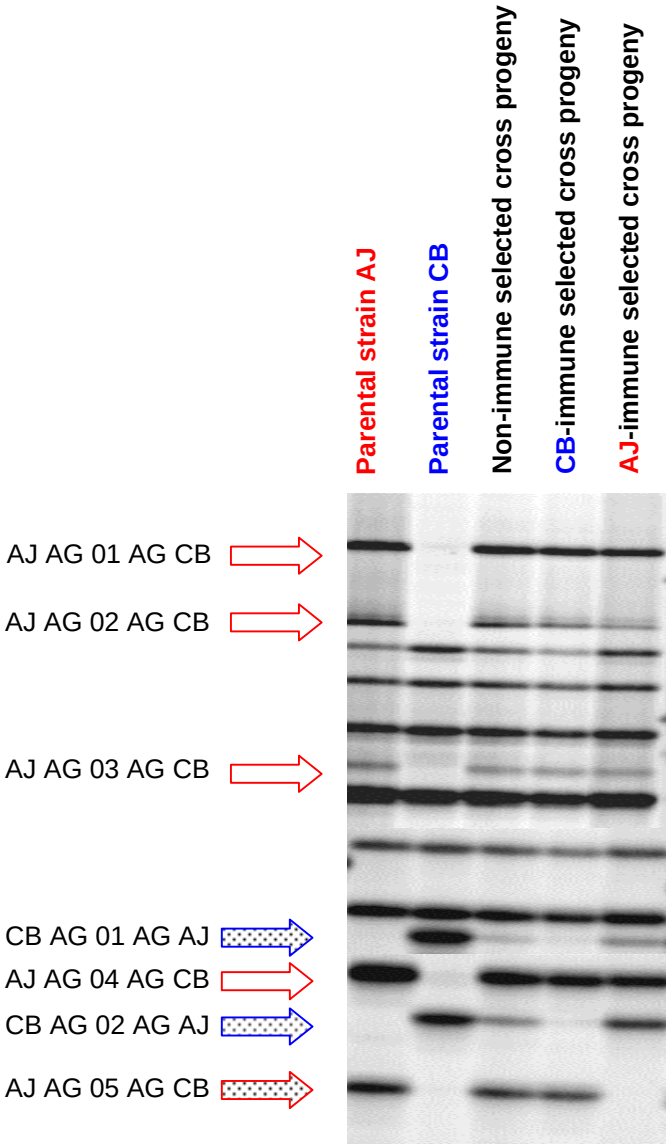

Figure 2.1. shows Amplified Fragment Length Polymorphism products using a single combination of selective primers, conducted on genomic DNA of *Plasmodium chabaudi chabaudi* strains AJ and CB of and the strain specific and non-immune selected progeny of a genetic cross between them. Polymorphic bands are detected on the gel as being present in one parental strain and absent in the other. These bands become parasite strain specific AFLP markers (red arrows, AJ markers; blue arrows CB markers). Of each AFLP marker, the first letters ("AJ" or "CB") indicates in which strain the identified marker band is visible. The last two letters indicates the strain in which the marker is absent. The second two letters (i.e. AG) corresponded to the selective bases used in the ^{33}P-radiolabelled *Eco*RI selective primers (Grech *et al.*, 2002). The two numbers in the middle identify the markers in decreasing size. The following two letters correspond to the selective bases used in the *Mse*I selective primers (Grech *et al.*, 2002). Strain specific AFLP markers that were strongly reduced following strain specific immune selection (filled arrows) are expected to be closely linked to the targets of Strain Specific Protective Immunity (SSPI) (see text). AFLP markers which were not reduced are indicated by open arrows.

2.3.7 Physical mapping of AFLP markers under SSPI selection

Strain specific AFLP markers that had CI reduced below 50% following the strain specific immune selection were identified and characterised by the method of Hunt *et al.*, (2004). The physical location of AFLP markers under SSPI selection was conducted as follows. Individual AFLP markers were excised and eluted from the polyacrylamide gel into distilled water to obtain DNA fragments of each marker. The extracts were PCR amplified using the same pairs of AFLP primers and conditions that were used to generate the original AFLP markers. PCR products were directly sequenced on both strands using the same AFLP primers. The sequencing reactions used ABI BigDye™ Terminator Chemistry on ABI3700 sequencing machine, according to manufacturer's instructions. For PCR products of AFLP markers less than 100 bp, DNA sequences were obtained by cloning with a TOPO TA cloning kit™ (Invitrogen). Plasmid DNA from each transformed bacterial colony was extracted using a QIAprep Spin Mini Prep kit (QIAGEN) and sequenced as described above. DNA sequences obtained from the forward and reverse primers were visualised using an EditView ABI automated DNA sequence viewer software (Perkin Elmer ABI) and assembled manually to generate a single contiguous sequence. The sequences of the specific AFLP primers were eliminated from the assembled DNA sequence prior to location of the AFLP marker onto the genome databases by BLAST search.

Eight-fold coverage contigs of the genome of *P. c. chabaudi* cloned strain AS (Hall *et al.*, 2005), http://www.sanger.ac.uk/cgi-bin/blast/submitblast/p_chabaudi, were searched using the BLASTN (DNA vs. DNA) option with sequences derived from the AFLP markers. Genomic contigs of *P. c. chabaudi* which contained DNA sequences corresponding to the relevant AFLP marker with lowest Probability (*E*) score (a cut-off of 90% sequence identity) were obtained. The chromosomal positions of these contigs have not been mapped physically in the *P. c. chabaudi* genome. Sequences of whole *P. c. chabaudi* contigs were, thereafter, used to locate orthologous loci in the *Plasmodium falciparum* clone 3D7 genome database (Gardner *et al.*, 2002) (http://www.ncbi.nlm.nih.gov/sutils/blast_table.cgi?taxid=Protozoa&database), using the BLASTP (DNA vs. Protein) option. The genomic locations of the *P. falciparum*

orthologues were, in turn, used to locate chromosomal positions of the AFLP markers in the genome of *P. c. chabaudi* through the conserved genetic synteny between the human malaria parasite *P. falciparum* and rodent malaria parasites (Kooij *et al.*, 2005).

2.3.8 Genetic mapping of AFLP markers

In addition to physical mapping, the strain specific were genetically located on a *P. c. chabaudi* genetic linkage map which was previously constructed with reference to the progeny of a genetic cross between *P. c. chabaudi* strains AS and AJ (Martinelli *et al.*, 2005b). The locations of 92 AJ AFLP markers identified in the present study on the *P. c. chabaudi* genetic linkage map are shown in Figure 2.4 and Table S1 (Appendix 1).

2.3.9 RTQ-PCR of CB and AJ alleles of *P. c. chabaudi msp*-1 and *ama*-1

RTQ-PCR analysis was performed on a LightCycler instrument (Roche Diagnostics) as described by Cheesman *et al.*, (2003) and Cheesman *et al.*, (2006) for allele specific amplification assays to measure in the mixtures of parasites the proportions of DNA of the CB or AJ allele of the *msp*-1 or *ama*-1 gene. The assays were standardized for accurate quantification of the CB or AJ alleles of *msp*-1 or *ama*-1 of the blood stage parasites, using artificial mixtures with known proportions of cloned strains CB and AJ as previously described by Cheesman *et al.*, (2003). DNA samples of (i) the blood stage mixed strain infections and (ii) the uncloned CB x AJ cross progeny grown in the strain specific immunised and non-immune mice were prepared as described in previous sections. DNA samples of the pure parental strains CB and AJ were 10-fold serially diluted in the range 66-0.0066 ng and used as quantification standards to construct a DNA concentration calibration curve. Oligonucleotide primers were designed to selectively amplify a strain specific region of the *msp*-1 or *ama*-1 gene (S. Cheesman, unpublished data). The *msp*-1 specific primers used in the assay were: the CB forward and reverse primers: 5'-CTGTTACAACCCAAACC-3' and 5'-AGTTG TTCCTGTGGCAG-3'; and the AJ forward and reverse primers: 5'-ACTGAAGCAACAACACCAGC-3' and 5'-GTTGTTGATGCACTTGCGGGTTC-3'. RTQ-PCR reactions for the CB and AJ

alleles of *msp*-1 were set up, as previously described (Cheesman *et al.*, 2006). The *ama*-1 specific primers used in the assay were: the CB forward and reverse primer 5'-AGGTTTCATTATTAACACGAG-3' and 5'-GATTACTTTTGTCATAAACAG CG-3'; and the AJ forward and reverse primer 5'-CTAAA TCATTCTTAGACCC-3' and 5'-GGCATAATTTTTATATTCTG-3'. The nucleotide positions in the forward primers that did not match the sequences of the CB and AJ *ama*-1 alleles are shown in bold. RTQ-PCR reactions for quantification of the CB and AJ alleles of *ama*-1 were performed in a 10 µl volume of standard buffer containing 4 µM of forward and reverse primers in 3 and 2.5 mM $MgCl_2$, respectively. The PCR conditions used were as follows: An initial "Hot Start" at 95 °C for 600 sec followed by 40 cycles of 95 °C with a 0 sec hold, cooling at 20 °C/sec to 58 °C with a 7 sec hold for CB or cooling at 20 °C/sec to 56 °C with a 7 sec hold for AJ, reheating at 20 °C/sec to 72 °C with a 15 sec hold for CB or 10 sec hold for AJ and finally heating at 20 °C/sec to 72 °C with a 0 sec hold. Melting curve data for each PCR run was produced, as follows: Heating at 20 °C/sec to 95 °C with 0 sec hold, cooling at 20 °C/sec to 65 °C with 30 sec hold and reheating at 0.2 °C/sec to 95 °C in a continuous data acquisition mode. The final step of RTQ-PCR was at 20°C/sec to 40 °C with a 60 sec hold. Data obtained from each LightCycler run were checked to ensure that the correct strain specific melting peak and no other non-specific amplicon was produced. The DNA concentrations measured on the LightCycler were converted into relative proportions of the AJ and CB alleles for *msp*-1 or *ama*-1 within each sample analysed.

2.4 Results

2.4.1 Characterisation of SSPI in mice for *Plasmodium chabaudi chabaudi* cloned strains CB and AJ

Immunity against the blood stages of *P. c. chabaudi* was induced in groups of female CBA mice, five to six weeks old on first infection, by two successive single strain blood stage-induced infections of either *P. c. chabaudi* CB or AJ, drug cured with mefloquine, as described in *Materials and Methods* (section 2.3.2). Sixteen weeks after the last mefloquine dose, two CB-immunised mice and two AJ-immunised mice were challenged with a mixture of an equal proportion of blood stage parasites of CB and AJ. A non-immune mouse, which was a batch mate of the immunised mice, was inoculated with the same mixture of blood stage parasites of CB and AJ. Total parasitaemias were measured daily by microscopic analysis of thin blood smears (Figure 2.2A). The proportions of parasites carrying the CB or AJ alleles of *P. c. chabaudi msp*-1 were determined using strain specific RTQ-PCR analysis of DNA samples obtained from the mixed strain infections between 5 and 8 days after inoculation (see *Materials and Methods*; section 2.3.9).

In mice previously immunised with either CB or AJ, the total parasitaemias following challenge with the mixed strain infections of *P. c. chabaudi* CB and AJ were greatly reduced relative to the total parasitaemia in a non-immune batch mate infected with the same mixture (Figure 2.2A). It is clear, therefore, that a strain-transcending immunity must have been present in the CB and AJ immunised mice as both reduced the absolute parasitaemias to a similar degree. Parasitaemias of the strain homologous to the immunising one were, however, consistently lower, and the parasites were eliminated more rapidly, than those of the heterologous strain in the immunised mice (Figure 2.2C and 2.2D). By contrast, CB and AJ were present in almost equal proportions throughout the period of observation in the non-immune mouse (days 5 to 8) (Figure 2.2B).

The differential effect in the strain specifically immunised mice was greatest on day 7 of infection when parasites of the homologous strain were undetectable (< 1% parasitaemia) by strain specific RTQ-PCR (see *Materials and Methods*; section

2.3.9), while parasites of the heterologous strain survived at parasitaemias of around 6% and 1 to 3% in CB- and AJ-immunised mice, respectively (Figure 2.2C and 2.2D). Single strain (CB or AJ) immunised batch mates of the mice tested in this experiment were used to apply SSPI selection pressure against the uncloned progeny of a genetic cross between CB and AJ, as described in the following section.

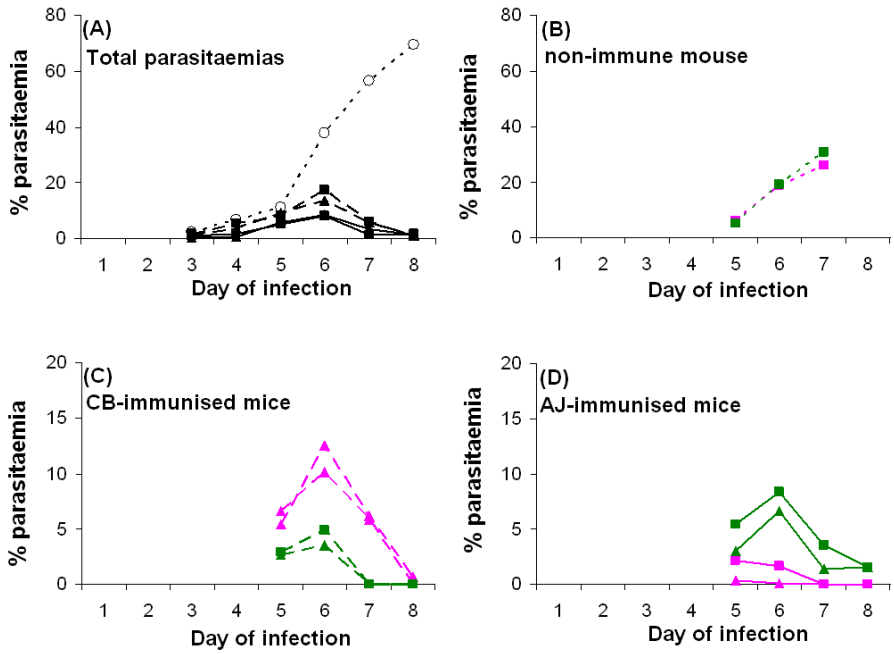

Figure 2.2. Mixed strain infections of *Plasmodium chabaudi chabaudi* CB and AJ in mice pre-immunised with either strain, or in a non-immune batch mate. (A) shows total parasitaemias during the course of mixed strain infections in immunised mice and a non-immune as measured by microscopic examination of thin blood smears stained with Giemsa's stain: non-immune mouse (dotted line with open circles), two CB-immunised mice (dashed lines with filled symbols), two AJ-immunised mice (unbroken lines with filled symbols). Strain specific parasitaemias (AJ in pink; CB in green) are shown (B) in the non-immune mouse, (C) in the two CB-immunised mice and (D) in the two AJ-immunised mice. The strain specific parasitaemias in the mixed strain infections were calculated from the total parasitaemias measured on thin blood smears stained with Giemsa's stain and from the proportions of CB and AJ parasites in the mixtures as determined using strain specific RTQ-PCR (see text). Squares and triangles represent mouse 1 and 2, respectively, in the CB and AJ immunised mice in (A), (C) and (D).

2.4.2 SSPI Selection of the uncloned cross progeny between *P. c. chabaudi* CB and AJ

A genetic cross between *P. c. chabaudi* CB and AJ was generated and yielded the predicted maximum number of independent recombinant lines of approximately 3,800 (Table 2.1), as described in *Materials and Methods* (section 2.3.3). Sporozoites were harvested from the mosquitoes containing the cross and inoculated into non-immune mice as uncloned cross progeny (see *Materials and Methods*, section 2.3.4). The blood stage parasites of the uncloned cross progeny were subinoculated into mice which had been immunised with one or the other of the parental strains. A non-immune mouse, which was a batch mate of the immunised mice, was inoculated with the same mixture of the uncloned CB x AJ cross progeny. The uncloned cross progeny was allowed to grow in the CB- and AJ-immunised mice and in the non-immune batch mate (Figure 2.3). The blood stage parasites in the CB- and AJ-immunised mice were harvested and expanded by subinoculation of 10^6 pRBC into groups of four non-immune female CBA mice on days 11 and 12 of infection, respectively. The cross progeny from the non-immune mouse was similarly expanded by subinoculation on day 9 of infection. These parasites were prepared for extraction of parasite genomic DNA for the subsequent analyses, as described in *Materials and Methods* (section 2.3.5).

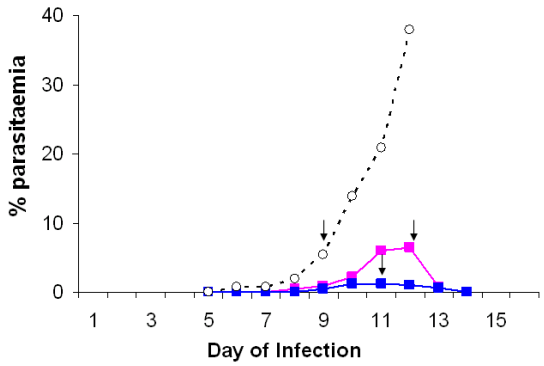

Figure 2.3. The course of blood stage-induced infection of the uncloned CB x AJ cross progeny grown in a non-immune mouse (dotted line with open circles), in a CB-immunised mouse (pink line filled squares) and in an AJ immunised mouse (blue line with filled symbols). Arrows indicate day of infection when the uncloned cross progeny grown in the immunised mice or the non-immune mouse were sub-inoculated for expansion into non-immune mice (see text).

2.4.3 Molecular Genetic Analysis of SSPI Selected Uncloned Cross Progeny

The genomic DNA of parasites derived from the CB- and AJ-immune selected cross progeny and from the cross progeny grown in the control non-immunised batch mate was typed with genome-wide quantitative genetic markers produced by Amplified Fragment Length Polymorphism (AFLP) (Grech *et al.*, 2002) (see *Materials and Methods*, section 2.3.6). From 97 combinations of selective AFLP primers, 350 polymorphic bands that differentiated CB and AJ strains of *P. c. chabaudi* were obtained. Of these bands, 197 were present only in AJ and 153 were present only in CB. These were used as AFLP markers for the strains AJ and CB, respectively. The Comparative Intensity (CI) (see *Materials and Methods*, section 2.3.6, for a definition) (Martinelli *et al.*, 2005a) was calculated for each AFLP marker in the CB and AJ-immune selected cross progeny (Table S1, Appendix 1).

Following AJ-specific immune selection, 24 of the 197 AFLP markers for strain AJ (AJ markers) (12.2% of AJ specific markers) had CI of less than 50% under AJ-specific immune selection (i.e. intensity reduction of greater than 50% relative to growth of the cross progeny in the non-immune batch mate), listed in Table 2.2. These AJ markers under SSPI selection were sequenced, as described in *Materials and Methods* (section 2.3.7). 19 contained a single sequence each, all of which were identified in the *P. c. chabaudi* genome database, at the website http://www.sanger.ac.uk/cgi-bin/blast/submitblast/p_chabaudi. The predicted orthologous loci of these *P. c. chabaudi* contigs were, thereafter, located within the *P. falciparum* genome. Because of the high level of conserved synteny between the genomes of *P. falciparum* and the rodent malaria parasites (Kooij *et al.*, 2005), the chromosomal locations of the *P. falciparum* orthologues of AJ markers could, in turn, be mapped to the equivalent predicted chromosomal positions in the *P. c. chabaudi* genome. The remaining 5 AJ markers under SSPI selection gave unreadable sequence data. Their physical locations in the *P. c. chabaudi* genome could not be determined (Table 2.2).

Of the 19 AJ markers whose predicted orthologues were successfully identified in the *P. falciparum* genome, one mapped to a region on *P. falciparum* chromosome 4 which is syntenic with a location on *P. c. chabaudi* chromosome 7. Nine other markers were located to a region on *P. falciparum* chromosome 9 which is syntenic with one on *P. c. chabaudi* chromosome 8. Three markers were located to a region on *P. falciparum* chromosome 11 which is syntenic with one on *P. c. chabaudi* chromosome 9. Five additional markers were located to a region on *P. falciparum* chromosome 12 which is syntenic with one on chromosome 14 of *P. c. chabaudi*. A final marker responding to SSPI selection was located to a region on *P. falciparum* chromosome 14 corresponding to one on *P. c. chabaudi* chromosome 12 (Table 2.2).

In addition, 92 of the 197 AJ markers were located onto ten *P. c. chabaudi* chromosomes and six unassigned *P. chabaudi* linkage groups of a *P. c. chabaudi* genetic linkage map previously generated from the progeny of a genetic cross between AS and AJ strains (Martinelli *et al.*, 2005b). The genetic position of the 92 markers is displayed versus its CI in Figure 2.4. Of 24 AJ AFLP markers having CI of less than 50% under AJ-specific immune selection, 21 AJ markers with CI of < 50% following AJ-specific immune selection successfully mapped onto three *P. c. chabaudi* chromosomes and two unassigned *P. c. chabaudi* linkage groups (Table 2.2). In most cases, the location of the markers by genetic mapping was in agreement with that of the physical mapping. However, five markers that were located to *P .c. chabaudi* chromosome 14 by physical mapping mapped genetically to *P. c. chabaudi* chromosome 7 (Table 2.2). The reason for this discrepancy is not known and will require further investigation. Four markers whose orthologues could not be identified in *P. falciparum* were genetically located to unassigned *P. c. chabaudi* linkage groups g2 (one marker) and g12 (three markers).

There are six AJ markers with CI of below 20% following the AJ-specific immune selection. These markers had the greatest reduction in intensity and were all located on *P. c. chabaudi* chromosome 8 by both physical and genetic mapping (represented in bold in Table 2.2). These AJ markers generally decreased in CI with deceasing physical distance (in *P. falciparum*) or genetic distance (in *P. c. chabaudi*) from the AJ allele of the *P. c. chabaudi msp*-1 locus (Table 2.2 and Figure 2.4). The

proportion of parasite DNA carrying the AJ allele of *msp*-1 was reduced to undetectable levels (<1%) as measured by strain specific RTQ-PCR in the cross progeny grown under the AJ-specific immune selection compared to its presence at a proportion of 60.0% in the cross progeny grown in the non-immune mouse (Table 2.2, Figure 2.4). Thus, the *P. c. chabaudi msp*-1 locus lies at the lowest point that has been detected in the strain specific immune-selected valley on *P. c. chabaudi* chromosome 8.

In addition, three of the 24 AJ markers with CI of < 50% under AJ-specific immune selection also formed a linkage group that contains the gene encoding the *P. c. chabaudi* AMA-1 (Table 2.2, Figure 2.4). An *ama*-1 strain specific RTQ-PCR assay for CB and AJ (see *Materials and Methods*; section 2.3.9) showed that 17.3% of the DNA of parasites in the AJ-immune selected cross progeny carried the AJ allele of *ama*-1 (Table 2.2), compared to 79.6% with the AJ allele of *ama*-1 in the cross progeny grown in the non-immune mouse.

In a reciprocal experiment the uncloned progeny of the cross between *P. c. chabaudi* strains CB and AJ were subjected to CB-specific immune selection. The intensities of most of the AFLP markers for strain CB (CB markers) were already very faint in the unselected (control) cross progeny compared to the intensities of the corresponding AFLP bands in the parental strain CB. The general band intensities of the CB markers were below the limit of detection by AFLP in the CB-immune selected cross progeny (Martinelli *et al.*, 2004). Consequently, we were unable to achieve a meaningful analysis of the CB-immune selected cross progeny by AFLP. We were, nevertheless, able to investigate the level of CB-specific immune selection against the CB allele at the *msp*-1 and *ama*-1 loci using RTQ-PCR. In DNA from parasites of the CB-immune selected cross progeny, the CB allele of *msp*-1 was undetectable (< 1 %) by RTQ-PCR, compared to 40.0% in the cross progeny grown in the non-immune mouse. At the *ama*-1 locus, 1.8% of the DNA of parasites carried the CB allele of *ama*-1 from cross progeny grown in the CB-immunised mouse, compared to 20.4% in the cross progeny grown in the non-immune mouse.

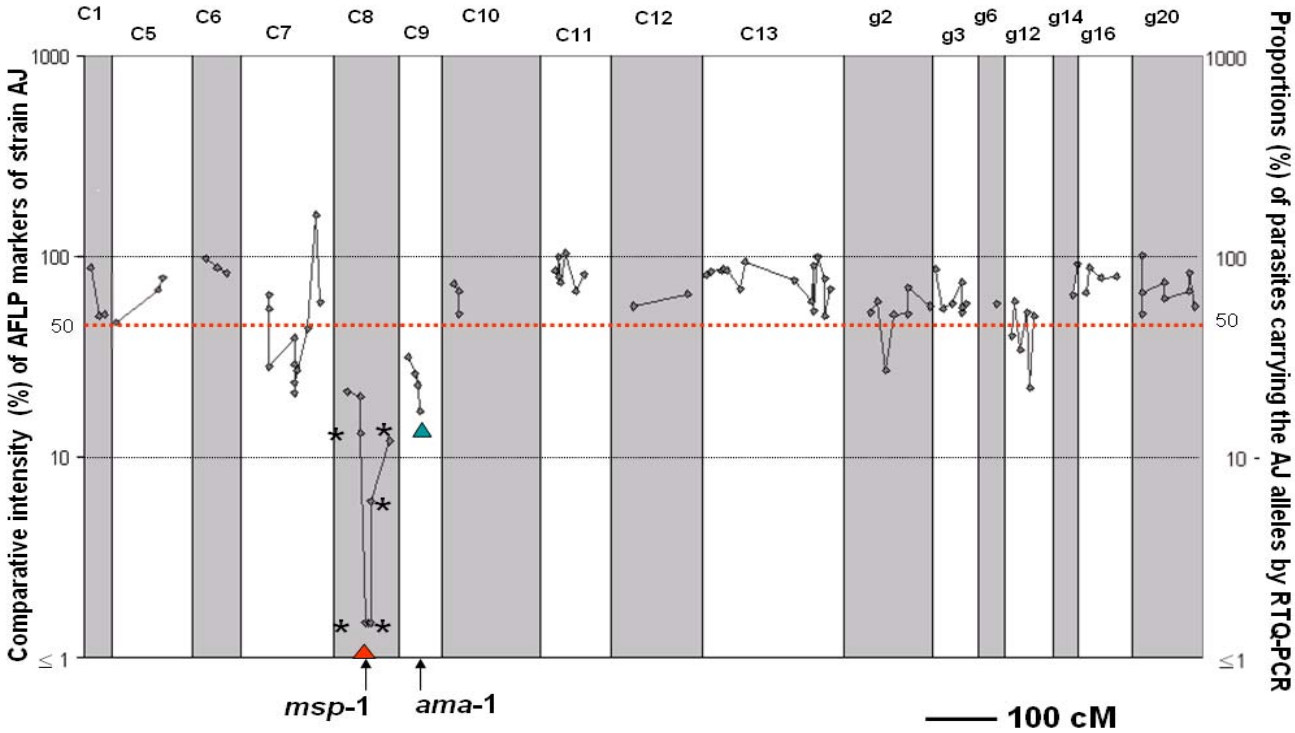

Figure 2.4. The Comparative Intensities of 92 AFLP markers of *Plasmodium chabaudi chabaudi* strain AJ from the progeny of a genetic cross between *P. c. chabaudi* strains CB-pyr10 and AJ following selection in mice immunised with strain AJ (see text). AJ-specific markers (indicated by black diamonds) were located on a *P. c. chabaudi* genetic linkage map, generated from a genetic cross between AS and AJ (Martinelli *et al.*, 2005b). Numbers after letter 'C' and 'g' represent *P. c. chabaudi* chromosome numbers and *P. c. chabaudi* unassigned linkage groups, respectively, in the genetic linkage map (Martinelli *et al.*, 2005b). Of the six AJ markers which were most reduced under AJ-specific immune selection (see Table 2.2), five (indicated by asterisks) could be located to *P. c. chabaudi* chromosome 8, forming a selection valley with the *P. c. chabaudi msp*-1 gene at its lowest point (see text). RTQ-PCR values for the proportions of the AJ-immune selected cross progeny carrying the AJ alleles of the Merozoite Surface Protein-1 (*msp*-1) are indicated by the red triangle and Apical Membrane Antigen-1 (*ama*-1) by the green triangle in the AJ-immune selected cross progeny. The red line indicates Comparative Intensity of 50%.

Table 2.2

Name of AJ markers	**CIs of the AJ markers in AJ immunised mouse**	**Physical location of *P. falciparum* orthologues of the AJ markers in *P. falciparum* genome** (Gardner *et al.*, 2002)	***P. falciparum* chromosomes**	**Chromosome on which the AJ marker is predicted to be located in *P. c. chabaudi* by physical mapping**	**Chromosome on which the AJ marker is predicted to be located in *P. c. chabaudi* by genetic mapping**	**Genetic distances along the *P. c. chabaudi* chromosomes in centiMorgan** (Martinelli *et al.*, 2005b)
AJ TG 03 AA CB	21.7	*pf* 9 - 378**	9	8	8	22.0
AJ TA 04 AT CB	20.2	*pf* 9 - 718	9	8	8	43.0
AJ GT 02 TA CB	**13.1**	***pf* 9 - 992**	**9**	**8**	**8**	**43.0**
AJ AT 01 AG CB	**10.4**	***pf* 9 - 1019**	**9**	**8**	**ND**	**ND**
AJ TT 03 AT CB	**12.15**	***pf* 9 - 1113**	**9**	**8**	**8**	**59.4**
AJ TA 07 TA CB	**6.01**	***pf* 9 - 1150**	**9**	**8**	**8**	**59.4**
AJ TC 01 TG CB	**1.5**	***pf* 9 - 1159**	**9**	**8**	**8**	**59.4**
AJ allele of *msp*-1	(0 %, 60%)*	*pf* 9 - 1201	9	8	8	50.8
AJ AG 05 AG CB	**1.5**	***pf* 9 - 1263**	**9**	**8**	**8**	**55.3**
AJ AT 03 AG CB	47.5	*pf* 9 - 1368	9	8	ND	ND
AJ AC 01 CT CB	31.8	*pf* 11-1084	11	9	9	51.7
AJ AG 01 TC CB	25.9	*pf* 11-1245	11	9	9	63.5
AJ AT 03 TA CB	22.7	*pf* 11-1084	11	9	9	67.2
AJ allele of *ama*-1	(17.3 %, 79.6%)*	*pf* 11-1290	11	9	9	71.1
AJ AT 01 GT CB	28.4	ND	ND	ND	7	37.3
AJ TG 01 AT CB	21.09	*pf* 12 -1259	12	14	7	77.7
AJ AT 01 TC CB	39.1	*pf* 12 -1267	12	14	7	77.7
AJ TA 06 TA CB	23.37	*pf* 12 -1290	12	14	7	77.7
AJ TG 01 TC CB	29.0	*pf* 12 -1346	12	14	7	77.7
AJ TT 02 TG CB	27.0	*pf* 12 -1355	12	14	7	81.4
AJ AG 01 CA CB	43.54	*pf* 4 - 787	4	7	7	99.6
AJ AG 02 AG CB	27.2	*pf* 14 -2756	14	12	ND	ND
AJ TT 01 TC CB	26.88	ND	ND	ND	g2	ND
AJ AG 01 TA CB	40.0	ND	ND	ND	g12	ND
AJ AT 02 TT CB	22.22	ND	ND	ND	g12	ND
AJ TG 02 AT CB	34.6	ND	ND	ND	g12	ND

Table 2.2. Physical and genetic locations of AFLP markers of strain AJ of *Plasmodium chabaudi chabaudi* with Comparative Intensity (CI) reduced below 50% in the progeny of the genetic cross between CB-pyr10 and AJ following selection in an AJ-immunised mouse (see text). The six AJ markers with CI of <20% in the AJ-immune selected cross progeny mapped to positions closely linked to the gene encoding the *P. c. chabaudi merozoite surface protein*-1 (MSP-1), indicated in bold. ND not determined.

* The first and second numbers in the bracket represents percentage of parasite DNA carrying the AJ alleles of *msp*-1 (pink background) and *ama*-1 (blue background) in the AJ-immune selected cross progeny and the non-immune selected cross progeny, respectively, as measured by RTQ-PCR (see text)

** Numbers after '*pf*' indicate the *Plasmodium falciparum* chromosome number and distance along the chromosome in kilo basepairs, respectively

2.5 Discussion

Linkage Group Selection is a molecular and genetic approach that applies a specific selection pressure to the uncloned recombinant progeny of a genetic cross between two genotypes (strains) of the same species of malaria parasites that differ in their phenotypic responses under the specified selection pressure (Culleton *et al.*, 2005; Carter *et al.*, 2007). Here we have used LGS in an attempt to identify the region(s) in the genome of the rodent malaria parasite *Plasmodium chabaudi chabaudi* which harbours the gene(s) that is the target of SSPI against the blood stages of this parasite. We have taken two genetically distinct strains of *P. c. chabaudi*, CB and AJ, which induce SSPI with respect to each other in laboratory mice, and crossed them to produce recombinant progeny. We then applied SSPI selection to the uncloned recombinant progeny by growing the cross progeny in mice made immune to one or the other of the two strains of the parasite (strain specific immunised mice). As a reference, the same uncloned progeny were also grown in a non-immune batch mate of the strain specific immunised mice. The cross progeny grown in the CB- or AJ-immunised mouse, or the non-immune batch mate, were screened for the presence and intensity of quantifiable genome-wide AFLP markers distinguishing CB and AJ.

Following growth of the cross progeny in the AJ-immunised mouse, a small proportion of markers of the immunising strain (AJ markers) was judged to be significantly reduced (CI of <50%) relative to the progeny grown in the non-immune mouse (Martinelli *et al.*, 2005a). These markers were located in the genome by either physical or genetic mapping, or both, as described in *Results* (section 2.4.3). Several AJ markers that were located to a region on *P. c. chabaudi* chromosome 8 formed the deepest identified selection valley in this analysis (Figure 2.4). Within this selection valley, the two AJ markers under the strongest AJ-specific immune selection spanned a predicted ~120 kilobase pair (kb) region (based upon the predicted physical locations of the orthologues of these two markers in the *P. falciparum* genome). This region contains a gene for the *P. c. chabaudi* MSP-1, a principle candidate for a target antigen of SSPI. RTQ-PCR analysis confirmed that the uncloned cross progeny carrying the AJ allele of the *msp*-1 gene was virtually eliminated after selection in the AJ-immunised mouse. The reciprocal result was

obtained after selection of the cross progeny in a CB-immunised mouse. In this case, parasites carrying the CB allele of the *P. c. chabaudi msp*-1 locus were virtually eliminated. These results demonstrate that parasites carrying AJ or CB alleles in the region of the *msp*-1 locus were under very strong selection in AJ- or CB-immunised mice, respectively. A previous LGS analysis of SSPI in the progeny of a genetic cross between strains AS-pyr1 and CB of *P. c. chabaudi* also found that the region containing the *msp*-1 locus was under the strongest detected strain specific protective immune selection for this combination of strains (Martinelli *et al.*, 2005a). Together, these results strongly support the view that the region on *P. c. chabaudi* chromosome 8 around the *msp*-1 locus contains a gene or genes encoding a major target antigen or antigens of SSPI.

In addition to those AJ markers associated with the selection valley around *msp*-1, several other AJ markers had CIs of less than 50% after selection in the AJ-immunised mouse (Table 2.2 and Figure 2.4), suggesting the possible presence of other targets of SSPI.

Several of these more weakly reduced AJ markers formed linkage groups having possible association with selection valleys. One such group, containing seven weakly reduced AJ markers, was located on *P. c. chabaudi* chromosome 7 and spanned a predicted ~ 62.3 centiMorgan (cM) of genetic distance (Table 2.2) by genetic mapping. However, there was discrepancy in the location of some markers in this group by physical mapping (Table 2.2). Two other such markers were located on *P. c. chabaudi* unassigned linkage groups 2 and 12 (Table 2.2). The reductions in intensity of all these markers after selection in the AJ-immunised mouse were, however, much less than those associated with the *msp*-1 locus, suggesting relatively small contributions toward SSPI. No obvious candidate gene for a target antigen of SSPI has been identified within these genomic regions.

Three of the relatively weakly affected AJ markers were located on *P. c. chabaudi* chromosome 9 and were linked to the gene for AMA-1 (Table 2.2), another

candidate target of SSPI (Crewther *et al.*, 1996). The results suggest that AMA-1, or the product of a gene closely linked to the *ama*-1 gene, may be a target of SSPI, but one that is less strongly affected than the target(s) associated with the gene for MSP-1.

As already noted we had previously conducted LGS analysis of SSPI with a combination of *P. c. chabaudi* strains, AS-pyr1 and CB (Martinelli *et al.*, 2005a). However, in contrast to the situation in the present cross between AJ and CB, the AS-pyr1 and CB strains have DNA sequences which are identical at the *ama*-1 locus for the extracellular domain of the AMA-1 protein (Cheesman and Carter, unpublished data). It is, therefore, highly unlikely that evidence of the involvement of AMA-1 in SSPI could have been found in the AS-pyr1 and CB cross, nor was it (Martinelli *et al.*, 2005a). In order to evaluate the involvement of AMA-1 in SSPI, in collaboration with Dr Robin Anders, La Trobe University, in Melbourne, Australia, we are planning to test the effects of vaccination with recombinant AMA-1 using constructs based on the AJ or CB allele of *P. c. chabaudi*. This would allow to us determine if vaccination with recombinant AMA-1 can induce strong SSPI in laboratory rodents. Should such an effect be observed, we will conduct LGS analysis upon the progeny of an AJ x CB genetic cross in mice immunised with an AJ and CB AMA-1 vaccine to address this question. Currently, the refolded recombinant AMA-1 proteins expressed in *E. coli* have been produced in Dr Anders' laboratory using their established protocols (Crewther *et al.*, 1996). Vaccination studies are now underway.

There is a possibility that there are other regions of the *P. c. chabaudi* genome that may be under SSPI selection pressure but have not been detected, as no AFLP markers have been mapped on *P. c. chabaudi* chromosomes 2, 3, 4 and 14 and only a few on chromosomes 10 and 12 (Figure 2.4). Nevertheless, quite large chromosomally unassigned linkage groups were identified (Figure 2.4). These almost certainly represent groups of linked markers on all, or most, of the missing chromosomes (Martinelli *et al.*, 2005b). Since no clear selection valley has been located on any of these unassigned linkage groups, it is unlikely that the present

analysis failed to identify any other major SSPI-associated selection valleys that might exist.

It is noteworthy, however, that there is a limitation of LGS to the discovery of genes encoding antigens for SSPI. For instance, LGS would not be able to identify genes coding for proteins exhibiting clonal antigenic variation to be the cause of the observed differences between any two parasite lines which appeared to show mutual SSPI in mice. The clonal antigenic variation in *P. c. chabaudi* is probably mediated by the *cir* gene family which codes for variant surface antigens (Janssen *et al.*, 2002). The main reason for this is that there will be a subpopulation of parasites that switch off the expression of alleles of the variant antigen which are targeted by protective immunity by antigenic switching but switch on the expression of different alleles not recognised by protective immunity. This phenomenon has been recently demonstrated in the rodent malaria parasite *Plasmodium yoelii yoelii* (Cunningham *et al.*, 2005).This process allows parasites to evade host protective immune response and allows parasites to maintain the alleles of the variant surface antigens conferring immune protection in their populations.

LGS analyses of SSPI conducted on the progeny of two different genetic crosses, one between AJ and CB-pyr10 (in this present study) and the other between AS-pyr1 and CB (the parental clone of CB-pyr10) (Martinelli *et al.*, 2005a) have consistently revealed prominent selection valleys at the same location on *P. c. chabaudi* chromosome 8. In both cases, the base of the SSPI-selected valley covered a region within ~ 60 kb on either side of the *msp*-1 locus. We conclude, therefore, that the *P. c. chabaudi msp*-1 locus, or a gene very closely linked to it, dominated the SSPI selection in both of these cases.

Although our work has not yet proved that the *P. c. chabaudi* MSP-1 protein is itself a target of SSPI in malaria, such a result would be consistent with the literature that indicates the involvement of MSP-1 in the strain specific protective immune response in the rodent malarias. Thus, on passive transfer into non-immune mice, monoclonal antibody (mAb) NIMP23, which had been raised against MSP-1 of *P. c.*

chabaudi strain AS and shown to bind the MSP-1_{19} portion of the protein (McKean *et al.*, 1993b), inhibited the growth of blood stages of the homologous strain (AS) (Boyle *et al.*, 1982), but had no effect on a heterologous challenge infection with the CB strain of *P. c. chabaudi* (Brown *et al.*, 1985). Following these studies, McKean and colleagues were able to identify an epitope to which mAb NIMP23 bound and which was located at the C-terminus of *P. c. chabaudi* MSP-1 (McKean *et al.*, 1993b). This part of MSP-1 was shown to be genetically distinct between strains AS and CB (McKean *et al.*, 1993a; S. Cheesman and R. Carter, unpublished data). The coding sequence in the same region of MSP-1 is also different between strains AJ and CB (McKean *et al.*, 1993a; S. Cheesman and R. Carter, unpublished data), which are the two parental strains used in the present LGS analysis of SSPI. However, between strains AS and AJ, two strains which show virtually no SSPI with respect to each other (R. Carter and A. Martinelli, unpublished data), there is no difference in the amino acid sequence of MSP-1 in this region (McKean *et al.*, 1993a). The sequence polymorphisms identified at the C-terminus of MSP-1 in *P. c. chabaudi* are, therefore, consistent with the involvement of MSP-1 in SSPI in this parasite.

Evaluating the involvement of MSP-1 in SSPI is now a priority for future research. One approach used to address this topic involves (1) cloning out the SSPI-selected cross progeny and (2) characterising the haplotypes at the base of the selection valley in the resulting cloned lines (Carter *et al.*, 2007) (see Figure 2.5). In theory, it is expected that in cloned lines of the SSPI-selected cross progeny, alleles of the sensitive parent at the locus containing the target of SSPI will be removed. Nevertheless, there will be some SSPI-resistant parasite progeny clones in which recombination events have taken place between the allele of the target locus of SSPI from the resistant parent (locus ‘B’) and markers (e.g. locus ‘A’ or ‘C’) from the sensitive parent that are closely linked to the target locus (Figure 2.5). Identification of such recombinant cloned lines of the SSPI selected cross progeny will enable us to pinpoint the locus under SSPI selection to the interval between these recombination events (Carter *et al.*, 2007). Should such a defined locus contain only the allele of the *msp*-1 gene, this will identify the *msp*-1 gene as a principle target of SSPI. Currently, there is an ongoing project in our laboratory which has taken this approach to

identify genes encoding targets of SSPI using the SSPI-selected progeny of a genetic cross between *P. c. chabaudi* AS-pyr1 and CB (Martinelli *et al.*, 2005a) (S. Cheesman, E. O' Mahony, and R. Carter, personal communication).

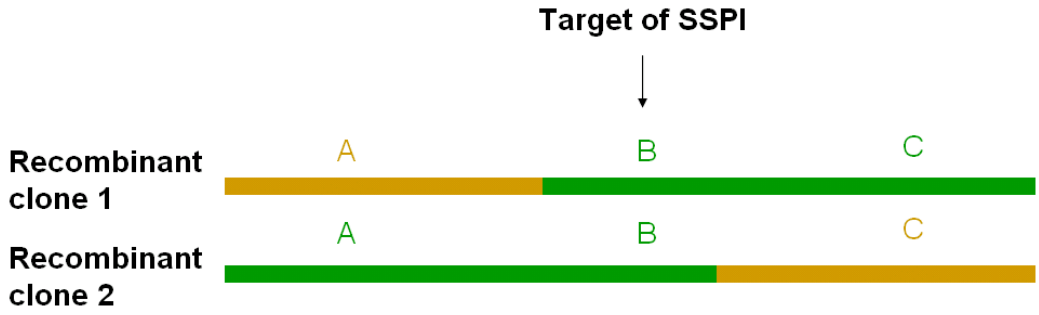

Figure 2.5 A schematic representation of haplotypes of two cloned progeny of a genetic cross between strain specific protective immune (SSPI)-resistant and sensitive lines which have recombination events within a region containing a target of SSPI selection. Locus 'B' represents the target of SSPI. Loci 'A' and 'C' represent genes whose the position is closely genetically linked to the target locus of SSPI. Alleles of the SSPI-resistant and sensitive parental clones are indicated by green and orange, respectively. (Figure adapted from Carter *et al.*, 2007).

Moreover, transfection systems could be used to evaluate the candidacy of MSP-1. This approach will involve a production of a transgenic parasite line of *P. c. chabaudi* whose original (wild-type) allele of the *msp*-1 gene is replaced with an allele from a genetically distinct strain. Subsequently, the transgenic parasite can be used for immunisation and testing of strain specificity of protective immunity in mice previously immunised with parasites that have a wide-type *msp*-1 allele. Should the wild-type strain be virtually eliminated and the transgenic *msp*-1 parasite, whose genetic background is identical to the wild-type parasites except for the *msp*-1 locus, survive following a challenge infection between these two lines, this would indicate that MSP-1 is an antigen that triggers SSPI. Currently, the transfection systems have been developed, and are now routinely used, for the rodent malaria parasites *Plasmodium berghei* (Waters *et al.*, 1997; Tomas *et al.*, 1998; Janse *et al.*, 2006) and *Plasmodium yoelii* (Jongco *et al.*, 2006), the primate malaria parasite *Plasmodium knowlesi* (van der Wel *et al.*, 1997) and the human malaria parasite *P. falciparum* (Crabb *et al.*, 1997; Waterkeyn *et al.*, 1999; Crabb *et al.*, 2004; Balu *et al.*, 2005).

This technology would be equally applicable for evaluating an involvement of MSP-1 in SSPI in *P. c. chabaudi*.

Alternatively, vaccination with recombinant *P. c. chabaudi* MSP-1 using constructs based on the AJ or CB alleles of *P. c. chabaudi* can be conducted to determine if immunisation with MSP-1 can elicit strong SSPI in the host animal. If such an effect is observed, an LGS analysis can be conducted upon the progeny of a genetic cross between *P. c. chabaudi* strains AJ and CB in mice immunised with an AJ or CB MSP-1 vaccine. Taken together, these approaches should allow us to identify *P. c. chabaudi* MSP-1 as the major target of SSPI in mice.

In conclusion, SSPI to blood stage malarial infections has been observed in malarias of rodents (Jarra *et al.*, 1985; Martinelli *et al.*, 2005a; Cheesman *et al.*, 2006). Using LGS analysis, we have located the region on *P. c. chabaudi* chromosome 8 which is under significant SSPI selection and which contains the gene for the *P. c. chabaudi* MSP-1 antigen. No other region in the *P. c. chabaudi* genome was revealed to contain a similarly strong target of SSPI.

CHAPTER 3: Congenicity and Genetic Polymorphism in Cloned Lines Derived from a Single Isolate of the Rodent Malaria Parasite *Plasmodium yoelii yoelii*

A part of this chapter has been published as: Pattaradilokrat S., Cheesman S.J., Carter R. (2008) Congenicity and Genetic Polymorphism in Cloned Lines Derived from a Single Isolate of a Rodent Malaria Parasite. *Mol Biochem Parasitol*, **157** (2): 244-247.

3.1 Abstract

Many of the most commonly studied lines of the rodent malaria parasite *Plasmodium yoelii yoelii* are derived from a single parasite isolate designated 17X. There are, however, large phenotypic (growth rate) and genetic differences among these lines of 17X that have been distributed to laboratories worldwide. We describe herein the results of a comparative genetic analysis between the cloned lines of 17X that are different in growth rate, based on (i) nucleotide sequence data from specific genes and (ii) restriction site polymorphism by Amplified Fragment Length Polymorphism (AFLP). Our findings indicate that there are two completely distinct genotypes among lines of *P. y. yoelii* 17X. Within one of these, genotype-1, represented by 17XNIMR, two growth phenotypes are present, a slow growth rate phenotype (e.g. 17XNIMR) and a fast growth rate phenotype (e.g. 17XYM). Within genotype-1, there were some genomic differences between lines with fast and slow growth rate, 17XYM and 17XNIMR, which are manifest as size variation of some chromosomes and a small number of AFLP polymorphism. The other genotype, genotype-2, represented by 17XA, has been found only with a slow growth rate phenotype.

3.2 Short Communication

Many of the most frequently used laboratory lines of the rodent malaria parasite *Plasmodium yoelii yoelii* are derived from a single parasite isolate, 17X, collected from the blood of a wild thicket rat *Thamnomys rutilans* captured near the field station at La Mobokè near Bangui in the Central Africa Republic in 1965 (Landau *et al.*, 1965) (see also Chapter 1, section 1.6). The parasites of isolate 17X were introduced into laboratory rodents and transmitted through laboratory-reared Anopheline mosquitoes (Landau *et al.*, 1966). Many lines of *P. y. yoelii* 17X have since been cloned and distributed to laboratories worldwide (Figure 3.1 and Table 3.1).

Amongst lines of 17X are parasites that vary in growth rate and in their ability to cause mild or lethal infections in the rodent host (Figure 3.1). Most lines of 17X have a slow growth rate and produce a typically mild, self-limited infection in mice (Figure 3.1). Such infections are characterised by a marked preference for immature red blood cell (reticulocyte) invasion (Landau *et al.*, 1978) and parasitaemia rises slowly from 1 to 5% on the third or fourth day of blood-induced infection to reach 15 to 20% after 2 to 3 weeks (Walliker *et al.*, 1973). The parasites are typically cleared in the third or fourth week and the host achieves full recovery. By contrast, three lines of a much faster growth rate have arisen independently on three separate occasions from the stock *P. y. yoelii* malaria designated 17X (Figure 3.1 and Table 3.1). These parasites retain their invasion preference for reticulocytes in early infection in mice, and rapidly deplete the reticulocytes from the circulation. Then, they tend to infect mainly mature red cells (normocytes) from the third and fourth day of infection (Yoeli *et al.*, 1975; Walliker *et al.*, 1976; Walliker 1981; Burns *et al.*, 1989). These parasites multiply at almost exponential rates through most of the period of infection, usually killing mice within 7 days (Yoeli *et al.*, 1975; Walliker *et al.*, 1976; Walliker 1981; Burns *et al.*, 1989).

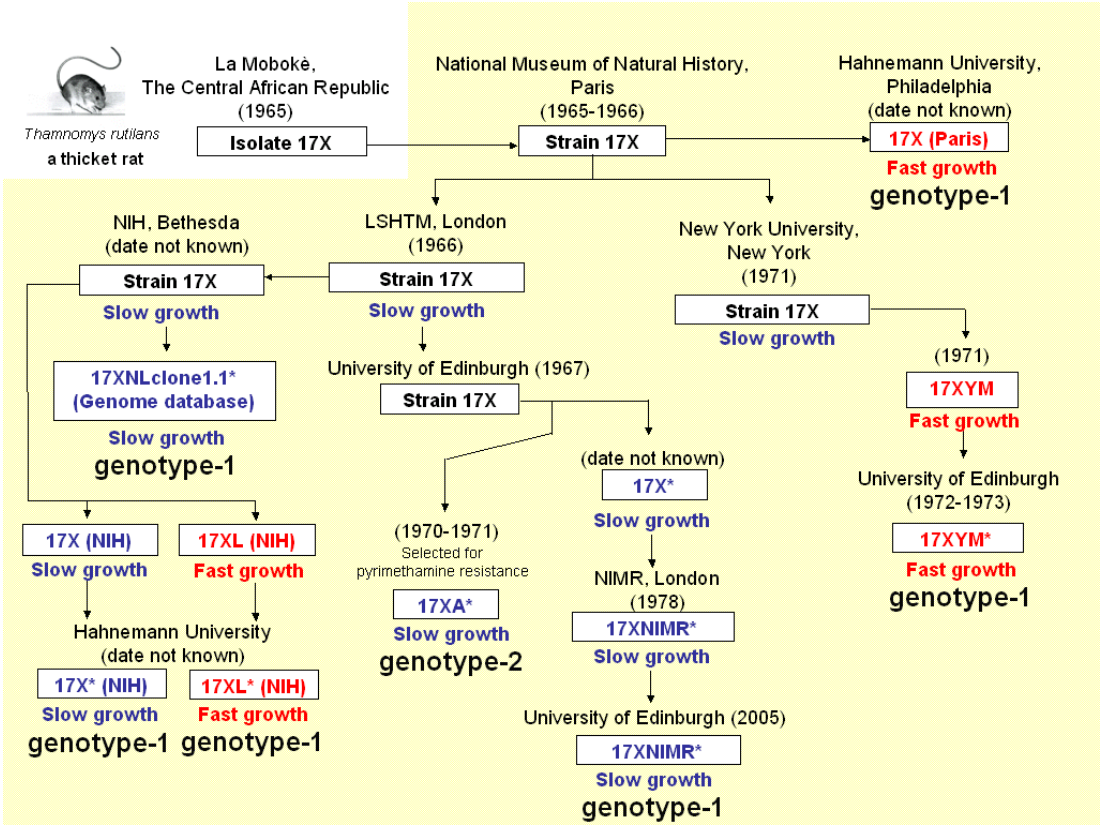

Figure 3.1. Summary history and genotype analyses of laboratory-cloned lines of the rodent malaria parasite *Plasmodium yoelii yoelii* derived from a single isolate called 17X. Parasite isolate 17X was obtained from a wild thicket rat, *Thamnomys rutilans*, called 17X captured in the Central African Republic in 1965 (Landau *et al.*, 1965). Parasites in the original isolate 17X consisted of two species originally named *Plasmodium chabaudi* and *Plasmodium berghei yoelii* (Landau *et al.*, 1966). *P. b. yoelii* was renamed *Plasmodium yoelii yoelii* in 1974 (Killick-Kendrick, 1974). Only lines of *P. y. yoelii* 17X are displayed here. Asterisks (*) indicate cloned lines. The cloned line 17XA was selected from strain 17X for resistance to pyrimethamine (see text). Fast or slow growth refers to the behaviour of the parasites in blood infections in laboratory mice (yellow background). Where known, these lines of 17X are characterised by one or the other of two genotypes (genotype-1 and -2) based upon sequence data of the genes for blood stage parasite proteins, Merozoite Surface Protein-1 and Apical Membrane Antigen-1 (see text). LSHTM, London School of Hygiene & Tropical Medicine (UK); NIH, National Institutes of Health (USA); NIMR, National Institute for Medical Research (UK).

Table 3.1. Source of the rodent malaria parasite *Plasmodium yoelii yoelii* isolate 17X and of lines derived from it. Cloned lines examined experimentally in the present work are indicated in bold. Asterisks (*) represent cloned lines. Fast or slow growth refers to the behaviours of the parasites in blood stage- induced infections in laboratory mice. LSHTM, London School of Hygiene & Tropical Medicine (UK); NIH, National Institutes of Health (USA); NIMR, National Institute for Medical Research (UK).

Isolate		Source of isolate	References
17X		Central African Republic in 1965 (see text)	Landau *et al.*, 1965; Landau *et al.*, 1966
Lines of Isolate 17X	**Growth Rate**	**Source of line**	**References**
17XA*	Slow	From strain 17X from LSHTM (London, UK) arrived in Edinburgh in Dec 1967. Subjected to selection for resistance to the anti-malarial drug (pyrimethamine) and cloned by D. Walliker (Edinburgh, UK) between 1970-1971.	Walliker *et al.*, 1971; Walliker *et al.*, 1973; Knowles *et al.*, 1980; a laboratory record of D. Walliker (The University of Edinburgh)
17XL (NIH)*	Fast	From strain 17X maintained in NIH. Parasites became suddenly virulent in the laboratory of J. Finerty (NIH, USA) (date not known). Cloned in the laboratory of C. A. Long (Hahnemann University, Philadelphia, USA) (date not known).	Finerty *et al.*, 1976; Finerty *et al.*, 1977; Burns *et al.*, 1989
17XNIMR*	Slow	From strain 17X from LSHTM (London, UK) arrived in Edinburgh (UK) in Dec 1967. Cloned by D. Walliker (unknown date), sent to R. Freeman (NIMR, London, UK), 24 Oct 1978, and returned to the laboratory of R. Carter (Edinburgh, UK), 20 Apr 2005.	A laboratory record of D. Walliker (The University of Edinburgh); Anthony A Holder (NIMR), personal communication
17X (NIH)*	Slow	From strain 17X maintained in NIH in the laboratory of J. Finerty (NIH, USA). Cloned in the laboratory of C. A. Long (Hahnemann University, Philadelphia, USA) (date not known).	Finerty *et al.*, 1976; Finerty *et al.*, 1977; Burns *et al.*, 1989
17XNLclone 1.1*	Slow	From strain 17X maintained in LSHTM (London, UK) between 1966-1968. Cloned in NIH (Bethesda, USA) (date not known). Clone 1.1 used to construct the *P. y. yoelii* Genome	Topley *et al.*, 1970; Weinbaum *et al.*, 1976; Carlton *et al.*, 2002
17XYM*	Fast	From strain 17X maintained in the National Museum of Natural History (Paris, France) by I. Landau and sent to M. Yoeli (New York University, USA) in 1971. Parasites became suddenly virulent following removal of a stabilate (after 110- day storage in the liquid nitrogen) and inoculation into CF1 mice. Strain 17XYM sent to G. Beale (Edinburgh, UK), 19 Oct 1972, and cloned by D. Walliker, 12 Dec 1973.	Yoeli *et al.*, 1975; Walliker *et al.*, 1976; Walliker 1981; a laboratory record of D. Walliker (The University of Edinburgh)
17X (Paris)	Fast	From strain 17X maintained in Paris by I. Landau at the National Museum of Natural History (Paris, France). Supplied to the laboratory of C. A. Long (Hahnemann University, Philadelphia) (date not known).	Burns *et. al.*, 1989

The purpose of the present work was to determine the genetic relationships between lines of *P. y. yoelii* 17X including those that differed in growth rate. These lines of *P. y. yoelii* studied here consisted of the fast-growing line, 17XYM, and the slow-growing lines, 17XNIMR, 17XA and 17XNLclone1.1 (used for nucleotide sequence comparison). 17XA was derived following selection for resistance to the anti-malarial drug pyrimethamine (Walliker *et al.*, 1971; Walliker *et al.*, 1973). The other three lines of 17X studied here are sensitive to this drug. The history of each line is shown in Table 3.1. The genetic comparisons made between them involved (1) nucleotide sequence comparisons of the genes encoding a part of *P. y. yoelii* Apical Membrane Antigen-1 (AMA-1) and Merozoite Surface Protein-1 (MSP-1) and (2) a genome-wide scan for restriction site-associated polymorphism using the method of Amplified Fragment Length Polymorphism (AFLP) (Grech *et al.*, 2002). The 5' and 3' nucleotide sequences of AMA-1 and MSP-1 were chosen because these loci have been previously shown to exhibit polymorphism between isolates, and strains, of *P. y. yoelii* (Daly *et al.* 1992; Kappe *et al.*, 1996; Benjamin *et al.* 1999).

The fast-growing line, 17XYM, and the slow-growing lines, 17XNIMR and 17XNLclone1.1, are identical in the 5' nucleotide sequence of the gene for AMA-1 and are designated genotype-1 in Figure 3.1. All of the above lines are genetically distinct from 17XA at 22 positions in this region of the AMA-1 gene, which is designated genotype-2 in Figure 3.1 (see also Table 3.2A and Figure 3.2, for methods). This represents 5.06% sequence difference between 17XA and any of the other three lines of 17X analysed (Table 3.2A). These results confirm the previous finding that 17XYM and 17XA are genetically distinct from each other at the *ama*-1 locus (Kappe *et al.*, 1996).

Similarly, there are no polymorphisms in the 3' nucleotide sequence of the gene for MSP-1 between 17XYM (Lewis, 1989; Benjamin *et al.*, 1999), 17XNIMR (A. Holder, personal communication) and 17XNLclone1.1 (Carlton *et al.*, 2002), all genotype-1 in Figure 3.1 (Table 3.2B and Figure 3.3). The nucleotide sequence of these parasite lines is also identical to the slow-growing cloned line 17X (NIH) (Daly *et al.*, 1992) and the two other fast-growing cloned lines, 17X (Paris) (Daly *et al.*,

1992) and 17XL (NIH) of *P. y. yoelii* (Burns *et al.*, 1988), indicated as genotype-1 in Figure 3.1 (Figure 3.3; see also Table 3.1 for the history of these lines). All of the above lines are genotypically distinct from 17XA (Daly *et al.*, 1992) at 22 positions in this region of the *msp*-1 gene, which is designated genotype-2 in Figure 3.1 (Table 3.2B and Figure 3.3).

(A) The numbers of polymorphic residues and percent nucleotide sequence difference in the 5' nucleotide sequences of the genes encoding AMA-1 between the four cloned lines of *P. y. yoelii*

	17XNLclone1.1	17XNIMR	17XYM	17XA
17XNLclone1.1	-			
17XNIMR	0 (0%)	-		
17XYM	0 (0%)	0 (0%)	-	
17XA	22 (5.06%)	22 (5.06%)	22 (5.06%)	-

(B) The numbers of polymorphic residues and percent nucleotide sequence difference in the 3' nucleotide sequences of the genes encoding MSP-1 between the four cloned lines of *P. y. yoelii*

	17XNLclone1.1	17XNIMR	17XYM	17XA
17XNLclone1.1	-			
17XNIMR	0 (0%)	-		
17XYM	0 (0%)	0 (0%)	-	
17XA	22 (7.72%)	22 (7.72%)	22 (7.72%)	-

Table 3.2. Pair wise analysis of (A) the 5' nucleotide sequences of the genes encoding Apical Membrane Antigen-1 and (B) the 3' nucleotide sequences of the genes encoding Merozoite Surface Antigen-1 between the four cloned lines, 17XNLclone1.1, 17XNIMR, 17XYM, 17XA of *Plasmodium yoelii yoelii*. (A) shows the numbers of polymorphic residues between each of the two lines identified in the 435 base pair nucleotide sequence of the gene for AMA-1 which corresponds to positions 94-528 of the nucleotide sequence of 17XNLclone1.1, deposited in the GenBank® database under the accession number XM_724270 (Carlton *et al.*, 2002) as well as in the *P. y. yoelii* genome database (The Institute for Genomic Research website) under the locus name PY01581 (see also Figure 3.2). (B) shows the numbers of polymorphic residues between each of the two lines identified in the 285 base pair nucleotide sequence of the gene for MSP-1 which corresponds to positions 4966-5250 of the nucleotide sequence of 17XNLclone1.1, deposited in the GenBank® database under the accession number XM_721164 (Carlton *et al.*, 2002) as well as in the *P. y. yoelii* genome database (The TIGR website) under the locus name PY05748 (see also Figure 3.3). Numbers in brackets represent percent nucleotide sequence difference, which is defined as the percentage of polymorphic residues present in each sequence analysed.

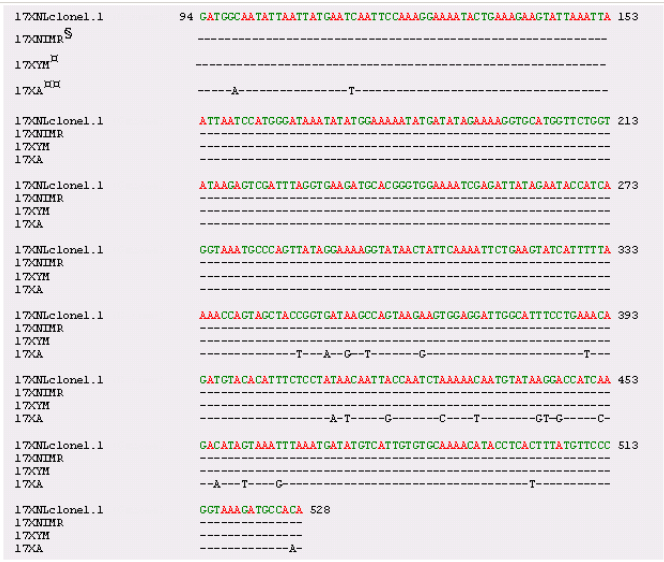

Figure 3.2. Multiple alignment of the 5' nucleotide sequences of the genes encoding Apical Membrane Antigen-1 (AMA-1) from the four cloned lines, 17XNLclone1.1, 17XNIMR, 17XYM and 17XA of the rodent malaria parasite *Plasmodium yoelii yoelii*. The residue numbering, shown along the side, corresponds to the nucleotide sequence from 17XNLclone1.1 as deposited in the GenBank under the accession number XM_724270 (Carlton *et al.*, 2002). The nucleotide sequences from 17XNIMR, 17XYM and 17XA were amplified by polymerase chain reaction (PCR) of genomic DNA of blood stage parasites for each line as follows. Oligonucleotide primers were 5'-GCTCTATTT ATCTAATAAACCTG-3' and 5'-GGATCATTACTACAATATCTTG-3', corresponding to positions 32-54 and 635-614 of the nucleotide sequence from *P. y. yoelii* 17XYM that was deposited in the GenBank® database under the accession number U45970 (Kappe *et al.*, 1996). PCR reactions were performed in a 50 µl volume consisting of 100 ng genomic DNA, 4 µM forward and reverse primers, 1.5 mM $MgCl_2$, 0.6 mM dNTPs, 5 U Immolase™ DNA polymerase (Bioline) and 1 x Immobuffer™ (Bioline). PCR conditions were as follows: one cycle of 95°C for 7 min, 46°C for 1 min, 72°C for 1 min, one cycle of 94°C for 3 min, 47°C for 1 min, 72°C for 1 min, followed by 26 cycles of 95°C for 1 min, 48°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. The PCR products were examined on Ethidium-Bromide stained agarose gels. Each sample produced single amplicons of the predicted length. The PCR products were purified with QIAquickPCR purification kit (Qiagen). The amplicons were sequenced using an ABI Prism® BigDye™ Terminator (Applied Biosystems) cycle sequencing ready reaction kit (version I) in the forward and reverse directions, using the primers described above. DNA sequences for each parasite cloned line were visualised using EditView ABI automated DNA sequence viewer software (Perkin Elmer ABI) and assembled manually to generate a single contiguous sequence. The nucleotide sequence for each parasite line was confirmed on both strands in duplicate. The 435-base pair nucleotide sequences obtained for each strain were aligned using the sequence alignment software provided by EMBL-EBI ClustalW (http://www.ebi.ac.uk/clustalw/). (-) indicates identical nucleotide sequence between the four cloned lines analysed.

§ The 5' nucleotide sequence of the gene encoding AMA-1 from 17XNIMR was deposited in the GenBank® database under the accession number EF690378.
¤ The 5' nucleotide sequence of the gene encoding AMA-1 from our Edinburgh 17XYM was deposited in the GenBank® database under the accession number EF989729. This sequence was found to be different from that of 17XYM in the laboratory of J. H. Adams (The University of Notre Dame, USA) as deposited in the GenBank® database under the accession number U45970 (Kappe *et al.*, 1996) at nucleotide positions 99 (base A to C), 285 (base G to A) and 295 (G to A). The first base corresponds to the nucleotides from our Edinburgh 17XYM sequence
¤¤ The 5' nucleotide sequence of the gene encoding AMA-1 from our Edinburgh 17XA was deposited in the GenBank® database under the accession number EF989730. This sequence was found to be different from that of 17XA in the laboratory of J. H. Adams (The University of Notre Dame, USA) as deposited in the GenBank® database under the accession number U45971 (Kappe *et al.*, 1996) at nucleotide positions 320 (base T to A) and 379 (base C to G). The first base corresponds to the nucleotides from our Edinburgh 17XA sequence.

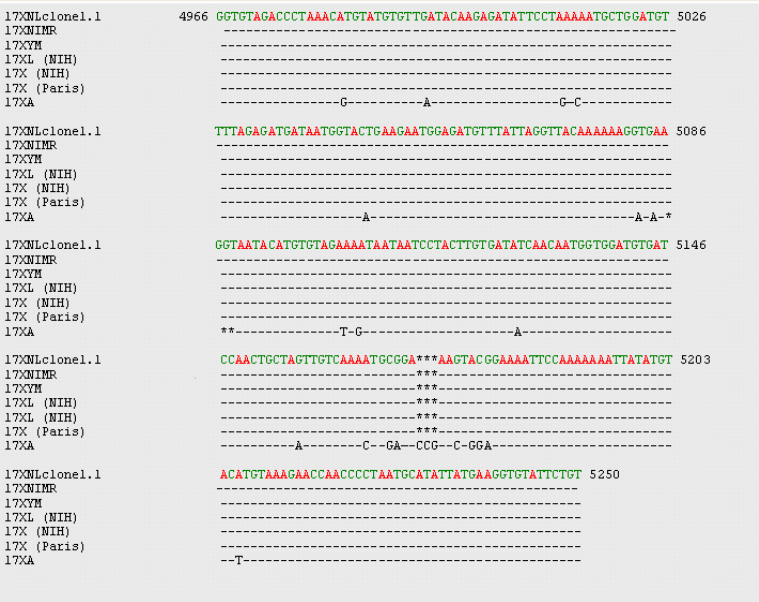

Figure 3.3 Multiple alignment of the 3' nucleotide sequences of the genes encoding Merozoite Surface Protein-1 from the six cloned lines, 17XNLclone1.1, 17XNIMR, 17XYM, 17XL (NIH), 17X (NIH) and 17XA and one strain 17X (Paris) of the rodent malaria parasites *Plasmodium yoelii yoelii*. The residue numbering, shown along the side, corresponds to the nucleotide sequence from 17XNLclone1.1 as deposited in the GenBank® database under the accession number XM_721164 (Carlton *et al.*, 2002). The nucleotide sequences from 17XYM, 17XL (NIH), 17X (NIH), 17X (Paris), 17XA are deposited in the GenBank® database under the accession numbers J04668 (Lewis, 1989), J03612 (Burns *et al.*, 1988), M87553 (Daly *et al.*, 1992), M87555 (Daly *et al.*, 1992) and M87556 (Daly *et al.*, 1992), respectively. The nucleotide sequence from 17XNIMR was provided by Dr. Anthony Holder (National Institute for Medical Research, UK) (personal communication). The 285-base pair sequences were aligned using the ClustalW software. (-) indicates identical nucleotide sequence between lines of 17X analysed. (*) indicates nucleotide insertions or deletions.

From 65 randomly chosen combinations of selective AFLP primers (Table 3.3 and Table S2.1-S2.6, Appendix 2) (Grech *et al.*, 2002), we found 279 AFLP markers that distinguished 17XYM (genotype-1) and 17XA (genotype-2). Of these markers, 130 were present only in 17XYM and 149 only in 17XA. 283 AFLP markers distinguished 17XNIMR (genotype-1) and 17XA (genotype-2). Of these markers, 136 were present only in 17XNIMR and 147 only in 17XA. In contrast, only six AFLP markers distinguished 17XYM (fast growth rate, genotype-1) and 17XNIMR (slow growth rate, genotype-1). Of these markers, all were found only in 17XNIMR. These results indicate that 17XYM and 17XNIMR are almost totally congenic, consistent with the complete sequence identity in the 5'nucleotide sequence of the gene for AMA-1 and the 3'nucleotide sequence of the gene for MSP-1 in 17XYM and 17XNIMR (see above). By contrast, 17XA differs from all the other lines of 17X analysed in this study. The results suggest that the fast-growing cloned line 17XYM arose from the genotype represented by 17XNIMR, and certainly not that represented by 17XA.

There are, however, a small number of differences in the AFLP bands between the fast-growing line 17XYM and the representative of its presumed progenitor, the slow-growing line 17XNIMR (Table 3.3). This suggests that significant genomic changes have occurred between them. In order to examine this at a karyotypic level, we performed PFGE analysis on genomic DNA prepared from the blood stage parasites of 17XNIMR and 17XYM (see Figure 3.4 for PFGE conditions and also Chapter 4, section 4.3.8 for the method), as previously described (Owen *et al.*, 1999; Khan *et al.*, 2001).

It was observed that a band in 17XNIMR representing co-migrating *P. y. yoelii* chromosomes 13 and 14 had higher molecular weight than that in 17XYM under the PFGE conditions for separation of high molecular weight chromosomes (Figure 3.4). This indicates that there are karyotypic and some other genomic differences, such as those we observed with AFLP, between these two apparently congenic lines of 17X that are different in growth rate.

AFLP bands present only in	17XNIMR	17XYM	17XA
versus			
17XNIMR	-	0	147
17XYM	6	-	149
17XA	136	130	-

Table 3.3. Summary of the numbers of parasite clone-specific polymorphic bands (Amplified Fragment Length Polymorphism markers) identified in the three cloned lines, 17XNIMR, 17XYM, 17XA of the rodent malaria parasite *Plasmodium yoelii yoelii* using 65 combinations of selective *Eco*RI and *Mse*I AFLP primers. AFLP was conducted on genomic DNA for each parasite line prepared from the blood-stage parasites, as previously described (Grech *et al*., 2002). Each sample was analysed in duplicate and only reproducible AFLP bands were counted in this analysis.

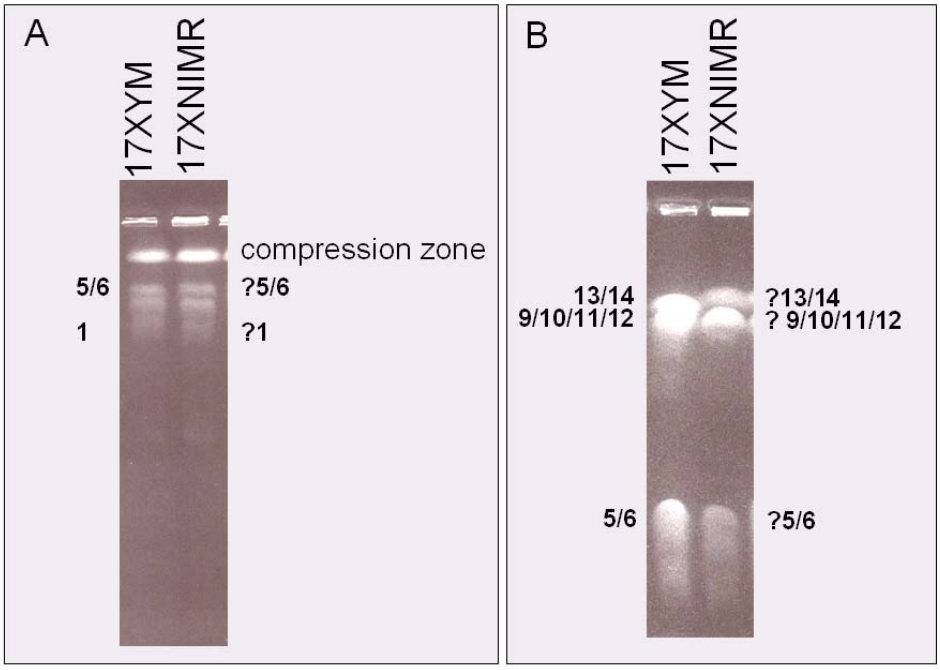

Figure. 3.4. Karyotypes of the cloned lines 17XNIMR and 17XYM of the rodent malaria parasite *Plasmodium yoelii yoelii* analysed by Pulsed Field Gel Electrophoresis (PFGE). Panels A and B present parasite chromosomes separated by two PFGE conditions optimised for separation of low and high molecular weight chromosomes, respectively. The chromosome numbers of 17XYM are shown on the left-hand side of the gels (Owen *et al.*, 1999; Khan *et al.*, 2001). The chromosome numbers of 17XNIMR, as shown in the right-hand side of the gels, have been tentatively assigned by comparison to 17XYM (hence the question marks at the side of the chromosome bands of 17XNIMR. In panel A, electrophoresis conditions were 50 v, 360-sec pulse time for 72 hours; 50 v, 720 sec pulses for 72 hours, using a contour-clamped homogenous electric field apparatus (CHEF-DRII, Bio-Rad) and 0.8% chromosomal grade agarose gel (Sigma). Large chromosomes migrate unresolved as a compression zone. In panel B, electrophoresis conditions were 100 v, 360-sec pulse time for 72 hours; 100 v, 720 sec pulses for 24 hours, using a CHEF-DRII and 0.7% agarose gel. Ethidium bromide-stained gels were visualised under UV transillumination and photographed.

3.3 Summary

The purpose of the present work was to make a comparative genetic study of cloned lines in the Edinburgh collection that vary in growth rate and are derived from the single parasite isolate 17X of *P. y. yoelii*. Comparisons made between these lines were conducted upon (1) nucleotide sequence analyses of the specific genes and (2) AFLP analysis. In the present work, we were able to demonstrate the existence of two genotypes within cloned lines derived from isolate 17X of *P. y. yoelii*. Genotype-1 is represented by a slow-growing line, e.g. 17XA.Genotype-2 is represented by a slow-growing line, e.g. 17XNIMR, and a fast-growing parasite, e.g. 17XYM. The latter genotype may probably include fast-growing lines, 17XL (NIH) and 17X (Paris), and slow-growing lines, 17X (NIH) and 17XNL.

Our result indicated that 17XA is genetically polymorphic from other lines of *P. y. yoelii* 17X analysed in this study. Our finding is in agreement with results from a few previous observations that indicated the extensive sequence polymorphism between 17XA and 17XYM in the genes for MSP-1 and AMA-1 (Daly *et al.* 1992; Benjamin *et al.* 1999; Kappe *et al.* 1996). Other studies also showed that the genome of 17XA was found to contain a different distribution and a different copy number of the genes coding for 235 kDa rhoptry proteins (Py235) from that of 17XYM (Khan *et al.*, 2001; Iyer *et al.*, 2006). Moreover, unpublished data from our laboratory, based on nucleotide sequence analysis of the gene for dihydrofolate reductase (DFHR), showed that 17XA carried a different allelic type of the *dhfr* gene from 17XYM. Based on these observations, it is highly unlikely, therefore, that, in spite of the shared isolate 17X origin, the fast-growing line 17XYM arose from the genetic stock represented by 17XA.

Rather, the results of the present work indicated that the 5' and 3' nucleotide sequences of the genes for AMA-1 and MSP-1 of the fast-growing line 17XYM and the slow-growing line 17XNIMR were identical. Furthermore, AFLP analysis between 17XYM and 17XNIMR showed that the majority of AFLP bands between these lines were similar. These observations have demonstrated for the first time the

genetic congenicity between the fast-growing line 17XYM and the slow-growing line 17XNIMR. This suggests, therefore, that 17XNIMR could be a representative of a genetic stock from which 17XYM arose. Since previous work proposed that the sudden appearance of 17XYM was probably due to a simple mutation in the genome of a slow-growing line 17X of *P. y. yoelii* (Walliker *et al.*, 1976), an identification of genes that are polymorphic between the fast and slow-growing congenic lines, 17XYM and 17XNIMR, would reveal likely or possible candidate genes associated with differences in growth rate in this parasite.

There is evidence, from work with the human malaria parasite *Plasmodium falciparum*, indicating that genomic and karypotypic variations are, indeed, related to variation in parasite proliferation rate in cultured red blood cells. Wellems and others (1987) showed that strain HB3 of *P. falciparum* which lacks a ~200 kb subtelomeric region on *P. falciparum* chromosome 13 containing the gene for histidine-rich protein had a lower proliferation rate of the blood stages relative to that of strain 3D7. Furthermore, in a genetic cross between these two parasite lines, all 15 of 15 progeny carried this subtelomeric sequence and they exhibited a proliferation advantage over the HB3 parent under *in vitro* culture conditions, suggesting this locus marks a linkage group that confers a strong growth advantage to the progeny (Wellemes *et al.*, 1987). Following this work, the gene coding for protein EBL-1 previously implicated in host erythrocyte invasion was mapped onto the subtelomeric region associated with high proliferation rate phenotypes in clones 3D7 and Dd2 of *P. falciparum*, but absent in clone HB3 which had lower proliferation rate. (Peterson *et al.*, 2000). Furthermore, in an analysis of two genetic crosses between 3D7 and HB3 and between Dd2 and HB3, progeny of the two crosses possessed the chromosome segment containing the *ebl-1* gene from the 3D7 or Dd2 parents had higher proliferation rate than HB3 (Peterson *et al.*, 2000). These observations indicate, therefore, that the genomic alternations on the subtelomoeric region *P. falciparum* chromosome 13 are strongly associated with growth rate differences in this malaria parasite.

In the present work, we have already observed that some genomic alternations between the fast and slow congenic lines, 17XYM and 17XNIMR. Results showed that 17XNIMR had additional 6 AFLP markers and a larger size of co-migrating chromosomes 13 and 14 than 17XYM. However, whether these differences are attributed to the chromosomal loss or translocation is currently unknown. These will require further investigation. Furthermore, it would be of interest to determine the possible significance of these genomic differences to variation in blood stage growth rate between the fast- and slow-growing lines of these parasites.

In conclusion, the parasites in worldwide circulation as *P. y. yoelii* 17X comprise two distinct genetic stocks. One is represented by 17XA. The other is represented by 17XNIMR and 17XYM as analysed in the present investigation. This group probably includes 17XNLclone1.1, 17X (NIH), 17XL (NIH) and 17X (Paris) based upon the partial coding sequence of the genes coding for MSP-1 and AMA-1. Within this group of parasites of probable congenic origin, the fast-growing line 17XYM has significant genomic alterations, such as the loss of a small number of AFLP markers and size differences in some chromosomes, both of which distinguish it from its associated congenic stock. As the fast-growing line 17XYM arose spontaneously during infection with a mild strain of *P. y. yoelii* 17X (Yoeli *et al.*, 1975), the identification of an apparently congenic parasite with a slow growth rate phenotype, 17XNIMR, will be useful in determining genes underlying growth rate differences in these malaria parasites. This information is particularly relevant following identification of a region of the parasite's genome containing genetic determinants of growth rate in *P. y. yoelii* 17XYM by LGS analysis (see Chapter 4).

CHAPTER 4: Linkage Group Selection: Towards Identifying Genes Controlling Growth in Malaria

4.1 Abstract

The clinical outcome of malarial infections in humans and other animal hosts can be highly variable. One factor positively correlated with the pathological severity of an infection is growth rate of a malaria parasite in the blood. The genetic determinants of growth rate are, however, not known. Here we have utilised a combined genetic and genomic approach, Linkage Group Selection (LGS), in attempt to locate regions in the genome of the rodent malaria parasite *Plasmodium yoelii yoelii* that contain genes controlling blood stage growth rate. Our approach is based upon crossing a slow-growing line, 33XC, and a faster-growing line, 17XYM, of *P. y. yoelii* and growing the entire uncloned progeny of a genetic cross in laboratory rodents. The blood stages of the uncloned cross progeny were then grown as three successive blood infections in mice. This is expected to select for those parasites of the uncloned cross progeny that have inherited the allele controlling the growth rate of the faster growing parental clone. The growth rate selected cross progeny were analysed with numerous quantifiable genetic markers to identify the signature of growth selection. In the present study, LGS analysis of growth rate has been conducted in the progeny of two independent genetic crosses between 33XC and 17XYM of *P. y. yoelii*. The results indicate that a single ~1 megabase pair region on *P. y. yoelii* chromosome 13 is under strong growth selection in both crosses, although there are also other regions of the *P. y. yoelii* genome, including that on co-migrating chromosome 5 or 6, which may be weakly affected by growth selection. This finding, which is agreement with the results of a classical linkage analysis by Walliker *et al.,* (1976), demonstrates that a major genetic determinant of growth rate in *P. y. yoelii* is found in a single region on chromosome 13.

4.2 Introduction

Malaria parasites of the same species exhibit phenotypic diversity in growth of the blood forms in both human and animal malarias (James *et al.*, 1932; Yoeli *et al.,* 1975; Simpson *et al.,* 2002). Such variation can be of medical importance because blood stage growth rate of a malaria parasite has been shown to be positively associated with an increase risk of pathological severity and fatality. For instance, a case study of the human malaria parasite *Plasmodium falciparum* in South East Asia indicated that parasites isolated from patients with complicated malaria and fatal cases had a faster growth rate than those with uncomplicated disease (Dondrop *et al.,* 2005), although this was not the case in *P. falciparum* malaria studied in children in Kenya and Mali (Deans *et al.*, 2006). Blood-induced infections with a fast growth line of the rodent malaria parasite *Plasmodium yoelii yoelii* cause a high parasite density in the blood, leading to severe anaemia and death of the host (Yoeli *et al.,* 1975; Walliker *et al.*, 1976; Walliker, 1981; Burns *et al.*, 1989). Growth rate of blood stage parasites is also considered to be a virulence factor that could determine the potential of a host's survival (Mackinnon *et al.*, 1999). The blood stage growth rate may also lead to differences in the competitive success (de Roode *et al.,* 2005a; Bell *et al.*, 2006) and the transmission of the parasites to mosquitoes (Ferguson *et al.,* 2003; Paul *et al.,* 2004; de Roode *et al.,* 2005b).

Variation in growth rate in the rodent malaria parasite *P. y. yoelii* is commonly related to erythrocyte invasion preference, i.e. the ability to invade red blood cells of different ages. Most lines have a slow growth rate and show a strong preference for immature erythrocytes (reticulocytes) (Carter *et al.*, 1977; Landau *et al.*, 1978). Since reticulocytes normally constitute between 1 to 2% of the total red blood cells (Bessman, 1990), the blood stage parasites of *P. y. yoelii* remain at relatively low densities during the course of infection. Parasites of this phenotype generally cause a mild, self-limited infection in laboratory mice (Walliker *et al.*, 1971; Walliker *et al.,* 1973). In contrast to the above, parasites of *P. y. yoelii* lines 17XYM (Walliker *et al.,* 1976; Knowles *et al.*, 1980; Walliker, 1981), 17XL (NIH) (Finerty *et al.,* 1976; Finerty *et al.,* 1977; Burns *et al.,* 1989) and 17X (Paris) (Burns *et al.,* 1989) have a much faster growth rate. Erythrocyte invasion of these parasites is strongly biased

toward reticulocytes only in early infection; after four or five days of infection it switches to invade exclusively mature erythrocytes (normocytes). In laboratory mice, parasitaemias usually reach 60 to 80% within a week of blood-induced infection, which causes rapid death of the animals (Yoeli *et al.*, 1975; Walliker *et al.*, 1976; Burns *et al.,* 1989).

Determining the genetic determinants of growth rate could provide insights into why some malaria parasites grow faster and become more virulent to their host than others. A genetic cross between the fast-growing line, 17XYM, and a slow-growing line, A/C, of *P. y. yoelii* (Walliker *et al.,* 1976) was conducted to investigate the genetic basis of blood stage growth rate in *P. y. yoelii*. The progeny clones showed recombination between the fast growth rate character and other genetic markers (isozyme characters and pyrimethamine resistant/sensitive characters). In the cloned progeny of a genetic cross the fast growth rate character was also found to segregate from the slow growth rate character. Thus, the fast and slow growth rate characters have undergone a pattern of inheritance typical of that expected for a simple nuclear gene mutation (Walliker *et al.,* 1976). However, the exact genetic determinant(s) of this trait could not be identified.

Here we describe the application of a combined genetic and genomic approach which we called Linkage Group Selection (LGS) (Culleton *et al.,* 2005; Carter *et al.,* 2007) to identify the regions in the genome of *P. y. yoelii* containing genes controlling growth rate differences in these malaria parasites. The approach characterises the uncloned progeny of a genetic cross between fast- and slow-growing lines, 17XYM and 33XC, grown for three rounds of infection in laboratory mice by quantitatively measuring proportions of genetic markers representing alleles of the slow-growing parent at genome-wide loci. The present study has identified a major genomic region on *P. y. yoelii* chromosome 13 under strong growth selection. There is also evidence that other regions, such as that identified on co-migrating *P. y. yoelii* chromosome 5 or 6, are weakly associated with the growth selection in these parasites. Consistent with the finding of the classical linkage analysis by Walliker *et al.,* (1976), the

present finding indicates, therefore, that a single genetic locus on *P. y. yoelii* chromosome 13 confers growth rate in this parasite.

4.3 Materials and Methods

4.3.1 Parasites, Mice and Mosquitoes

The cloned lines of *P. y. yoelii* used here are denoted 17XYM and 33XC. Parasites of 17XYM line have a fast growth rate phenotype and cause a fulminating and fatal infection (Walliker *et al.,* 1976; Walliker, 1981; see also Chapter 3, for the detailed history of 17XYM). Parasites of this phenotype emerged in a single step during passage of 17X, a mild strain of *P. y. yoelii* (Yoelii *et al.,* 1975). Parasites of 33XC line have a slow growth rate phenotype and cause a mild infection (Walliker *et al.,* 1973). Both lines were independently derived from wild thicket rats *Thamnomys rutilans*, captured in the Central African Republic in 1965 (Landau *et al.*, 1965; Landau *et al.*, 1966). Both lines have been known to be genetically distinct from each other based on electrophoretic forms of the enzyme glucose phosphate isomerase (Walliker *et al.,* 1973; Yoelii *et al.,* 1975) and nucleotide sequences of the genes coding for Merozoite Surface Protein-1 (Benjamin *et al.,* 1999) and Apical Membrane Antigen-1 (AMA-1) (unpublished data). The methods employed for routine blood and mosquito passage and for deep freezing blood forms were similar to those described previously (Walliker *et al.,* 1973; Walliker *et al.,* 1975).

Hosts were inbred CBA/Ca and C57B1/6J female mice, aged 6 to 8 weeks at the time of a primary infection. For simplicity, the mouse strains CBA/Ca and C57B1/6J will be referred to here as CBA and C57, respectively. Mice were maintained as described elsewhere (de Roode *et al.,* 2004; see also Chapter 2, section 2.3.1). Mosquitoes were *Anopheles stephensi* from a laboratory-bred colony and maintained in a dedicated insectary, as previously described (Ferguson *et al.,* 2002; see also Chapter 2, section 2.3.1). All experiments in this study were conducted in accordance with the Animals (Scientific Procedures) Act 1986 (UK).

4.3.2 Single and mixed clone infections with 17XYM and 33XC

Blood stage parasites of 17XYM and 33XC were initiated from deep frozen stabilates by intra-peritoneal (i.p.) injection into donor CBA mice. The infections

were monitored daily by microscopic analysis of Giemsa stained thin tail blood smears and red blood cell densities were measured using a Coulter Counter (Beckman Coulter), according to the manufacturer's instructions. For inoculations, the blood stage parasites were suspended in physiological citrate saline [0.9% (w/v) Sodium Chloride, 1.5% (w/v) Trisodium Citrate dihydrate, pH 7.2] at 1 x 10^6 parasitised red blood cells (pRBC) per 0.1 ml volume. Mice were allocated at random to four experimental groups. Two groups, each containing four mice, were i.p. inoculated with a mixture consisting of 17XYM and 33XC in proportions of 1:1 and 1:9, respectively. Each mouse received 1 x 10^6 pRBC. A further two groups, each containing five mice, were i.p. inoculated with the same number of either 33XC or 17XYM. The infections were monitored daily by microscopy as before. A 20 µl volume of tail blood was collected daily from each mixed clone infected mouse. DNA was extracted from infected mouse blood as previously described (Cheesman *et al.,* 2003; Cheesman *et al.,* 2006).

4.3.3 Pyrosequencing

Pyrosequencing™ (PSQ) was performed on DNA prepared from blood samples taken from the mixed-clone infections (see above) with slight modifications from the methods previously described (Cheesman *et al.,* 2007). An assay was designed to estimate proportions of parasites of 17XYM and 33XC lines based on an allele-specific, single nucleotide polymorphism (SNP) within the gene coding for AMA-1. The assays utilised the following primers and conditions. The forward and reverse primers were 5'-AGGTGCATGGTTCTGGTATAAGAG-3' and 5'-Biotinylated-GGAAATGCCAATCCTCCACTT-3'. PCR reactions were carried out in a 50 µl volume consisting of 100 ng DNA, 1.5 mM $MgCl_2$, 0.3 mM forward and reverse primers, 2 mM dNTPs, 1 unit of Bio-X-ACT™ Polymerase (Bioline) in 1 x Optibuffer™ (Bioline). PCR conditions were as follows: 1 cycle of an initial 'hot start' at 95˚C for 7 min, 64˚C for 1 min, 72˚C for 1 min, followed by 1 cycle of 95˚C for 3 min, 64˚C for 1 min, 72˚C for 1 min, followed by 40 cycles of 95˚C for 30 sec, 64˚C for 1 min, 72˚C for 1 min and a final elongation step heating at 72˚C for 10 min. PCR products were run on 1.5% agarose gels. Each sample produced single

amplicons of the predicted length. PCR products (175 base pairs in size) were, thereafter, subjected to a sequencing reaction according to the manufacturer's instructions (Biotage), with a sequencing primer 5'-GGTATAACTATTCAAAATTC -3'. Nucleotide-specific light signals generated by the sequencing reactions were detected on a Biotage Pyrosequencer HS96A. Light signals at the SNP position were transformed into relative peak heights, representing proportions of parasites carrying the 17XYM and 33XC alleles of the gene for AMA-1 present in a sample analysed. PSQ assays were standardised to accurately measure proportions of parasites in a mixture within ±<5% (Cheesman *et al.,* 2007).

To obtain clone-specific parasitaemias proportions of 33XC and 17XYM determined by PSQ, as just described, were multiplied by the absolute parasitaemias of mixed clone infections on the same day. The 95% confidence intervals (2 x Standard Error of Mean) were calculated. Analysis of variance (ANOVA) was performed using SPSS version 11.5. Differences were deemed significant with a P value < 0.01.

4.3.4 Genetic cross

We conducted two genetic crosses between *P. y. yoelii* 17XYM and 33XC on two separate occasions as follows. Single clone infections of 17XYM and 33XC were grown individually in donor mice. Blood from these mice was mixed to produce inocula containing equal proportions of the two clones. Blood inocula were i.p. inoculated into three CBA and C57 mice that were used to carry out the first and second genetic crosses, respectively. Each mouse received 1 x 10^6 pRBC. Infectivity to mosquitoes was similar, and high, using both strains of mice (see below). Four days post inoculation, parasitaemias were approximately 20% and 6% in CBA and C57 mice, respectively, and the presence of gametocytes of both sexes was microscopically confirmed in mice. Mosquitoes were allowed to feed on the mice, as previously described (Culleton *et al.,* 2005). Seven days post feed, six female mosquitoes from each batch were dissected and examined for the presence of oocysts in their midguts. In the first cross, 5 of the 6 dissected mosquitoes were infected and had a mean of 33 oocysts per midgut. In the second cross, all of the 6 dissected

mosquitoes were infected and had a mean of 50 oocysts per midgut. Seventeen days post feed, the presence of sporozoites in salivary glands of the mosquitoes was microscopically confirmed. Salivary glands were dissected from 200 and 359 mosquitoes from the first and second batches, respectively, for harvesting sporozoites, as previously described (Pattaradilokrat *et al.*, 2007; see also Chapter 2, section 2.3.3). Sporozoites harvested from each cross were i.p. inoculated into three and four CBA mice, respectively, to obtain blood stage parasites for growth selection (see the following section). From the number of mosquitoes dissected for each cross, we calculated the likely maximum number of independent recombinant clones which each cross could have generated (Table 4.1), as previously described (Pattaradilokrat *et al.*, 2007; see also Chapter 2, section 2.3.3)

	First Genetic Cross	**Second Genetic cross**
Mean numbers of oocysts per mosquito midgut (number of mosquitoes dissected)	33 (6)	50 (6)
Numbers of mosquitoes dissected for sporozoites	200	359
The predicted total numbers of oocysts represented	6,600	17,950
The predicted maximum numbers of independent recombinant lines that could have been generated	**13,200**	**35,900**

Table 4.1. Parameters used to calculate the predicted numbers of independent recombinant lines of parasites from two independent genetic crosses between the cloned lines 17XYM and 33XC of *Plasmodium yoelii yoelii*. Parameters used to predict the maximum numbers of the recombinant lines in each uncloned progeny are calculated, as previously described (Pattaradilokrat *et al.*, 2007; see also Chapter 2, section 2.3.3)

4.3.5 Growth selection of 17XYM and 33XC cross progeny

When the sporozoite-induced infections reached average parasitaemias of 1 to 2%, the blood forms were harvested and pooled in physiological citrate saline to produce a blood inoculum containing $1x10^6$ pRBC per 0.1 ml. A total of $1x10^6$ blood stage parasites of the first cross were grown for three rounds of infections in female CBA mice, aged 6 to 8 weeks. Three mice were used in each round. The infections were monitored daily by microscopy. The uncloned progeny parasites were allowed to develop 20% parasitaemias or above before the blood stage parasites were harvested and/or serially passaged into new host. Based upon the observations from the mixed infection studies that growth rate of parasites of 17XYM was significantly different from growth rate of parasites of 33XC on day 5 post infection at which absolute parasitaemias were above 20% (see Results, section 4.4.1), this is expected to select against those parasites that have a slow growth rate phenotype. Parasites of the first cross were harvested 4 days post inoculation in each of the three blood passages. Blood stage parasites of the second genetic cross were, likewise, grown in three successive blood infections in groups, each containing four CBA mice. Parasites of the second genetic cross were harvested 6 days post inoculation after the first passage and 5 days post inoculation after the second and third passages. Parasite DNA was extracted prior to Amplified Fragment Length Polymorphism (AFLP), as previously described (Grech *et al.,* 2002).

4.3.6 Proportional AFLP

DNA samples prepared from the growth rate selected cross progeny and from the two parental clones, 17XYM and 33XC, were analysed by AFLP as previously described by Grech *et al.,* (2002). Briefly, ^{33}P-labelled AFLP products were separated on 6% denaturing polyacrylamide gel and exposed to a Phosphoscreen (Molecular Dynamics) and processed using a Storm 860 Phosphorimager (Molecular Dynamics), as previously described (Martinelli *et al.,* 2004). The resulting images were analysed using ImageQuant™ version 1.2 software (Molecular Dynamics) according to the manufacturer's instructions. Polymorphic bands could then be visualised on the gel as being present in one parental clone and absent in the other, thereby defining parasite clone-specific AFLP markers.

AFLP band intensities for the slow-growing parental clone 33XC present in the cross progeny after three serial passages were quantitatively measured (Martinelli *et al.*, 2004) and expressed relative to the intensity of a non-polymorphic band within the same lane, defining the 'Intensity Index' (II) (Martinelli *et al.*, 2004). The II of the AFLP band in the cross progeny after three passages was divided by the II of the corresponding AFLP band of the parental clone 33XC. This ratio defines the 'Relative Intensity Index' (RII) (Martinelli *et al.*, 2004). It has been shown that the RII of an individual AFLP marker is directly proportional to the ratio of parasites carrying that marker in a complex genetic mixture (Martinelli *et al.*, 2004). Comparison of the RIIs of the AFLP bands of the first and second growth rate selected cross progeny in each of the three serial passages was performed using Microsoft Excel 2000 (Microsoft).

4.3.7 Physical mapping of AFLP markers

AFLP markers were located on the *P. y. yoelii* genome as follows. DNA sequences were obtained from the markers, as previously described (Hunt *et al*., 2004; see also Chapter 2, section 2.3.7). All AFLP markers produced a single sequence as confirmed on both strands in triplicate. Five-fold (5X) coverage contigs of the *P. y. yoelii* 17XNLclone1.1 genome (Carlton *et al.*, 2002), http://www.tigr.org/tdb/e2k1/pya1/, were searched using the BLASTN (DNA vs. DNA) option with sequences derived from the AFLP markers. The genomic contig of *P. y. yoelii* which contained DNA sequences corresponding to the relevant AFLP marker with lowest Probability (*E*) score (a cut-off of 90% sequence identity) were obtained. Because the physical locations of these contigs in the *P. y. yoelii* genome have not been identified, sequences of the *P. y. yoelii* contigs containing the AFLP marker were then used to identify orthologues in the *P. falciparum* clone 3D7 genome (Gardner *et al.*, 2002) (http://www.ncbi.nlm.nih.gov/sutils/blast_table.cgi?taxid=Protozoa&database), using the BLASTP (DNA vs. Protein) option. The genomic locations of the *P. falciparum* orthologues were subsequently used for physical mapping to chromosomal locations in the genome of *P. y. yoelii* through the conserved genetic synteny between the

human malaria parasite *P. falciparum* and the rodent malaria parasites (Kooij *et al.,* 2005).

4.3.8 Pulsed Field Gel Electrophoresis

Host cell free-parasite pellets were prepared from the blood stages of *P. y. yoelii* 17XYM and 33XC, as described by Grech *et al.,* (2002). The pellets were resuspended with an appropriate volume of PBS pH 7.2 and mixed with an equal volume of 2.0% low melting temperature (LMT) agarose (Sigma). The mixtures were added into 10 x 5 x 1.5 mm^3 molds (BioRad) and allowed to set at 4˚C for 20 min. The solidified agarose blocks were then released into lysis buffer [0.5 M EDTA, 10 mM Tris-HCl pH 9.5, 1% (v/v) N-lauroylsarcosine (Sigma), 0.2 mg ml^{-1} ProteinaseK (Sigma)] and incubated at 42˚C for 48 hr with one change of the buffer after 24 hr. Blocks were stored in 0.05 M EDTA pH 8.0 at 4˚C until use.

PFGE was performed on the contour-clamped homogeneous electric field (CHEF)-DRII apparatus (Carle *et al.*, 1984; Carle *et al.*, 1987). A half slice of the agarose blocks was washed three times in DNase-free wash buffer [20 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0] at room temperature. The slices were embedded in 0.7% LMT agarose gel (Sigma) in 0.5 x TBE buffer [45 mM Tris-Borate, 1 mM EDTA] and allowed to set at room temperature for 1 hour. CHEF chamber buffer was 0.5 x TBE buffer and pre-circulated at a constant temperature of 8˚C. PFGE conditions were optimised for separation of high molecular weight chromosomes. Electrophoresis conditions were as follows: 100 volts, 360-sec pulse time for 72 hr; 100 volts, 720-sec pulses for 24 hr, using a CHEF-DRII and 0.7% agarose gel (Owen *et al.*, 1999; Khan *et al.*, 2001). The gels were stained with 1 μg ml^{-1} Ethidium Bromide at room temperature for 1 hour, de-stained in 0.5 x TBE for 30 min with gentle agitation and visualised under UV transillumination and photographed.

4.3.9 Preparation of DNA probes

The genomic contigs of the *P. y. yoelii* strain 17XNLclone1.1 genome, corresponding to the *P. y. yoelii* 33XC AFLP markers, were used to design DNA

probes. Primers were designed using a web-based primer designed tool called Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) (Rozen *et al.*, 2000) to amplify specific loci of *P. y. yoelii* 33XC. The primers used for each of the AFLP markers and conditions for PCR are given in Table 4.2. PCR products of the predicted length were purified using a PCR purification kit (QIAGEN) and sequenced using ABI BigDye Terminator Chemistry on ABI3700 sequencing machine, according to manufacturer's instructions (Applied Biosystem). All PCR products contained sequences that matched those of the AFLP markers. The PCR products were then radiolabelled with ~ 3000 Ci mmol^{-1} α-[^{32}P] dATP (Amersham) using a Prime-It® Random Primer labelling kit (Stratagene) and purified with a NucTrap® Probe purification Column (Stratagene).

4.3.10 Hybridisation

The *P. y. yoelii* chromosomes separated by PFGE were transferred onto Hybond N+ membrane (Amersham), following the manufacturer's protocols. Southern blots were incubated in QuikHyb® Hybridisation solution (Stratagene) for 30 min before the addition of the ^{32}P-radiolabelled probe. Pre-hybridisation and hybridisation incubation temperatures were kept constant at 62 ˚C. Hybridisations were conducted using a hybridisation oven (Hybaid). The labelled probe was mixed with 100 µl of 10 mg ml^{-1} sonicated herring sperm DNA (Invitrogen) and 1 ml of QuikHyb® Hybridisation solution, and the mixture was boiled for 2 min. The probe was added immediately and hybridised onto the pre-incubated blot for 1 hr. Blots were washed twice for 15 min at room temperature with a 2 x SSC buffer [0.3 M sodium chloride, 30 mM Trisodium Citrate dehydrate pH 7.0], and washed twice for 30 min at 62˚ C with pre-warmed wash solution [0.2 x SSC buffer and 0.1% (w/v) sodium dodecyl sulfate] for a high-stringency wash. The washed blots were exposed to a Phosphoscreen overnight prior to visualisation with ImageQuant™ software (Molecular Dynamics). The blots were stripped and stored as recommended by the manufacturer's instruction (Amersham).

Table 4.2. A summary of primers used to generate DNA probes for four *Plasmodium yoelii yoelii* 33XC AFLP markers most reduced in cross progeny following growth selection, as indicated by bold letters, and one unreduced marker and their PCR conditions

AFLP marker names	*P. y. yoelii* genomic contig ID corresponding to the AFLP markers	Primers	Annealing Temperature
33XC AG01AG 17XYM	AABL01002010	5'-TTGTTACAATATCCAAAAAGATGGTT-3' 5'-TCAAGAGATTAAAGATATTGCAGATGA-3'	59.0
33XC AG01TA 17XYM	AABL01001623	5'-GCAATTACACTTTTGTGTGAAA-3' 5'-TCCTTTAGAGATACTGCAACATT-3'	55.0
33XC CA05TT 17XYM	AABL01000278	5'-CACAATTTTCCCCATTTTTGTT-3' 5'-AAATGATGAAATTCAAAATGCTG-3'	58.0
33XC CA05TA 17XYM	AABL01002306	5'-GACAAGGAAAGAAAAAGGAATCA-3' 5'-GGAAAGAGTGGCAAAAGTC-3'	60.0
33XC AA01CT 17XYM	AABL01000468	5'-GTTTTCAATGGGTAGCCAAAAC-3' 5'-TTGCTGAAGATTGTAATCCCACT-3'	59.0

PCR reactions for each marker were carried out in a 150 µl volume consisting of 300 ng genomic DNA of *P. y. yoelii* 33XC, 2.5 mM $MgCl_2$, 0.3 mM forward and reverse primers (as above), 400 µM dNTPs, 5 U *Taq* DNA polymerase in storage Buffer B (Promega) and 1 x *Taq* polymerase 10 x Buffer, Magnesium free (Promega). PCR conditions were as follows: 30 cycles of 94˚C for 45 sec, 55-60˚C (depending on the primers, see Annealing Temperature above) for 1 min, 72˚C for 1 min, and a final elongation step heating at 72˚C for 10 min. PCR products were run on 1.5% agarose gel. Each sample produced single amplicon of the predicted length, ~600 to 800 base pairs).

4.4 Results

4.4.1 Growth of 17XYM and 33XC in the single and mixed clone infections

Growth of 17XYM and 33XC in the single and mixed clone infections was studied in CBA mice, aged 6 to 8 weeks old. Two groups of mice (n = 4) were i.p. inoculated with 1 x 10^6 pRBC mixtures consisting of 17XYM and 33XC in proportions of 1:1 and 1:9, respectively. A further two groups of batch mates (n = 5) were i.p. inoculated with the same numbers of the blood stage parasites of either 17XYM or 33XC. The daily parasitaemias of the single and mixed clone infections in mice are shown in Figure 4.1A to 4.1C. The proportions of 17XYM and 33XC in the mixed clone infections were determined using a quantitative SNP-based assay Pyrosequencing™ (Cheesman *et al.,* 2007) for the 17XYM and 33XC alleles of the gene coding for AMA-1 (see *Materials and Methods*, section 4.3.3). The clone specific parasitaemias of 17XYM and 33XC in the mixed clone infections (Figure 4.1B and 4.1C) were compared on each day of infection using an analysis of variance (ANOVA) (*see Materials and Methods*, section 4.3.3).

In the single clone infections, the percent parasitaemia of parasites of 17XYM rose to a mean of 20% by day 4 post infection and reached a mean of 70% by day 6 (n = 5). By contrast, the percent parasitaemia of parasites of 33XC rose slightly to a mean of 1-2% by day 4 post infection. This remained constant at day 6, the last day of sampling for this group. It is clear, therefore, that parasites of 17XYM had a much faster growth rate than those of 33XC from days 4 onwards ($P < 0.01$) (Figure 4.1A).

In mixed clone infections containing proportions of 1:1 (Figure 4.1B) and 1:9 (Figure 4.1C) parasites of 17XYM achieved much higher parasitaemias than those of 33XC from days 5 and day 6 onwards ($P < 0.01$) when the total parasitaemias reached a mean of around 25% and 20%, respectively. Although the parasitaemias of parasites of 17XYM in the 1:9 YM:33XC mixed infection increased more slowly than in the 1:1 YM:33XC mixed infection, the parasitaemias of parasites of 33XC in both mixed infections remained stable at around 1-2% throughout a period of observation. This result was due purely to the fact that the number of parasites of 17XYM in the 1:1

YM:33XC mixed infection was higher than in the 1:9 YM:33XC mixed infection. These experiments demonstrate, therefore, that parasites of 17XYM had a faster growth rate than those of 33XC in the single and mixed-cloned infections containing both proportions.

From this data, it is possible to select for uncloned progeny parasites of a genetic cross between 17XYM and 33XC that have a fast growth rate by allow them to develop to a mean parasitaemia of 20% or above in laboratory mice in LGS experiments.

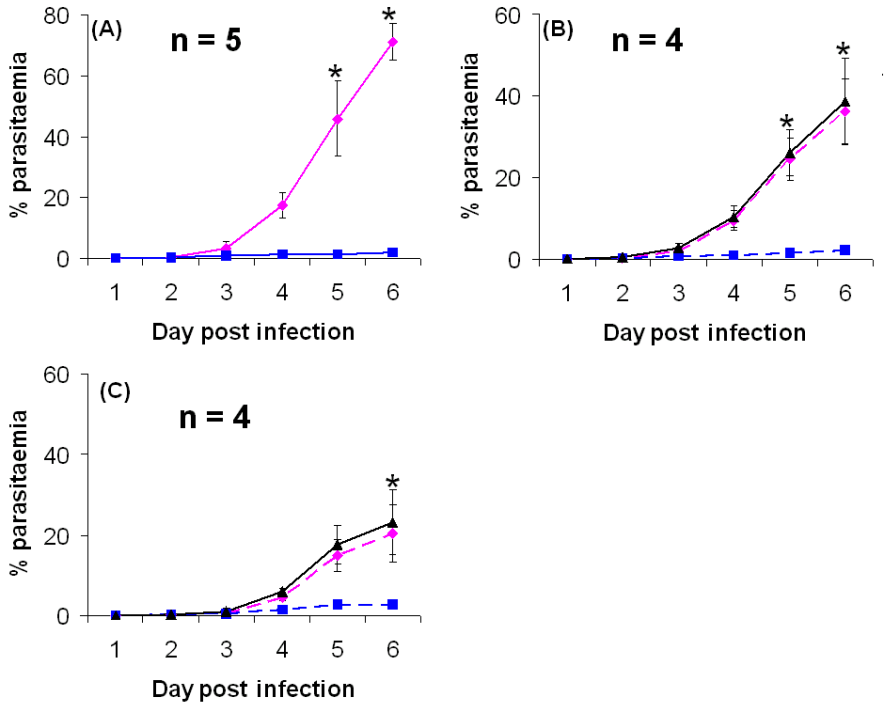

Figure 4.1. Growth of (A) single and (B and C) mixed clone infections of 17XYM and 33XC of the rodent malaria parasite *Plasmodium yoelii yoelii*. The parasitaemias during the course of single clone infections with either 17XYM (pink solid line) or 33XC (blue solid line) are shown in panel A. The clone specific parasitaemias (17XYM, pink dashed lines; 33XC, blue dashed lines) and the absolute parasitaemias (black solid lines) in mixed clone infections with parasites of 17XYM and 33XC in proportions of 1:1 and 1:9 are shown in panels B and C, respectively. Error bars give the 95% confidence intervals of the means. 'n' is the total number of mice used in the experimental groups. Asterisks (*) represent statistically significant differences ($P < 0.01$) in parasitaemia between 17XYM and 33XC.

4.4.2 Growth of the uncloned 17XYM and 33XC cross progeny

Two independent genetic crosses between 17XYM and 33XC were made, as described in *Materials and Methods* (section 4.3.4). The highest numbers of independent recombinant lines that could have been obtained in the first and second genetic crosses were estimated to be approximately 13,200 and 35,900 (Table 4.1). Blood stage parasites of the uncloned progeny of the first and second genetic crosses were each grown in three successive infections in groups of three and four CBA mice, respectively, aged 6 to 8 weeks, as described in *Materials and Methods* (section 4.3.5). The infections were examined daily by microscopy (Figure 4.2). The surviving parasites from each round of growth selection were harvested, as described in *Materials and Methods* (section 4.3.5). Parasite DNA was prepared prior to the molecular genetic analysis by AFLP (Grech *et al.*, 2002).

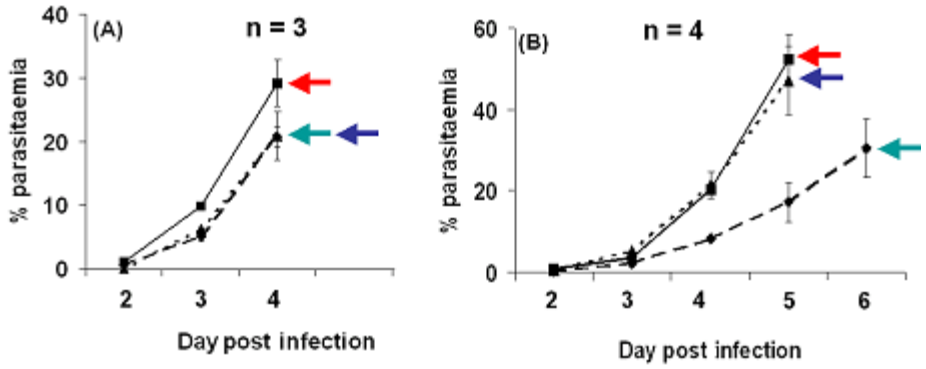

Figure 4.2. Growth of the uncloned progeny of the two genetic crosses between *Plasmodium yoelii yoelii* lines 17XYM and 33XC in three successive passages in laboratory mice. The absolute parasitaemias of the uncloned progeny of (A) the first genetic cross and (B) the second cross after first passage (dashed line), second passage (dotted line) and third passage (solid line) were measured by microscopy. Days post infection on which blood stage parasites in first, second and third passages were harvested for LGS analysis are indicated by green, blue and red arrows, respectively. Error bars give the 95% confidence intervals of the means. The letter 'n' represents the number of mice in the experimental groups in each successive blood-induced infection.

4.4.3 Identification of AFLP markers under strong growth selection

Parasite DNA of the uncloned progeny from the two genetic crosses in the three successive passages was typed with a large number of quantitative genetic markers generated by AFLP, as described in *Materials and Methods* (section 4.3.6). From 68 randomly chosen combinations of selective AFLP primers, we identified 197 parasite clone specific polymorphic AFLP bands (AFLP markers) between 17XYM and 33XC. Of these bands, 89 were present only in 17XYM and 108 in 33XC and used as markers for the fast-growing parental clone 17XYM and the slow-growing parental clone 33XC, respectively. Following growth selection of cross progeny, the RIIs of 108 AFLP markers of the slow-growing parent 33XC were determined (Table S3, Appendix 3; see *Materials and Methods*, section 4.3.6, for the definition of the RII). The RIIs of individual markers in the two crosses were plotted against each other in Figure 4.3 for each of the three passages.

Five 33XC AFLP markers had RIIs of below 0.20 following three rounds of growth selection, i.e. intensity of an AFLP marker carried by parasites of the growth rate selected progeny reduced by 80% relative to its intensity of parasites of the parental clone (Figure 4.3 and Table 4.3A). Three of the five affected 33XC markers including 33XC AG01TA 17XYM, 33XC AG01AG 17XYM and 33XC CA05TT 17XYM were already reduced in the first passage in the growth rate selected uncloned progeny of the two genetic crosses (Figure 4.3 and Table 4.3A). The remaining two 33XC markers, 33XC CA05TA 17XYM and 33XC GT02TT 17XYM, were strongly reduced only following the second and third passages, respectively.

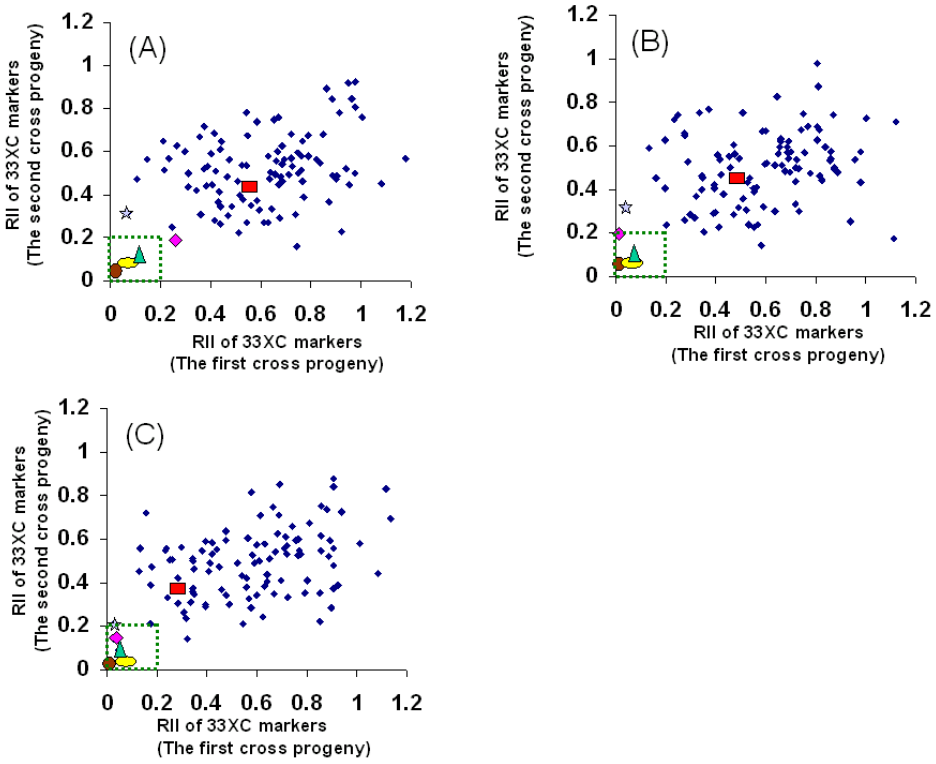

Figure 4.3. Pairwise comparison of the Relative Intensity Indices (RIIs) of *Plasmodium yoelii yoelii* 33XC AFLP markers from the uncloned progeny of the two genetic crosses between *P. y. yoelii* 17XYM and 33XC following growth selection in laboratory mice. (A), (B) and (C) show the RIIs of 108 *P. y. yoelii* 33XC markers in the progeny of the two genetic crosses in the first, second and third passages, respectively. The X-and Y-axes represent the RIIs of the *P. y. yoelii* 33XC markers of the first and second cross progeny, respectively. Five *P. y. yoelii* 33XC markers were consistently and strongly reduced under growth selection in both crosses (green squares). These markers were as follows: 33XC CA05TT 17XYM, brown circle; 33XC AG01AG 17XYM, yellow oval; 33XC AG01TA 17XYM, green triangle; 33XC CA05TA 17XYM, pink diamond. Each of these five markers and one marker, 33XC AA01CT 17XYM, red rectangle, that was not under growth selection were genetically mapped.

4.4.4 Physical Mapping of AFLP markers under selection

The five 33XC markers under growth selection were located to the *P. y. yoelii* genome as follows. DNA sequences of the five affected 33XC markers were obtained and *P. y. yoelii* 17XNLclone1.1 genomic contigs containing DNA sequences that matched those of the affected markers were identified by BLASTN searching of the *P. y. yoelii* genome (the Institute for Genomic Research), as described in *Materials and Methods* (section 3.3.7). The predicted orthologues of the genes on the identified *P. y. yoelii* genomic contigs were, thereafter, located within the *P. falciparum* genome. Because of the high level of conserved synteny between the genomes of *P. falciparum* and the rodent malaria parasites (Kooij *et al.,* 2005), the chromosomal locations of the *P. falciparum* orthologues of *P. y. yoelii* 33XC markers could, in turn, be mapped to the equivalent predicted chromosomal positions in the *P. y. yoelii* genome (Table 4.3B).

Two of the five strongly reduced 33XC markers were found in *P. y. yoelii* contigs containing *P. y. yoelii*-specific genes (Table 4.3B) that had no orthologues in the *P. falciparum* genome (Kooij *et al.,* 2005). One 33XC marker, 33XC CA05TA 17XYM, which was strongly reduced only following the second passage, was located in a gene of a multigene family coding for the telomerase reverse transcriptase (TERT). The other, 33XC GT02TT 17XYM, which was strongly reduced only following the third passage, was found in a gene coding for putative YIR3, a member of the multigene family coding for the Yoelii Interspersed Repeats (YIR). The *P. y. yoelii* chromosomal locations of these two affected markers were not known and were indicated as not determined in Table 4.3B.

The other three 33XC AFLP markers that were strongly reduced following the first passage were successfully located to a single region on *P. y. yoelii* chromosome 13 (Table 4.3B). One marker, 33XC CA05TT 17XYM, had an orthologue on *P. falciparum* chromosome 13, while the other two markers, 33XC AG01TA 17XYM and 33XC AG01AG 17XYM, had orthologues on *P. falciparum* chromosome 14. Both locations were syntenic with a single region on *P. y. yoelii* chromosome 13. The *P. falciparum* genomic regions containing the orthologues of the three most reduced

33XC markers on *P. y. yoelii* chromosome 13 (see above) defines approximately ~1 megabase pairs (Mb) of sequence (Table 4.3B and Figure 4.4). This region contains at least 254 predicted genes in *P. falciparum* (data from September 2007; http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=36329&query=) and 204 predicted genes in *P. y. yoelii* as deduced from the synteny between them (Kooij *et al.*, 2005).

We also identified a *P. y. yoelii* 33XC AFLP marker, 33XC AA01CT 17XYM, which was not reduced following growth selection (Figure 4.3). However, an orthologue of this marker was located on *P. falciparum* chromosome 13, which was syntenic with a region on *P. y. yoelii* chromosome 13 and lay approximately ~ 152 kilobase paris (kb) from the nearest location on the linkage group containing the three strongly and consistently reduced 33XC markers (Figure 4.4). This marker thereby places the outer limit of the genomic region under strong growth selection.

(4.3A)

Name of 33XC AFLP markers	RIIs of most selected 33XC markers of the uncloned progeny of the first 17XYM x 33XC genetic cross			RIIs of most selected 33XC markers of the uncloned progeny of the second 17XYM x 33XC genetic cross		
	First passage	Second passage	Third passage	First passage	Second passage	Third passage
33XC AG01AG 17XYM	0.073	0.067	0.065	0.096	0.077	0.038
33XC AG01AT 17XYM	0.104	0.056	0.045	0.103	0.067	0.089
33XC CA05TT 17XYM	0.007	0.001	0.004	0.045	0.061	0.033
33XC CA05TA 17XYM	0.255	0.010	0.030	0.198	0.207	0.144
33XC GT02TT 17XYM	0.070	0.038	0.027	0.314	0.315	0.199

(4.3B)

Name of 33XC AFLP markers	*P. y. yoelii* genomic contig§ corresponding to the 33XC markers	*P. y. yoelii* gene ID§ nearest to the position of the 33XC markers	*P. y. yoelii* gene annotation۞	*P. falciparum* gene ID orthologous to the *P. y. yoelii* genes identified by the 33XC markers§	Physical locations in the *P. falciparum* genome§	*P. y. yoelii* Chromosome to which 33XC markers are predicted to be located according to synteny#
33XC AG01AG 17XYM	AABL01002010	XM_721545	Hypothetical protein	PF14_0454	*pf*14 - 1946*	13
33XC AG01AT 17XYM	AABL01001623	XM_720491	Hypothetical protein	PF14_0580	*pf*14 - 2476	13
33XC CA05TT 17XYM	AABL0100278	XM_723337	Hypothetical protein	MAL13P1.124	*pf*13 - 943	13
33XC CA05TA 17XYM	AABL01002306	XM_722303	TERT protein	No orthologue	not determined	not determined
33XC GT02TT 17XYM	AABL01000153	XM_720217	Putative YIR3 protein	No orthologue	not determined	not determined

Table 4.3. Summary of (A) the Relative Intensity Indices (RIIs) and (B) the physical locations of the *Plasmodium yoelii yoelii* 33XC AFLP markers of the uncloned progeny from the two genetic crosses between 17XYM and 33XC which were most strongly reduced after growth selection. ID, identity

§ The genomic contigs and the gene ID of *P. y. yoelii* (Carlton *et al.*, 2002) and the gene ID and physical locations in the *Plasmodium falciparum* genome (Gardner *et al.*, 2002) as described in the GenBank®.

۞ The gene annotation based upon the *P. y. yoelii* 17XNLclone1.1 genome (the TIGR website; http://www.tigr.org/tdb/e2k1/pya1/pya1.shtml).

Detailed syntenic relationships between *P. falciparum* and *P. y. yoelii* were described by Kooij *et al*, (2005).

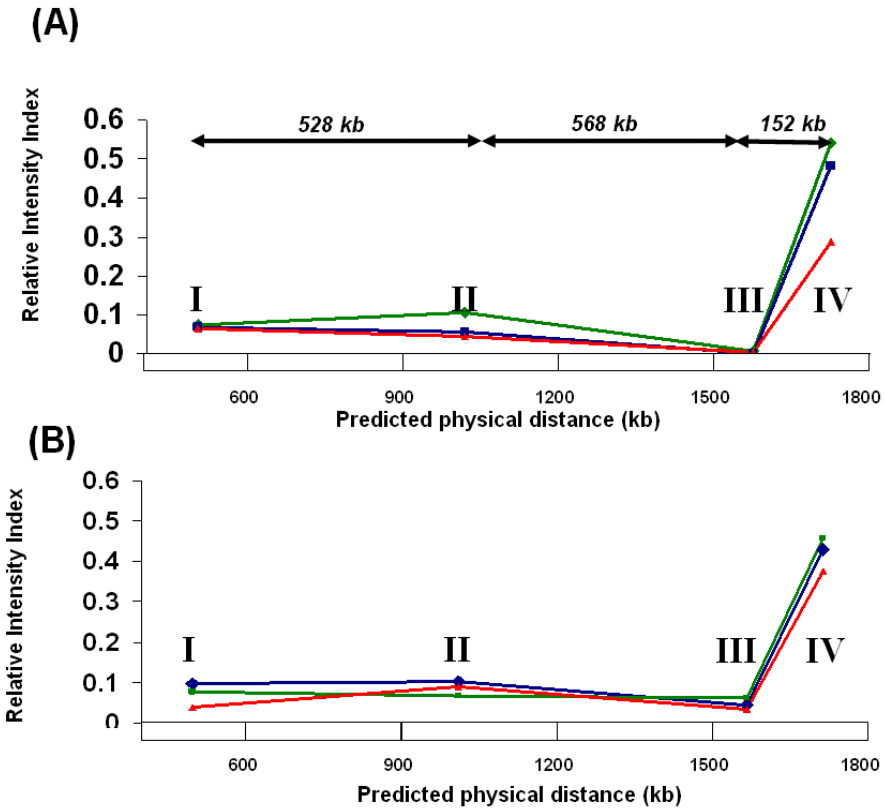

Figure 4.4. The Relative Intensity Indices (RIIs) of the four *Plasmodium yoelii yoelii* 33XC AFLP markers on chromosome 13 following growth selection of the progeny of the genetic cross between 17XYM and 33XC. The vertical axis represents the RII. The horizontal axis represents the physical distance on *P. y. yoelii* chromosome 13 in kilobase pair (kb). The RIIs of the 33XC markers in the first, second, and third passages are indicated by green, blue and red colours, respectively. Of the four 33XC markers, three markers including (**I**) 33XC AG01AG 17XYM, (**II**) 33XC AG01TA 17XYM, (**III**) 33XC CA05TT 17XYM were strongly reduced following growth selection and the other marker, (**IV**) 33XC AA01CT 17XYM, which was a non-reduced marker (see text). The RII data shown here was obtained from the growth selected uncloned progeny of the first genetic cross (A) and the second genetic cross (B) (see Supplementary data). Numbers above the double-headed arrows indicate distances between each of the 33X markers in kb based on the orthologues of the 33XC markers in *P. falciparum* genome. Extrapolation to *P. y. yoelii* chromosome 13 is from synteny between these two species as previously described in *Materials and Methods* (section 4.3.7).

4.4.5 Confirmation of genomic regions of selection

To confirm the locations of the 33XC markers that were mapped by genetic mapping on *P. y. yoelii* chromosome 13, we hybridised ^{32}P-radiolabelled sequences derived from each of the 33XC markers onto a chromosome blot containing chromosomes of the two parental clones 17XYM and 33XC, separated by the method of pulsed field gel electrophoresis (PFGE), as described in *Materials and Methods* (section 4.3.8 to 4.3.10). All four 33XC markers hybridised to a single band corresponding to the co-migrating *P. y. yoelii* chromosomes 13 and 14 (Figure 4.5) of 17XYM and 33XC clones.

We attempted to hybridise the two other affected 33XC AFLP markers whose locations could not be identified by physical mapping, due to the absence of the orthologues in *P. falciparum*. One marker, 33XC CA05TA 17XYM, found in the gene for TERT protein, hybridised to a band corresponding to the co-migrating *P. y. yoelii* chromosomes 5 and 6 (Figure 4.5). However, we were not able to perform hybridisation for the other marker, 33XC GT02TT 17XYM. The main reason for this was that we were not able to design a specific DNA probe for this marker. This was due to the fact that the marker was found in the gene coding for putative YIR3 protein, a member of the largest gene family in *P. y. yoelii* (Carlton *et al.*, 2002). The chromosomal location of this marker in the *P. y. yoelii* genome was not known by either physical mapping or PFGE and hybridisation analysis.

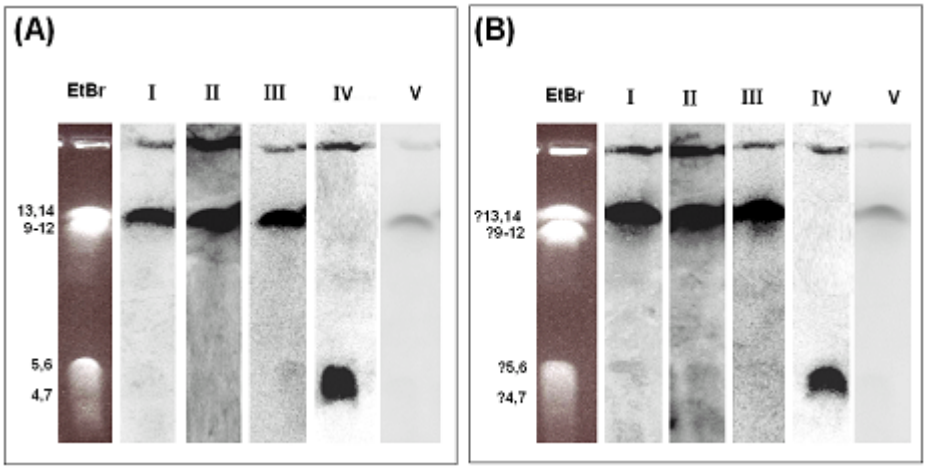

Figure 4.5. Physical locations of *Plasmodium yoelii yoelii* 33XC AFLP markers on chromosome blots of (panel A) 17XYM and (panel B) 33XC, separated by pulsed field gel electrophoresis (PFGE). The chromosome numbers are shown on the left-hand side of the gels in each panel (Owen *et al*, 1999; Khan *et al.*, 2001). The chromosome numbers of 33XC have been tentatively assigned by comparison to 17XYM (hence, the question marks at the side of the chromosome bands of 33XC). Chromosome blots were hybridised with DNA sequences corresponding to the *P. y. yoelii* 33XC AFLP markers as follows: (**I**) 33XC AG01AG 17XYM, (**II**) 33XC AG01TA 17XYM, (**III**) 33XC CA05TT 17XYM, (**IV**) 33XC CA05TA 17XYM and (**V**) 33XC AA01CT 17XYM (see *Materials and Methods*, section 4.3.8-4.3.10).

4.5 Discussion

Using the Linkage Group Selection (LGS) approach, we have identified regions in the rodent malaria parasite *P. y. yoelii* genome containing genes that determine growth rate of the blood stages of these parasites. First, we crossed two genetically distinct lines of *P. y. yoelii* that differ in blood stage growth rate, 17XYM (fast growth) and 33XC (slow growth), to generate recombinant progeny. We then grew blood stage parasites of uncloned cross progeny for three rounds of infections in naïve mice. The uncloned cross progeny in each infection was then allowed to develop high parasitaemias. This is expected to select for those parasites which possess the alleles determining growth rate of the faster growing parental line. Parasite DNA from the uncloned progeny of the two genetic crosses after the three successive passages was screened for the presence and intensity of genome-wide AFLP markers that allowed measuring of the proportions of 17XYM and 33XC in the mixture. Following growth of the progeny of the two genetic crosses in the three successive infections, a small number of AFLP markers of the slow-growing parent, 33XC, were consistently and strongly reduced in intensity, with RIIs below 0.2. The locations of these most reduced markers were searched for in the *P. y. yoelii* genome, as described in *Results* (see section 4.4.3).

Three of the five most strongly selected 33XC markers were already strongly reduced in the first passage following growth selection of both crosses. All these markers were located to a region on *P. y. yoelii* chromosome 13 and formed a linkage group that spanned a predicted ~1 Mb of genetic distance. Closely linked to it was a 33XC marker that was not reduced under growth selection. This marker thus places an outer limit to the genomic region under strong growth selection on *P. y. yoelii* chromosome 13. Consistent with the above, CHEF and hybridisation results showed these four 33XC markers hybridised to a single position on chromosome blot corresponding to co-migrating *P. y. yoelii* chromosomes 13 and 14. These results, therefore, have identified a single non-recombined region on *P. y. yoelii* chromosome 13 which is under strong growth selection. It may be that this entire linkage group must be inherited as a non-recombined genetic unit, in order to confer fast growth rate upon the parasites. Alternatively, it may be that, for some other reason, this

section of *P. y. yoelii* chromosome 13 cannot recombine between the alleles of the two parental clones. Within this is at least one gene which determines a fast growth trait.

The two other markers under strong selection were only reduced after the second and third passages respectively. Both markers were found in *P. y. yoelii*-specific multigene families, one in the family coding for TERT and the other in the family coding for YIR. Within *P. y. yoelii* genome data, 6 *tert* genes and 838 *yir* genes (693 full genes and 145 partial genes) are present and the exact roles of these gene families are not known (Carlton *et al.*, 2002; see also the TIGR *P. y. yoelii* genome database http://www.tigr.org/tdb/e2k1/pya1/LocusNameSearch.shtml). Due to the absence of orthologues in *P. falciparum*, according to the genetic synteny map of Kooij *et al.,* (2005), these markers could not be located in the *P. y. yoelii* genome by physical mapping. Nevertheless, CHEF and Southern hybridisation analysis indicated that the marker found in the gene for TERT was located to the position of co-migrating *P. y. yoelii* chromosomes 5 and 6, suggesting that at least one of these chromosomes also contains a genomic region under growth selection. The other marker, found in the gene for YIR, could not be located by either physical mapping or CHEF and hybridisation analysis. Its location in the *P. y. yoelii* genome is currently not known.

The effects of growth selection on the two markers found in the *P. y. yoelii*-specific gene families, TERT and YIR, were less pronounced than those upon the three markers that formed a single linkage group on *P. y. yoelii* chromosome 13. This ~1 Mb linkage group appears, therefore, to contain the major genetic determinant(s) of growth rate in *P. y. yoelii* 17XYM. The original description of the 17XYM line reported that parasites having fast growth rate had emerged in a single step during blood passage of 17X, a mild line of *P. y. yoelii* (Yoeli *et al.,* 1975). In a genetic cross between 17XYM and another slow-growing line of *P. y. yoelii*, line A/C, the fast and slow growth characters recombined with other genetic markers, and segregated in the cloned progeny, implicating a single locus for genes conferring these growth characters (Walliker *et al.,* 1976). This is consistent with the present

finding by LGS of a single region of the *P. y. yoelii* genome that determines growth rate differences between 17XYM and 33XC.

It is noteworthy, however, that, in the present study, we were able to locate only one 33XC marker which was not under growth rate selection on *P. y. yoelii* chromosome 13. Thus, we will need additional quantifiable markers on this chromosome 13 in order to precisely locate the affected region under growth rate selection. One approach is to generate additional AFLP markers from the two parental lines with different combinations of selective AFLP primers (Grech *et al.*, 2002). If necessary, SNP-based PSQ assays can be developed to increase the number of quantifiable genetic markers on *P. y. yoelii* chromosome 13 (Cheesman *et al.*, 207). These assays would enable us to accurately quantify proportions of the parasites carrying alleles representing SNPs within the region under growth rate selection, or other regions of the genome under investigation. With a further tens of quantifiable genetic markers developed across the *P. y. yoelii* chromosome 13, we would be able to narrow the location under selection and increase a possibility of identifying loci associated with growth rate differences in the parental strains used in the cross.

More recently, we identified a slow-growing line of 17X, 17XNIMR (Pattaradilokrat *et al.*, 2008), which appeared to be from the same genetic stock as 17XYM. This could be useful in identifying candidate genes controlling growth rate through detailed genetic comparison of these two putatively congenic lines. Identification of candidate genes controlling growth rate within the region on *P. y. yoelii* chromosome 13 under strong growth selection involves the sequencing of all such genes in 17XYM and 17XNIMR. This process would yield genes for the individual proteins that are polymorphic between them. This could, in principle, be no more than a single nucleotide polymorphism. Subsequently, likely or possible candidate genes can be tested further in transfection experiments, which have been developed for *P. y. yoelii* (Jongco *et al.*, 2006). Moreover, bioinformatic analysis, such as prediction of membrane location and stage-specific expression profile (which can be assessed at the PlasmoDB and EBI websites http://www.plasmodb.org/plasmo/home.jsp and

http://us.expasy.org/sprot/), may also be used to predict a possible function of the identified gene. Together, these approaches should lead to identification and validation of the gene, or genes, that determine growth rate in the linkage group under strong growth selection on *P. y. yoelii* chromosome 13.

It has been proposed that candidate genes determining growth rate differences in this malaria parasite could be those coding for (i) proteins associated with differential induction of host proinflamatory cytokine responses in the early infection (Omer *et al.*, 2003), (2) proteins involved in different rates of DNA replication (Reilly *et al.*, 2007), or (3) proteins expressed on the merozoites surface and involved in recognition and invasion of host erythrocytes (Grüner *et al.*, 2004; Iyer *et al.*, 2007a).

First, it may be that growth rate differences between strains of *P. y. yoelii* are related to differences in their ability in suppressing innate immune responses during the course, especially the early course, of an infection. Thus, Omer and others (2003) showed that C57BL/6 mice infected with a slow-growing strain 17X (NIH) strain of *P. y. yoelii* produced TGF-β from 5 days post infection; this was correlated with resolution of parasitemia and full recovery. In contrast, infection with the lethal strain 17XL (NIH) induced high levels of circulating TGF-β within 24 h; this was associated with delayed proinflamatory cytokine IFN-γ and TNF-α responses, failure to clear parasites, and 100% mortality (Omer *et al.*, 2003). However, candidate genes coding proteins inducing the differential cytokine production in this parasite have not yet been identified.

Second, there may be genotype-specific differences in the duration, or the number of rounds, of DNA replication in each erythrocytic cycle [i.e. a faster DNA replication rate would lead to shorter erythrocytic cycles and could be associated with faster growth rate]. However, there is no proof that such differences are associated with differences in growth rate in *P. y. yoelii*, although a study by Reilly and colleagues (2007) showed that the human malaria parasite *P. falciparum* strain Dd2 has shorter erythrocytic cycle duration and produces more merozoites per schizont than *P. falciparum* strain HB3. This subject will require further investigation in *P. y. yoelii*.

Third, growth rate differences in this malaria parasite could also be associated with differences in how easily merozoites can invade the host erythrocytes (Preiser *et al.*, 1999) and differences in ability to invade and develop in erythrocytes of different ages (Walliker *et al.*, 1976; Jayawardena *et al.*, 1983; Burns *et al.*, 1989). Previous work indicated that a multigene family coding for 235 kDa rhoptry proteins (Py235) located at the rhoptries of merozoites (Oka *et al.*, 1984) might be involved in erythrocyte invasion preference and, in turn, blood stage growth of this malaria parasite (Freeman *et al.*, 1980; Holder *et al.*, 1981). For instance, monoclonal antibodies (mAbs) specific for a Py235 member can interfere with parasite invasion of mature erythrocytes (Freeman *et al.*, 1980), and immunisation with Py235 protected mice against a lethal challenge infection with parasites of 17XYM (Holder *et al.*, 1981). Subsequently, several studies have focused on examining antigenic polymorphisms and/or differences in the copy number of the Py235 gene family between the fast growing line, 17XYM, and a small number of slow-growing lines derived from isolate *P. y. yoelii* 17X from which 17XYM arose, including 17XNIMR (Owen *et al.*, 1999; Narum *et al.*, 2001), 17XA (Khan *et al.*, 2001; Iyer *et al.*, 2006) and 17XNL (Iyer *et al.*, 2006). The genome of 17XYM appeared to contain one less member of the Py235 family than that of 17XA (Khan *et al.*, 2001) and of 17XNIMR (Owen *et al.*, 1999). However, in an analysis of cloned progeny from a genetic cross between 17XNIMR and 17XYM, the presence or absence of this additional locus did not correlate with the fast or slow growth phenotype in these malaria parasites (Owen *et al.*, 1999). Furthermore, Iyer and others (2006) showed that the overall copy number of Py235 detected in 17XYM is similar to that of 17XNL 1.1 (Iyer *et al.*, 2006). It is highly unlikely, therefore, that the antigenic diversity and differences in the copy number of the genes for Py235 are associated with variation in growth rate in these parasite strains.

On the other hand, Preiser and Jarra (1998) showed that expression patterns of Py235 proteins were notably different between the fast- and slow-growing lines, 17XYM and 17XA, of *P. y. yoelii*. They suggested that differential expressions of specific members of Py235 in this malaria parasite might facilitate switching of erythrocyte invasion preference and an increase in blood stage growth rate. Using quantitative

real-time PCR and Western blot analysis Iyer *et al.* (2007b) demonstrated that expression levels of a small number of members of Py235 in the fast growing line 17XYM are much greater than those of the slow growing strain 17XA. Thus, this data leads a hypothesis that specific members of the Py235 multigene family express proteins that mediate host red blood cell selection and, hence, increased blood stage growth rate. However, we were not able to use LGS analysis to address the above question. The main reason for this is that Py235 genes exhibit a newly discovered form of clonal antigenic variation, whereby each individual merozoite derived from a single parent schizont has the propensity to express different Py235 proteins (Preiser *et al.*, 1999; Snounou *et al.*, 2000). Should such phenotypic switching account for the observed differences in growth rate between two parental parasite lines, LGS would fail to reveal selection valleys in response to selection for growth rate. On the other hand, where genotypic differences do underline growth rate between the two parental lines of a genetic cross, LGS should be able to uncover the relevant genes as already demonstrated in the present work.

In conclusion, phenotypic diversity in blood stage growth rate and virulence in the host is commonly found in malarial parasites infecting humans and rodents (James *et al.*, 1932; Yoeli *et al.,* 1975; Simpson *et al.,* 2002). LGS analysis of growth rate has identified a genomic region on *P. y. yoelii* chromosome 13 under strong growth rate selection. A weaker growth selection was also found in a region on co-migrating *P. y. yoelii* chromosome 5 or 6. No other regions in the genome of *P. y. yoelii* were identified under the strength of growth selection comparable to that on *P. y. yoelii* chromosome 13. The present finding, which is consistent with the classical genetic analysis of Walliker *et al.,* (1976), indicates that growth rate in *P. y. yoelii* is likely to be mainly determined by a single region on *P. y. yoelii* chromosome 13.

CHAPTER 5: Discussion and Summary

The identification of genes in malaria parasites which control medically important phenotypes, such as drug resistance, transmissibility, strain specific protective immunity (SSPI) and growth rate in the vertebrate host, would assist the development of strategies for malaria control. Existing methods for genetic studies of malaria parasites, including classical linkage analysis and a quantitative trait loci analysis, have led to the successful identification of the malaria parasite genes conferring drug resistance (Carlton *et al.*, 1998a; Wellems *et al.*, 1990; Wellems *et al.*, 1991; Su *et al.*, 1997; Wang *et al.*, 1998) and gametocytogenesis (Vaidya *et al.*, 1995; Guinet *et al.*, 1996). The work involved, however, is expensive and extremely labour intensive. Therefore, a newly devised genetic approach of Linkage Group Selection (LGS) that bypasses the laborious procedure of cloning and characterising cross progeny is used here towards gene discovery. This approach involves (1) the application of a specific selection pressure, e.g. SSPI, to the uncloned recombinant progeny of a genetic cross between two members of a single species that differ phenotypically and measurably in their responses to the selection pressure, and (2) evaluation of the effects of the selection upon the progeny at genome-wide loci using quantitative genetic markers (Culleton *et al.*, 2005; Carter *et al.*, 2007).

The focus of the present study was to search for genetic determinants controlling strain specific protective immunity (SSPI) and growth rate in the rodent malaria parasites *Plasmodium chabaudi chabaudi* and *Plasmodium yoelii yoelii*, respectively. These two phenotypes were chosen for study because both phenotypes were likely to be interrelated and involved in determining the survival of malaria parasites in the host. This, in turn, contributes to the clinical outcome of a malarial infection. Parasite growth rate is likely to be related to the immunogenicity of the malaria parasite. This is because strains of malaria parasites that differ in growth rate may differ in their efficiency in inducing protective immunity during the course of an infection. In other words, the immune system of the host might respond differently to an infection with slow- or fast-growing strains of malaria parasites.

In the cross between the two genetically distinct strains, AJ and CB-pyr10 of *P. c. chabaudi*, that showed strong SSPI, the genetic determinants of growth rate could not, however, have been evaluated by LGS as these two strains have similar growth rate throughout the course of infection in a non-immune mouse (see Chapter 2, section 2.4.1). Nevertheless, growth rate differences in rodent malaria are well documented between lines of *P. y. yoelii*, such as lines derived from a single isolate 17X (see Chapter 3). In a classical genetic study by Walliker *et al.*, (1976) using a cross between the fast-growing strain 17XYM and a slow-growing strain A/C of *P. y. yoelii*, the fast growth character segregated from the slow growth character in the cloned progeny, suggesting a single locus for genes conferring these growth characteristics. Therefore, in the present study, we performed LGS analysis of growth rate in a new cross between 17XYM and a genetically unrelated, slow-growing line 33XC, to discover genes that are responsible for growth rate in this malaria parasite.

The outcomes of the LGS analyses of SSPI and growth rate are discussed as follows:

5.1 Linkage Group Selection analysis of Strain Specific Protective Immunity

A previous LGS analysis of SSPI conducted using a genetic cross between *P. c. chabaudi* lines AS-pyr1 and CB (Martinelli *et al.*, 2005a) identified an SSPI-selected valley in a region on *P. c. chabaudi* chromosome 8 whose base was found to contain the gene for Merozoite Surface Protein-1 (MSP-1), a known major candidate as a target of SSPI. However, the possible involvement of another candidate for SSPI, *P. c. chabaudi* Apical Membrane Antigen-1 (Crewther *et al.*, 1996) which is located on chromosome 9, could not have been evaluated in this cross. This was due to the fact that there were no polymorphisms within the cell surface, ectodomain region of this protein between AS-pyr1 and CB. In the present study, LGS analysis of SSPI was made on a new genetic cross between *P. c. chabaudi* lines AJ and CB-pyr10 in which genes for MSP-1 and AMA-1 are both polymorphic. The present study has again identified a region associated with the gene for MSP-1 which is under strong SSPI selection. The results showed that the *msp*-1 gene in *P. c. chabaudi* was the locus at which the strongest detected reduction under strain specific immune selection

occured. Nevertheless, the region containing the AMA-1 gene may contain a target of SSPI, but one that is less strongly affected than the target associated with the gene for MSP-1. Therefore, these results suggest that the gene for MSP-1, or genes closely linked to it, is a major target of SSPI.

One of the important questions concerning the application of LGS to the discovery of genes for SSPI is the influence of the host genotype, and/or the physiological state of the host, on strain-specific immune components against malaria. Since LGS analyses of SSPI on the progeny of the AJ and CB-pyr10 and AS-pyr1 and CB crosses have been conducted hitherto using a single genetic type and condition of host animal (female CBA/Ca mice, 4-5 month old at the time of challenge with the cross progeny for LGS studies), it would be of great interest to determine whether the identified target loci are the same or different in host of different genetic background, age or sex. Should the host genotype be found to affect the results obtained by LGS analysis, it would be possible to further explore the influence of host genotype using MHC congenic mouse strain, i.e. mice having different haplotypes at the MHC locus against the same genetic background. Currently, there is an ongoing project that aims to induce and determine the strain specificity of protective immunity in laboratory mouse strains Balb/c and C57 (Cheesman S. and Carter R., personal communication).

In our laboratory, there are also a number of additional strain combinations of *P. c. chabaudi* that have been characterised for their mutual strain-specific immune relationships (Cheesman *et al.*, 2006; Cheesman S. and Carter R., unpublished data). Crosses of selected combinations of the strains which show solid SSPI can be analysed by LGS and the loci encoding antigens of SSPI will be identified and compared with our present findings. Furthermore, LGS will equally applicable for studies of the target loci of SSPI in other malaria species, such as the human malaria parasite *P. falciparum* – the most deadly and medically significant species. The LGS approach is ideally suited to this malaria parasite due to the existence of strain specificity of protective immunity in humans (Jeffery *et al.*, 1969; Cardigan *et al.*, 1969), the availability of the genetic crosses between combinations of strains that

vary in these traits (Walliker *et al.*, 1976; Wellems *et al.*, 1990; Wellems *et al.*, 1991; Su *et al.*, 2007), the high-throughput methodology for genotyping the parasites and for analysing the quantitative traits of the clones of a genetic cross (Su *et al.*, 2007). Such experiments may be also conducted in chimpanzee or Aotus monkeys in which the genetic crosses have been grown. The information from such studies will lead to a greater understanding of immunity to malaria in general and it will crucially inform decision on the choice of candidate antigens for vaccine developments. In addition, LGS can be exploited to further investigate the target loci of SSPI in other Apicomplexan parasites, which are important pathogens of domestic animals. An example is the coccidian parasite *Eimeria tenella*, whose genetics is conventionally Mendelian and in which mutual SSPI has been well documented (Blake *et al.*, 2004).

5.2 Congenicity and Genetic Polymorphism between Cloned lines of *Plasmodium yoelii yoelii* 17X

In Chapter 3, experiments were described in which a comparative genetic analysis was made between cloned lines of *Plasmodium yoelii yoelii* that differed in growth rate and were derived from a single isolate designated 17X. The results indicate that there are two completely different genotypes among lines of *P. y. yoelii* 17X. Within these two genotypes, genotype-1 is represented by parasites with a slow growth rate phenotype, e.g. 17XA. The other genotype, genotype-2, is represented by parasites with either a slow growth rate phenotype, e.g. 17XNIMR, or a fast growth rate phenotype, e.g. 17XYM. This finding is consistent with results from previous studies, based upon nucleotide sequence analyses of the gene for MSP-1, that indicate that 17XA is completely genetically distinct from other lines of *P. y. yoelii* 17X (Daly *et al.*, 1992, Benjamin *et al.*, 1999).

It is noteworthy that only parasites of genotype-1 have only been found in isolate 17X of *P. y. yoelii* maintained at Edinburgh only. By contrast, parasites of genotype-2 have been present in all isolates of 17X worldwide. A likely explanation is that parasites of genotype-1 and genotype-2 are both common in isolate 17X, but

parasites of genotype-2 might be present at a much lower proportion than parasites of genotype-1. The main reason for this was that parasites of genotype-2, e.g. 17XA, were derived after selection for resistance to the anti-malarial drug pyrimethamine (Walliker *et al.*, 1971; Walliker *et al.*, 1973), while parasites of genotype 1 were obtained by cloning out directly from the isolate 17X of *P. y. yoelii* (Yoeli *et al.*, 1975; Burns *et al.*, 1989; Carlton *et al.*, 2002; Walliker D., unpublished data). This is supported by the finding that parasites of genotype-1 were shown to be sensitive to pyrimethamine (Yoelii *et al.*, 1975; Walliker *et al.*, 1976). A possibility that 17XA (genotype-2) were derived from a cross-contamination or mislabelling can, nevertheless, not be entirely ruled out unless parasites of the normal isolate 17X are subjected to pyrimethamine drug selection and are characterised for the presence of genotype-2 in the drug-selected parasite population. Should genotype-2 parasites be detected in the pyrimethamine-selected population, this would suggest the co-existence of parasites of genotype-1 and 2 in the original isolate 17X.

The presence of two genotypes within the *P. y. yoelii* strain 17X has an important implication for studies of the genetic basis of growth rate in this malaria parasite. The discovery of a slow-growing line 17X, e.g. 17XNIMR, congenic to the fast-growing line 17XYM can be useful in determining candidate genes underlying growth rate differences in these malaria parasites since the fast-growing line 17XYM arose suddenly during blood infection with a slow-growing strain of *P. y. yoelii* 17X (Yoeli *et al.*, 1975), probably due to a single mutation (Walliker *et al.*, 1976). A whole genome sequencing approach could lead to discovery of all polymorphic genes between the fast and slow congenic parasite lines. Nevertheless, we were able to conduct LGS analysis of growth rate in the cross between two genetically unrelated parasites that differed in growth rate, to narrow regions of the parasite's genome to be sequenced. In the present work, we have performed LGS analysis of growth rate between 17XC and 33XYM and identified that a region on *P. y. yoelii* chromosome 13 contain a major candidate gene(s) controlling growth rate in this malaria parasite. From this information, we will only need to sequence the chromosome 13 between 17XYM and 17XNIMR to obtain all candidate genes controlling growth rate. Subsequently, transfection systems can be used to test the involvement of candidate

genes in growth rate control and these are now available in *P. y. yoelii* (Jongco *et al.*, 2006). These approaches should allow us to identify the gene, or genes, that determine growth rate in the linkage group under strong growth selection on *P. y. yoelii* chromosome 13.

5.3 Linkage Group Selection analysis of Growth Rate

As described in Chapter 4, LGS analysis of growth rate was conducted with a genetic cross between a fast-growing line, 17XYM, and a genetically unrelated, slow-growing line, 33XC, of *Plasmodium yoelii yoelii*. A major unexpected result was the finding that it was not possible to identify a typical selection valley in any of the chromosomes of this parasite. Rather, strong growth selection acted on a group of genetically linked markers that were mapped across a 1-megabase pair (Mb) region on *P. y. yoelii* chromosome 13, i.e. about a half of the estimated size of the chromosome. This implies that a major genetic determinant, or genetic determinants, of growth rate in this parasite was mapped onto a single large non-recombined section of *P. y. yoelii* chromosome 13. This finding is consistent with the result of the classical genetic study by Walliker *et al.*, (1976), which indicates that growth rate in this parasite is conferred by a single genetic locus.

Currently, there is an ongoing project in our laboratory using the LGS approach to identify genes controlling differences in growth rate in another rodent malaria species, *Plasmodium chabaudi adami* (Gadsby N. J. and Carter R., personal communication). The study conducted on a genetic cross between a fast-growing line (DS) and a slow-growing line (DK) of *P. c. adami* has also identified a large non-recombined region of more than half a megabase pair, which is under strong growth selection. Evidence from these two independent LGS studies indicates, therefore, that large genomic regions inherited in these parasites, each as a single non-recombined genetic locus, are under growth selection pressure and contain a genetic determinant(s) of blood stage growth rate.

There are two possible explanations for these observations. First, it could be that this region contains a group of genes that are functionally linked in determining growth

rate such that this entire linkage group must be inherited as a non-recombined genetic unit in order to confer fast or slow growth rate upon the parasites. Alternatively, it may be that alleles of genes of the two parental clones within this region of *P. y. yoelii* chromosome 13 cannot recombine with each other for structural chromosomal reasons.

In order to test these hypotheses, the progeny of a genetic cross between slow- and fast-growing lines would have to be cloned out before the growth selection had taken place in the mice. This would allow us to obtain slow-growing cloned progeny of the genetic cross, which would otherwise be eliminated by growth selection pressure. Then, such fast- and slow-growing cloned progeny would need to be characterised for their haplotypes within the linkage group on *P. y. yoelii* chromosome 13 which is selected by growth selection. If these clones, derived before growth selection, possess the alleles of both parents within the linkage group on chromosome 13 under growth selection, then this would indicate that genetic recombination can occur within this region. In this case, the reason why, in the growth rate selected cross progeny, this linkage group on chromosome 13 appears not to recombine must have been due to its selection as an unrecombined unit by the growth selection pressure. However, if, in the clones derived before the selection, alleles only from one or the other parental type are present in this region, then this would indicate that recombination within this linkage group on chromosome 13 is not possible. Such investigation is now being performed in our laboratory.

At present, there are genetically distinct strains of the malaria parasites of rodent and human which have marked differences in blood stage growth rate and their underlying genetic determinants are not yet identified. For instance, *P. c. chabaudi* strain AS produces a very mild infection compared to *P. c. chabaudi* strain ER, which achieves higher peak parasitaemias and causes more severe pathology to the host (Cheesman S. and Carter R., unpublished data). Likewise, the *P. falciparum* strain Dd2 has a shorter ring stage, generates more merozoites per schizont, invades new RBCs at a higher rate and, hence, has a faster growth rate than HB3 strain (Reilly *et al.*, 2007). Since genetic crosses have been made between these lines

(Wellems *et al.*, 1990; Wellems *et al.*, 1991), the high-throughput methods for genotyping (Grech *et al.*, 2002; Martinelli *et al.*, 2004; Su *et al.*, 2007) and the complete genome sequence data are available (Gardner *et al.*, 2002 and Hall *et al.*, 2005), it will be relatively simple to apply LGS to investigate genetic basis of growth rate in this malaria parasite. Together with the findings of the present work, the information on genes controlling growth rate will be relevant to our understanding of pathogenicity of a malarial infection.

5.4 Limitations of LGS

Our present results demonstrate the success of LGS in the location of genes that are responsible for two major selectable phenotypes, SSPI and blood stage growth rate, of malaria parasites. There are, nevertheless some limitations concerning the application of LGS in the present study. For example, LGS analysis of SSPI could not be used to determine whether clonal antigenic variation in the expression of the proteins expressed on the infected erythrocyte surface accounts for the differences between two parasite lines that appeared to show strong SSPI (see *Discussion* in Chapter 2). Furthermore, in LGS analysis of growth rate, LGS could fail to determine whether the difference expression levels of specific members of a multigene family are associated with the differences between two parental lines used in generating a genetic cross (see *Discussion* in Chapter 4). However, where genotype-differences were involved, LGS should be able to identify the relevant genes.

.

5.5 Executive summary

The principle findings of the present genetic studies are as follows:

1. SSPI in *Plasmodium chabaudi chabaudi* in mice is mainly determined by a 120-kb region on chromosome 8 containing the gene for Merozoite Surface Protein-1.

2. Lines of *Plasmodium yoelii yoelii* strain 17X consist of two completely distinct genotypes. One of these genotypes, genotype-1, is represented by a slow-growing line 17XNIMR and a fast-growing line 17XYM. Within genotype-1 there are, however, a small number of genomic differences between lines with slow and fast growth rate. The other, genotype-2, is represented by a slow-growing line 17XA.

3. The fast blood stage growth rate phenotype in *Plasmodium yoelii yoelii* is mainly determined by a single 1-Mb region on chromosome 13.

References

Aide, P., Bassat, Q., Alonso, P.L. (2007) Towards an effective malaria vaccine. *Arch Dis child* **92**(6):476-9.

Aidoo, M., Terlouw, D.J., Kolczak, M.S., McElroy, P.D., ter Kuile, F.O., *et al.*, (2002) Protective effects of the sickle cell gene against malaria morbidity and mortality. *Lancet* **359**(9314):1311-2.

Allison, A.C. (1954) Protection afforded by sickle-cell trait against subtertian malareal infection. *Br Med J* **1**(4857):290-4.

Alonso, P.L., Sacarlal, J., Aponte, J.J., Leach, A., Macete, E., *et al.* (2004) Efficacy of the RTS,S/AS02A vaccine against *Plasmodium falciparum* infection and disease in young African children randomised controlled trial. *Lancet* **364** (9443):1411-20.

Alonso, P.L., Sacarlal, J., Aponte, J.J., Leach, A., Macete, E., *et al.* (2005) Duration of protection with RTS,S/AS02A malaria vaccine in prevention of *Plasmodium falciparum* disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial. *Lancet* **366** (9502):2012-8.

al-Yaman, F., Genton, B., Anders, R.F., Falk, M., Triglia, T., *et al.* (1994) Relationship between humoral response to *Plasmodium falciparum* merozoite surface antigen-2 and malaria morbidity in a highly endemic area of Papua New Guinea. *Am J Trop Med Hyg* **51**(5):593-602.

Aponte, J.J., Aide, P., Renom, M., Mandomando, I., Bassat, Q., *et al.* (2007) Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial. *Lancet* **370**(9598):1543-51.

Amino, R., Thiberge, S., Martin, B., Celli, S., Shorte, S., *et al.* (2006) Quantitative imaging of Plasmodium transmission from mosquito to mammal. *Nat Med* **12**(2):220-4

Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408**(6814): 796-815

Audran, R., Cachat, M., Lurati, F., Soe, S., Leroy, O., *et al.* (2005) Phase I malaria vaccine trial with a long synthetic peptide derived from the merozoite surface protein 3 antigen. *Infect Immun* **73**(12):8017-26.

Babiker, H.A., Ranford-Cartwright, L.C., Walliker, D. (1999) Genetic structure and dynamics of *Plasmodium falciparum* infections in the Kilombero region of Tanzania. *Trans R Soc Trop Med Hyg* **93**(Suppl 1):11-4.

Balu, B., Shoue, D.A., Fraser, M.J. Jr, Adams, J.H. (2005) High-efficiency transformation of *Plasmodium falciparum* by the lepidopteran transposable element piggyBac. *Proc Natl Acad Sci U S A* **102**(45):16391-6.

Beale, G.H., Carter, R., Walliker, D. (1978) "Genetics". In: Killick-Kendrick, R., Peters, W., editors. *Rodent Malaria*. London: Academic Press. pp. 213-45.

Beales, P.F., Gilles, H.M. (2002) "Rationale and technique of malaria control". In: Warrell, D.A., Gilles, H. M., editors. *Essential Malariology*. 4th edition. London: Arnold, pp.107-90.

Beet, E.A. (1946) Sickle cell disease in the Balovale district of Northern Rodesia. *East Afr Med J* **23**:75-86.

Bejon, P., Mwacharo, J., Kai, O., Mwangi, T., Milligan, P., *et al.* (2006) A phase 2b randomised trial of the candidate malaria vaccines FP9 ME-TRAP and MVA ME-TRAP among children in Kenya. *PLoS Clin Trials* **1**(6):e29.

Bell, A.S, de Roode, J.C., Sim, D., Read, A.F. (2006) Within-host competition in genetically diverse malaria infections: parasite virulence and competitive success. *Evolution* **60**(7): 1358-71.

Benjamin, P.A., Ling, I.T., Clottey, G., Valero, L.M., Ogun, S.A., *et al.*, (1999) Antigenic and sequence diversity at the C-terminus of the merozoite surface protein-1 from rodent malaria isolates, and the binding of protective monoclonal antibodies. *Mol Biochem Parasitol* **104**(2):147-56.

Bessman, J. D. (1990). "Reticulocyte". In: Walker, H.K.; Hall, W.D.; Hurst, J.W.; editors. *Clinical Methods: The History, Physical and Laboratory Examination*. 3rd edition. Butterworth Publishers, pp. 735-8.

Binka., F., Akweongo, P. (2006) Prevention of malaria using ITNs: potential for achieving the millennium development goals. *Curr Mol Med* **6**(2):261-7.

Bjorkman, A., Phillips-Howard, P.A. (1990) The epidemiology of drug-resistant malaria. *Trans R Soc Med Hyg* **84**(2):177-80.

Black, R.E., Morris, S.S., Bryce, J. (2003) Where and why are 10 million children dying every year? *Lancet* **361** (9376):2226-34.

Blake, D.P., Hesketh, P., Archer, A., Carroll, F., Smith, A.L., *et al.* (2004) Parasite genetics and the immune host: recombination between antigenic types of *Eimeria maxima* as an entrée to the identification of protective antigens. *Mol Biochem Parasitol* **138**(1):143-52.

Bojang, K.A., Milligan, P.J.M., Pinder, M., Vigneron, L., Alloeche, A. (2001) Efficacy of RTS,S/AS02 malaria vaccine against *Plasmodium falciparum* in semi-immune adult men in The Gambia: a randomised trial. *Lancet* **358** (9297):1927-34.

Bojang, K.A. (2006) RTS,S/AS02A for malaria. *Expert Rev Vaccines* **5**(5):611-5.

Boudreau, E. F., Webster, H.K., Pavanand, K., Thosingha, L. (1982) Type II mefloquine resistance in Thailand. *Lancet* **320**(8311):1335.

Bowman, B.R., Grant, P.T., Kermack, W. O. (1960) The metabolism of *Plasmodium berghei*, the malaria parasite of rodents. I. The preparation of the erythrocyte form of *P. berghei* separated from the host cell. *Exp Parasitol* **9**(2):131-136.

Boyd, M. F., Stratman-Thomas, W. K., Kitchen, S. F. (1936) On acquired immunity to *Plasmodium falciparum*. *Am J Trop Med* **s1-16**(2):139-45

Boyle, D.B., Newbold, C.I., Smith, C.C., Brown, K.N. (1982) Monoclonal antibodies that protect *in vivo* against *Plasmodium chabaudi* recognize a 250,000-Dalton parasite polypeptide. *Infect Immun* **38**(1):94-102.

Branch, O.H., Udhayakumar, V., Hightower, A.W., Oloo, A.J., Hawley, W.A., *et al.* (1998) A longitudinal investigation of IgG and IgM antibody responses to the merozoite surface protein-1 19-kiloDalton domain of *Plasmodium falciparum* in pregnant women and infants: associations with febrile illness, parasitemia, and anemia. *Am J Trop Med Hyg* **58**(2):211-9.

Bremen, J. (2001) The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. *Am J Trop Med Hyg* **64**(Sup 1,2): 85-96.

Brooker, S., Guyatt, H., Omumbo, J., Shretta, R., Drake, L., *et al.* (2000) Situation analysis of malaria in school-aged children in Kenya: what can be done? *Parasitol Today* **16**(5):183-6.

Brown, K.N., Jarra, W., Newbold, C.I., Schryer, M. (1985) Variability in parasite protein antigen structure and protective immunity to malaria. *Ann Inst Pasteur Immunol* **136C**:11-23.

Bruce-Chwatt, L.T. (1948) Infection of reticulocytes by *Plasmodium falciparum* and *Plasmodium malariae* in hyperendemic indogenous malaria. *Annal Trop Med Parasitol* **42**:101-112.

Bryce, J., Boschi-Pinto, C., Shibuya, K., Black, R.E., WHO Child Health Epidemiology Reference Group. (2005) WHO estimates of the causes of death in children. *Lancet* **365** (9465):1147-52.

Bull, P.C., Berriman, M., Kyes, S., Quail, M.A., Hall, N., *et al.* (2005) *Plasmodium falciparum* variant surface antigen expression patterns during malaria. *PLoS Pathog* **1**(3):e26.

Burns, J.M. Jr, Daly, T.M., Vaidya, A.B., Long, C.A. (1988) The 3' portion of the gene for a *Plasmodium yoelii* merozoite surface antigen encodes the epitope recognized by a protective monoclonal antibody. *Proc Natl Acad Sci USA* **85**(2):602-6.

Burns, J.M., Parke, L.A., Daly, T.M., Cavacini, L.A., Weidanz, W.P., *et al.*, (1989) A protective monoclonal antibody recognizes a variant-specific epitope in the precursor of the major merozoite surface antigen of the rodent malarial parasite *Plasmodium yoelii*. *J Immunol* **142**(8):2835-40.

Cardigan, F.C. Jr, Chaicumpa, V. (1969) *Plasmodium falciparum* in the white-handed gibbon: protection afforded by previous infection with homologous and heterologous strains obtained in Thailand. *Mil Med* **134**(10):1135-9.

Carle, G.F., Olson, M.V. (1984) Separation of chromosomal DNA molecules from yeast by orthogonal-field-alternation gel electrophoresis. *Nucleic Acids Res* **12**(14):5647-64.

Carle, G.F., Olson, M.V. (1987) Orthogonal-field-alternation gel electrophoresis *Methods Enzymol* **155**:468-82.

Carlton, J.M.R., Howard, J., Jensen, J.B., Walliker, D.A. (1995) A rapid technique for the detection of DNA polymorphisms in *Plasmodium*. *Exp Parasitol* **80**(1):163-6.

Carlton, J. Mackinnon, M., Walliker, D. (1998a). A chloroquine locus in the rodent malaria parasite *Plasmodium chabaudi*. *Mol Biochem Parasitol* **93**(1):57-72.

Carlton, J.M., Vinkenoog, R., Waters, A.P., Walliker, D. (1998b) Gene synteny in species of *Plasmodium*. *Mol Biochem Parasitol* **93**(2):285-94.

Carlton, J.M., Galinski, M.R., Barnwell, J.W., Dame, J.B. (1999) Karyotype and synteny among the chromosomes of all four species of human malaria parasite. *Mol Biochem Parasitol* **101**(1-2):23-32.

Carlton, J.M., Angiuoli, S.V., Suh, B.B., Kooij, T.W., Pertea, M., *et al.* (2002) Genome sequence and comparative analysis of the model rodent malaria parasite *Plasmodium yoelii yoelii*. *Nature* **419**(6906):512-9.

Carter, R. (1970) Enzyme variation in *Plasmodium berghei*. *Trans R Soc Trop Med Hyg* **64**(3):401-6.

Carter, R. (1972) Effect of PABA on chloroquine resistance in *Plasmodium berghei yoelii*. *Nature* **238**(5359):98-9.

Carter, R. (1973) Enzyme variation in *Plasmodium berghei* and *Plasmodium vinckei*. *Parasitology* **66**(2):297-307.

Carter, R., McGregor, I.A. (1973) Enzyme variation in *Plasmodium falciparum* in the Gambia. *Trans R Soc Trop Med Hyg* **67**(6):830-7.

Carter, R., Walliker, D. (1975a) New observations on the malaria parasites of rodents of the Central African Republic - *Plasmodium vinckei petteri* subsp. nov. and *Plasmodium chabaudi* Landau, 1965. *Ann Trop Med Parasitol* **69**(2):187-96.

Carter, R., Voller, A. (1975b) The distribution of enzyme variation in populations of *Plasmodium falciparum* in Africa. *Trans R Soc Trop Med Hyg* **69**(4):371-6.

Carter, R., Walliker, W. (1976). Malaria parasites of rodents of the Congo (Brazzaville): *Plasmodium chabaudi adami* subsp. Nov. and *Plasmodium vinckei lentum* Landau, Michel, Adam, and Boulard, 1970. *Ann Parasitol Hum Comp* **51**(6):637-46.

Carter, R., Diggs, C. L. (1977) "Plasmodia of Rodent". In: Kreier, J., editor. *Parasitic Protozoa*. *Volume III: Gregaines, Haemogregarines, Coccidia, Plasmodia, and Haemoproteids.* 2nd edition. New York: Academic Press, pp. 359–465.

Carter, R. (1978) Studies on enzyme variation in the murine malaria parasites *Plasmodium berghei*, *P. yoelii*, *P. vinkei* and *P. chabaudi* by starch gel electrophoresis. *Parasitology* **76**(3): 241-67.

Carter, R., Hunt, P., Cheesman, C. (2007) Linkage Group Selection - A fast approach to the genetic analysis of malaria parasites. *Int J Parasitol* **37**(3-4): 285-93.

Casals-Pascual, C., Roberts, D.J. (2006) Severe malarial anaemia. *Curr Mol Med* **6**(2):155-68.

Cavanagh, D.R., Dodoo, D., Hviid, L., Kurtzhals, J.A., Theander, T.G., *et al.* (2004) Antibodies to the N-terminal block 2 of *Plasmodium falciparum* merozoite surface protein 1 are associated with protection against clinical malaria. *Infect Immun* **72**(11):6492-502.

Chang, S.P., Case, S.E., Gosnell, W.L., Hashimoto, A., Kramer, K.J., (1996) A recombinant baculovirus 42-kilodalton C-terminal fragment of *Plasmodium falciparum* merozoite surface protein 1 protects Aotus monkeys against malaria. *Infect Immun* **64**(1):253-61.

Cheesman, S.J., de Roode, J., Read, A., Carter, R. (2003) Real-time quantitative PCR for analysis of genetically mixed infections in malaria parasites: technique validation and applications. *Mol Biochem Parasitol* **131**(2):83-91.

Cheesman, S., Raza, A., Carter, R. (2006) Mixed strain infections and strain specific protective immunity in the rodent malaria parasite *Plasmodium chabaudi chabaudi* in mice. *Infect Immun* **74**(5):2996-3001.

Cheesman, S., Creasey, A., Degnan, K., Kooij, T., Afonso, A., *et al.* (2007) Validation of Pyrosequencing for accurate and high throughput estimation of allele frequencies in malaria parasites *Mol Biochem Parasitol* **152**(2):213-9.

Chen, P.Q., Li, G.Q., Guo, X.B., He, K.R., Fu, Y.X., *et al.* (1994) The infectivity of gametocytes of *Plasmodium falciparum* from patients treated with artemisinine. *Chin Med J (Engl)* **107**(9): 709-11.

Chen, Q., Schlichtherle, M., Wahlgren, M. (2000). Molecular aspects of severe malaria. *Clin Microbiol Rev* **13**(3):439-50.

Cheng, Q., Saul, A. (1994a) The dihydrofolate reductase domain of rodent malarias: point mutations and pyrimethamine resistance. *Mol Biochem Parasitol* **65**(2):361-3

Cheng, Q., Saul. A. (1994b) Sequence analysis of the apical membrane antigen I (AMA-1) of *Plasmodium vivax.* *Mol Biochem Parasitol* **65**(1):183-7

Cheng, Q., Cloonan, N., Fischer, K., Thompson, J., Waine, G., *et al.* (1998) *stevor* and *rif* are *Plasmodium falciparum* multicopy gene families which potentially encode variant antigens. *Mol Biochem Parasitol* **97**(1-2):161-76.

Choi, H.J., Lee, S.Y., Yang, H., Bang, J.K. (2004) Retinal haemorrhage in *vivax* malaria. *Tran R Soc Med Hyg* **98**(6):387-9.

Chotivanich, K., Udomsangpetch, R., Simpson, J.A., Newton, P., Pukrittayakamee, S. *et al.* (2000) Parasite multiplication potential and the severity of Falciparum malaria. *J Infect Dis* **181**(3):1206-9.

Chotivanick, K., Sattabongkot, J., Udomsangpetch, R., Looareesuwan, Day, N.P., *et al.* (2006). Transmission-blocking activities of quinine, primaquine, and artesunate. *Antimicrob Agents Chemother* **50**(6):1927-30.

Ciuca, M., Ballif, L., Chelarescu Vieru, M. (1934) Immunity in Malaria. *Trans R Soc Trop Med Hyg* **27**(6)**:**619-22

Coleman, R. E., Clavin, A. M., Milhous, W. K. (1992) Gametocytocidal and sporontocidal activity of antimalarials against *Plasmodium berghei* ANKA in ICR mice and *Anopheles stephensi* mosquitoes. *Am J Trop Med Hyg* **46**(2)**:**169 -82.

Collins, W.H., Aikawa, M. (1993). "Plasmodia of non-human primates". In: Kreier, J., editor. *Parasitic Protozoa*. *Volume 5*. 2nd edition. New York: Academic Press, pp. 105-33.

Conway, D.J., Greenwood, B.M., McBride, J.S. (1991a) The epidemiology of multiple-clone *Plasmodium falciparum* infections in Gambian patients. *Parasitology* **103**(1):1-6.

Conway, D.J., McBride, J.S. (1991b) Population genetics of *Plasmodium falciparum* within a malaria hyperendemic area. *Parasitology* **103**(1):7-16.

Conway, D.J., Cavanagh, D.R., Tanabe, K., Roper, C., Mikes ZS, *et al.* (2000) A principal target of human immunity to malaria identified by molecular population genetic and immunological analyses. *Nat Med* **6**(6):689-92.

Cox, F.E. (1978) "Concomitant Infections". In: Killick-Kendrick, R., Peters, W., editors. *Rodent Malaria*. London: Academic Press. pp. 309-43.

Cox, F. E. (1988). "Major animal models in malaria research: rodent". In: Wernsdorfer, W.H., McGregor, I. editors. *Malaria: Principles and Practice of Malariology. Volume II*. New York: Churchill Livingstone, pp. 1503-43.

Cox, F.E. (1993). "Plasmodia of rodents". In: Kreier, J., editor. *Parasitic Protozoa: Babesia and Plasmodia. Volume 5*. 2nd edition. New york: Academic Press, pp. 49-104.

Cox, F.E. (2001) Concomitant infections, parasites and immune responses. *Parasitology*. **122**(Suppl):S23-38.

Craik, R. (1920) A note on the erythrocytes in malaria. *Lancet* **195**(5047):1110.

Crabb, B.S., Triglia, T., Waterkeyn, J.G., Cowman, A.F.(1997) Stable transgene expression in *Plasmodium falciparum*. *Mol Biochem Parasitol* **90**(1):131-44.

Crabb, B.S., Rug, M., Gilberger, T.W., Thompson, J.K., Triglia, T., *et al.* (2004) Transfection of the human malaria parasite *Plasmodium falciparum*. *Methods Mol Biol* **270**:263-76.

Creasey, A.M., Ranford-Cartwright, L.C., Moore, D.J., Williamson, D.H., Wilson, R.J., *et al.* (1993) Uniparental inheritance of the mitochondrial gene cytochrome *b* in *Plasmodium falciparum*. *Curr Genet* **23**(4):360-4.

Creasey, A., Mendis, K., Carlton, J., Williamson, D., Wilson, I., *et al.* (1994) Maternal inheritance of extrachromosomal DNA in malaria parasites. *Mol Biochem Parasitol* **65**(1):95-8.

Crewther, P.E., Matthew, M.L., Fleg, R.H., Anders, R.F. (1996) Protective immune responses to apical membrane antigen 1 of *Plasmodium chabaudi* involve recognition of strain specific epitopes. *Infect Immun* **64**(8):3310-7

Culleton, R., Martinelli, A., Hunt, P., Carter, R. (2005) Linkage group selection: rapid gene discovery in malaria parasites. *Genome Res* **15**:92-7.

Daly, T.M., Burns, J.M. Jr, Long, C.A. (1992) Comparison of the carboxy-terminal, cysteine-rich domain of the merozoite surface protein-1 from several strains of *Plasmodium yoelii*. *Mol Biochem Parasitol* **52**(2):279-82.

Darko, C.A., Angov, E., Collins, W.E., Bergmann-Leitner, E.S., *et al.* (2005) The clinical-grade 42-kilodalton fragment of merozoite surface protein 1 of *Plasmodium falciparum* strain FVO expressed in *Escherichia coli protects Aotus nancymai* against challenge with homologous erythrocytic-stage parasites. *Infect Immun* **73**(1):287–97.

Day, K.P., Marsh, K. (1991) Naturally acquired immunity to *Plasmodium falciparum*. *Immunol Today* **12**(3):A68-71.

Deans, A.M., Lyke, K.E., Thera, M.A., Plowe, C.V., Koné, A., *et al.* (2006) Low multiplication rates of African *Plasmodium falciparum* isolates and lack of association of multiplication rate and red blood cell selectivity with malaria virulence. *Am J Trop Med Hyg* **74**(4):554-63.

Delcher, A.L., Phillippy, A., Carlton, J., Salzberg, S.L. (2002) Fast algorithms for large-scale genome alignment and comparison. *Nucleic Acids Res* **30**(11):2478-83.

del Portillo, H.A., Fernandez-Becerra, C., Bowman, S., Oliver, K., Preuss, M., *et al.* (2001) A superfamily of variant genes encoded in the subtelomeric region of *Plasmodium vivax*. *Nature* **410**(6830):839-42.

de Roode, J.C., Culleton, R., Cheesman, S.J., Carter, R., Read, A.F. (2004) Host heterogeneity is a determinant of competitive exclusion or coexistence in genetically diverse malaria infections. *Proc Biol Sci* **271**(1543):1073-80.

de Roode, J.C., Helinski, M.E., Anwar, M.A., Read, A.F. (2005a) Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. *Am Nat* **166**(5):531-42.

de Roode, J.C., Pansini, R., Cheesman, S.J., Helinski, M.E., Huijben, S., *et al.* (2005b) Virulence and competitive ability in genetically diverse malaria infections. *Proc Natl Acad Sci U S A* **102**(21):7624-8.

Dondorp, A.M., Desakorn, V., Pongtavornpinyo, W., Sahassananda, D., Silamut, K., *et al.* (2005) Estimation of the total parasite biomass in acute *falciparum* malaria from plasma PfHRP2. *PLoS Medicine* **2**(8):e204.

Druilhe, P., Spertini, F., Soesoe, D., Corradin, G., Mejia, P., *et al.* (2005) A malaria vaccine that elicits in humans antibodies able to kill *Plasmodium falciparum*. *PLoS Med* **2**(11):e344.

Dunachie, S.J., Hill, A.V. (2003) Prime-boost strategies for malaria vaccine development. *J Exp Biol* **206**(21):3771-9.

Dunachie, S.J., Walther, M., Epstein, J.E., Keating, S., Berthoud, T., *et al.* (2006) A DNA prime-modified vaccinia virus ankara boost vaccine encoding thrombospondin-related adhesion protein but not circumsporozoite protein partially protects healthy malaria-naive adults against Plasmodium falciparum sporozoite challenge. *Infect Immun* **74**(10):5933-42.

Edeson, J.F.B. (1950) Proguanil resistant falciparum malaria in Malaya. *Br Med J* **1**(4646):147-51.

Egan, A.F., Morris, J., Barnish, G., Allen, S., Greenwood, B.M., *et al.* (1996) Clinical immunity to *Plasmodium falciparum* malaria is associated with serum antibodies to the 19-kDa C-terminal fragment of the merozoite surface antigen, PfMSP-1. *J Infect Dis* 173(3):765-9.

Enserink, M. (2005) Infectious diseases. Source of new hope against malaria is in short supply. *Science* **307**(5706):33.

Fandeur, T., Volney, B., Peneau, C., de Thoisy, B. (2000) Monkeys of the rainforest in French Guiana are natural reservoirs for *P. brasilianum*/*P. malariae* malaria. *Parasitology* **120**(1):11-21.

Feagin, J.E. (1992) The 6-kb element of *Plasmodium falciparum* encodes mitochondrial cytochrome genes. *Mol Biochem Parasitol* **52**(1):145-8.

Felger, I., Marshal, V.M., Reeder, J.C., Hunt, J.A., Mgone, C.S., *et al.* (1997) Sequence diversity and molecular evolution of the merozoite surface antigen 2 of *Plasmodium falciparum*. *J Mol Evol* **45**(2):154-60.

Felger, I., Genton, B., Smith, T., Tanner, M., Beck, H.P. (2003) Molecular monitoring in malaria vaccine trials. *Trends Parasitol* 19(2):60-3.

Fenton, B., Walker, A., Walliker, D. (1985) Protein variation in clones of *Plasmodium falciparum* detected by two-dimensional electrophoresis. *Mol Biochem Parasitol* **16**(2):173-83.

Ferguson, H.M., Read, A.F. (2002) Genetic and environmental determinants of *Plasmodium* virulence in vectors. *Proc Biol Sci* **269**(1497):1217-24.

Ferguson, H.M., Mackinnon, M.J., Chan, B.H., Read, A.F. (2003) Mosquito mortality and the evolution of malaria virulence. *Evolution Int J Org Evolution* **57**(12):2792-804

Fernando, S.D., Gunawardena, D.M., Bandara, M.R.S.S., De Silva, D., Carter, R., *et al.* (2003) The impact of repeated malaria attacks on the school performance of children. *Annal Trop Med Hyg* **69**(9):582-8.

Flück, C., Schöpflin, S., Smith, T., Genton, B., Alpers, M.P., *et al.* (2007) Effect of the malaria vaccine Combination B on merozoite surface antigen 2 diversity. *Infect Genet Evol* **7**(1):44-51.

Fidock, D.A., Nomura, T., Talley, A.K., Cooper, R.A., Dzekunov, S.M., *et al.* (2000). Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol Cell* **6**(4):861-71.

Finerty, J.F., Krehl, E.P. (1976) Cyclophosphamide pretreatment and protection against malaria. *Infect Immun* **14**(4):1103-5.

Finerty, J.F., Krehl, E.P. (1977) Delayed immune reactions in mice immunized with malarial antigen. *Am J Trop Med Hyg* **26**(3):377-81.

Freeman, R.R., Trejdosiewicz, A.J., Cross, G.A.M. (1980) Protective monoclonal antibodies recognising stage specific merozoite antigens of a rodent malaria parasite. *Nature* **284**(5754):366–368.

Freitas-Junior, L., Bottius, E., Pirrit, L.A., Deitsch, K.W., Scheidig, C., *et al.* (2000) Frequent ectopic recombination of virulence factor genes in telomeric chromosome clusters of *P. falciparum*. *Nature* **407**(6807): 1018-22.

Foth, B.J., Ralph, S.A., Tonkin, C.J., Struck, N.S., Fraunholz, M., *et al.* (2003) Dissecting apicoplast targeting in the malaria parasite *Plasmodium falciparum*. *Science* **299**(5607):705-8.

Galinski, M.M., Dluzewski, A. R., Barnwell, J. W. (2005) "A Mechanistic Approach to Merozoite Invasion of Red Blood Cells: Merozoite Biogenesis, Rupture and Invasion of Erthrocytes". In: Sherman, I.W., editor. *Molecular approaches to malaria*. Washington: ASM Press, pp. 113-168.

Gallup, J.L., Sachs, J.D. (2001) The economic burden of malaria. *Am J Trop Med Hyg* **64**(Sup 1,2):85-96.

Gardner, K.J., Hall, N., Fung, E., White, O., Berriman, M., *et al.* (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* **419**(6906):498-511.

Garnham, P.C.C. (1966). Malaria Parasites and other haemosporidia. Oxford: Blackwell Scientific Publications. 1114 p.

Genton, B., Bbetuela, I., Felger, I, Al-Yaman, F., Anders, R.F., *et al.* (2002) A Recombinant Blood-Stage Malaria Vaccine Reduces *Plasmodium falciparum* Density and Exerts Selective Pressure on Parasite Populations in a Phase 1-2b Trial in Papua New Guinea. *J Infect Dis* **185**(6):820-7.

Genton, B., Reed, R.Z. (2007) Asexual blood-stage malaria vaccine development: facing the challenges. *Curr Opin Infect Dis* **20**(5):467-75.

Georghiou, G.P. (1990) "The effect of agrochemicals on vector populations". In: Roush, R.T., Tabashinik, B.E., editors. *Pesticide resistance in Arthropod*. 1st edition. New York: Chapman and Hall, pp. 183-202.

Girard, M.P., Reed, Z.H., Friede, M., Kieny, M.P. (2007) A review of human vaccine research and development: malaria. *Vaccine* **25**(9):1567-80.

Grech, K., Martinelli, A., Pathirana, S., Walliker, D., Hunt, P., *et al.* (2002) Numerous, robust genetic markers for *Plasmodium chabaudi* by the method of amplified fragment length polymorphism. *Mol Biochem Parasitol* **123**(2):95-104.

Greenberg, J., Trembley H.L. (1954) The apparent transfer of pyrimethamine resistance from the BI strain of *Plasmodium gallinaceum* to the M strain. *J Parasitol* **40**(6):667-72.

Greenwood, B., Bojang, K., Whitty, C.J., Targett, G.A. (2005) Malaria. *Lancet* **365**(9649):1487-98.

Grüner, A.C., Snounou, G., Fuller, K., Jarra, W., Rénia, L., *et al*. (2004) The Py235 proteins: glimpses into the versatility of a malaria multigene family. *Microbes Infect* **6**(9):864-73.

Guerra, C.A., Snow R.W., Hay, S.I. (2006) Mapping the global extent of malaria in 2005. *Trends Parasitol* **22**(8):353-8

Guinet, F., Dvorak, J.A., Fujioka, H., Keister, D.B., Muratova, O., *et al.* (1996) A developmental defect in *Plasmodium falciparum* male gametogenesis. *J Cell Biol* **135**(1):269-78.

Guinovart, C., Navia, M.M., Tanner, M., Alonso, P.L. (2006) Malaria: burden of disease. *Curr Mol Med* **6**(2):137-40.

Hall, N., Karras, M., Raine, J.D., Carlton, J.M., Kooij, T.W., *et. al.* (2005) A comprehensive survey of the *Plasmodium* life cycle by genomic, transcriptomic, and proteomic analyses. *Science* **307**(5706):82-6.

Hisaeda, H., Saul, A., Reece, J.J., Kennedy, M.C., Long, C.A., *et al.* (2002) Merozoite surface protein 3 and protection against malaria in *Aotus nancymai* monkeys. *J Infect Dis* 185(5):657-64.

Herrera, S., Corradin, G., Arevalo-Herrera, M. (2007) An update on the search for *Plasmodium vivax* vaccine. *Trends Parasitol* **23**(3):122-8.

Hill, A. V. (2006) Pre-erythrocytic malaria vaccines: towards greater efficacy. *Nature Rev. Immunol* **6**:21–32.

Hunt, P., Martinelli, A., Fawcett, R., Carlton, J., Carter, R., *et al.* (2004) Gene synteny and chloroquine resistance in *Plasmodium chabaudi*. *Mol Biochem Parasitol* **136**(2):157-64.

Hunt, P., Afonso, A., Creasey, A., Culleton, R., Sidhu, A.B., *et al.* (2007) Gene encoding a deubiquitinating enzyme is mutated in artesunate- and chloroquine-resistant rodent malaria parasites. *Mol Microbiol* **65**(1):27-40.

Hoffman, S.L., Bancroft, W.H., Gottlieb, M., James, S.L., Burroughs, E.C., *et al.* (1997) Funding for malaria genome sequencing. *Nature* **387**(6634):647.

Hoffman, S.L., Goh, L.M., Luke, T.C., Schneider, I., Le, T.P., *et al.* (2002) Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. *J Infect Dis* **185**(8):1155-64.

Hogh, B., Marbiah, N.T., Burghaus, P.A., Andersen, P.K., *et al.* (1995) Relationship between maternally derived anti-*Plasmodium falciparum* antibodies and risk of infection and disease in infants living in an area of Liberia, west Africa, in which malaria is highly endemic. *Infect Immun* **63**(10):4034-8.

Holder, A.A., Freeman, R.R. (1981) Immunisation against blood-stage rodent malaria using purified parasite antigens. *Nature* **294**(5839):361-4.

Holder, A.A. (1988) The precursor to major merozoite surface antigens: structure and role in immunity. *Prog Allergy* **41**:72-97.

Holder, A.A., Riley, E.M. (1996) Human immune response to MSP-1. *Parasitol Today* **12**(5):173-4;

Homewood, C.A., Neame, K.D. (1976) A comparison of methods used for the removal of white cells from malaria-infected blood. *Ann Trop Med Parasitol* **70**(2):249-51

Iyer, J.K., Fuller, K., Preiser, P.R. (2006) Differences in the copy number of the *py*235 gene family in virulent and avirulent lines of *Plasmodium yoelii*. *Mol Biochem Parasitol* **150**(2):186-91.

Iyer, J., Grüner, A.C., Rénia, L., Snounou, G., Preiser, P.R. (2007a) Invasion of host cells by malaria parasites: a tale of two protein families. *Mol Microbiol* **65**(2):231-49.

Iyer, J.K., Amaladoss, A., Genesan, S., Preiser, P.R. (2007b) Variable expression of the 235 kDa rhoptry protein of *Plasmodium yoelii* mediate host cell adaptation and immune evasion. *Mol Microbiol* **65**(2):333-46.

Jacobs, R.L. (1964) Role of *p*-aminobenzoic acid in *Plasmodium berghei* infection in the mouse. *Exp Parasitol* **15**(3):213-25.

James, S.P., Nicol, W.D., Shute, P.G. (1932) A study of induced malignant tertian malaria. *Proc R Soc Med* **25**:1153-86**.**

Janse, C.J., Carlton, J.M., Walliker, D., Waters, A.P. (1994) Conserved location of genes on polymorphic chromosomes of four species of malaria parasites. *Mol Biochem Parasitol* **68**(2):285-96.

Janse, C. J., Waters, A. P. (2005) "Sexual Development of Malaria Parasites". In: Waters, A.P., Janse, C.J., editors. Malaria Parasites: *Genomes and Molecular Biology*. Norfolk: Caister Academic Press, pp. 445-474.

Janse, C.J., Ramesar, J., Waters, A.P. (2006) High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite *Plasmodium berghei*. *Nat Protoc* **1**(1):346-56.

Janssen, C.S., Barrett, M.P., Turner, C.M., Phillips, R.S. (2002) A large gene family for putative variant antigens shared by human and rodent malaria parasites. *Proc Biol Sci* **269**(1489):431-6.

Janssen, C.S., Phillips, R.S., Turner, C.M., Barrett, M.P. (2004) *Plasmodium* interspersed repeats: the major multigene superfamily of malaria parasites. *Nucleic Acids Res* **32**(19):5712-20.

Jarra, W., Brown, K.N. (1985) Protective immunity to malaria: studies with cloned lines of *Plasmodium chabaudi* and *P. berghei* in CBA/Ca mice. I. The effectiveness and inter- and intra-species specificity of immunity induced by infection. *Parasite Immunol* **7**(6):595-606.

Jayawardena, A.N., Mogil, R., Murphy, D.B., Burger, D., Gershon, R.K. (1983) Enhanced expression of H-2K and H-2D antigens on reticulocytes infected with *Plasmodium yoelii*. *Nature* **302**(5909):623-6.

Jeffrey, G.M. (1966) Epidemiological significance of repeated infections with homologous and heterologous strains and species of *Plasmodium*. *Bull World Health Organ* **35**(6):873-82.

Jones, T.R., Obaldia, N. 3rd, Gramzinski, R.A., Hoffman, S.L. (2000) Repeated infection of *Aotus* monkeys with *Plasmodium falciparum* induces protection against subsequent challenge with homologous and heterologous strains of parasite. *Am J Trop Med Hyg* **62**(6):675-80.

Jongco, A.M., Ting, L.M., Thathy, V., Mota, M.M., Kim, K. (2006) Improved transfection and new selectable markers for the rodent malaria parasite *Plasmodium yoelii*. *Mol Biochem Parasitol* **146**(2):242-50.

Jongwutiwes, S., Putaporntip, C., Iwasaki, T., Sata, T., Kanbara, H. (2004) Naturally acquired *Plasmodium knowlesi* malaria in human, Thailand. *Emerg Infect Dis* **10**(12):2211-3.

Kang, Y., Long, C.A. (1995) Sequence heterogeneity of the C-terminal, Cys-rich region of the merozoite surface protein-1 (MSP-1) in field samples of *Plasmodium falciparum*. *Mol Biochem Parasitol* **73**(1-2):103-10.

Kanoi, B.N., Egwang, T.G. (2007) New concepts in vaccine development in malaria. *Curr Opin Infect Dis* **20**(3):311-6.

Kappe, S.H., Adams, J.H. (1996) Sequence analysis of the apical membrane antigen-1 genes (*ama*-1) of *Plasmodium yoelii yoelii* and *Plasmodium berghei*. *Mol Biochem Parasitol* **78**(1-2):279-83.

Kester, K.E., McKinney, D.A., Tornieporth, N., Ockenhouse, C.F., Grey Heppner, D., *et al.* (2001). Efficacy of Recombinant Circumsporozoite Protein Vaccine Regimens against Experimental *Plasmodium falciparum* Malaria. *J Infect Dis* **183**(4):640-7.

Kester, K.E., McKinney, D.A., Tornieporth, N., Ockenhouse, C.F., Grey Heppner Jr, D., *et al.* (2007). A phase I/IIa safety, immunogenicity, and efficacy bridging randomised study of a two-dose regimen of liquid and lyophilised formulations of the candidate malaria vaccine RTS,S/AS02A in malaria-naïve adults. *Vaccine* **25**(29):5359-66.

Khan, S.M., Jarra, W., Bayele, H., Preiser, P.R. (2001) Distribution and characterisation of the 235 kDa rhoptry multigene family within the genomes of virulent and avirulent lines of *Plasmodium yoelii*. *Mol Biochem Parasitol* **114**(2):197-208.

Khan, S.M., Waters, A.P. (2004) Malaria parasite transmission stages: an update. *Trends Parasitol.* 20(12):575-80.

Killick-Kendrick, R. (1973) Parasitic Protozoa of the blood of rodents. 1. The life-cycle and zoogeography of *Plasmodium berghei nigeriensis* subsp. nov. *Annal Trop Med Parasitol* **67**(3):261-77.

Killick-Kendrick, R. (1974) Parasitic protozoa of the blood of rodents. A revision of *Plasmodium berghei*. *Parasitology* **69**(2):225-37.

Killick-Kendrick, R. (1978) "Taxonomy, Zoogeography and Evolution". In: Killick-Kendrick R., Peters W., editors. *Rodent Malaria*. London: Academic Press. pp. 1-52.

Knowles, G., Walliker, D. (1980) Variable expression of virulence in the rodent malaria parasite *Plasmodium yoelii yoelii*. *Parasitology* **81**(1):211-9.

Kumar, N., Zheng, H. (1990) Stage-specific gametocytocidal effect *in vitro* of the antimalaria drug qinghaosu on *Plasmodium falciparum*. *Parasitol Res* **76**(3):214-8.

Kooij, T.W., Carlton, J.M., Bidwell, S.L., Ramesar, J., Janse, C.J., *et al.* (2005) A *Plasmodium* whole-genome synteny map: indels and synteny breakpoints as foci for species-specific genes. *PLoS Pathog* **1**(4):e44.

Kooij, T.W., Janse, C.J., Waters, A.P. (2006) *Plasmodium* post-genomics: better the bug you know? *Nat Rev Microbiol* **4**(5):344-57.

Kooij, T.W., Matuschewski, K. (2007) Triggers and tricks of Plasmodium sexual development. *Curr Opin Microbiol* **10**(6):547-53.

Kyes, S.A., Rowe, J.A., Kriek, N., Newbold, C.I.. (1999) Rifins: a second family of clonally variant proteins expressed on the surface of red cells infected with *Plasmodium falciparum*. *Proc Natl Acad Sci U S A* **96**(16): 9333– 8

Kyes, S., Horrocks, P., Newbold, C. (2001) Antigenic variation at the infected red cell surface in malaria. *Annu Rev Microbiol* **55**:673-707.

Landau, I. (1965) Description de *Plasmodium chabaudi* n. sp., parasite de rongeur africains. *C R Acad Sci Hebd Seances Acad Sci D* **260**:3758-61.

Landau, I., Chabaud, A. (1965) Natural infection by 2 plasmodia of the rodent *Thamnomys rutilans* in the Central African Republic. *C R Acad Sci Hebd Seances Acad Sci D* **260**:230-2.

Landau, I., Killick-Kendrick, R. (1966) Rodent plasmodia of the République Centrafricaine: The sporogony and tissue stages of *Plasmodium chabaudi* and *P. berghei yoelii*. *Trans R Soc Trop Med Hyg* **60**(5):633-49.

Landau, I., Michel, J.C., Adam, J.P. (1968) Cycle biologique au laboratorie de *P. beghei killicki* n. subsp. *Ann Parasitol Hum Comp* **43**:545-550.

Landau, I., Boulvard, Y. (1978) "Life Cycles and Morphology". In: Killick-Kendrick, R., Peters, W., editors. *Rodent Malaria*. London: Academic Press. **3**:53-84.

Landau, I., Chabaud, A. (1994) *Plasmodium* species infecting *Thamnomys rutilans*: a zoological study. *Adv Parasitol* **33**:49-90.

Landau, I., Gautret, P. (1998) "Animal Models: Rodents". In: Sherman, I.R., editor. *Malaria: parasite biology, pathogenesis, and protection*. Washington D.C.: ASM Press, pp. 401-17.

Lawrence, G.W., Cheng, Q., Reed, C., Taylor, A., Stowers, A., *et al.* (2000) Effect of vaccination with 3 recombinant asexual-stage malaria antigens on initial growth rates of *Plasmodium falciparum* in non-immune volunteers. *Vaccine* **18**(18):1925–31.

Lewis, A.P. (1989) Cloning and analysis of the gene encoding the 230-kilodalton merozoite surface antigen of *Plasmodium yoelii*. *Mol Biochem Parasitol* **36**(3):271-82.

Li, L., Zhang, Y., Sullivan, M., Hong, L., Huang, L., Lu, F., *et al.* (2007) Typing *Plamodium yoelii* microsatellites using a simple and affordable fluorescent labeling method. *Mol Biochem Parasitol* **155**(2):94-102.

Looareesuwan, S. Viravan, C., Webster, H.K., Kyle, D.E., Hutchinson, D.B. *et al.* (1996) Clinical studies of atovaquone, alone or in combination with other antimalarial drugs, for treatment of acute uncomplicated malaria in Thailand. *Am J Trop Med Hyg* **54**(1):62-6.

Maberti, S. (1960) Desarrollo de resistencia a la pirimetamina. Presentacion de 15 casos estudiados en Trujiilo, Venezuela. *Archivos Venezolanos de Medicina Triopocal y Parasitologia Medica* **3**:23-59, cited by Wernsdorfer, W.H., Payne, D. (1991) The dynamics of drug resistance in *Plasmodium falciparum*. *Pharmocol Ther* **50**:95-121.

Mackinnon, M.J., Read, A.F. (1999) Genetic relationships between parasite virulence and transmission in the rodent malaria *Plasmodium chabaudi*. *Evolution* **53**(3):689-703.

Mackinnon, M.J., Gaffney, D.J., Read, A.F. (2002) Virulence of rodent malaria parasites: host genotype by parasite genotype experiments. *Infect Genet Evol* **36**(4): 287-96.

Mackinnon, M.J., Read, A.F. (2004) Virulence in malaria: an evolutionary viewpoint. *Phil Trans R Soc Lond B* **359**(1446):965-86.

Magesa, S.M., Mdira, K.Y., Babiker, H.A., Alifrangis, M., Fernert, A., *et al.* (2002) Diversity of *Plasmodium falciparum* clones infecting children living in a holoendemic area in north-eastern Tanzania. *Acta Trop* **84**(2): 83-92.

Malaney, P., Spielman, A., Sachs, J. (2004) The malaria gap. *Am J Trop Med Hyg* **71**(Sup 2):141-6.

Malkin, E., Dubovsky, F., Moree, M. (2006) Progress towards the development of malaria vaccines. *Trends Parasitol* **22**(7):292-5.

Malkin, E., Long, C.A., Stowers, A.W., Zou, L., Singh, S., et al. (2007) Phase 1 study of two merozoite surface protein 1 (MSP1(42)) vaccines for *Plasmodium falciparum* malaria. *PLoS Clin Trials* **2**(4):e12.

Mangoni, E.D., Severini, C., Menegon, M., Romi., R., Ruggiero, G., *et al.* (2003). Case report: An unusual late relapse of *Plasmodium vivax* malaria. *Am J Trop Med Hyg* **68**(2):159-60.

Marshall, V.M., Coppel, R.L., Anders, R.F., Kemp, D.J. (1992) Two novel alleles within subfamilies of the merozoite surface antigen 2 (MSA-2) of *Plasmodium falciparum*. *Mol Biochem Parasitol* **50**(1):181-4.

Marshall, V.M., Zhang, L., Anders, R.F., Coppel, R.L. (1996) Diversity of the vaccine candidate AMA-1 of *Plasmodium falciparum*. *Mol Biochem Parasitol* **77**(1):109-13.

Marshall, V. M., Tieqiao, W., Coppel, R.L. (1998) Close linkage of three merozoite surface protein genes on chromosome 2 of *Plasmodium falciparum*. *Mol Biochem Parasitol* **94**(1):13-25.

Martinelli, A., Hunt, P., Cheesman, S.J., Carter, R. (2004) Amplified fragment length polymorphism measures proportions of malaria parasites carrying specific alleles in complex genetic mixtures. *Mol Biochem Parasitol* **136**(2):117-22.

Martinelli, A., Cheesman, S., Hunt, P., Culleton, R., Raza, A., *et al.* (2005a) A genetic approach to the *de novo* identification of targets of strain specific immunity in malaria parasites. *Proc Natl Acad Sci U S A* **102**(3):814-9.

Martinelli, A., Hunt, P., Fawcett, R., Cravo, P.V., Walliker, D., *et al.* (2005b) An AFLP-based genetic linkage map of *Plasmodium chabaudi chabaudi*. *Malar J* **4**(1):11

Matuschewski, K., Mueller, A.K. (2007) Vaccines against malaria - an update. *FEBS J*. **274**(18):4680-7.

McBride, J.S., Walliker, D., Morgan, G. (1982) Antigenic diversity in the human malaria parasite *Plasmodium falciparum*. *Science* **217**(4556):254-7.

McColl, D.J,., Silva, A., Foley, M., Kun, J.F., Favaloro, J.M., *et al.* (1994) Molecular variation in a novel polymorphic antigen associated with *Plasmodium falciparum* merozoites. *Mol Biochem Parasitol* **68**(1):53-67.

McCutchan, T.F., Dame, J.B., Miller, L.H., Barnwell, J. (1984) Evolutionary Relatedness of *Plasmodium* species as Determined by the structure of DNA. *Science* **225**(4662):625-8.

McGregor, I.A., Gilles, H.M., Walters, J.H., Davies, A.H., Pearson, F.A. (1956) Effects of heavy and repeated malarial infections on Gambian infants and children; effects of erythrocytic parasitization. *Br Med J* **2**:686-92.

McGregor, I.A. (1974) Mechanisms of acquired immunity and epidemiological patterns of antibody responses in malaria in man. *Bull World Health Organ* **50**(3-4):259-66.

McKean, P.G., O' Dea, K., Brown, K.N. (1993a) A single amino acid determines the specificity of a monoclonal antibody which inhibits *Plasmodium chabaudi* AS *in vivo*. *Mol Biochem Parasitol* **62**(2):211-21.

McKean, P.G., O' Dea, K., Brown, K.N. (1993b) Nucleotide sequence analysis and epitope mapping of the merozoite surface protein 1 from *Plasmodium chabaudi chabaudi* AS. *Mol Biochem Parasitol* **62**(2):199-209.

Mendis, K.N., David, P.H., Carter, R. (1991) Antigenic polymorphism in malaria: is it an important mechanism for immune evasion? *Immunol Today* **12**(3):A34-7.

Mendis, K.N., Sina, B.J., Marchesini, P., Carter, R (2001) The neglected burden of *Plasmodium vivax* malaria. *Am J Trop Med Hyg* **64** (Supp 1), 97-106

Metzger, W.G., Okenu, D.M., Cavanagh, D.R., Robinson, J.V., Bojang, K.A., *et al.* (2003) Serum IgG3 to the *Plasmodium falciparum* merozoite surface protein 2 is strongly associated with a reduced prospective risk of malaria. *Parasite Immunol* **25**(6):307-12.

Mikolajczak, S.A., Aly, A.S.I., Kappe, S.H.I. (2007) Preerythrocytic malaria vaccine development. *Curr Opin Infect Dis* **20**(5):461-6.

Miller, J.M. Korenromp, E.L., Nahlen, B.L., W Steketee, R. (2007) Estimating the number of insecticide-treated bed nets required by African households to reach continent-wide malaria coverage targets. *JAMA* **297**(20):2241-50.

Miller, L.H., Mason, S.J., Clyde D.F., McGinniss, M.H. (1976) The resistance factor to *Plasmodium vivax* in blacks. The Duffy-blood-group genotype, FyFy, *N Engl J Med* **295**:302–4.

Miller, L.H., Roberts, T., Shahabuddin, M., McCutchan, T.F. (1993) Analysis of sequence diversity in the *Plasmodium falciparum* merozoite surface protein-1 (MSP-1). *Mol Biochem Parasitol* **59**(1):1-14.

Miller, L.H., Baruch, D.I., Marsh, K., Doumbo, O.K. (2002) The pathogenic basis of malaria. *Nature* **415**(6872):673-9.

Nahlen, B.L., Korenromp, E.L., Miller, J. M., Shibuya, K. (2005) Malaria risk: estimating clinical episodes of malaria. *Nature* **437**(7056):E3.

Najera, J.A. (1989) Malaria and the work of WHO. *Bull World Health Organ* **67**:229-43.

Narum, D.L., Green, J.L., Ogun, S.A., Holder, A.A. (2001) Sequence diversity and antigenic polymorphism in the *Plasmodium yoelii* p235 high molecular mass rhoptry proteins and their genes. *Mol Biochem Parasitol* **112**(2):193-200.

Nussenzweig, R.S., Vanderberg, J., Most, H., Orton, C. (1967) Protective immunity produced by the injection of x-irradiated sporozoites of *Plasmodium berghei*. *Nature* **216**:160–2.

Ockenhouse, C.F., Angov, E., Kester, K. E., *et al.* (2006) Phase 1 safety and immunogenicity trial of FMP1/AS02A, a *Plasmodium falciparum* MSP-1 asexual blood stage vaccine. *Vaccine* **24**(17):3009–17.

Oeuvray, C., Bouharoun-Tayoun, H., Grass-Masse, H., Lepers, J.P., Ralamboranto, L., Tartar, A., *et al.* (1994a). A novel merozoite surface antigen of *Plasmodium falciparum* (MSP-3) identified by cellular-antibody cooperative mechanism antigenicity and biological activity of antibodies. *Mem Inst Oswaldo Cruz* **89**(Suppl 2):77-80.

Oeuvray, C., Bouharoun-Tayoun, H., Gras-Masse, H., Bottius, E., Kaidoh, T., *et al.* (1994b) Merozoite surface protein-3: a malaria protein inducing antibodies that promote *Plasmodium falciparum* killing by cooperation with blood monocytes. *Blood* **84**(5):1594-602.

Oka, M., Aikawa, M., Freeman, R.R., Holder A.A., Fine, E. (1984) Ultrastructural localization of protective antigens of *Plasmodium yoelii* merozoites by the use of monoclonal antibodies and ultrathin cryomicrotomy. *Am. J. Trop. Med. Hyg* **33**:342-6.

Omer, F.M., de Souza, J.B., Riley, E.M. (2003) Differential induction of TGF-beta regulates proinflammatory cytokine production and determines the outcome of lethal and nonlethal Plasmodium yoelii infections. *J Immunol* 171(10):5430-6.

Osier, F.H., Polley, S.D., Mwangi, T., Lowe, B., Conway, D.J., *et al.* (2007) Naturally acquired antibodies to polymorphic and conserved epitopes of *Plasmodium falciparum* merozoite surface protein 3. *Parasite Immunol* **29**(8):387-94.

Owen, C.A., Sinha, K.A., Keen, J.K., Ogun, S.A., Holder, A.A. (1999) Chromosomal organisation of a gene family encoding rhoptry proteins in *Plasmodium yoelii*. *Mol Biochem Parasitol* **99**(2):183-92.

Pain, A., Ferguson, D.J., Kai, O., Urban, B.C., Lowe, B., *et al.* (2001) Platelet-mediated clumping of *Plasmodium falciparum*-infected erythrocytes is a common adhesive phenotype and is associated with severe malaria. *Proc Natl Acad Sci U S A* **98**(4):1805-10.

Pattaradilokrat, S., Cheesman, S. J., Carter, R. (2007) Linkage Group Selection: Towards Identifying Genes Controlling Strain Specific Protective Immunity in Malaria. *PLoS One* **2**(9):e857.

Pattaradilokrat, S., Cheesman, S.J., Carter, R. (2008) Congenicity and genetic polymorphism in cloned lines derived from a single isolate of a rodent malaria parasite. *Mol Biochem Parasitol* **157**(2):244-7.

Paul, R.E., Lafond, T., Müller-Graf, C.D., Nithiuthai, S., Brey, P.T., *et al.* (2004) Experimental evaluation of the relationship between lethal or non-lethal virulence and transmission success in malaria parasite infections. *BMC Evol Biol* **4**:30

Perraut, R., Marrama, L., Diouf, B., Sokhna, C., Tall, A., *et al.* (2005) Antibodies to the conserved C-terminal domain of the *Plasmodium falciparum* merozoite surface protein 1 and to the merozoite extract and their relationship with in vitro inhibitory antibodies and protection against clinical malaria in a Senegalese village. *J Infect Dis* **191**(2):264-71.

Peterson, D.S., Wellems, T.E. (2000) EBL-1, a putative erythrocyte binding protein of *Plasmodium falciparum*, maps within a favored linkage group in two genetic crosses. *Mol Biochem Parasitol* **105**(1):105-13.

Pichyangkul, S., Gettayacamin, M., Miller, R.S., Lyon, J.A., Angov, E., *et al.* (2004) Pre-clinical evaluation of the malaria vaccine candidate *P. falciparum* MSP1(42) formulated with novel adjuvants or with alum. *Vaccine* **22**(29-30):3831-40.

Polley, S.D., Conway, D.J. (2001) Strong diversifying selection on domains of the *Plasmodium falciparum* apical membrane antigen 1 gene. *Genetics* **158**:1505-12.

Polley, S.D., Tetteh, K.K., Cavanagh, D.R., Pearce, R.J., Lloyd, J.M., *et al.* (2003a) Repeat sequences in block 2 of *Plasmodium falciparum* merozoite surface protein 1 are targets of antibodies associated with protection from malaria. *Infect Immun* **71**(4):1833-42.

Polley, S.D., Chokejindachai, W., Conway, D.J. (2003b) Allele frequency-based analyses robustly map sequence sites under balancing selection in a malaria vaccine candidate antigen. *Genetics* **165**:555-61.

Polley, S.D., Mwangi, T., Kocken, C.H., Thomas, A.W., Dutta, S., *et al.* (2004) Human antibodies to recombinant protein constructs of *Plasmodium falciparum* Apical Membrane Antigen 1 (AMA1) and their associations with protection from malaria. *Vaccine* **23**(5):718-28.

Polley, S.D., Conway, D.J., Cavanagh, D.R., McBride, J.S., Lowe, B.S., *et al.*(2006) High levels of serum antibodies to merozoite surface protein 2 of *Plasmodium falciparum* are associated with reduced risk of clinical malaria in coastal Kenya. *Vaccine* **24**(19):4233-46.

Polley, S.D., Tetteh, K.K., Lloyd, J.M., Akpogheneta, O.J., Greenwood, B.M., *et al.* (2007) *Plasmodium falciparum* merozoite surface protein 3 is a target of allele-specific immunity and alleles are maintained by natural selection. *J Infect Dis* **195**(2):279-87.

Powell, R.D.J., McNamara, J.V., Rieckman, K.H. (1972) Clinical aspects of acquisition of immunity to Falciparum malaria. *Proc Helminthol Soc Wash* **39**:51-68.

Preiser, P.R., Wilson, R.J., Moore, P.W., McCready, S., Hajibagheri, M.A., *et al.* (1996) Recombination associated with replication of malarial mitochondrial DNA. *EMBO J* **15**(3):684-93.

Preiser, P.R., Jarra, W. (1998) *Plasmodium yoelii*: differences in the transcription of the 235-kDa rhoptry protein multigene family in lethal and nonlethal lines. *Exp Parasitol* **89**(1):50-7.

Preiser, P.R., Jarra, W., Capiod, T., Snounou, G. (1999) A rhoptry-protein-associated mechanism of clonal phenotypic variation in rodent malaria. *Nature* **398**(6728):618-22.

Prudêncio, M., Rodriguez, A., Mota, M.M. (2006) The silent path to thousands of merozoites: the Plasmodium liver stage. *Nat Rev Microbiol* 4(11):849-56.

Ranford-Cartwright, L., C., Balfe, P., Carter, R., Walliker, D. (1993) Frequency of cross fertilisation in the human malaria parasite *Plasmodium falciparum*. *Parasitology* **107**(1):11-8.

Ranford-Cartwright, L.C., Taylor, R.R., Asgari-Jirhandeh, N., Smith, D.B., Roberts, P.E. *et al.* (1996) Differential antibody recognition of FC27-like *Plasmodium falciparum* merozoite surface protein MSP2 antigens which lack 12 amino acid repeats. *Parasite Immunol* **18**(8):411-20.

Reilly, H.B., Wang, H., Steuter, J.A., Marx, A.M., Ferdig, M.T. (2008) Quantitative dissection of clone-specific growth rates in cultured malaria parasites. *Int J Parasitol* 37(14):1599-607.

Renia, L., I. T. Ling, M. Marussig, F. Miltgen, A. A. Holder, and D. Mazier. (1997) Immunization with a recombinant C-terminal fragment of *Plasmodium yoelii* merozoite surface protein 1 protects mice against homologous but not heterologous *P. yoelii* sporozoite challenge. *Infect Immun* **65**(11):4419-23.

Rodhain, J. (1952) *Plasmodium vinckei* n. sp. Un deuxième *Plasmodium* de rongeurs sauvage au Katanga. *Annls Soc belge Med Trop* **32**:275-80.

Rosario, V.E. (1981) Cloning of naturally occurring mixed infections of malaria parasites. *Science* **212**(4498):1037-8.

Rosenberg, R., Wirtz., R.A., Scneider, I., Burge, R. (1990) An Estimation of the Number of Malaria Sporozoites Ejected by a Feeding Mosquito. *Trans R Soc Trop Med Hyg* **84**(2):209-12.

Rotman, H. L., Daly, T. M., Long. C. A. (1999) *Plasmodium*: immunization with carboxyl-terminal regions of MSP-1 protects against homologous but not heterologous blood-stage parasite challenge. *Exp Parasitol* **91**(1)**:**78-85.

Roussilhon, C., Oeuvray, C., Müller-Graf, C., Tall, A., Rogier, C., *et al.* (2007) Long-term clinical protection from falciparum malaria is strongly associated with IgG3 antibodies to merozoite surface protein 3. *PLoS Med* **4**(11):e320.

Rowe, A.K., Rowe, S.Y., Snow, R.W., Korenromp, E.L., Schellenberg, J.R., *et al.* (2006) The burden of malaria mortality among African children in the year 2000. *Int J Epidemiol* **35**(3):691-704.

Rozen, S., Skaletsky, H. (2000) Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* **132**:365-86.

Rubio, J.M., Berzosa, P.J., Benito, A. (2001) Amplified fragment length polymorphism (AFLP) protocol for genotyping the malarial parasite *Plasmodium falciparum*. *Parasitology* **123**(4):331-6.

Sabatinelli, G., Ejov, M., Joergensen, P. (2001) Malaria in the WHO European Region (1971-1999). *Euro Surveill* **6**(4):61-5.

Sachs, J., Malaney, P. (2002) The economic and social burden of malaria. *Nature* **415**(6872):680-5.

Sambrook, J., Fritsch E. F., Maniatis T. (1989) *Molecular cloning*: *A laboratory manual*. *Volume I*. 2nd edition. New York: Cold Spring Harbour Laboratory Press.

Saul, A. (2007) Mosquito stage, transmission blocking vaccines for malaria. *Curr Opin Infect Dis* **20**(5):476-81.

Sayles, P.C., Wassom, D.L. (1988) Immunoregulation in murine malaria. Susceptibility of inbred mice to infection with *Plasmodium yoelii* depends on the dynamic interplay of host and parasite genes. *J Immunol* **141**(1):241-8.

Shai, S., Blackman, M.J., Holder, A.A. (1995) Epitopes in the 19kDa fragment of the *Plasmodium falciparum* major merozoite surface protein-1 (*Pf*MSP-1(19)) recognized by human antibodies. *Parasite Immunol* **17**(5):269-75.

Simpson, J.A., Aarons, L., Collins, W.E., Jeffery, G.M., White, N.J. (2002) Population dynamics of untreated *Plasmodium falciparum* malaria within the adult human host during the expansion phase of the infection. *Parasitology* **124**(3):247-63

Sinden, R.E. (1997). "Infection of mosquitoes with rodent malaria". In: Cramptom, J.M., Beard, C.M., Louis, C., editors. *Molecular Biology of Insect Disease Vectors: A Methods Manual*. London: Chapman and Hall, pp. 67-91.

Sinden, R.E., Gilles, H.M. (2002) "The malaria parasites". In: Warrell, D.A., Gilles, H. M., editors *Essential Malariology*. 4th edition. London: Arnold, pp.8-34.

Sinden, R.E., Hartley, R.H. (1985) Identification of the meiotic cell division of malaria parasites. *J Protozool* **32**(4):742-44.

Singh, B., Kim Sung, L., Matusop, A., Radhakrishnan, A., Shamsul, S.S., *et al.* (2004) A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. *Lancet* **363**(9414):1017-24.

Skarbinski, J., James, E.M., Causer, L.M., Barber, A.M., Mali, S., *et al.* (2006) Malaria surveillance–United States, 2004. *MMWR Surveill Summ* **55**(4):23-37.

Smith, J.D, Chitnis, C.E., Craig, A.G., Roberts, D.J., Hudson-Taylor, D.E., *et al.* (1995) Switches in expression of *Plasmodium falciparum var* genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell* **82**(1):101-10.

Smythe, J.A., Coppel, R.L., Brown, G.V., Ramasamy, R., Kemp, D.J., *et al.* (1988) Identification of two integral membrane proteins of *Plasmodium falciparum*. *Proc Natl Acad Sci U S A* **85**(14):5195-9.

Smythe, J.A., Peterson, M.G., Coppel, R.L., Saul, A.J., Kemp, D.J., *et al.* (1990) Structural diversity in the 45-kilodalton merozoite surface antigen of *Plasmodium falciparum*. *Mol Biochem Parasitol* **39**(2):227-34.

Smythe, J.A., Coppel, R.L., Day, K.P., Martin, R.K., Oduola, A.M., *et al.* (1991) Structural diversity in the *Plasmodium falciparum* merozoite surface antigen 2. *Proc Natl Acad Sci U S A* **88**(5):1751-5

Snounou, G., Jarra, W., Preiser, P.R. (2000) Malaria multigene families: the price of chronicity. *Parasitol Today* **16**(1):28-30.

Snow, R.W., Guerra, C.A., Noor, A.M., Myint, H.Y., Hay, S.I. (2005) The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature* **434**(7030): 214-7.

Soe, S., Theisen, M., Roussilhon, C., Aye, K.S., Druilhe, P. (2007) Association between protection against clinical malaria and antibodies to merozoite surface antigens in an area of hyperendemicity in Myanmar: complementarity between responses to merozoite surface protein 3 and the 220-kilodalton glutamate-rich protein. *Infect Immun* **72**(1):247-52.

Stafford, W.H., Blackman, M.J., Harris, A., Shai, S., Grainger, M., *et al.* (1994) N-terminal amino acid sequence of the *Plasmodium falciparum* merozoite surface protein-1 polypeptides. *Mol Biochem Parasitol* **66**(1):157-60.

Stafford, W.H., Günder, B., Harris, A., Heidrich, H.G., Holder, A.A,. *et al.* (1996) A 22 kDa protein associated with the *Plasmodium falciparum* merozoite surface protein-1 complex. *Mol Biochem Parasitol* **80**(2):159-69.

Stoute, J.A., Slaoui, M., Heppner, G., Momin, P., Kester, K.E., *et al.* (1997) A preliminary evaluation of a recombinant circumsporozoite protein vaccine against *Plasmodium falciparum* malaria. *N Eng J Med* **336**(2):86-91.

Stoute, J.A., Kester, K.E., Krzych, U., Wellde, B.T., Hall, T., *et al.* (1998) Long-term efficacy and immune responses following immunization with the RTS,S malaria vaccine. *J Infect Dis* **178**(4):1139-44.

Stoute, J.A., Gombe, J., Withers, M.R., Siangla, J., McKinney, D., *et al.* (2007) Phase 1 randomized double-blind safety and immunogenicity trial of *Plasmodium falciparum* malaria merozoite surface protein FMP1 vaccine, adjuvanted with AS02A, in adults in western Kenya. *Vaccine* **25**(1):176-84.

Su, X.Z., Heatwole, V.M., Wertheimer, S.P., Guinet, F., Herrfeldt, J.A. (1995) The large diverse gene family *var* encodes proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected erythrocytes. *Cell* **82**(1):89-100.

Su, X., Wellems, T.E. (1996) Toward a high-resolution *Plasmodium falciparum* linkage map: polymorphic markers from hundreds of simple sequence repeats. *Genomics* **33**(3):430-44.

Su, X., Kirkman, L.A., Fujioka, H., Wellems, T.E. (1997) Complex polymorphisms in an approximately 330 kDa protein are linked to chloroquine-resistant *P. falciparum* in Southeast Asia and Africa. *Cell* **91**(5):593-603.

Su, X., Ferdig, M.T., Huang, Y., Huynh, C.Q., Liu, A., *et al.* (1999) A genetic map and recombination parameters of the human malaria parasite *Plasmodium falciparum*. *Science* **286**(5443):1351-3.

Su, X.Z., Wootton, J.C. (2004) Genetic mapping in the human malaria parasite *Plasmodium falciparum*. *Mol Microbiol* **53**(6):1573-82.

Sutherland, C. (2007) A challenge for the development of malaria vaccines: polymorphic target antigens. *PLoS Med* **4**(3):e116.

Takala, S.L., Coulibaly, D., Thera, M.A., Dicko, A., Smith, D.L., *et al.* (2007) Dynamics of polymorphism in a malaria vaccine antigen at a vaccine-testing site in Mali. *PLoS Med* **4**(3):e93.

Tanabe, K., Mackay, M., Goman, M., Scaife, J.G. (1987) Allelic dimorphism in a surface antigen gene of the malaria parasite *Plasmodium falciparum*. *J Mol Biol* **195**(2):273-87.

Taylor, R.R., Allen, S.J., Greenwood, B.M., Riley, E.M. (1998) IgG3 antibodies to *Plasmodium falciparum* merozoite surface protein 2 (MSP2): increasing prevalence with age and association with clinical immunity to malaria. *Am J Trop Med Hyg* **58**(4):406-13.

Telford, S.R. (1994). "Plasmodia of Reptiles". In: Kreier, J.P., editor. *Parasitic Protozoa*: *Plasmodia, Babesia and Theileria*. *Volume 7*. 2nd edition. New York: Academic Press, pp. 1–71.

Todryk, S.M., Hill, A.V. (2007) Malaria vaccines: the stage we are at. *Nat Rev Microbiol* **5**(7):487-9.

Tolle, R., Bujard, H., Cooper, J.A. (1995) *Plasmodium falciparum*: variations within the C-terminal region of merozoite surface antigen-1. *Exp Parasitol* **81**(1):47-54.

Tomas, A.M., van der Wel, A.M., Thomas, A.W., Janse, C.J., Waters, A.P. (1998) Transfection systems for animal models of malaria. *Parasitol Today* **14**(6):245-9.

Topley, E., Bruce-Chwatt, L.J., Dorrell, J. (1970) Haematological study of a rodent malaria model. *J Trop Med Hyg* **73**(1):1-8.

Topolska, A.E., Wang, L., Black, C.G., Coppel, R.L. (2005) "Merozoite Cell Biology". In: Waters, A.P., Janse, C.J., editors. *Malaria Parasites: Genomes and Molecular Biology.* Norfolk: Caister Academic Press, pp. 365-444.

Thomas, A.W., Waters, A.P., Carr, D. (1990) Analysis of variation in PF83, an erythrocytic merozoite vaccine candidate antigen of *Plasmodium falciparum*. *Mol Biochem Parasitol* **42**(2):285-7.

Vaidya, A.B., Arasu, P. (1987) Tandemly arranged gene clusters of malarial parasites that are highly conserved and transcribed. *Mol Biochem Parasitol* **22**(2-3):249-57.

Vaidya, A.B., Akella, R., Suplick, K. (1989) Sequences similar to genes for two mitochondrial proteins and portions of ribosomal RNA in tandemly arrayed 6-kilobase-pair DNA of a malarial parasite. *Mol Biochem Parasitol* **35**(2):97-107.

Vaidya, A.B., Morrisey, J., Plowe, C.V., Kaslow, D.C., Wellems, T.E. (1993) Unidirectional dominance of cytoplasmic inheritance in two genetic crosses of *Plasmodium falciparum*. *Mol Cell Biol* **13**(12):7349-57.

Vaidya, A.B., Muratova, O., Guinet, F., Keister, D., Wellems, T.E., *et al.* (1995) A genetic locus on *Plasmodium falciparum* chromosome 12 linked to a defect in mosquito-infectivity and male gametogenesis. *Mol Biochem Parasitol* **69**(1):65-71.

van der Wel, A.M., Tomás, A.M., Kocken, C.H., Malhotra, P., Janse, C.J, *et al.* (1997) Transfection of the primate malaria parasite *Plasmodium knowlesi* using entirely heterologous constructs. *J Exp Med* **185**(8):1499-503.

van Riper, C., Atkinson, C.T., Seed, T.M. (1994). "Plasmodia of birds". In: Kreier, J., *editor. Parasitic Protozoa: Plasmodia, Babesia, and Theileria. Volume 7.* 2nd edition. *New* York: Academic Press, pp. 73–140.

Verdrager, J., Riche, A., Cheang, C.M. (1967) Traitement du paludism à *Plasmodium falciparum* par l' association sulformethoxine-pyrimethamine. Geneva: WHO, mimeographed document WHO/MAL 67.629, cited by Bjorkman, A., Phillips-Howard, P.A. (1990) The epidemiology of drug-resistant malaria. *Trans R Soc Med Hyg* **84**(2):177-80.

Vincke, I.H., Lips, M. (1948) Un nouveau *Plasmodium* d' un rongeur sauvage du Congo, *Plasmodium berghei* n. sp. *Annls Soc belge Med Trop* **28**:97-104.

Vlachou, D., Schlegelmilch, T., Runn, E., Mendes, A., Kafatos, F.C. (2006) The developmental migration of *Plasmodium* in mosquitoes. *Curr Opin Genet Dev* **16**(4):384-91.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Vandelee, T. *et al.* (1995) *AFLP: a new technique for DNA fingerprinting*. *Nucleic Acid Res* **23**(21):4407-14.

Walker-Jonah, A.S., Dolan, S.A., Cwadz, R.W., Panton, L.J., Wellems, T.E. (1992). An RFLP map of the *Plasmodium falciparum* genome, recombination rate and favoured linkage groups in a genetic cross. *Mol Biochem Parasitol* **51**(2):313-20.

Walliker, D., Carter, R., Morgan, S. (1971) Genetic recombination in malaria parasites. *Nature* **232**(5312):561-2.

Walliker, D., Carter, R., Morgan, S. (1973) Genetic recombination in *Plasmodium berghei*. *Parasitology* **66**(2):309-20.

Walliker, D., Carter, R., Sanderson, A. (1975) Genetic studies on *Plasmodium chabaudi*: recombination between enzyme markers. *Parasitology* **70**(1):19-24.

Walliker, D., Sanderson, A., Yoeli, M., Hargreaves, B. (1976) A genetic investigation of virulence in a rodent malaria parasite. *Parasitology* **72**(2):183-94.

Walliker, D. (1981) "Genetics of virulence in *Plasmodium yoelii*". In: Canning, E.U., editor. *Parasitological Topics. A Presentation Volume to P.C.C. Garnham, F.R.S., on the Occasion of His 80th Birthday*. Kansas: Allen Press, **1**:260-5.

Walliker, D. (1983). The Genetic Basis of Diversity in Malaria Parasites. *Adv Parasitol* **22**:217-59.

Walliker, D., Quakyi, I.A., Wellems, T.E., McCutchan, T.F., Szarfman, A., *et al.* (1987) Genetic analysis of the human malaria parasite *Plasmodium falciparum*. *Science* **236**(4809):1661-6.

Walliker, D., Habiker, H., Randford-Cartwrigth, L. (1998). "The Genetic Structure of Malaria Parasite Population". In: Sherman, I.R., editor. *Malaria: parasite biology, pathogenesis, and protection*. Washington D.C.: ASM Press, pp. 235-52.

Walliker, D. (2005). The hitchhiker's guide to malaria parasite genes. *Trends Parasitol* **21**(11):489-93.

Wang, P., Read, M., Sims, P.F., Hyde, J.E. (1997) Sulfadoxine resistance in the human malaria parasite *Plasmodium falciparum* is determined by mutations in dihydropteroate synthetase and an additional factor associated with folate utilization. *Mol Microbiol* **23**(5):979-86.

Warrell, D.A. (2002) "Clinical features of malaria". In: Warrell, D.A., Gilles, H. M., editors *Essential Malariology*. 4th edition. London: Arnold, pp.191-205.

Waters, A.P., Thomas, A.W., van Dijk, M.R., Janse, C.J. (1997) Transfection of malaria parasites. *Methods* **13**(2):134-47.

Webster, D. P., Dunachie, S., Vuola, J.M., Berthoud, T., Keating, S., *et al*. (2005) Enhanced T cell-mediated protection against malaria in human challenges by using the recombinant poxviruses FP9 and modified vaccinia virus Ankara. *Proc Natl Acad Sci USA* **102**(13): 4836–41.

Weinbaum, F.I., Evans, C.B., Tigelaar, R.E. (1976) An *in vitro* assay for T cell immunity to malaria in mice. *J Immunol* **116**(5):1280-3.

Wellems, T.E., Walliker, D., Smith, C.L., do Rosario, V.E., Maloy, W.L., *et al*. (1987) A histidine-rich protein gene marks a linkage group favored strongly in a genetic cross of *Plasmodium falciparum*. *Cell* **49**(5):633-42.

Wellems, T.E., Panton, L.J., Gluzman, I.Y., do Rosario, V.E., Gwadz, R.W., *et al.* (1990) Chloroquine resistance not linked to mdr-like genes in a *Plasmodium falciparum* cross. *Nature* **345**(6272):253-5.

Wellems, T.E., Walker-Jonah, A., Panton, L.J. (1991) Genetic mapping of the chloroquine-resistance locus on *Plasmodium falciparum* chromosome 7. *Proc Natl Acad Sci U S A* **88**(8):3382-6.

Wernsdorfer, W.H., Payne, D. (1991) The dynamics of drug resistance in *Plasmodium falciparum*. *Pharmacl Ther* **50**:95-121.

White, N. (1999) Antimalarial drug resistance and combination chemotherapy. *Philos Trans R Soc Lond B Biol Sci* **354**(1384):739-49.

Williamson, D.H. (1998). "Extrachromosomal DNA: the Mitochrondrian". In: Sherman, I.R., editor. *Malaria: parasite biology, pathogenesis, and protection*. Washington D.C.: ASM Press, pp. 267-75.

Wilson, R.J. (1998). "Extrachromosomal DNA: Plastid DNA". In: Sherman, I.R., editor. *Malaria: parasite biology, pathogenesis, and protection*. Washington D.C.: ASM Press, pp. 277-83.

Wilson, R.J., Denny, P.W., Preiser, P.R., Rangachari, K., Roberts, K., *et al.* (1996) Complete gene map of the plastid-like DNA of the malaria parasite *Plasmodium falciparum*. *J Mol Biol.* **261**(2):155-72.

Wilson, R.J., Williamson DH. (1997) Extrachromosomal DNA in the Apicomplexa. *Microbiol Mol Biol Rev* **61**(1):1-16.

Wood, V., Rutherford, K. M., Ivens, A., Rajandream, M.A., Barrell B., A Re-Annotation of the *Saccharomyces Cerevisiae* Genome. *Comp Funct Genom* **2**(3):143-154.

Wood, V., Gwilliam, R., Rajandream, M.A., Lyne, M., Lyne, R., *et al.* (2002) The genome sequence of *Schizosaccharomyces pombe*. *Nature* **415**(6874):871-880.

Wongsrichanalai, C., Pickard, A.L., Wernsdorfer, W.H., Meshnick, S.R. (2002) Epidemiology of drug-resistant malaria. *Lancet Infect Dis* **2**(4):209-18.

World Health Organisation (2005) World Malaria Report. Geneva. WHO http://www.rollbackmalaria.org/wmr2005/pdf/WMReport_lr.pdf
Reference Type: Electronic citation

World Health Organisation (2006) Guidelines for the Treatment of Malaria. Geneva. WHO
http://www.who.int/malaria/docs/TreatmentGuidelines2006.pdf
Reference Type: Electronic citation

Yeung, S., W. Pongtavornpinyo, I. M. Hastings, A. J. Mills, and N. J. White. (2004) Antimalarial drug resistance, artemisinin-based combination therapy, and the contribution of modeling to elucidating policy choices. *Am J Trop Med Hyg* **71**(2 suppl)**:**179-86.

Yoeli, M., Hargreaves, B., Carter, R., Walliker, D. (1975) Sudden increase in virulence in a strain of *Plasmodium berghei yoelii*. *Annal Trop Med Parasitol* **69**(2):173-8.

Appendix 1

Table S1. The Relative Intensity Indices of the 197 AFLP markers of strain AJ of *Plasmodium chabaudi chabaudi* of the AJ x CB cross progeny grown in an AJ-immunise mouse and a non-immune batch mate and their Comparative Intensities. AFLP markers were named as previously described in Figure 2.1 (Chapter 2). 92 AJ markers identified in the present study had positions corresponding to those of AFLP markers, which differentiated AJ from AS, on the *P. c. chabaudi* genetic linkage map (Martinelli *et al.*, 2005b). 24 AJ AFLP markers that had CIs of below 50% were considered to be under SSPI selection, as shown in pink background. Calculations of the RII and CI were done, as described in *Materials and Methods* in Chapter 2 (section 2.3.6).

Name of AJ AFLP markers identified in the AJ x CB genetic cross	Corresponding AJ markers on the the *P. c. chabaudi* AS x AJ genetic linkage map (Martinelli *et al.* 2005b)	Relative Intensity Index		Comparative Intensity (%)
		Non-immune selected progeny	AJ-immune selected progeny	
AJ AA 01 AA CB		0.941	0.486	51.619
AJ AA 02 AA CB		0.917	0.736	80.180
AJ AA 03 AA CB		0.955	0.828	86.743
AJ AA 01 AC CB	AJ AA 02 AC	0.890	0.450	50.562
AJ AA 02 AC CB		0.740	0.380	51.351
AJ AA 01 AG CB	AJ AA 01 AG	1.000	0.690	69.000
AJ AA 01 AT CB		0.700	0.440	62.857
AJ AA 02 AT CB	AJ AA 02 AT	0.860	0.500	58.140
AJ AA 03 AT CB		0.840	0.600	71.429
AJ AA 01 TA CB		0.910	0.700	76.923
AJ AA 02 TA CB	AJ AA 01 TA	1.070	0.640	59.813
AJ AA 03 TA CB	AJ AA 02 TA	1.331	0.699	52.486
AJ AA 01 TC CB		0.790	0.540	68.354
AJ AA 02 TC CB	AJ AA 01 TC	0.880	0.460	52.273
AJ AA 03 TC CB	AJ AA 02 TC	0.620	0.350	56.452
AJ AA 01 TG CB	AJ AA 01 TG	0.970	0.740	76.289
AJ AA 02 TGCB		0.840	0.420	50.000
AJ AA 01 TT CB	AJ AA 02 TT	0.708	0.612	86.495
AJ AA 03 TT CB	AJ AA 02 TT	0.818	0.499	61.757
AJ AA 01 CA CB	AJ AA 01 CA	0.589	1.066	180.952
AJ AA 02 CA CB		0.473	0.764	161.538
AJ AA 03 CA CB	AJ AA 02 CA	0.937	1.266	135.135
AJ AA 01 CT CB		1.758	0.909	51.706
AJ AA 02 CT CB	AJ AA 01 CT	0.725	1.375	89.655
AJ AA 01 GA CB	AJ AA 01 GA	0.576	0.315	54.733
AJ AA 01 GT CB		0.794	0.427	53.833
AJ AC 02 AA CB	AJ AC 02 AA	0.941	0.486	51.619
AJ AC 01 AC CB		0.917	0.736	80.180
AJ AC 02 AC CB		0.955	0.828	86.743
AJ AC 01 AG CB		0.890	0.450	50.562
AJ AC 02 AG CB	AJ AC 01 AG	0.740	0.380	51.351
AJ AC 03 AG CB		1.000	0.690	69.000
AJ AC 01 AT CB	AJ AC 01 AT	0.700	0.440	62.857
AJ AC 02 AT CB		0.860	0.500	58.140
AJ AC 03 AT CB		0.840	0.600	71.429
AJ AC 01 TA CB	AJ AC 01 TA	0.910	0.700	76.923
AJ AC 02 TA CB		1.070	0.640	59.813
AJ AC 02 TA CB	AJ AC 04 TA	1.331	0.699	52.486

Name of AJ AFLP markers identified in the AJ x CB genetic cross	Corresponding AJ markers on the *P. c. chabaudi* genetic linkage map (Martinelli *et al.* 2005b)	Relative Intensity Index		Comparative Intensity (%)
		Non-immune selected progeny	AJ-immune selected progeny	
AJ AC 01 TT CB	AJ AC 01 TT	0.966	0.652	67.442
AJ AC 02 TT CB		1.171	0.641	54.745
AJ AC 03 TT CB	AJ AC 02 TT	1.020	0.740	72.549
AJ AC 04 TT CB		0.830	0.630	75.904
AJ AC 05 TT CB		0.780	0.580	74.359
AJ AC 01 CA CB		0.910	0.470	51.648
AJ AC 02 CA CB	AJ AC 01 CA	0.820	0.430	52.439
AJ AC 01 CT CB	AJ AC 01 CT	0.720	0.229	31.801
AJ AC 01 GA CB		0.740	0.380	51.351
AJ AG 01 AA CB		0.600	0.600	100.000
AJ AG 02 AA CB	AJ AG 01 AA	0.770	0.830	107.792
AJ AG 01 AC CB	AJ AG 02 AC	0.650	0.330	50.769
AJ AG 02 AC CB	AJ AG 03 AC	1.020	0.530	51.961
AJ AG 01 AG CB	AJ AG 01 AG	0.840	0.540	64.286
AJ AG 02 AG CB		0.650	0.177	27.231
AJ AG 03 AG CB		0.610	0.350	57.377
AJ AG 04 AG CB	AJ AG 04 AG	0.720	0.430	59.722
AJ AG 05 AG CB	AJ AG 05 AG	0.860	0.010	1.513
AJ AG 06 AG CB		0.450	0.240	53.333
AJ AG 01 AT CB		0.700	0.360	51.429
AJ AG 02 AT CB	AJ AG 02 AT	0.670	0.350	52.239
AJ AG 03 AT CB	AJ AG 03 AT	0.800	0.450	56.250
AJ AG 04 AT CB		0.820	0.410	50.000
AJ AG 05 AT CB		0.750	0.410	54.667
AJ AG 06 AT CB		0.760	0.450	59.211
AJ AG 01 TA CB	AJ AG 02 TA	0.750	0.300	40.000
AJ AG 01 TC CB	AJ AG 01 TC	0.810	0.210	25.926
AJ AG 01 TG CB	AJ AG 01 TG	0.620	0.440	70.968
AJ AG 01 CA CB	AJ AG 01 CA	0.713	0.311	43.548
AJ AG 02 CA CB		0.600	1.100	183.333
AJ AG 01 CT CB	AJ AG 01 CT	0.410	0.250	60.976
AJ AG 01 GT CB		0.820	0.760	92.683
AJ AG 02 GT CB	AJ AG 01 GT	0.979	0.495	50.520
AJ AT 01 AA CB	AJ AT 01 AA	0.890	0.920	103.371
AJ AT 02 AA CB		0.732	0.470	64.208
AJ AT 03 AA CB	AJ AT 02 AA	0.730	0.740	101.370
AJ AT 04 AA CB		0.900	0.850	94.444
AJ AT 05 AA CB		0.747	0.813	108.929
AJ AT 01 AC CB	AJ AT 01 AC	0.790	0.520	65.823
AJ AT 02 AC CB	AJ AT 03 AC	1.000	0.660	66.000
AJ AT 01 AG CB		0.860	0.090	10.465
AJ AT 02 AG CB		0.645	0.330	51.163
AJ AT 03 AG CB		1.010	0.480	47.525
AJ AT 04 AG CB		0.700	0.450	64.286

Name of AJ AFLP markers identified in the AJ x CB genetic cross	Corresponding AJ markers on the *P. c. chabaudi* genetic linkage map (Martinelli *et al.* 2005b)	Relative Intensity Index		Comparative Intensity (%)
		Non-immune selected progeny	AJ-immune selected progeny	
AJ AT 01 AT CB	AJ AT 02 AT	0.790	0.400	50.633
AJ AT 02 AT CB		1.040	0.970	93.269
AJ AT 01 TA CB		0.790	0.680	86.076
AJ AT 02 TA CB		0.930	0.600	64.516
AJ AT 03 TA CB	AJ AT 04 TA	0.806	0.183	22.705
AJ AT 01 TC CB	AJ AT 02 TC	0.690	0.270	39.130
AJ AT 01 TG CB	AJ AT 01 TG	1.402	0.856	61.081
AJ AT 02 TG CB	AJ AT 02 TG	0.773	0.536	69.284
AJ AT 03 TG CB		0.750	0.660	88.000
AJ AT 01 TT CB	AJ AT 01 TT	0.930	0.850	91.398
AJ AT 02 TT CB	AJ AT 03 TT	1.120	0.249	22.232
AJ AT 01 CA CB	AJ AT 01 CA	0.780	0.510	65.385
AJ AT 02 CA CB	AJ AT 02 CA	0.843	0.504	59.813
AJ AT 03 CA CB		1.077	1.431	132.857
AJ AT 01 CT CB	AJ AT 03 CT	0.990	0.840	84.848
AJ AT 03 CT CB		0.880	0.580	65.909
AJ AT 01 GA CB		0.870	0.610	70.115
AJ AT 02 GA CB	AJ AT 02 GA	0.670	0.400	59.701
AJ AT 01 GT CB	AJ AT 01 GT	1.274	0.363	28.495
AJ AT 02 GT CB		0.720	0.540	75.000
AJ TA 01 AC CB	AJ TA 01 AC	0.790	0.520	65.823
AJ TA 02 AC CB	AJ TA 03 AC	0.760	0.430	56.579
AJ TA 01 AG CB	AJ TA 01 AG	0.700	0.660	94.286
AJ TA 01 AT CB	AJ TA 01 AT	0.579	0.319	55.152
AJ TA 02 AT CB	AJ TA 02 AT	0.619	0.359	57.906
AJ TA 03 AT CB		0.419	0.291	69.388
AJ TA 04 AT CB	AJ TA 04 AT	0.946	0.190	20.198
AJ TA 05 AT CB		0.872	0.508	58.256
AJ TA 01 TA CB		0.944	0.500	52.941
AJ TA 02 TA CB	AJ TA 01 TA	0.815	0.543	66.667
AJ TA 03 TA CB		0.983	1.069	108.772
AJ TA 04 TA CB		1.017	0.826	81.301
AJ TA 05 TA CB		0.690	0.490	71.020
AJ TA 06 TA CB	AJ TA 02 TA	1.070	0.250	23.366
AJ TA 07 TA CB	AJ TA 03 TA	0.970	0.058	6.016
AJ TA 01 TC CB	AJ TA 01 TC	1.030	1.030	100.000
AJ TA 01 TG CB		0.830	0.480	57.831
AJ TA 02 TG CB		0.760	0.590	77.632
AJ TA 01 TT CB		1.022	0.826	80.851
AJ TA 02 TT CB		1.262	0.839	66.488
AJ TA 03 TT CB	AJ TA 01 TT	0.505	0.402	79.612
AJ TA 01 CT CB		0.780	0.470	60.256
AJ TA 01 GA CB		1.040	0.530	50.962
AJ TA 02 GA CB		0.840	0.660	78.571
AJ TA 03 GA CB		1.330	1.330	100.000

Name of AJ AFLP markers identified in the AJ x CB genetic cross	Corresponding AJ markers on the *P. c. chabaudi* genetic linkage map (Martinelli *et al.* 2005b)	Relative Intensity Index		Comparative Intensity (%)
		Non-immune selected progeny	AJ-immune selected progeny	
AJ TC 01 AA CB		0.179	0.175	97.765
AJ TC 02 AA CB		0.780	0.540	69.231
AJ TC 03 AA CB		1.050	0.540	51.429
AJ TC 01 AG CB		1.060	0.600	56.604
AJ TC 02 AG CB		1.400	0.900	64.286
AJ TC 01 AT CB	AJ TC 01 AT	1.050	0.600	57.143
AJ TC 02 AT CB		0.760	0.530	69.737
AJ TC 03 AT CB		0.820	0.760	92.683
AJ TC 04 AT CB		0.940	0.580	61.702
AJ TC 01 TA CB	AJ TC 02 TA	0.770	0.400	51.948
AJ TC 01 TG CB	AJ TC 01 TG	0.590	0.009	1.525
AJ TC 01 CA CB		1.290	1.030	79.845
AJ TC 02 CA CB		0.900	0.640	71.111
AJ TC 03 CA CB		1.320	1.170	88.636
AJ TC 04 CA CB		1.080	0.920	85.185
AJ TC 01 CT CB		0.670	0.340	50.746
AJ TC 01 GT CB	AJ TC 01 GT	0.740	0.650	87.838
AJ TC 02 GT CB		0.810	0.550	67.901
AJ TG 01 AA CB	AJ TG 01 AA	1.580	0.810	51.266
AJ TG 02 AA CB		1.460	0.930	63.699
AJ TG 03 AA CB	AJ TG 03 AA	1.150	0.250	21.739
AJ TG 04 AA CB		1.200	1.020	85.000
AJ TG 05 AA CB		0.550	0.430	78.182
AJ TG 06 AA CB		0.710	0.380	53.521
AJ TG 01 AT CB	AJ TG 02 AT	1.280	0.270	21.094
AJ TG 02 AT CB	AJ TG 03 AT	0.780	0.270	34.615
AJ TG 03 AT CB		0.750	0.590	78.667
AJ TG 04 AT CB		0.800	0.800	100.000
AJ TG 05 AT CB		0.940	0.880	93.617
AJ TG 06 AT CB		0.770	0.480	62.338
AJ TG 07 AT CB		0.700	0.460	65.714
AJ TG 01 TC CB	AJ TG 01 TC	0.616	0.179	29.032
AJ TG 01 CT CB	AJ TG 01 CT	0.676	0.454	67.172
AJ TG 02 CT CB		2.456	1.463	59.561
AJ TG 03 CT CB	AJ TG 02 CT	0.985	0.634	64.376
AJ TG 01 GT CB		0.670	0.370	55.224
AJ TG 02 GT CB	AJ TG 01 GT	0.890	0.780	87.640
AJ TT 01 AA CB		1.220	0.700	57.377
AJ TT 02 AA CB	AJ TT 01 AA	0.760	0.600	78.947
AJ TT 03 AA CB	AJ TT 02 AA	0.600	0.390	65.000
AJ TT 04 AA CB	AJ TT 03 AA	1.022	0.760	74.364
AJ TT 05 AA CB		0.617	0.530	85.900
AJ TT 06 AA CB		0.880	0.650	73.864
AJ TT 07 AA CB		0.786	0.650	82.697
AJ TT 08 AA CB		1.090	0.610	55.963

Name of AJ AFLP markers identified in the AJ x CB genetic cross	Corresponding AJ markers on the *P. c. chabaudi* genetic linkage map (Martinelli *et al.* 2005b)	Relative Intensity Index		Comparative Intensity (%)
		Non-immune selected progeny	AJ-immune selected progeny	
AJ TT 01 AC CB		0.790	0.870	110.127
AJ TT 02 AC CB	AJ TT 01 AC	0.760	0.440	57.895
AJ TT 01 AT CB		0.850	0.910	107.059
AJ TT 02 AT CB		0.850	0.550	64.706
AJ TT 03 AT CB	AJ TT 02 AT	0.979	0.119	12.155
AJ TT 04 AT CB		1.080	0.640	59.259
AJ TT 05 AT CB		1.180	1.150	97.458
AJ TT 01 TA CB		0.849	0.580	68.316
AJ TT 02 TA CB		0.690	0.660	95.652
AJ TT 03 TA CB	AJ TT 02 TA	0.770	0.660	85.714
AJ TT 01 TC CB	AJ TT 02 TC	1.090	0.293	26.881
AJ TT 01 TG CB	AJ TT 02 TG	0.940	0.850	90.426
AJ TT 02 TG CB	AJ TT 03 TG	1.000	0.271	27.083
AJ TT 01 TT CB		0.920	0.480	52.174
AJ TT 02 TT CB	AJ TT 02 TT	1.070	0.570	53.271
AJ TT 01 CA CB	AJ TT 01 CA	1.060	0.880	83.019
AJ TT 02 CA CB	AJ TT 02 CA	0.840	0.470	55.952
AJ TT 01 CT CB		1.150	0.730	63.478
AJ TT 02 CT CB	AJ TT 02 CT	1.440	0.849	58.958
AJ TT 03 CT CB		0.720	0.430	59.722
AJ TT 04 CT CB	AJ TT 03 CT	0.730	0.370	50.685
AJ TT 01 GA CB		3.140	1.660	52.866
AJ GT 01 TA CB	AJ GT 01 TA	0.535	0.606	113.158
AJ GT 02 TA CB	AJ GT 02 TA	0.455	0.060	13.187
AJ GT 03 TA CB		0.722	0.417	57.692

Appendix 2

Table S2.1 The number of parasite clone-specific bands (AFLP markers) differentiating 17XA from 17XYM of the rodent malaria parasite *Plasmodium yoelii yoelii* generated by 65 combinations of selective *Eco*RI and *Mse*I AFLP primers (Grech *et al.*, 2002). Subtotals of the numbers of AFLP markers are shown for each pair of the specific *Eco*RI and *Mse*I PCR selective primers together with the grand total number of AFLP markers for each cloned line. ND, not determined

AFLP markers for 17XA versus 17XYM

	Selective bases at *Mse*I primer												
	AA	AC	AG	AT	TA	TC	TG	TT	CA	CT	GA	GT	Total
Selective bases at *Eco*RI primer													
AA	4	0	ND	ND	7	ND	0	ND	ND	2	0	1	14
AC	1	ND	1	2	5	ND	ND	ND	2	ND	1	ND	12
AG	4	1	1	ND	2	0	ND	5	ND	ND	ND	ND	13
AT	ND	0	ND	ND	ND	ND	2	5	1	ND	ND	4	12
TA	ND	1	ND	ND	5	0	3	ND	2	ND	2	1	14
TC	ND	ND	2	ND	ND	ND	ND	3	1	0	ND	ND	6
TG	2	ND	ND	4	3	ND	ND	2	ND	0	ND	ND	11
TT	3	ND	2	ND	3	ND	ND	2	ND	3	ND	1	14
CA	ND	ND	ND	2	4	2	ND	5	1	ND	ND	ND	14
CT	ND	ND	ND	7	3	3	1	ND	ND	ND	ND	ND	14
GA	ND	ND	1	ND	5	1	1	2	1	ND	ND	ND	11
GT	3	ND	ND	3	ND	ND	4	4	ND	ND	ND	ND	14
Total	17	2	7	18	37	6	11	28	8	5	3	7	149

Table S2.2 The number of parasite clone-specific bands (AFLP markers) differentiating 17XYM from 17XA of the rodent malaria parasite *Plasmodium yoelii yoelii* generated by 65 combinations of selective *Eco*RI and *Mse*I AFLP primers (Grech *et al*., 2002). Subtotals of the numbers of AFLP markers are shown for each pair of the specific *Eco*RI and *Mse*I PCR selective primers together with the grand total number of AFLP markers for each cloned line. ND, not determined

AFLP marker for 17XYM versus 17XA

	Selective bases at *Mse*I primer												
	AA	AC	AG	AT	TA	TC	TG	TT	CA	CT	GA	GT	Total
Selective bases at *Eco*RI primer													
AA	5	1	ND	ND	5	ND	1	ND	ND	1	1	2	16
AC	2	ND	2	0	2	ND	ND	ND	0	ND	0	ND	6
AG	2	1	2	ND	2	0	ND	4	ND	ND	ND	ND	11
AT	ND	0	ND	ND	ND	ND	3	4	1	ND	ND	2	10
TA	ND	1	ND	ND	2	1	2	ND	5	ND	3	2	16
TC	ND	ND	2	ND	ND	ND	ND	1	0	0	ND	ND	3
TG	1	ND	ND	5	2	ND	ND	3	ND	1	ND	ND	12
TT	2	ND	3	ND	5	ND	ND	2	ND	3	ND	0	15
CA	ND	ND	ND	1	3	2	ND	5	1	ND	ND	ND	12
CT	ND	ND	ND	3	3	2	1	ND	ND	ND	ND	ND	9
GA	ND	ND	2	ND	2	1	1	2	1	ND	ND	ND	9
GT	1	ND	ND	5	ND	ND	2	3	ND	ND	ND	ND	11
Total	13	3	11	14	26	6	10	24	8	5	4	6	130

Table S2.3 The number of parasite clone-specific bands (AFLP markers) differentiating 17XA from 17XNIMR of the rodent malaria parasite *Plasmodium yoelii yoelii* generated by 65 combinations of selective *Eco*RI and *Mse*I AFLP primers (Grech *et al.*, 2002). Subtotals of the numbers of AFLP markers are shown for each pair of the specific *Eco*RI and *Mse*I PCR selective primers together with the grand total number of AFLP markers for each cloned line. ND, not determined

AFLP marker for 17XA versus 17XNIMR

	Selective bases at *Mse*I primer												
	AA	AC	AG	AT	TA	TC	TG	TT	CA	CT	GA	GT	Total
Selective bases at *Eco*RI primer													
AA	4	0	ND	ND	7	ND	0	ND	ND	2	0	1	14
AC	1	ND	1	2	5	ND	ND	ND	2	ND	1	ND	12
AG	4	1	1	ND	2	0	ND	5	ND	ND	ND	ND	13
AT	ND	0	ND	ND	ND	ND	2	5	1	ND	ND	4	12
TA	ND	1	ND	ND	5	0	3	ND	2	ND	1	1	13
TC	ND	ND	2	ND	ND	ND	ND	3	1	0	ND	ND	6
TG	2	ND	ND	4	3	ND	ND	2	ND	0	ND	ND	11
TT	3	ND	2	ND	3	ND	ND	2	ND	3	ND	1	14
CA	ND	ND	ND	2	4	2	ND	5	1	ND	ND	ND	14
CT	ND	ND	ND	7	3	3	1	ND	ND	ND	ND	ND	14
GA	ND	ND	1	ND	5	1	1	2	1	ND	ND	ND	11
GT	3	ND	ND	3	ND	ND	4	3	ND	ND	ND	ND	13
Total	17	2	7	18	37	6	11	27	8	5	2	7	147

Table S2.4. The number of parasite clone-specific bands (AFLP markers) differentiating 17XNIMR from 17XA of the rodent malaria parasite *Plasmodium yoelii yoelii* generated by 65 combinations of selective *Eco*RI and *Mse*I AFLP primers (Grech *et al.*, 2002). Subtotals of the numbers of AFLP markers are shown for each pair of the specific *Eco*RI and *Mse*I PCR selective primers together with the grand total number of AFLP markers for each cloned line. ND, not determined

AFLP marker for 17XNIMR versus 17XA

	Selective bases at *Mse*I primer												
	AA	AC	AG	AT	TA	TC	TG	TT	CA	CT	GA	GT	Total
Selective bases at *Eco*RI primer													
AA	5	1	ND	ND	5	ND	1	ND	ND	1	1	2	16
AC	2	ND	2	0	3	ND	ND	ND	0	ND	0	ND	7
AG	2	1	2	ND	2	0	ND	4	ND	ND	ND	ND	11
AT	ND	0	ND	ND	ND	ND	3	4	1	ND	ND	2	10
TA	ND	1	ND	ND	2	1	2	ND	5	ND	3	2	16
TC	ND	ND	2	ND	ND	ND	ND	1	0	0	ND	ND	3
TG	1	ND	ND	5	2	ND	ND	3	ND	1	ND	ND	12
TT	2	ND	3	ND	5	ND	ND	2	ND	5	ND	1	18
CA	ND	ND	ND	1	3	2	ND	5	1	ND	ND	ND	12
CT	ND	ND	ND	4	3	2	1	ND	ND	ND	ND	ND	10
GA	ND	ND	2	ND	2	2	1	2	1	ND	ND	ND	10
GT	1	ND	ND	5	ND	ND	2	3	ND	ND	ND	ND	11
Total	13	3	11	15	27	7	10	24	8	7	4	7	136

Table S2.5. The number of parasite clone-specific bands (AFLP markers) differentiating 17XYM from 17XNIMR of rodent malaria parasite *Plasmodium yoelii yoelii* generated by 65 combinations of selective *Eco*RI and *Mse*I AFLP primers (Grech *et al.*, 2002). No AFLP markers are found. ND, not determined.

AFLP marker for 17XYM versus 17XNIMR

	Selective bases at *Mse*I primer												
	AA	AC	AG	AT	TA	TC	TG	TT	CA	CT	GA	GT	Total
Selective bases at *Eco*RI primer													
AA	0	0	ND	ND	0	ND	0	ND	ND	0	0	0	0
AC	0	ND	0	0	0	ND	ND	ND	0	ND	0	ND	0
AG	0	0	0	ND	0	0	ND	0	ND	ND	ND	ND	0
AT	ND	0	ND	ND	ND	ND	0	0	0	ND	ND	0	0
TA	ND	0	ND	ND	0	0	0	ND	0	ND	0	0	0
TC	ND	ND	0	ND	ND	ND	ND	0	0	0	ND	ND	0
TG	0	ND	ND	0	0	ND	ND	0	ND	0	ND	ND	0
TT	0	ND	0	ND	0	ND	ND	0	ND	0	ND	0	0
CA	ND	ND	ND	0	0	0	ND	0	0	ND	ND	ND	0
CT	ND	ND	ND	0	0	0	0	ND	ND	ND	ND	ND	0
GA	ND	ND	0	ND	0	0	0	0	0	ND	ND	ND	0
GT	0	ND	ND	0	ND	ND	0	0	ND	ND	ND	ND	0
Total	0	0	0	0	0	0	0	0	0	0	0	0	0

Table S2.6. The number of parasite clone-specific bands (AFLP markers) differentiating 17XNIMR from 17XYM of rodent malaria parasite *Plasmodium yoelii yoelii* generated by 65 combinations of selective *Eco*RI and *Mse*I AFLP primers (Grech *et al.*, 2002). Subtotals of the numbers of AFLP markers are shown for each pair of the specific *Eco*RI and *Mse*I PCR selective primers together with the grand total number of AFLP markers for each cloned line. ND, not determined

AFLP marker for 17XNIMR versus 17XYM

	Selective bases at *Mse*I primer												
	AA	AC	AG	AT	TA	TC	TG	TT	CA	CT	GA	GT	Total
Selective bases at *Eco*RI primer													
AA	0	0	ND	ND	0	ND	0	ND	ND	0	0	0	0
AC	0	ND	0	0	1	ND	ND	ND	0	ND	0	ND	1
AG	0	0	0	ND	0	0	ND	0	ND	ND	ND	ND	0
AT	ND	0	ND	ND	ND	ND	0	0	0	ND	ND	0	0
TA	ND	0	ND	ND	0	0	0	ND	0	ND	1	0	1
TC	ND	ND	0	ND	ND	ND	ND	0	0	0	ND	ND	0
TG	0	ND	ND	0	0	ND	ND	1	ND	0	ND	ND	1
TT	0	ND	0	ND	0	ND	ND	0	ND	0	ND	0	0
CA	ND	ND	ND	0	0	1	ND	0	0	ND	ND	ND	1
CT	ND	ND	ND	0	1	0	0	ND	ND	ND	ND	ND	1
GA	ND	ND	0	ND	0	0	0	0	0	ND	ND	ND	0
GT	0	ND	ND	0	ND	ND	0	1	ND	ND	ND	ND	1
Total	0	0	0	0	2	1	0	2	0	0	1	0	6

Appendix 3

Table S3. The Relative Intensity Indices (RIIs) of the 108 AFLP markers of *Plasmodium yoelii yoelii* cloned line 33XC in relation to cloned line 17XYM obtained from parasite DNA of the uncloned progeny of the two genetic crosses between 17XYM and 33XC in three successive passages. AFLP markers were named as follows (Grech *et al.*, 2002). The first letters '33XC' indicate the clone of *P. y. yoelii* in which the AFLP band is present. The last letters '17XYM' indicate the clone of *P. y. yoelii* in which the AFLP band is absent. The second two letters (i.e. AA, AG) correspond to the selective bases in the *Eco*RI AFLP selective primers. The numbers in the middle identify the size of the band relative to other polymorphic bands in the same lane. The numbers are increased in the decreasing size. The following two letters correspond to the selective bases used in the *Mse*I AFLP selective primers (Grech *et al.*, 2002). Five of the 108 33XC AFLP markers were consistently and strongly reduced in intensity following growth selection, as indicated in pink background. Calculations of the RIIs were performed, as described in *Materials and Methods*.

(Table see overleaf)

		RIIs of 33XC markers of the growth rate selected progeny of the first genetic cross			RIIs of 33XC markers of the growth rate selected progeny of the second genetic cross		
	Marker name	the 1st passage	the 2nd passage	the 3rd passage	the 1st passage	the 2nd passage	the 3rd passage
1	33XC AA 01 AC 17XYM	0.295434925	0.47847385	0.232443509	0.599105315	0.564342244	0.546446374
2	33XC AA 01 TA 17XYM	0.312272248	0.19640257	0.17557996	0.498160389	0.403175736	0.391998188
3	33XC AA 02 TA 17XYM	0.884420331	0.74046781	0.765556469	0.489140513	0.676219429	0.599033939
4	33XC AA 01 TG 17XYM	0.436696876	0.41204717	0.315470266	0.265476283	0.227795479	0.235638853
5	33XC AA 01 GA 17XYM	0.38566183	0.34493504	0.283288919	0.5285334	0.400873304	0.307254402
6	33XC AA 01 GT 17XYM	0.749726446	0.40709072	0.260274123	0.592531101	0.557326194	0.504605992
7	33XC AA 01 CT 17XYM	0.541581602	0.481811676	0.285606969	0.427946034	0.457548264	0.375347564
8	33XC AA 02 CT 17XYM	0.672092261	0.806467891	0.671533542	0.760906893	0.624775477	0.542046557
9	33XC AC 01 AG 17XYM	0.664423174	0.693721017	0.612728128	0.602324063	0.621071377	0.712015122
10	33XC AC 01 AT 17XYM	0.612050266	0.591197313	0.450740947	0.269833625	0.31870032	0.473319654
11	33XC AC 01 TA 17XYM	0.147733607	0.133726276	0.129590811	0.564358282	0.586879203	0.455193495
12	33XC AC 02 TA 17XYM	0.938543406	0.887003672	0.800476144	0.519736469	0.478354337	0.408469036
13	33XC AC 04 TA 17XYM	0.835698116	0.695843431	0.611111422	0.547597714	0.481409128	0.499831106
14	33XC AC 01 CA 17XYM	0.756890577	0.660314044	0.60074651	0.581857749	0.61967758	0.574136706
15	33XC AC 01 GA 17XYM	1.242250885	0.97959603	1.248866234	0.353685777	0.42940646	0.505240955
16	33XC AC 02 GA 17XYM	1.080050259	0.872527374	1.010426474	0.453172281	0.50057966	0.578584093
17	33XC AG 01 AA 17XYM	0.585626	0.553178	0.765408	0.373034234	0.39006597	0.524492172
18	33XC AG 01 AC 17XYM	0.848289	0.957341	0.923375	0.681963461	0.53803381	0.391529736
19	33XC AG 01 AG 17XYM	0.073746	0.066953	0.06465	0.096490145	0.076961342	0.038234506
20	33XC AG 01 TT 17XYM	0.861749	1.120956	0.879934	0.89134788	0.71016381	0.737286105
21	33XC AG 02 TT 17XYM	0.652961	0.813365	0.629911	0.748391245	0.67374435	0.577639419
22	33XC AG 03 TT 17XYM	0.772854	0.732723	0.898194	0.389838331	0.29952681	0.286011008
23	33XC AG 01 TA 17XYM	0.1041225	0.05612534	0.04525252	0.102599196	0.06680967	0.08926207
24	33XC AT 01 AA 17XYM	0.210952	0.19584	0.180646	0.645354701	0.626968992	0.475318255
25	33XC AT 01 AC 17XYM	0.341526	0.394581	0.475094	0.417408187	0.421457491	0.342400392

(to be continued)

		RIIs of 33XC markers of the growth rate selected progeny of the first genetic cross			RIIs of 33XC markers of the growth rate selected progeny of the second genetic cross		
	Marker name	the 1st passage	the 2nd passage	the 3rd passage	the 1st passage	the 2nd passage	the 3rd passage
26	33XC AT 02 AC 17XYM	0.694807	0.719761	0.66969	0.562560924	0.497555042	0.349438092
27	33XC AT 01 TT 17XYM	0.789647	0.75634	0.907525	0.578266401	0.569191449	0.559241204
28	33XC AT 02 TT 17XYM	0.469553	0.387864	0.392147	0.302598359	0.291722003	0.29746128
29	33XC AT 01 GT 17XYM	0.404256	0.54969	0.558608	0.445485804	0.406723976	0.377766707
30	33XC AT 02 GT 17XYM	0.403647	0.276431	0.249699	0.591385375	0.657458535	0.506014505
31	33XC AT 03 GT 17XYM	0.374208	0.335887	0.476909	0.716297918	0.753220172	0.592751318
32	33XC TA 01 AC 17XYM	0.463158842	0.526202217	0.639659121	0.482022179	0.43415725	0.438869338
33	33XC TA 01 TA 17XYM	0.905203234	0.85570362	0.908410382	0.490283484	0.52911913	0.477206215
34	33XC TA 01 TC 17XYM	0.685044442	0.693513342	0.563770488	0.624939826	0.53959334	0.605121974
35	33XC TA 01 TG 17XYM	0.415790022	0.369791861	0.343064098	0.684228156	0.76769665	0.498583158
36	33XC TA 02 TG 17XYM	0.264865565	0.234979505	0.299556279	0.626165937	0.72004058	0.561956319
37	33XC TA 02 CA 17XYM	0.354095568	0.246297983	0.156877113	0.666726616	0.743177089	0.718541546
38	33XC TA 03 CA 17XYM	0.766752239	0.648425594	0.682207699	0.460401287	0.562379171	0.625383396
39	33XC TA 01 GA 17XYM	0.976089793	1.202508549	1.117296081	0.925933688	0.735666737	0.831056485
40	33XC TA 02 GA 17XYM	0.436863924	0.585463502	0.574954692	0.610948873	0.666208609	0.815763458
41	33XC TA 01 GT 17XYM	0.358540913	0.199420367	0.171928441	0.306032944	0.238596739	0.20933619
42	33XC TA 02 GT 17XYM	0.421734126	0.416752938	0.419222704	0.511196505	0.53562992	0.583772289
43	33XC TA 03 GT 17XYM	0.674439052	0.470690892	0.460230012	0.289828323	0.282870109	0.366099033
44	33XC TC 01 CA 17XYM	0.680970861	0.533626269	0.441328594	0.484071607	0.453595725	0.550206728
45	33XC TC 01 CT 17XYM	0.960828714	1.002441505	0.907118195	0.846010446	0.726945015	0.84218504
46	33XC TC 01 TT 17XYM	0.713736012	0.81469639	0.768405145	0.493713896	0.464952676	0.547478082
47	33XC TC 02 TT 17XYM	0.760495627	0.8616995	0.891704523	0.513129917	0.57459051	0.596000312
48	33XC TC 03 TT 17XYM	0.654911516	0.730929306	0.649783497	0.491883854	0.494950007	0.513156286
49	33XC TG 01 AA 17XYM	0.685874112	0.454111078	0.641131935	0.542046	0.553651	0.404132
50	33XC TG 02 AA 17XYM	0.669118039	0.561612064	0.907664021	0.444775	0.408136	0.373513

(to be continued)

		RIIs of 33XC markers of the growth rate selected progeny of the first genetic cross			RIIs of 33XC markers of the growth rate selected progeny of the second genetic cross		
	Marker name	the 1st passage	the 2nd passage	the 3rd passage	the 1st passage	the 2nd passage	the 3rd passage
51	33XC TG 01 AT 17XYM	0.591653836	0.506506762	0.344320436	0.331760998	0.355838001	0.373880712
52	33XC TG 02 AT 17XYM	0.63166513	0.51986367	0.725296011	0.268760091	0.302913066	0.331906224
53	33XC TG 03 AT 17XYM	0.485798266	0.399323358	0.541330756	0.347491607	0.295881106	0.431063759
54	33XC TG 04 AT 17XYM	0.527004623	0.34820648	0.377933845	0.378662387	0.452880304	0.549306948
55	33XC TG 01 TT 17XYM	0.819234085	0.859539844	0.414300743	0.564123	0.54378	0.491351
56	33XC TG 01 CT 17XYM	0.586227145	0.507273011	0.81017762	0.736612	0.751917	0.673346
57	33XC TT 01 AA 17XYM	0.563348351	0.491815623	0.579242134	0.351914391	0.315871372	0.282664566
58	33XC TT 01 AG 17XYM	0.511289866	0.552028936	0.391839378	0.222722504	0.227237049	0.288440415
59	33XC TT 01 TA 17XYM	0.214785423	0.161139868	0.132651745	0.512461161	0.452612863	0.557048214
60	33XC TT 01 TT 17XYM	0.869822485	0.978295668	0.691790606	0.36452747	0.571731789	0.854574176
61	33XC TT 01 CT 17XYM	0.429276171	0.274341351	0.243654387	0.364521069	0.259444869	0.330266288
62	33XC TT 02 CT 17XYM	0.308559822	0.296694631	0.284861888	0.43576148	0.282326367	0.42276297
63	33XC CA 01 TC 17XYM	0.977630137	0.777167539	0.855887798	0.497339	0.474408	0.614526
64	33XC CA 01 TT 17XYM	0.414696529	0.635296601	0.567245204	0.282153182	0.298756055	0.326381723
65	33XC CA 02 TT 17XYM	0.640007723	0.724708839	0.58215214	0.503554504	0.538016186	0.525078377
66	33XC CA 03 TT 17XYM	0.724975072	0.663664212	0.697496118	0.533501734	0.509871123	0.413045069
67	33XC CA 04 TT 17XYM	0.684872629	0.721237408	0.521557113	0.461171697	0.435497005	0.488767443
68	33XC CA 05 TT 17XYM	0.007194894	0.001036464	0.003879107	0.044666553	0.061155652	0.033252342
69	33XC CT 01 AT 17XYM	0.64195752	0.72011289	0.488115518	0.33442408	0.332133026	0.297841584
70	33XC CT 02 AT 17XYM	0.742926087	0.583617045	0.31890988	0.157731504	0.140800281	0.144455462
71	33XC CT 01 TA 17XYM	0.922438267	0.939232556	0.79066993	0.226447409	0.255040734	0.354416245
72	33XC CT 02 TA 17XYM	1.257104291	1.111488934	0.850330653	0.197733268	0.173613423	0.219760564
73	33XC CT 04 TA 17XYM	0.544284526	0.568478098	0.543150293	0.272574545	0.236101429	0.209635744
74	33XC CT 05 TA 17XYM	0.541747859	0.470357893	0.4772943	0.780058129	0.60619238	0.638794
75	33XC CT 01 TG 17XYM	0.381809991	0.431386053	0.3496277	0.440011122	0.376686572	0.34858395

(to be continued)

		RIIs of 33XC markers of the growth rate selected progeny of the first genetic cross			RIIs of 33XC markers of the growth rate selected progeny of the second genetic cross		
	Marker name	the 1st passage	the 2nd passage	the 3rd passage	the 1st passage	the 2nd passage	the 3rd passage
76	33XC GA 01 AG 17XYM	0.232035828	0.290831949	0.343484412	0.568077355	0.525749915	0.512142338
77	33XC GA 02 AG 17XYM	0.66160615	0.637108623	0.714080897	0.558265184	0.511953395	0.609408875
78	33XC GA 03 AG 17XYM	0.245351161	0.350743801	0.334255937	0.2510511	0.205620774	0.311716516
79	33XC GA 01 TA 17XYM	0.432497472	0.344513369	0.34807883	0.415847	0.46497	0.465391
80	33XC GA 02 TA 17XYM	0.897929072	0.873430788	0.851980676	0.473479	0.494354	0.354886
81	33XC GA 03 TA 17XYM	1.0036122	0.869887591	1.136237076	0.760736034	0.740487945	0.692729985
82	33XC GA 01 TC 17XYM	0.632956807	0.678211569	0.592453033	0.47515	0.484336	0.487382
83	33XC GA 02 TC 17XYM	0.883258123	0.647600054	0.687287846	0.845098	0.823981	0.709666
84	33XC GA 01 TG 17XYM	1.177861787	0.661085636	0.886876412	0.570736	0.630009	0.522531
85	33XC GA 01 TT 17XYM	0.444776812	0.275508635	0.23807108	0.64959	0.645708	0.559742
86	33XC GT 01 AA 17XYM	0.526296127	0.422376426	0.408521001	0.534201343	0.470671995	0.452420757
87	33XC GT 02 AA 17XYM	0.545202618	0.496093424	0.422675807	0.537907433	0.54446494	0.533399465
88	33XC GT 03 AA 17XYM	0.474281496	0.596854797	0.59790204	0.564506465	0.515492789	0.34037125
89	33XC GT 01 AT 17XYM	0.760191778	0.80747966	0.743010406	0.577980139	0.687380476	0.66002888
90	33XC GT 02 AT 17XYM	0.719658154	0.701329705	0.717319775	0.56901366	0.572127793	0.569013275
91	33XC GT 01 TT 17XYM	0.54641755	0.598514784	0.666044366	0.676150208	0.666263665	0.749077598
92	33XC GT 02 TT 17XYM	0.070570447	0.037635575	0.027217906	0.314625621	0.314496936	0.209739016
93	33XC GT 03 TT 17XYM	0.631882933	0.654663983	0.757957817	0.576390342	0.59649233	0.59545653
94	33XC GT 01 TG 17XYM	0.767947567	0.689259706	775089587	0.648131146	0.570187875	0.53399253
95	33XC GT 02 TG 17XYM	0.912000272	0.809782679	0.937755203	0.779790082	0.872338031	0.726466632
96	33XC GT 03 TG 17XYM	0.950253795	0.808049223	0.904729419	0.9207224	0.977335018	0.88046966
97	33XC AT 01 TG 17XYM	0.73924	0.533192	0.62309	0.307233979	0.234622668	0.243095278
98	33XC AT 01 CA 17XYM	0.747788	0.60341	0.557197	0.497588884	0.519522817	0.423208543
99	33XC AT 02 CA 17XYM	0.103179	0.826477	1.084847	0.473910828	0.440412306	0.440178708
100	33XC TC 01 AG 17XYM	0.789757463	687793033	0.856961312	0.673807792	0.592723943	0.751854236

(to be continued)

		RIIs of 33XC markers of the growth rate selected progeny of the first genetic cross				RIIs of 33XC markers of the growth rate selected progeny of the second genetic cross		
	Marker name	the 1st passage	the 2nd passage	the 3rd passage		the 1st passage	the 2nd passage	the 3rd passage
101	33XC CA 01 TA 17XYM	0.954482586	0.838665847	0.720757353		0.446397599	0.595229585	0.529580045
102	33XC CA 02 TA 17XYM	0.719232177	0.752935087	0.71083489		0.68053972	0.745157176	0.558885599
103	33XC CA 03 TA 17XYM	0.505416466	0.394182484	0.630102041		0.459670311	0.405538227	0.386237017
104	33XC CA 04 TA 17XYM	0.977630137	0.77167539	0.855887798		0.806818421	0.68971622	0.548187081
105	33XC CA 05 TA 17XYM	0.25533402	0.01041729	0.02965208		0.198091153	0.207230116	0.164140052
106	33XC CA 01 CA 17XYM	0.514112082	0.321950775	0.342092757		0.397485	0.370914	0.356542
107	33XC AC 03 TA 17XYM	0.439498787	0.31785305	0.308933858		0.345971746	0.269218164	0.260620725
108	33XC TT 01 GT 17XYM	0.373811153	0.459256158	0.393995728		0.521933037	0.50152333	0.589858372

190