Cross-fertilisation in the malaria parasite *Plasmodium falciparum*

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Declaration

I declare that this thesis is my own composition and that the research described herein is my own work.

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Abstract

The objective of this work has been to investigate the frequency of cross-fertilisation between gametes of genetically distinct clones of the human malaria parasite Plasmodium falciparum. Previous genetic experiments involving both rodent malaria parasites in vivo and human malaria parasites in vitro have demonstrated higher than expected numbers of recombinants among the progeny of crosses. It has been suggested that this could be due to a favouring of cross-fertilisation over self-fertilisation in the mosquito phase of the life-cycle. The work has involved examining the genotypes of individual oocysts (derived from individual zygotes) resulting from mixed infection of two clones in mosquitoes.

In preliminary work using the mouse malaria parasite P. yoelii nigeriensis attempts were made to examine the chromosomes of single oocysts using pulsed field gradient gel electrophoresis. However there was insufficient DNA in an oocyst to allow chromosomes to be visualised using this technique.

The bulk of the work has been concerned with P. falciparum. In the first stage oligonucleotide primers suitable for use in the polymerase chain reaction (PCR) were designed to allow amplification of repetitive regions of two polymorphic antigen genes, denoted MSP1 and MSP2. The two clones of P. falciparum used in the crossing experiments possessed a different allele of each

gene. These alleles were found to be recognisable as size differences of the PCR-amplified fragments on agarose gels.

Gametocytes of the two clones were grown _in vitro_. Mixtures of gametocytes of each clone were made and fed to _Anopheles stephensi_ or _A. gambiae_ mosquitoes through membrane feeders. 9 to 10 days later the mosquitoes were dissected and their midguts were examined for the presence of oocysts. Individual oocysts were dissected from the midguts and the DNA extracted from them.

128 oocysts were examined from mosquitoes fed on nine separate occasions with a mixture of equal quantities of gametocytes of the two clones. Following PCR amplification of part of the MSP1 gene, 54 were identified as hybrid oocysts by their possession of both parental alleles. 108 of the 128 oocysts were analysed for the allelic form(s) of the MSP2 gene, and 46 were identified as hybrids.

The application of Hardy-Weinberg equilibrium law to these results suggests that gametes in the original mixtures fed to mosquitoes undergo random mating i.e. there was no favouring of cross-fertilisation over self-fertilisation.

A preliminary trial of this technique in the field has shown that it is applicable to studies on the frequency of cross-fertilisation in natural populations of _P.falciparum_.

List of Abbreviations

ADA	adenosine deaminase
bp	base pairs
Bq	becquerels
BSA	bovine serum albumin
c^7dGTP	7-deaza-2'-deoxyguanosine 5'-triphosphate
CHEF	Contour-clamped homogenous electric field electrophoresis
Ci	curies
CPD	citrate-phosphate-dextrose
cpm	counts per minute
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddATP	2'3'-dideoxyadenosine 5'-triphosphate
ddCTP	2'3'-dideoxycytidine 5'-triphosphate
ddGTP	2'3'-dideoxyguanosine 5'-triphosphate
ddTTP	2'3'-dideoxythymidine 5'-triphosphate
d.f.	degrees of freedom
dGTP	2'-deoxyguanosine 5'-triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
DTT	DL-dithiothreitol
DTE	dithioerythritol
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
EMBL	European Molecular Biology Laboratory
GII	gut infection index
GLB	gel loading buffer
GPI	glucose-6-phosphate isomerase
GPI	glycosyl phosphatidylinositol
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonicacid]
HPLC	High pressure liquid chromatography
HRP	Histidine-rich protein
i.p.	intra-peritoneally
kb	kilobase(s)
KCl	potassium chloride
kDa	kilodalton
LDH	lactose dehydrogenase
$MgCl_2$	magnesium chloride
MRC	Medical Research Council
MSA-1	merozoite surface antigen 1
MSA-2	merozoite surface antigen 2
MSP1	precursor to the major merozoite surface antigen (PMMSA/MSA-1/p190/gp195/PSA)
MSP2	a second merozoite surface antigen (GYMSSA/MSA-2/gp35-36)
NEW	NaCl/ethanol/water wash
NIH	National Institutes of Health
NO	Nitric oxide
OFAGE	orthogonal field alternation gel electrophoresis
PABA	4-aminobenzoic acid

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFGE	pulsed field gradient gel electrophoresis
PMMSA	polymorphic major merozoite surface antigen
PSA	polymorphic schizont antigen
RBC	red blood cell(s)
RFLP	restriction fragment length polymorphism
RH	relative humidity
RPMI	RPMI-1640 medium
s.d.	standard deviation
SDS	sodium dodecyl sulphate
SHARP	Small histidine- & alanine-rich protein
SSC	saline-sodium citrate buffer
TBE	tris-borate-EDTA buffer
TDR	special programme for research and training in tropical diseases
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TNF	Tumour necrosis factor
Tris	tris(hydroxymethyl)aminomethane
U-V	ultra-violet
UWGCG	University of Wisconsin Genetics Computer Group
WHO	World Health Organisation
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis

<u>Single letter amino acid code</u>

A	alanine	M	methionine
C	cysteine	N	asparagine
D	aspartic acid	P	proline
E	glutamic acid	Q	glutamine
F	phenylalanine	R	arginine
G	glycine	S	serine
H	histidine	T	threonine
I	isoleucine	V	valine
K	lysine	W	tryptophan
L	leucine	Y	tyrosine

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I. INTRODUCTION

The principal purpose of this study has been to investigate the frequency of cross-fertilisation between gametes of two clones of the malaria parasite *Plasmodium falciparum*. The work has involved characterising allelic variants of two antigen genes of two clones, and studying their pattern of inheritance in the mosquito stage of the life-cycle.

I.2 Introduction to the parasite and the disease

Malaria parasites (*Plasmodium*) belong to the protozoan phylum Apicomplexa [Levine *et al.*, 1980]. All members of this phylum are parasitic, and most produce male and female gametes at some point in their life-cycle. The malaria parasites belong to the suborder Haemosporina, within the order Eucoccidiidae, in the subclass Coccidia, within the class Sporozoea. Within the Haemosporina members of the family Plasmodiidae are represented by the single genus *Plasmodium*, and are characterised by stages of asexual multiplication (schizogony) as well as gametocyte production in the host erythrocyte.

In the Haemosporina two hosts are involved in the life-cycle. Sexual development begins with the formation of gametocytes in the blood cells of a vertebrate species. Gametogenesis and fertilisation take place in the vector species, a blood sucking insect, after

ingestion of the gametocytes. All known vectors of the Plasmodiidae are species of mosquito (Culicidae). The avian malarias are transmitted by mosquitoes of various genera, including Culex, Aedes and Anopheles. Mammalian Plasmodiidae are transmitted only by Anopheles species.

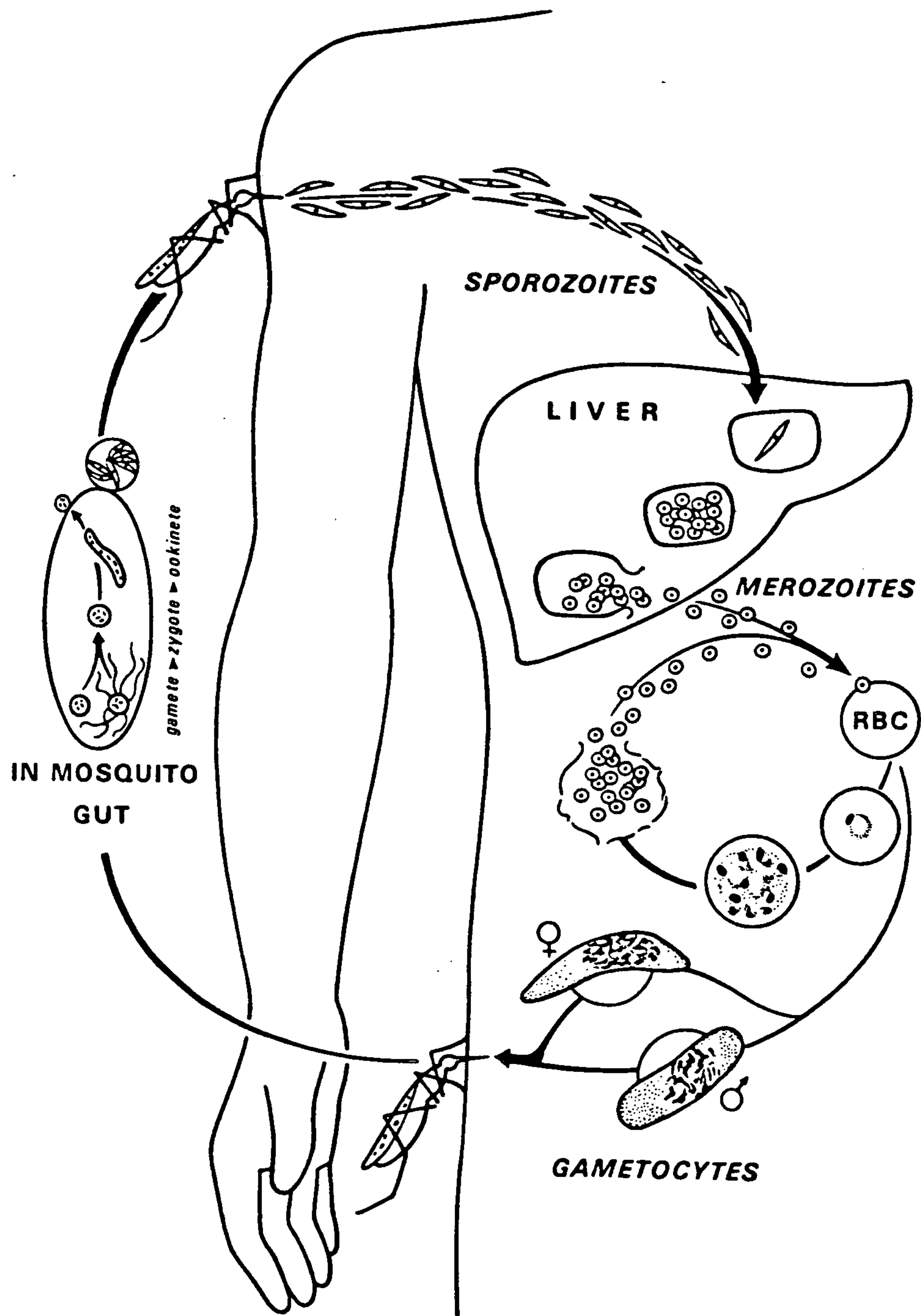
Four Plasmodium species affect man - P. falciparum, P. vivax, P. ovale and P. malariae. Of these, P. vivax is the most widely distributed while P. falciparum is the most common species in tropical and sub-tropical areas [WHO, 1989].

The characteristic features of the morphology of P. falciparum are the crescent shape of its gametocytes and their slow rate of growth, and the pattern of pigment around the nucleus of these gametocytes.

P. falciparum is the most pathogenic of the human malaria species, and is responsible for much morbidity and mortality. The disease which it causes is known as malignant tertian malaria.

I.2.i Life-cycle (Figure 1)

When an infective anopheline mosquito takes a blood meal sporozoites pass with the saliva into the skin of the vertebrate host. These sporozoites travel quickly to the liver and here they invade hepatocytes. The parasite first undergoes a phase of rapid division in the liver, termed exoerythrocytic schizogony. In P. falciparum this stage is completed very rapidly, within 5½ days after the introduction of sporozoites. Merozoites resulting from


SPOROZOITES
LIVER
MEROZOITES
RBC
GAMETOCYTES
IN MOSQUITO GUT
gamete ▸ zygote ▸ ookinete

these divisions are released into the blood to invade red blood cells. In P. falciparum a single liver cell contains a very large number of merozoites, perhaps as many as 30 000 [Garnham, 1988]. P. falciparum merozoites are the smallest exoerythrocytic merozoites of any primate malaria species. There is no evidence of subsequent generations of exoerythrocytic schizogony.

Following rupture of the exo-erythrocytic schizont, merozoites invade red blood cells. Within the red cell the parasite undergoes further divisions, a process known as erythrocytic schizogony. In P. falciparum, only the initial ring stages appear in the peripheral blood of the host, except in severe infections when schizonts may be seen. Schizogony normally occurs in the capillaries and sinusoids of deep organs, notably the brain. This stage is followed by the development of gametocytes, although gametocytes may also arise directly from the liver forms. The gametocytes are the terminal stages of parasite development in the vertebrate host. In P. falciparum immature gametocytes sequester markedly in the spleen and bone marrow [Garnham, 1931 ; Thomson & Robertson, 1935 ; Smalley et al., 1980]. Mature forms appear in the blood 8 to 10 days after the start of parasitaemia. The mature macrogametocyte is crescent-shaped, while the mature microgametocyte is more sausage shaped, both having characteristic dark perinuclear pigment.

The factors which determine the commitment of a parasite to gametocytogenesis are complex [Inselberg,

1983 ; Bruce <u>et al.</u>, 1990]. Nothing is known of the factors which determine the sex of gametocytes. Females generally predominate over males in number, typically in a ratio of between 5 and 10 females to one male. Cloned parasites give rise to both male and female gametocytes. Since all stages of the parasite in the vertebrate host are haploid, the different sexes of the gametocytes in a clone are derived from genetically identical cells.

Mature gametocytes are taken up into the <u>Anopheles</u> vector upon feeding. Following ingestion, numerous structural and shape changes occur, including disruption and loss of the host erythrocyte membrane (emergence), resulting in the transformation of the gametocytes to fertile gametes and thus to fertilisation. The male gametocyte undergoes a complex transformation known as exflagellation, resulting in the production of around 8 flagellated motile microgametes. Fertilisation between male and female gametes occurs almost immediately [Carter & Miller, 1979]. Following fusion of gametes, the resulting zygote transforms over about 18 hours to a motile ookinete stage. During the next 12 hours ookinetes traverse the midgut wall and develop into young oocysts between the midgut basement membrane and the basal lamella adjacent to the haemocoele. Further divisions occur within the oocyst, resulting in the production of sporozoites. Sporozoite differentiation takes from 4 to 21 days to complete, depending on the species of parasite

and the temperature at which the infected mosquito is maintained [Vanderberg, 1988].

A mature oocyst measures 40 to 60μm in diameter and contains around 3000 sporozoites [Rosenberg & Rungsiwongse, 1991] although up to 10 000 sporozoites have been recorded [Pringle, 1965]. These pass through the oocyst wall and travel through the haemocoelic fluid to accumulate in the acinal cells of the mosquito salivary glands. The sporozoites become infective, and, following a mosquito bite, pass with the saliva into their new host and hence to the liver.

I.2.ii The disease

The precise extent of malaria infections is difficult to assess. Perhaps 100 million people are affected per year, of whom 1 million die [WHO, 1989]. Malaria is often the major cause of infant mortality in tropical areas. It is endemic in 102 countries, with over half the population of the world at risk. P. falciparum is clinically the most important of the four species that affect man, causing, in some cases, cerebral malaria and death.

Clinical symptoms of malaria develop from 8 to 30 days after inoculation of sporozoites. Typical symptoms include chills, fever and sweating. The conventional explanation for cerebral malaria is obstruction of small blood vessels by parasitised red cells, leading to an inadequate blood supply to the brain [for a review see

Warrell, 1987]. There is increasing evidence for the involvement of cytokines, such as TNF, perhaps through the induction of nitric oxide (NO) release [for a review see Clark, I.A. et al., 1991]. NO is one of the major regulators of vascular tone, and is also essential in the processes initiated by activation of neurotransmitters in the brain. Increased levels of NO could induce temporary changes in cerebral function and intracranial pressures, which could give rise to the symptoms of cerebral malaria.

I.3 Malaria antigens

The work described in this thesis concerns the inheritance of allelic variants of two antigens of blood forms of *P. falciparum*. Characteristics of these antigens, and of their gene sequences, are given in this section.

I.3.i Malaria antigens : nomenclature

Many antigen genes of *P. falciparum* have been cloned and sequenced over the last 10 years, and a large amount of sequence data has accumulated. Most of the genes studied encode polypeptides which are antigens during natural infections. Diversity in these genes has obvious implications for vaccine design.

Some of the first identified malaria antigens were named according to their function or structure, and referred to by a corresponding set of initials, e.g. HRP

(Histidine-rich protein) [Kilejan, 1974]. Antigens have also been named after their molecular weights, e.g. the 195K protein [Holder & Freeman, 1982]. However, this system is problematic because of the anomalous migration of proteins on SDS-PAGE gels, leading to estimations of size that are often grossly in error, perhaps by up to 30%. Also, many polypeptides vary in size between isolates, and may exist as a series of processed fragments, often overlapping in size with other antigens.

Overall, a system of letters is perhaps less confusing. Where different laboratories have carried out studies on the same antigen a large variety of different names may exist. For example, the merozoite surface protein MSP1 described in the next section has been given at least 7 different names.

I.3.ii Merozoite surface protein 1 (MSP1)

(a) Characteristics of the protein

This antigen is also called MSA-1, PMMSA, PSA, gp195, p190 by different authors. It is one of the most widely studied plasmodial antigens. It is synthesised by blood- and liver-stage schizonts [Holder & Freeman, 1982; Szarfman _et al._, 1988] and varies in size in different parasite lines from 180 to 220 kDa. It is attached to the outer face of the parasite membrane by a glycosyl phosphatidylinositol anchor [Haldar, Ferguson & Cross, 1985]. The antigen is encoded by a single gene located on chromosome 9 [Kemp _et al._, 1987]. Each haploid parasite

therefore produces one allelic type of this protein [McBride, Newbold & Anand, 1985 ; Howard, R.F. et al., 1986 ; McBride & Heidrich, 1987]. The protein is believed to be cleaved into at least 4 smaller fragments of approximately 80-83 kDa, 28-31 kDa, 36-45 kDa and 40-45 kDa (figure 2) shortly before merozoite release from schizonts [Holder & Freeman, 1984 ; Heidrich et al., 1983] and these fragments can be identified on the surface of free merozoites [Holder et al., 1987]. McBride and Heidrich (1987) provided evidence for the association of at least 2 of the fragments as a non-covalently associated complex.

The C-terminal 40-45kDa fragment is further cleaved into a 16-19kDa fragment on the merozoite surface [Holder et al., 1987 ; McBride & Heidrich, 1987] and a 33kDa fragment that is released into culture supernatants [Blackman et al., 1991a]. Blackman et al. (1990) suggested that the 16-19kDa fragment is retained by the merozoite after invasion, since epitopes on this fragment can be detected by specific monoclonal antibodies in indirect immunofluoresence studies on early ring stages. A site-specific chymotrypsin-like activity on the 42kDa C-terminal fragment of P. falciparum cleaves this fragment to 33kDa and 19kDa fragments [Blackman et al., 1991a]. This cleavage occurs extracellularly and at the same time as the shedding of the 33 kDa fragment from the merozoite surface [Blackman et al., 1991b].

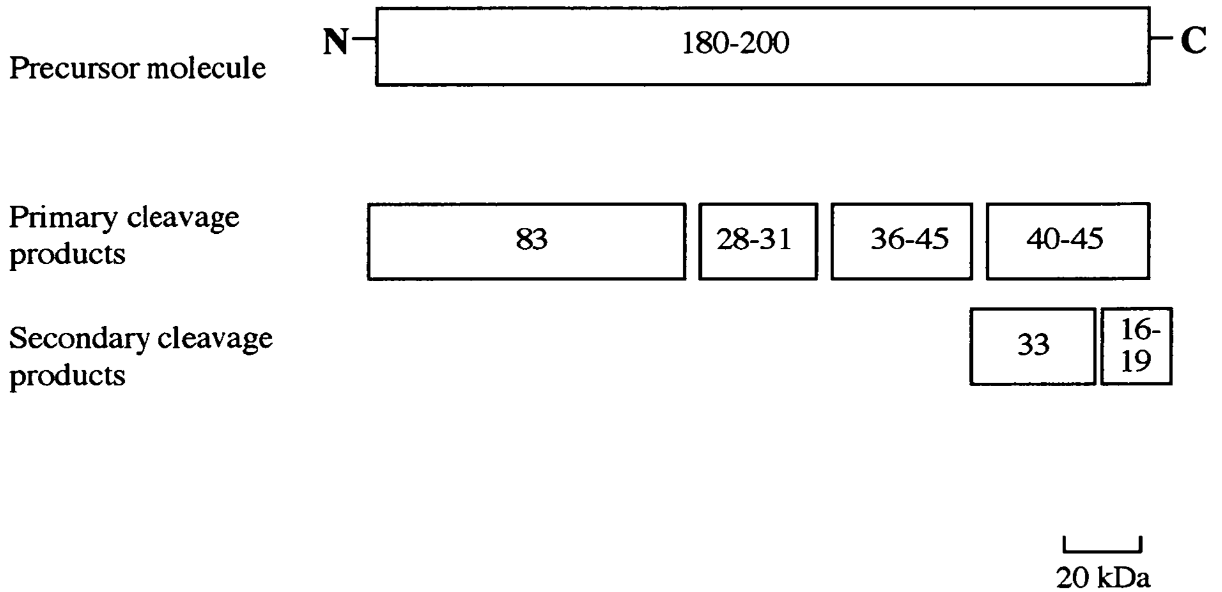


Figure 2 : Diagram representing the natural processing of the precursor to the major merozoite surface protein, MSP1 [Holder, 1988 ; Blackman *et al.*, 1991a]. Approximate sizes of the molecule and its processed fragments are shown in kilodaltons (kDa).

The N-terminal 80-83kDa fragment is apparently shed from the merozoite surface around the time of invasion, and can be recovered from in vitro culture supernatants [Holder et al., 1985]. The fate of the other fragments is not known, as they are not uniquely recognised by any monoclonal antibody [Holder, 1988].

The processing of the MSP1 has been described independently by different workers [Lyon et al., 1986 ; Holder et al., 1987], although one report [Pirson & Perkins, 1985] claimed that the 200kDa precursor remains unprocessed on the surface of merozoites.

The function of the MSP1 is unknown, although its position on the merozoite surface suggests a role in attachment to or invasion of the red blood cell. Perkins & Rocco (1988) demonstrated binding of the MSP1 to glycophorin molecules of human red blood cells, although they implied that the MSP1 was in an unprocessed state on the surface of the merozoites. Blackman et al.(1991a) found that the 19kDa fragment, derived from secondary processing of the 42kDa fragment, contains a cysteine-rich domain with two epidermal growth factor (EGF)-like motifs. They suggested that this fragment could have a role in receptor binding or other cell surface interactions, protein adherence or signalling. Antibodies to this part of MSP1 inhibit the growth of P. falciparum in vitro [Blackman et al., 1990] or protect against P. yoelii in vivo [Burns et al., 1989].

MSP1 is an important candidate antigen for inclusion in a vaccine against malaria because immunisation studies with purified MSP1 gave partial protection to challenge infection in Saimiri monkeys [Siddiqui et al., 1987]. The P. yoelii homologue (230kDa) gave species-specific immunity in mice [Holder & Freeman, 1981].

MSP1 has been examined for epitopes conferring protection in humans. T and B cell epitopes have been identified at particular domains [Sinigaglia et al., 1988, 1990] and the antigen is recognised by antibodies in sera from individuals in malaria endemic areas [Fruh et al., 1991 ; Riley et al., in press].

The extensive polymorphism of the MSP1 was originally characterised using a panel of monoclonal antibodies, some to polymorphic epitopes of the molecule [McBride et al., 1985 ; Holder, 1988]. The probable epitopes recognised by antibodies have been identified by a correlation of serological and sequence polymorphisms amongst different isolates or clones [Conway & McBride, 1991].

(b) Sequences of MSP1 alleles

The MSP1 gene is a single exon, encoding a protein with an N-terminal signal sequence, a hydrophobic sequence, and cluster of cysteine residues near to the C-terminus. Sequencing of the MSP1 gene from different P. falciparum clones and isolates has revealed the extent of the allelic polymorphism [Hall et al., 1984 ; Holder et

al., 1985 ; Mackay et al., 1985 ; Weber et al., 1986 ; Cheung et al., 1986 ; Howard, R.F. et al., 1986 ; Kemp et al., 1986 ; Tanabe et al., 1987 ; Certa et al., 1987 ; Peterson et al., 1988a, 1988b ; Chang et al., 1988 ; Scherf et al., 1989 ; Ranford-Cartwright et al., 1991a]. By comparing nucleotide sequences, Tanabe et al.(1987) identified polymorphic domains (<40% homology at the amino acid level), conserved domains (>87% homology at the amino acid level) and intermediate or 'semi-conserved' domains. On this basis the gene was divided into 17 domains or 'blocks' (Figure 3).

The second domain of MSP1, denoted block 2, contains tripeptide repeats and exhibits considerable polymorphism. This block differs by either sequence or by numbers of repeats in almost every allele investigated so far. Most alleles contain a number of Ser-X-X repeats. However in two clones, RO33 (Ghana) and CSL2 (Thailand), block 2 does not contain any repeats [Certa et al., 1987; Peterson et al., 1988b]. Three basic forms of block 2 have been defined on the basis of sequence and are named after the isolates from which they were originally described : the MAD20-type, the K1-type and the RO33-type.

With regard to the remainder of the molecule, block 4 has at least 2 alternative sequences [Tanabe et al., 1987 ; Certa et al., 1987]. All polymorphic and semi-conserved blocks downstream of block 5 have essentially only 2 alternative sequences, giving rise to the

Figure 3 : Diagrammatic representation of the structure of the merozoite surface protein MSP1 (after Tanabe *et al.*, 1987). The molecule can be divided into blocks on the basis of polymorphism between isolates. MSP1 alleles can be divided into two ('dimorphic') types on the basis of the non-repetitive sequences downstream of block 5. These are the MAD20 type and the K1 type. Block 2 contains highly polymorphic tripeptide repeats. The number and sequence of these repeats varies extensively between isolates. Three types of sequence have been reported for this block - the K1, MAD20 and RO-33 types ; the RO-33 type lacks repetitive sequences.

"dimorphic" allele structure proposed by Tanabe et al. (1987). Very few sites in this region have a third alternative amino acid. It has been suggested that intragenic recombination events at sites between blocks 2 and 5 could easily give rise to the range of alleles seen in natural isolates [Peterson et al., 1988a]. If, for example, isolates MAD20 and K1 are considered to be the two dimorphic "prototypes", then the alleles found in two isolates denoted 'Camp' and 'Wellcome' could have been readily generated by such intragenic recombination [Tanabe et al., 1987]. As the MSP1 gene is unique in the haploid genome, this recombination could only have occurred during meiosis of a heterozygote containing both MAD20- and K1-type alleles.

I.3.iii Merozoite surface protein 2 (MSP2)

(a) Characteristics of the protein

This antigen is also denoted GYMSSA, MSA-2, gp35-36 and GP3. It is synthesised in blood-stage schizonts and, like MSP1, is incorporated on the schizont surface, and on the surface of free merozoites [Stanley, Howard & Reese, 1985 ; Miettinen-Bauman et al., 1988]. It may also be attached via a glycosyl phosphatidylinositol membrane anchor [Smythe et al., 1988]. MSP2 was first defined by monoclonal antibodies [Stanley, Howard & Reese, 1985 ; Ramasamy, 1987 ; Epping et al., 1988 ; Fenton et al., 1989], although it was found to vary greatly in size from 35 to 56 kDa in different isolates.

Sequencing of the MSP2 gene of a clone from isolate FC27 [Smythe *et al.*, 1988] identified the antigen as a distinct integral membrane protein, and not a fragment of MSP1. The predicted size of MSP2 from deduced amino acid sequences was 262-347 amino acids [Smythe *et al.*, 1990 ; Thomas *et al.*, 1990 ; Fenton *et al.*, 1991 ; Smythe *et al.*, 1991], considerably smaller than the size estimated in earlier studies, probably because of glycosylation [Stanley *et al.*, 1985 ; Smythe *et al.*, 1988 ; Clark, J.T. *et al.*, 1989].

The function of MSP2 is unknown. It is known to be one of the antigens present in immune complexes that form at the surface of merozoites when antibodies in immune serum inhibit merozoite dispersal [Thomas *et al.*, 1990]. No challenge immunisation studies have yet been performed, and no homologues have been described for other *Plasmodium* species.

MSP2 is recognised by sera and T-cells from adults living in malaria endemic areas [Smythe *et al.*, 1990 ; Rzepczyk *et al.*, 1990] and is known to be immunogenic in rabbits [Miettinen-Bauman *et al.*, 1988] and monkeys [Stanley *et al.*, 1985]. Monoclonal antibodies can be raised against MSP2 in mice, and at least 3 of these have been shown to inhibit merozoite invasion of red blood cells *in vitro* [Epping *et al.*, 1988 ; Clark J.T. *et al.*, 1989 ; Ramasamy *et al.*, 1990]. All available monoclonal antibodies appear to recognise polymorphic epitopes. It has been suggested that the protein is encoded by a gene

at a single locus which exhibits considerable allelic polymorphism [Fenton et al., 1989]. This locus has been mapped to chromosome 2 [Kemp et al., 1987].

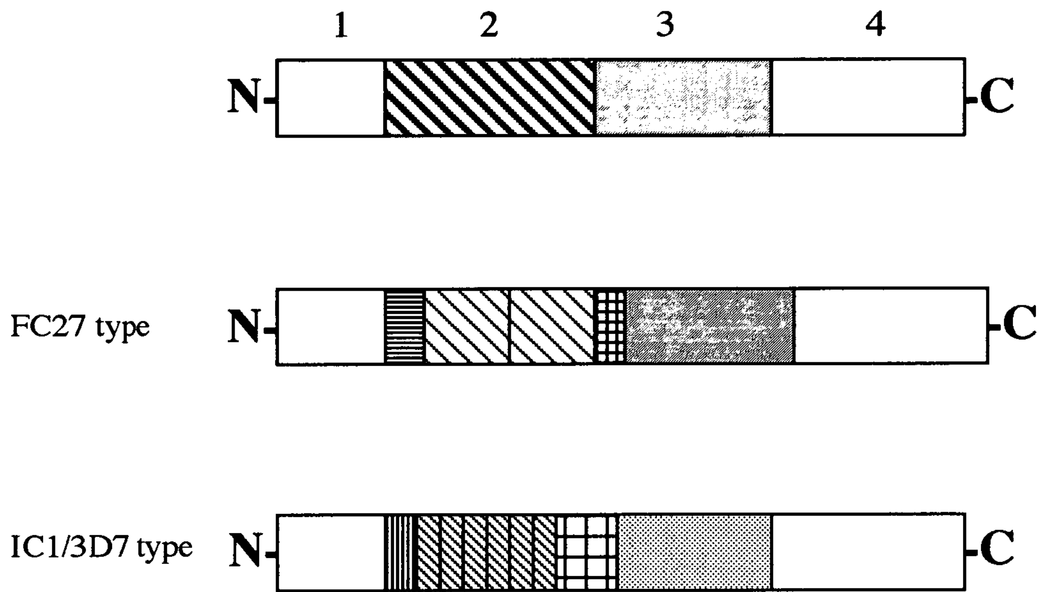
Comparison of the sequences from several isolates has allowed the structure of the gene to be determined. The 43 N-terminal amino acids and 74 C-terminal amino acids are very highly conserved. There is a 5 amino acid hydrophobic region flanked by charged residues at the N-terminal end [Smythe et al., 1988]. Sequencing has also revealed a stretch of 17 hydrophobic amino acids, homologous to a region of the variant surface glycoprotein in Trypanosoma brucei which is believed to be the signal for addition of a GPI anchor [Smythe et al., 1988].

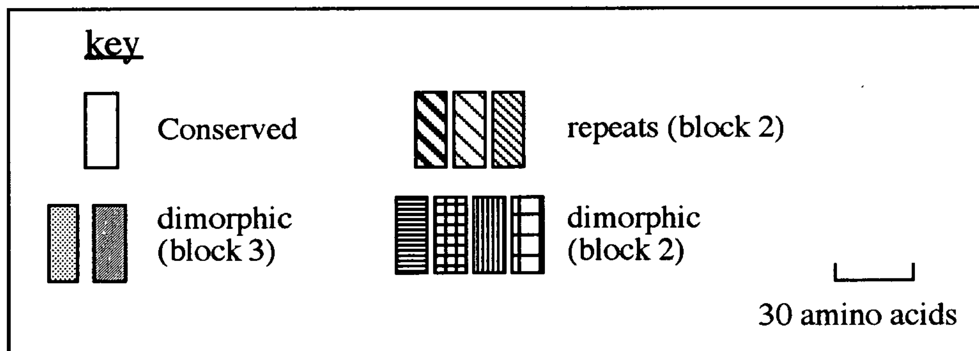
Extensive sequence diversity occurs in the central region, including a region of polymorphic repeats [Thomas et al., 1990 ; Smythe et al., 1990, 1991 ; Fenton et al., 1991] (Figure 4).

(b) Sequences of MSP2 alleles

Polymorphism of the MSP2 gene has been investigated using the polymerase chain reaction (PCR), or by cloning, and sequencing. The repetitive part of the molecule exhibits marked polymorphism, including the absence of repeats in block 2 in one clone, CAMP [Thomas et al., 1990]. This may be comparable to the lack of repeats in block 2 of the MSP1 gene of the clones RO33 and CSL2. It has been suggested that all of the alleles of MSP2 so far

Figure 4 : Diagrammatic representation of the structure of the merozoite surface protein MSP2 (after Thomas et al., 1990). The sequences in blocks 1 and 4 are very highly conserved. Block 2 consists of polymorphic repeat sequences and some non-repetitive polymorphic regions. Block 3 contains polymorphic or 'dimorphic' non-repetitive regions. MSP2 alleles can be divided into two distinct families on the basis of non-repetitive sequences. These families are denoted the FC27 family and the IC1/3D7 family (Smythe et al., 1991).


1
2
3
4
N-
-C
FC27 type
N-
-C
IC1/3D7 type
N-
-C


key
Conserved
repeats (block 2)
dimorphic
(block 3)
dimorphic
(block 2)
30 amino acids

examined can be divided into two families, defined by the non-repetitive region 3 [Thomas et al., 1990]. This is similar to the dimorphic model proposed for MSP1 [Tanabe et al., 1987].

Two recent papers [Marshall et al., 1991 ; Snewin et al., 1991] suggested that intragenic recombination between the two allelic families of MSP2 may generate new alleles, similar to the mechanism proposed for MSP1 [Tanabe et al., 1987]. An isolate NIG60 was found by sequencing to possess the N-terminal non-repetitive variable region of the IC1 type and some repeats of this family [Marshall et al., 1991]. The remaining variable region encodes a sequence resembling that of the FC27 allelic family. Thus a cross-over event in the middle of the variable repetitive region at meiosis could be responsible for the new allele. In the second paper, Snewin and colleagues describe the sequence of an MSP2 allele from a Colombian isolate Col8, and suggest that it could have been generated by recombination between an IC1-type allele such as T9-96 and an FC27-type allele. The allele is of the FC27 type, but contains a short IC1-type sequence in block 2 and at the beginning of block 3. These recombinational events are postulated to have occurred in the variable repetitive region, and therefore differ from the cross-over events suggested for the generation of MSP1 alleles, which have been proposed to occur in the conserved regions.

I.4 Genetics of Plasmodium

I.4.i The genome

The genome of P. falciparum consists of 14 chromosomes [Kemp et al., 1987 ; Wellems et al., 1987]. The chromosomes cannot be seen by conventional light microscopy, as they do not condense individually during mitosis. This is somewhat surprising as basic proteins (histones) were described in the parasite nucleus in early studies by Bahr & Mikel,(1972), and the DNA is known to be packaged into nucleosomes [Wunderlich et al., 1980]. Electron microscopy studies have identified up to 14 pairs of kinetochores, the attachment points of the chromosomes to spindle microtubules, in serial sections of budding sporozoite nuclei [Sinden & Strong, 1978], and of dividing nuclei of young schizonts [Prensier & Slomianny, 1986] of P. falciparum.

Pulsed field gradient gel electrophoresis (PFGE) was first used to separate the chromosomes of P. falciparum by Kemp et al.(1985) and Van der Ploeg et al.(1985). Subsequent studies have allowed visualisation of all 14 P. falciparum chromosomes by PFGE [Wellems et al., 1987 ; Kemp et al., 1987], as well as the chromosomes of P. berghei, P. chabaudi and P. vinckei [Sharkey et al., 1988; Janse et al., 1989 ; Sheppard et al., 1989].

Chromosomes of Plasmodium vary considerably in size, from 800 to 3500 kb, and in natural isolates of P. falciparum there is variation in size between homologous chromosomes [Corcoran et al., 1986]. Genes for antigens

and other proteins have been allocated to specific chromosomes by hybridising gene probes to blots of PFGE gels. There is also variation between natural isolates of P. falciparum with respect to the presence or absence of certain genes on particular chromosomes, and also in the quantity of a repetitive sequence, rep20, present (usually sub-telomerically) on different chromosomes. The linear order of genes has been determined for some chromosomes by restriction digestion of individual chromosomes and hybridisation of the fragments produced with gene probes [Corcoran et al., 1986 ; Wellems et al., 1987].

Chromosome size variation can also occur during asexual multiplication of clones, for example, in P. berghei following multiple passage of clones through mice [Janse et al., 1989 ; Ponzi et al., 1990], and in clones of P. falciparum following prolonged culture of blood forms [Corcoran et al., 1988 ; Wellems et al., 1988]. It has been suggested that this is due to recombination at repetitive regions in the genome during mitotic divisions. For a haploid organism this could only occur by sister chromatid exchange.

I.4.ii Ploidy, meiosis and mitosis

The parasite in the vertebrate host is entirely haploid. Studies on the inheritance of isoenzymes and other characters in P. chabaudi [Walliker, Carter & Sanderson, 1975] and in P. falciparum [Walliker et al.,

1987] have supported haploidy for the erythrocytic stages; exo-erythrocytic stage haploidy was demonstrated by studies on the inheritance of antigens of these forms [Szarfman et al., 1988].

Microfluorometric studies on DNA synthesis in P. berghei have demonstrated that sporozoites, ring forms, young trophozoites and mature microgametes possess a similar quantity of DNA, assumed to be the haploid amount [Janse et al., 1986]. Mature macrogametes were found to possess approximately 50% more DNA than the haploid quantity, possibly because of amplification of gametocyte-specific genes.

The only diploid phase in the Plasmodium life-cycle is the zygote (ookinete) in the mosquito stomach. Meiotic division in the zygote nucleus was detected within 2½ hours of fertilisation, in electron microscope studies of P. berghei [Sinden & Hartley, 1985]. Synthesis of approximately 4 times the haploid DNA quantity occurs at this time [Janse et al., 1986], consistent with duplication of the diploid chromosome set at the first stage of meiosis. This duplication of the diploid chromosome set is also supported by evidence of 4 chromatids in structures considered to be synaptonemal complexes (counted on the basis of kinetochore number) as would be expected in the pachytene stage of meiosis [Sinden & Hartley, 1985]. This is in contrast to the related sporozoan parasite, Eimeria, in which the fertilised zygote appears to pass from the diploid 2n to

the haploid n amount of DNA at the first meiotic division [Canning & Morgan, 1975].

Extensive electron microscope work on the developing zygote [Howells & Davies, 1971 ; Sinden & Strong, 1978 ; Sinden & Hartley, 1985] has revealed condensation of chromatids, which become embedded in attachment plaques in the nuclear envelope (thought to be leptotene prophase) followed rapidly by pairing (zygotene). Synapsis, characterised by the appearance of a central element between lateral DNA arms, has been demonstrated. There is a correlation between the presence of such synaptonemal complexes and meiosis (pachytene, meiosis I); however in Plasmodium none of the complexes yet observed have contained the nucleating centre considered to be responsible for chiasma formation.

The synaptonemal complex structure seems to persist unchanged into metaphase, retaining tightly paired chromosomes, and so there appear to be no separate phases of diplotene or diakinesis within the first meiotic division. Metaphase and anaphase are completed rapidly, and by telophase no chromosomes are visible on the kinetochores, now clustered around the retracted spindle poles.

The subsequent meiotic division, in which reduction of a 4n genome to the haploid n amount would be expected, is unclear, and cannot be distinguished from mitotic divisions in the young oocyst. No further condensation of

chromosomes around the prominent kinetochores has been seen.

Subsequent mitotic oocyst nuclear divisions in P. berghei [Howells & Davies, 1971 ; Schrèvel et al., 1977] appear to follow three distinct phases : proliferation, attenuation of nuclear lobes, and final division into sporozoites. P. falciparum nuclear divisions are similar [Sinden & Strong, 1978], with the appearance of multiple spindles in each nuclear lobe. Although the chromosomes do not condense individually during mitosis, DNA has been localised to mitotic spindles by DNAse digestion [Aikawa, 1972], by peripheral Feulgen staining in the nuclei of sporulating oocysts [Vanderberg et al., 1967], and by condensation of the whole genome (DNA and other nucleic acids, proteins) and the spindle at the final microgametic division [Sinden et al., 1976].

Mitotic division in the developing oocyst is rapid and synchronous. As mentioned above (section I.2.i), the number of sporozoites in a mature oocyst has been estimated as between 3000 and 10 000 [Rosenberg & Rungsiwongse, 1991 ; Pringle, 1965]. To achieve this, a division rate of 1.4 divisions per day is necessary [Sinden & Strong, 1978]. At any one time, 33-50% of oocysts are seen to be in mitosis ; thus each division is thought to take from 6 to 8 hours [Sinden & Strong, 1978].

I.4.iii Genetic crosses and recombination

The traditional basis of genetic studies, to obtain data on segregation and recombination of genes, is the genetic cross. Such crosses may allow the allocation of genes to particular chromosomes, and the determination of the linear order of genes on chromosomes.

Malaria parasites are crossed by allowing mosquitoes to feed on a mixture of gametocytes of two cloned lines differing in a number of genetic markers. This allows cross-fertilisation to occur between gametes of each clone. Self-fertilisation events are also expected. Assuming that (i) each clone produces equal numbers of male and female gametes and (ii) fertilisation occurs randomly, then equal numbers of hybrid and parental zygotes will be produced.

During meiosis of hybrid zygotes, each member of a given pair of chromosomes segregates randomly into the haploid progeny. In addition, crossing-over events may occur at this stage between homologous chromosomes. Genes located on different chromosomes will assort randomly in the progeny of crosses ; linked genes (those on the same chromosome) will segregate together, unless separated by crossing-over events during meiosis. The term 'recombination' is used here to denote the production of parasites with novel combinations of parent genes, either by independent assortment of unlinked genes, or by crossing-over between linked genes.

In allowing development of the zygotes to sporozoites, infections can then be established in suitable vertebrate hosts, and the resultant blood infections examined for forms exhibiting recombination between parental characters. Recombinants can only be derived from zygotes produced by cross-fertilisation. Such recombinants can be isolated by cloning.

Crossing experiments between malaria parasites have been carried out with species affecting birds, rodents and humans. Initial studies were carried out with P. gallinaceum [Greenberg & Trembley, 1954] and the rodent species P. yoelii and P. chabaudi [Walliker et al., 1973, 1975], since vertebrate hosts were readily available. In 1987, Walliker et al. performed the first human malaria crossing experiment, using the P. falciparum clones 3D7 (the Netherlands) and HB3 (Honduras), and chimpanzees (Pan troglodytes) as the vertebrate host. A further P. falciparum cross was carried out by Wellems et al. (1990) using the clones Dd2 (Indochina) and HB3 (Honduras).

All crosses have shown frequent production of recombinant parasites. Examples are given in the following sections.

I.4.iii(a) Chromosome re-assortment

In a P. chabaudi cross [Sharkey et al., 1988] independent assortment of chromosomes was demonstrated in progeny clones. The two parent clones used in this cross, denoted AS and CB, differed in the relative sizes of two

of their chromosomes ; chromosome 4 in clone AS is larger than that of clone CB, and chromosome 5 is larger in clone CB than in clone AS. Following crossing of the two clones, 6 progeny clones were isolated, 5 of which possessed parental karyotypes. The sixth clone however had chromosome 4 characteristic of parent clone CB, and chromosome 5 of parent clone AS.

Similar chromosome segregation has been demonstrated for P. falciparum [Wellems et al., 1987 ; Corcoran et al., 1988] in the 3D7/HB3 cross. Chromosome 1 of 3D7 possesses a SmaI site not found on the same chromosome of HB3. Chromosome 13 of 3D7 possesses a histidine-rich protein gene, SHARP/HRPIII absent on chromosome 13 of HB3. Following cloning of the progeny of the cross one clone, X5, was found to have a chromosome 1 characteristic of the HB3 parent (no SmaI site) but a chromosome 13 characteristic of the 3D7 parent (presence of HRPIII).

I.4.iii(b) Crossing-over events

In P. falciparum crosses, novel-sized chromosomes are readily generated at meiosis. Chromosome 4 in the 3D7 parent is approximately 1490 kb. and that of HB3 about 1200 kb.. In four of twelve progeny clones derived from the 3D7/HB3 cross, chromosome 4 was found to be intermediate in size. Seven of twelve progeny clones exhibited no change in chromosome size compared to the parent clones [Sinnis & Wellems, 1988]. Restriction

mapping of chromosome 4 from one of these clones, XP5, revealed that the chromosome had restriction sites characteristic of the left end of the 3D7 chromosome 4 and the right end of the HB3 chromosome 4. This strongly suggested crossing-over in the central region of the parental chromosomes, presumably during meiosis of a 3D7/HB3 heterozygote.

A further example from the first P. falciparum cross involved a size change in chromosome 2. Some progeny clones were found to have a chromosome 2 which was larger than that of either parent [Walliker et al., 1987]. Examination of two of these clones, XP2 and X5, revealed an increase in the quantity of a repetitive DNA sequence (rep20) at a subtelomeric site [Corcoran et al., 1988], thought to be due to an unequal crossing-over event in the rep20 sequence during meiosis. Subtelomeric regions of chromosomes of many organisms are known to be subject to great variation and genetic rearrangements. A study on the inheritance of a telomere-related sequence, Pftel.1, in 12 progeny clones of the 3D7/HB3 P. falciparum cross revealed novel, non-parental sized fragments of DNA hybridising to this gene probe [Vernick et al., 1988]. Some of these fragments were shown to be at internal chromosome sites as well as at subtelomeric locations. Such sites were suggested to be genetically unstable at meiosis - and perhaps to be "hotspots" of recombination.

I.4.iii(c) Recombination of enzymes, proteins and antigens.

Many antigens, enzymes and other proteins exist as variable forms in parasite populations [for review, see Beale & Walliker, 1988]. Variations in enzymes are revealed as charge differences by electrophoretic techniques [Carter, 1978 ; Sanderson et al., 1981]. For other proteins, the technique of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been particularly useful for demonstrating forms of a protein differing in size and/or charge [Fenton et al., 1985]. Differences between specific antigens are most conveniently studied by their reactions with monoclonal antibodies in immunofluorescence tests [Conway & McBride, 1991].

The inheritance of these characters has been much studied in crossing experiments. For example, in the P.falciparum cross described above, the parent clones 3D7 and HB3 could be distinguished by variant forms of one enzyme (adenosine deaminase (ADA)), by five 2D-PAGE proteins, and by the antigens MSP1 and MSP2. Examination of progeny clones derived from this cross showed that segregation of each form of each of these characters had occurred during the crossing procedure, as well as recombination between them.[Walliker et al., 1987 ; Fenton & Walliker, 1990]. This showed that the variant forms of each enzyme, protein and antigen were determined by allelic variants of their respective genes.

The gene for MSP1 is known from PFGE studies to be located on chromosome 9, and that for MSP2 to be on chromosome 2 [Kemp et al., 1987]. Recombination seen between these characters is thus due to simple independent assortment of each parental chromosome in different progeny clones [Walliker et al., 1987, 1988]. The chromosomal loci for the other enzyme and protein markers used as parent clone markers are still not known. The fact that they appear to undergo frequent recombination suggests that they are unlinked. However, the parental forms of MSP2 and the 2D-PAGE protein C co-segregated in the cross [Fenton & Walliker, 1990], suggesting that these genes could be linked.

Tanabe et al.(1987) suggested that the diverse forms of MSP1 could be generated by intragenic recombination during meiosis of zygotes heterozygous for this antigen. Definitive evidence for this is not yet available from crossing experiments, since this process is likely to occur very rarely, and large numbers of progeny clones would therefore need to be analysed.

I.4.iv Frequency of recombinant forms among progeny of crosses

Crossing experiments with both rodent and human malaria parasites have demonstrated that recombination occurs readily and at a greater frequency than expected. At least 50% of the progeny of a cross are expected to derive from self-fertilisation events and thus to exhibit

parental phenotypes and genotypes. Yet in the 3D7/HB3 P. falciparum cross, only three of 22 progeny clones had the characteristics of the parent clones with respect to the 10 markers used [Walliker et al., 1987 ; Fenton & Walliker, 1990]. In the Wellems Dd2/HB3 cross, none of the 76 progeny clones examined exhibited either of the parental combinations of markers [Wellems et al., 1990]. This study used over 30 single copy RFLP markers to individual chromosomes as well as drug susceptibility and other phenotypic markers. Sixteen independent recombinant types were identified in the progeny.

This excess of recombinants in the progeny of a cross could be explained by:

(i) selection in the mosquito, chimpanzee or during cloning favouring certain recombinant genotypes,

(ii) disproportions in the numbers of micro- and macrogametes produced by each parent clone,

(iii) a favouring of cross-fertilisation over self-fertilisation.

I.5 Present study

In this study, an attempt has been made to explain the excess of recombinants over parental types in the progeny of the two P. falciparum crosses made. The work involves examining the genotypes of individual oocysts in mosquitoes which have been fed on parasite mixtures. Since a single oocyst is believed to contain all of the meiotic products from a single fertilisation event,

hybrid oocysts should contain both types of parental markers, whereas oocysts from self-fertilisation events will only contain the markers from one of the parents.

Analysis of single oocysts has not previously been attempted for a number of reasons. Firstly, the amount of parasite material in an oocyst is small. The haploid Plasmodium genome contains about 2-4 x 10^7bp., or around 25fg [Janse et al., 1986]. The amount of DNA available is thus too small for conventional analysis by RFLP markers etc. Secondly, mosquitoes are difficult to infect with cultured P. falciparum gametocytes, and thirdly, the oocysts themselves are small (40 to 60μm in diameter) and therefore difficult to isolate.

Initial work in this study was performed on the rodent parasite P. yoelii, because of the relative ease with which mosquitoes can be infected and oocysts obtained.

The first approach to distinguish hybrid oocysts (from cross-fertilisation events) from parental oocysts (from self-fertilisation events) was to visualise the chromosomes of single oocysts using pulsed field gradient gel electrophoresis (PFGE) and by probing southern blots of these gels with whole DNA. If the chromosome banding patterns of the parent clones differed from one another, then hybrid oocysts would be expected to contain both banding patterns, whereas parental oocysts would only have one type of banding pattern.

The second approach made use of the application of the Polymerase Chain Reaction (PCR) [Saiki et al., 1985], to analyse the alleles of two antigen genes in individual oocysts for homozygous or heterozygous patterns, which would indicate, respectively, self- or cross-fertilisation events.

Preliminary work on the murine malaria parasite *Plasmodium yoelii nigeriensis*

II Materials and Methods

II.1 Growth and maintenance of parasites in vivo

Blood forms of *P. y. nigeriensis* [Killick-Kendrick, 1973], isolate N67, were taken an ampoule stored under liquid nitrogen and injected intra-peritoneally (i.p.) into outbred mice. Three days later, when parasites were visible in blood smears from tail snips, blood was transferred from the tail of an infected mouse to an uninfected mouse by i.p. injection [Killick-Kendrick, 1971]. This was repeated as necessary to maintain the parasite line *in vivo*. Mice were lethally anaesthetised with ether or chloroform before they developed malarial symptoms.

Mice infected with *P. y. nigeriensis* were given water supplemented with 4-aminobenzoic acid (PABA) [Ferone, 1977] to enhance the growth of the parasite.

II.2 Infection of mosquitoes

3 to 5 days post-infection blood from infected mice was taken, a small drop placed under a coverslip, and examined using phase contrast optics for exflagellation of male gametocytes. Exflagellation was usual within 6 to 14 minutes at 16°C [Landau & Boulard, 1978]. If exflagellation was seen, the mice were anaesthetised with

ether and placed inside small netting cages containing female Anopheles stephensi 'Dutch' mosquitoes [Vanderberg & Gwadz, 1980]. The mosquitoes were allowed to engorge on the mice. The mice were then removed and lethally anaesthetised.

Mosquitoes were maintained at 26°C and 80% relative humidity (RH). They were dissected 7 days after the infectious feed, and the mid-guts examined for the presence of oocysts.

II.3 Preparation of pulsed field gel blocks.

Oocysts were dissected from the mosquito mid-guts and low-gelling point agarose blocks for pulsed field gradient gel electrophoresis (PFGE) were made. Single oocysts, a group of oocysts, or whole guts with many oocysts, were embedded in 1.5 to 2% low gelling point agarose in 0.5 x TBE buffer to form blocks of 1.6 x 5 x 5 mm. The blocks were then treated with proteinase K (2mg ml^{-1}) and sarkosyl (1%) in 50:1 TE (50mM Tris-HCl, 1mM EDTA) for 48 hours at 42°C as described by Kemp et al., (1985), and stored at 4°C.

II.4 Pulsed field gradient gel electrophoresis (PFGE).

PFGE [Schwarz & Cantor, 1984] separates at least 10 large DNA fragments in P. chabaudi [Sharkey et al., 1988] and 14 chromosome-sized bands in P. falciparum [Kemp et al., 1987]. In PFGE an electric field is applied in alternate pulses first in one direction and then in a

direction oriented at an angle to the first. DNA molecules re-align with the field, and as the time taken to re-orient is a function of size, the molecules can be resolved if the pulse time is adjusted appropriately.

Using conditions similar to those used for _P. berghei_ [Sharkey _et al._, 1988] 1.5% agarose gels were run in 0.5 x TBE at 150V for 48 hours at 11°C with a pulse time of 200 seconds, using orthogonal-field alternation gel electrophoresis (OFAGE) [Carle & Olson, 1984]. Alternatively, gels were run on an apparatus allowing contour-clamped homogenous electric field electrophoresis (CHEF) [Chu _et al._, 1986], at 300V with an 82 second pulse for 24 hours. After electrophoresis gels were stained with ethidium bromide and photographed as in section V.15.

II.5 Southern blotting of PFGE gels.

PFGE gels were blotted onto nylon transfer membrane [Southern, 1975] for probing with whole parasite DNA to detect chromosome banding patterns.

The DNA was partially depurinated by soaking the gel for 25 minutes in 0.25M hydrochloric acid, in order to improve the efficiency of transfer of large DNA fragments [Wahl _et al._, 1979]. The gel was then denatured in 0.2M NaOH/ 0.6M NaCl for 15 minutes at room temperature, and neutralised in 1M Tris (pH8.0)/ 1.5M NaCl for 1 hour at room temperature with 3 changes of buffer. The nylon transfer membrane, GeneScreen™ (NEN Dupont), was cut to

the exact size of the gel, placed on top of the gel, and the DNA transferred for 12 to 24 hours as described in Maniatis et al.(1982).

The DNA was crosslinked to the nylon membrane by U-V irradiation [Church & Gilbert, 1984]. The immobilised DNA was completely denatured in 0.4M NaOH for 60 seconds and neutralised in 1M Tris pH8.0/ 1.5M NaCl for 60 seconds. The membrane was irradiated for 3 to 5 minutes with a 254nm shortwave U-V source at a distance of 15cm, and either stored dry or prehybridised immediately for probing.

II.6 Extraction of P. yoelii nigeriensis DNA from infected mice.

Mice infected with P. y. nigeriensis 3 to 4 days earlier were anaesthetised with ether, and blood was extracted in citrate (2mls citrate per ml blood) to prevent clotting. The blood was passed through a CF11 column, previously wetted with citrate, to remove the white blood cells, and the RBC collected from the bottom of the column. These were centrifuged at 2000g for 5 minutes and the supernatant removed. The pellet was incubated at 37^{0}C with saponin (0.15% in PBS) at 0.5-1 volume of packed cells for 20 minutes, until lysis of the RBC occurred. The saponin solution was then diluted with an excess (4 volumes) of PBS and the mixture centrifuged at 4000g for 10 minutes. The supernatant was removed and

the washing repeated until the supernatant remained clear.

The pellet was resuspended in 10 volumes of lysis buffer B (50mM NaCl, 50mM EDTA, 10mM Tris pH8.0, 2% sarkosyl) and mixed. Following lysis of the parasites an equal volume of phenol saturated with 10:1 TE was added, mixed and centrifuged. The DNA was re-extracted twice with phenol, once with a 1:1 mixture of phenol and chloroform, and once with chloroform [Maniatis et al., 1982) and then the DNA was precipitated with 0.1 volumes of 3M potassium acetate and 2 volumes of ice-cold absolute ethanol, and kept at -20°C for 30 minutes. The DNA was pelleted by centrifugation at 10 000g, the supernatant removed and the pellet dried before resuspending in 50μl 10:1 TE, pH7.5. The DNA was stored at -20°C.

II.7 Radiolabelling of P. y. nigeriensis DNA.

Parasite DNA was radiolabelled for hybridisation to southern blots of pulsed field gradient gels of oocysts, to detect chromosome banding patterns. DNA was labelled with ^{32}P by random primed labelling. This method of radiolabelling DNA [Feinberg & Vogelstein, 1983] is based on the hybridisation of a mixture of all possible hexanucleotides to the DNA to be labelled. The complementary strand is synthesised from the 3'-OH termini of the random hexanucleotide primer using Klenow enzyme, incorporating radiolabelled deoxynucleoside

triphosphates into the newly synthesised complementary strand. Random priming of DNA enables labelling to very high specific activities of minimal amounts of DNA, and is very useful for Southern hybridisations. Labelling involves denaturation of the DNA to be labelled and synthesis of the complementary strand with Klenow enzyme and labelled deoxyribonucleotides.

Random primed labelling of the P. y. nigeriensis DNA was performed using the Random primed DNA labelling kit from Boehringer Mannheim, according to the manufacturer's protocol. Briefly, 25ng of parasite DNA was denatured by boiling for 10 minutes, and then immediately snap-cooled on ice. 1μl each of 0.5mM dATP, dGTP and dCTP (25nM) and 2μl of the 10x hexanucleotide mixture supplied (containing 0.5M Tris-Cl, 0.1M $MgCl_2$, 1mM DTE, 2mg ml^{-1} BSA, 62.5 A_{260} units ml^{-1} primer 'random' pH7.2) were added. 50μCi (1.85MBq) of [α-^{32}P]dCTP (3000Ci (110TBq) $mmol^{-1}$, Amersham) were added along with sterile glass distilled water to make the final volume 19μl. 1μl (2 units) of Klenow enzyme was added and the mixture incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 2μl 0.5M EDTA pH8.0. Removal of unincorporated dNTPs was not necessary as the DNA was only used for hybridisation to Southern blots.

II.8 Hybridisation of labelled DNA to Southern blots.

The nylon membranes (GeneScreen™) were prehybridised at 60°C for 2 to 4 hours in sealed bags with 0.2ml

hybridisation solution (6 x SSC, 2% SDS, 5 x Denhardts, 0.01M EDTA, 200μg ml^{-1} sonicated salmon sperm DNA) for each cm^2 area of membrane. The solution was then replaced with a fresh aliquot of hybridisation solution (50μl cm^{-2} membrane) containing 100-500 cpm ml^{-1} of the denatured radiolabelled DNA probe, and incubated for 12-16 hours at 60°C.

Following hybridisation the membranes were washed in 2 x SSC, 0.5% SDS for 20 minutes at room temperature, and then in 2 x SSC, 3% SDS for 40 minutes at 60°C, with one change of buffer. Finally the membranes were washed with 0.1 x SSC, 3% SDS for 40 minutes at 60°C, again with one change of buffer [Maniatis et al., 1982]. Membranes were then sealed wet in saranwrap and exposed to Xray film (Kodak X-Omat XAR5 or Agfa Curix) for 24 to 72 hours at -70°C. Autoradiographs were developed as described in section V.16.vi.

The GeneScreen™ membranes were boiled in 0.1 x SSC, 1% SDS for 20 minutes to remove the bound probe, and allowed to dry before storage at room temperature or reprobing.

III Results

III.1 Pulsed field gradient gels

PFGE of the parasite DNA was carried out under two different running conditions, as described in the legends to figures 5, 6 and 7. After staining with ethidium

bromide the gels were photographed, as shown in figures 5a, 6a and 7a. The gels were then blotted onto nylon membranes and probed with whole P. y. nigeriensis DNA labelled with ^{32}P by random priming. The membranes were exposed with Xray film (Kodak X-Omat XAR-5 or Agfa Curix) for 24 hours before developing. Results are shown in figures 5b, 6b and 7b).

Gel 1 (figure 5)

Pulsed field gradient gels of P. y. nigeriensis oocysts (a) stained with ethidium bromide and photographed on a U-V transilluminator, and (b) blotted onto Genescreen membrane and probed with whole P. y. nigeriensis DNA labelled with ^{32}P.

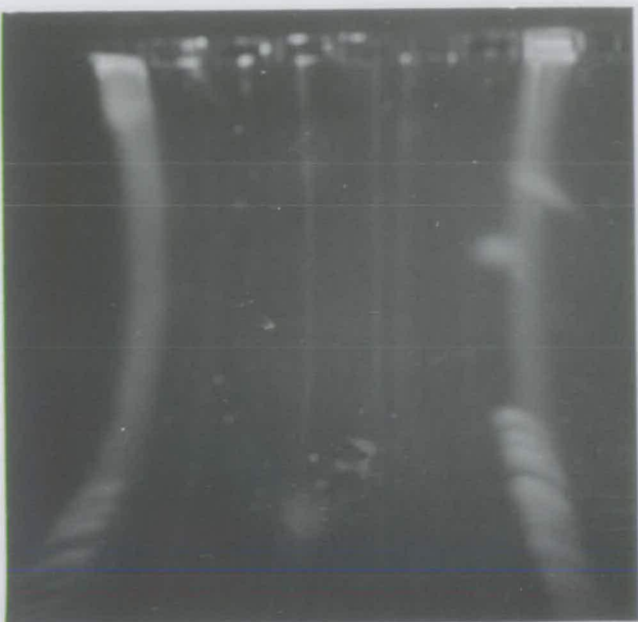
Running conditions:

Current/Voltage:	95mA / 150V
Pulse time:	200 seconds
Run time:	48 hours
Run temperature:	11°C
Gel used:	1.5% Agarose
Buffer used:	0.5 x TBE
Apparatus:	CHEF

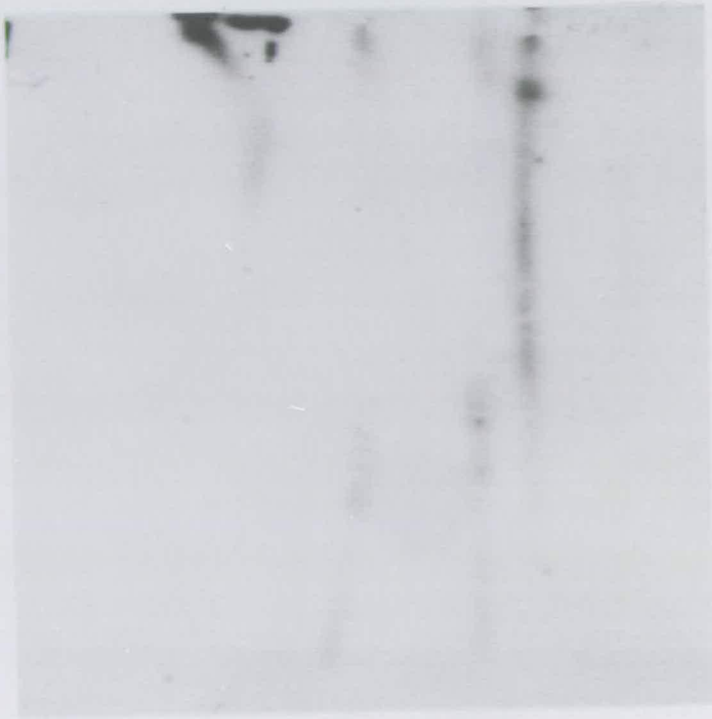
Loading :

Track	
1	blank
2	Yeast chromosomes
3	50 oocysts/ 1 midgut
4	30 oocysts/ 1 midgut
5	20 oocysts/ 1 midgut
6	20 oocysts/ 1 midgut
7	20 oocysts/ 1 midgut
8	10 oocysts/ 1 midgut
9	Yeast chromosomes
10	blank

a



b



Gel 2 (figure 6)

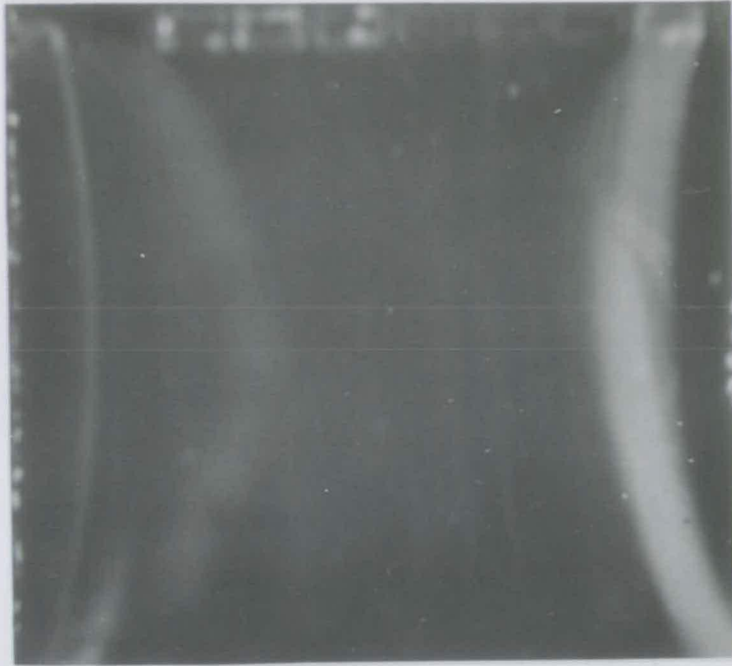
Pulsed field gradient gels of P. y. nigeriensis oocysts (a) stained with ethidium bromide and photographed on a U-V transilluminator and

(b) blotted onto nitrocellulose and probed with whole P. y. nigeriensis DNA labelled with ^{32}P.

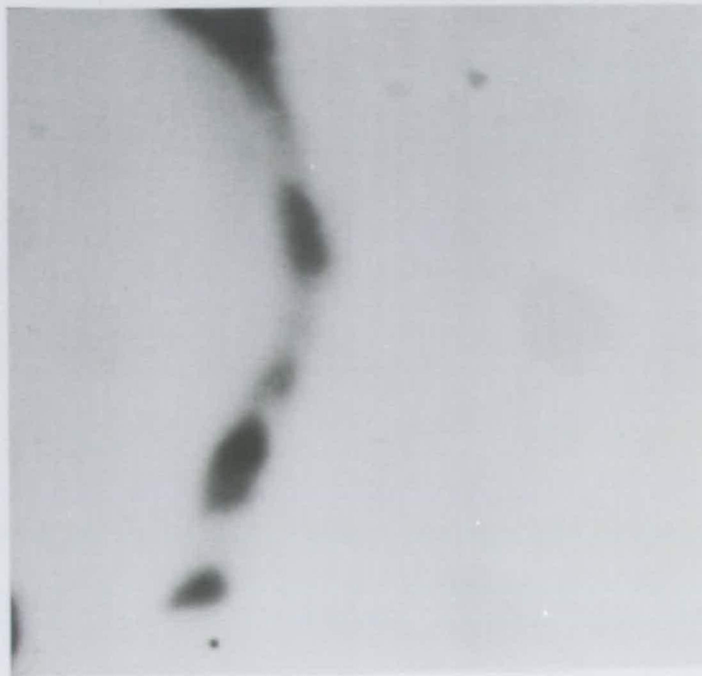
Running conditions:		Track	Loading:
Current/Voltage:	95mA / 170V	1	Yeast chromosomes
Pulse time:	200 seconds	2	3D7 asexual (marker)
Run time:	48 hours	3	many oocysts/ 1 midgut
Run temperature:	11°C	4	many oocysts/ 1 midgut
Gel used:	1.5% Agarose	5	4 oocysts
Buffer used:	0.5 x TBE	6	many oocysts/ 1 midgut
Apparatus:	CHEF	7	many oocysts/ 1 midgut
		8	1 oocyst
		9	Yeast chromosomes

1 2 3 4 5 6 7 8 9

a



b



Gel 3 (figure 7)

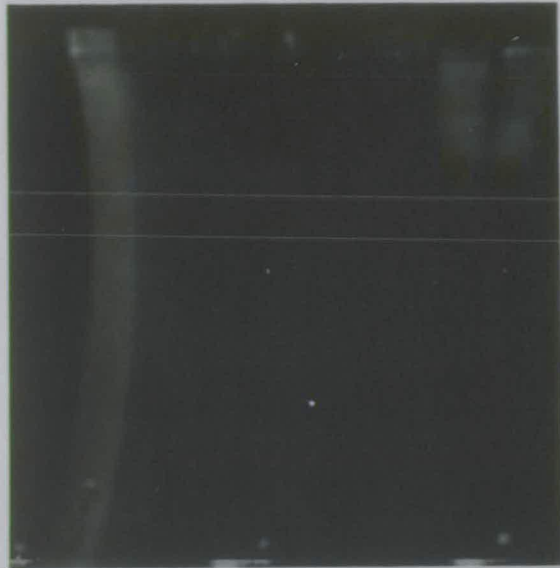
Pulsed field gradient gels of *P. y. nigeriensis* oocysts (a) stained with ethidium bromide and photographed on a U-V transilluminator and

(b) blotted onto nitrocellulose and probed with whole *P. y. nigeriensis* DNA labelled with ^{32}P.

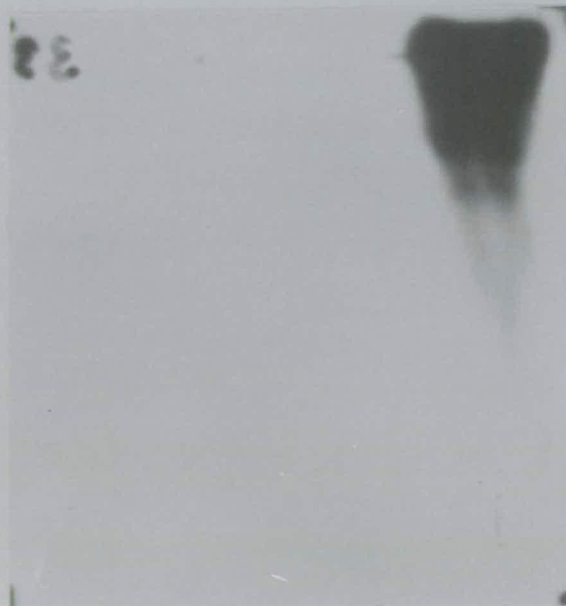
Running conditions:		Track	Loading:
Current/Voltage:	175mA / 300V	1	blank
Pulse time:	82 seconds	2	Yeast chromosomes
Run time:	24 hours	3	10 oocysts/ 1 midgut
Run temperature:	11°C	4	5 oocysts/ 1 midgut
Gel used:	1.5% Agarose	5	4 oocysts/ 1 midgut
Buffer used:	0.5 x TBE	6	many oocysts/ 1 midgut
Apparatus:	OFAGE	7	uninfected midgut
		8	*P.berghei* 265L(control)
		9	*P.berghei* 268L(control)

N.B. *P. berghei* chromosome blocks used as markers/controls in tracks 8 and 9 were kindly donated by Dr. V.E. do Rosario and Prof. D. Walliker, University of Edinburgh.

a



b



IV Conclusions and Discussion

Oocyst chromosomal material separated on three pulsed field gradient gels was not visible when the DNA was stained with ethidium bromide and examined under U-V transillumination (figures 5a-7a). When the gels were blotted onto nylon membranes and probed with whole P. y. nigeriensis DNA, labelled with ^{32}P, no DNA was visible with single oocysts. However, a faint smear could be seen on autoradiographs of tracks containing many oocysts (with the midgut of the mosquito vector also present), although no chromosome-like bands could be detected.

Since the amount of DNA contained in a single oocyst is very small, it is likely that this failure to detect chromosomes by PFGE and probing of PFGE blots with labelled whole DNA was due to an insufficient amount of DNA per oocyst for this technique.

It was decided instead to apply the Polymerase Chain Reaction (PCR) to examine single oocysts taken from an infected midgut. To design PCR primers for amplification of DNA requires knowledge of gene sequence. Information about sequence diversity of P. y. nigeriensis genes was inadequate to allow parent-specific primers to be designed. However, such information is available for the human malaria parasite P. falciparum, and crossing experiments have been performed using this species. The major part of this study has therefore been to examine oocysts of P. falciparum, obtained by feeding mosquitoes on cultured gametocytes.

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Work on the human malaria parasite *Plasmodium falciparum*

V : Materials and Methods

The parasites used in this work are shown in Table 1. They are maintained in the WHO Registry of Standard Strains of Malaria Parasites in the Genetics Department of the University of Edinburgh.

For the purposes of this work, a clone is defined as a line of parasites which originated from a single parasite. An isolate consists of uncloned parasites taken from a patient on a specific occasion and maintained thereafter in culture. A strain is a somewhat non-specific term, normally denoting an isolate which has been in continuous culture for a long period, and which may have undergone a particular procedure in the laboratory, e.g., selection for drug resistance or production of gametocytes.

Isolate	Geographical Origin	Clone	Method of Cloning	References
NF54[a]	The Netherlands	3D7	Limiting Dilution*	Ponnudurai et al., 1981 Ponnudurai et al.,1982b Walliker et al,1987
H1	Honduras	HB3	Microscopic-selection**	Bhasin & Trager,1984

Table 1 : Parasite clones used in this work.

[a]This isolate was derived from a patient living near Schipol Airport, Amsterdam, who had never left The Netherlands, and was probably infected by a mosquito imported on an aircraft from a tropical country. There is some evidence that the parasite is of African origin (Collins et al., 1986).

* Rosario, 1981
**Trager et al., 1981

V.1 Incomplete medium

This medium is based on that used by Trager and Jensen (1976), consisting of RPMI 1640 (Gibco BRL) supplemented with 25mM HEPES buffer (5.94g l^{-1}) and with 50mg ml^{-1} of hypoxanthine for gametocyte cultures [Ifediba and Vanderberg, 1981]. The incomplete medium was filtered through 0.22μM Nalgene filters and stored at 4°C for up to four weeks.

V.2 Complete medium

Complete RPMI medium was made by the addition of 0.2% w/v freshly made and filtered $NaHCO_3$ (42ml l^{-1} 5% $NaHCO_3$) and of 10% v/v pooled, heat-inactivated human serum to incomplete medium immediately before use. Complete medium was used within one week, and was kept at 37°C for 24 hours before use.

V.3 Erythrocytes

Fresh whole blood, group O, Rhesus group positive, was obtained weekly from the Edinburgh and South East Scotland Blood Transfusion Service in citrate-phosphate-dextrose (CPD) adenine packs. The blood was washed and centrifuged at 1500g three times in incomplete RPMI medium to remove the citrate. The 'buffy coat' of white blood cells was removed from the red cell pellet, which was resuspended in complete medium to give a haematocrit of 50%. The washed red blood cells (RBC) were kept at 4°C for up to one week.

V.4 Cryopreservation of parasites

Parasites were preserved in liquid nitrogen at -196°C [Jensen et al., 1979]. Cultures containing a large proportion of ring stages and at a parasitaemia of at least 2% were centrifuged at 1500g for 5 minutes. The supernatants were removed and the volume of the red cell pellet was measured. An equal volume of deep freeze solution (28% glycerol, 3% sorbitol, 0.65% NaCl) was

added slowly. 0.3 to 0.5ml aliquots of this cell suspension were placed in screw-capped, polypropylene ampoules (Nunc), which were immersed immediately in liquid nitrogen.

V.5 Retrieval of parasites from liquid nitrogen

Stabilates were thawed at room temperature by a method based on Aley et al. (1984). Initially, the frozen ampoule was thawed quickly at $37^{o}C$. The volume, V, of the parasite material was measured, and 0.2 x V of a 12% NaCl solution was added slowly with mixing. This suspension was allowed to stand at room temperature for 2 minutes to draw out the freezing mixture. Following this, 10 x V of a 1.6% NaCl solution was added, and following gentle mixing the RBC were pelleted by centrifugation at 1500g for 5 minutes. The supernatant was removed and 10 x V of a 0.2% dextrose, 0.9% NaCl solution was added, again slowly and with mixing. The suspension was centrifuged once more at 1500g for 5 minutes and the supernatant removed. The RBC pellet containing thawed parasites was then put into culture.

V.6 In vitro culturing of P. falciparum.

V.6.i Asexual parasites

Freshly thawed parasites were resuspended in 3 or 5mls of complete medium containing washed RBC, to initiate a new culture at 5% haematocrit in 25ml culture

flasks (J. Bibby Science products Ltd.) [Haynes et al., 1976; Trager and Jensen, 1976]. The flasks were gassed with a mixture of 1% O_2, 3% CO_2 and 96% N_2, and maintained at 37°C. The medium was replaced with fresh pre-warmed medium daily.

Small quantities of blood were removed in a pasteur pipette, smeared on a slide, stained with Giemsa's stain at pH 7.2 (Sorensen's buffer), and examined microscopically to measure the parasitaemia and health of the culture. Whenever the parasitaemia had increased to 5-6% the parasites were subcultured, with the addition of fresh RBC to reduce the parasite density to around 1%.

V.6.ii Gametocytes

(a) Choice of parasite clones for gametocyte production

The clones routinely used in this work were 3D7 and HB3, which were known to produce good numbers of gametocytes in *in vitro* culture [Ponnudurai et al., 1982b; Bhasin & Trager, 1984]. Since gametocytogenesis in culture has been reported to wane after a period of 3-4 months [Jensen, 1979 ; Ponnudurai et al., 1982b] fresh stabilates of these clones were thawed every three months in order to set up new gametocyte-producing cultures.

(b) Manual culturing of gametocytes

15ml cultures were set up at 6% haematocrit in 75ml culture flasks (J. Bibby Science Products Ltd.) [Ifediba

and Vanderberg, 1981]. Parasitised RBC from asexual stock cultures at parasitaemias above 4% were chosen to ensure a high rate of commitment to sexual development. These were used to give a starting parasitaemia in the cultures of 0.5 to 0.7%. Cultures were gassed as for asexual parasites (section V.6.i), and were maintained at 37°C. The medium was replaced daily with prewarmed medium at 37°C.

4 to 5 days after the start of culturing when a high parasitaemia had been reached samples of the parasites were examined microscopically, for changes in morphology associated with gametocyte development. These included features such as ring forms and early trophozoites becoming somewhat triangular in shape. When such features were observed, the haematocrit was reduced to 3.6% by the addition of 25ml of complete RPMI medium instead of 15ml. The gametocyte cultures were then maintained at 25mls until mature gametocytes (stage V in the classification of Hawking *et al.*, 1971) were present. Mature gametocytes were usually harvested 14 or 17 days after the start of these cultures.

(c) Automated gametocyte cultures.

An automated culture system (the "tipper") was purchased from the Institute of Medical Parasitology, University of Nijmegen, The Netherlands [Ponnudurai *et al.*, 1982a]. 12ml volumes of parasite suspensions were prepared at 6% haematocrit, 0.5 to 0.7% parasitaemia as

for manual gametocyte cultures. Each culture was injected into a glass culture vessel through the latex rubber septum. Complete RPMI medium was stored in the medium reservoir at 4°C. Medium was changed twice daily by pumping fresh medium through a peristaltic pump to replace that drawn off automatically. The culture vessels were maintained at 37°C on the tipping table within the incubator. Gassing was continuous throughout the period of culture. The gas mixture was the same as for manual cultures but was passed through a humidifier at 37°C prior to flowing through the culture vessels. Sampling of the cultures was carried out using long, autoclavable, hypodermic needles and disposable syringes via the rubber septum on each culture flask.

V.7 Infection of mosquitoes with gametocytes from cultures.

Mosquitoes were fed on gametocytes through membrane feeders. For each experiment, one batch of mosquitoes was fed 3D7 gametocytes only, another batch was fed HB3 gametocytes only, and a third batch was fed a 1:1 mixture of 3D7 and HB3 gametocytes. The feeding procedure is outlined in figure 8.

V.7.i Counting gametocytes and estimation of mix ratios.

Thin blood smears from the culture flasks were taken regularly to assess development of gametocytes and to ensure that bacterial contamination had not occurred. The

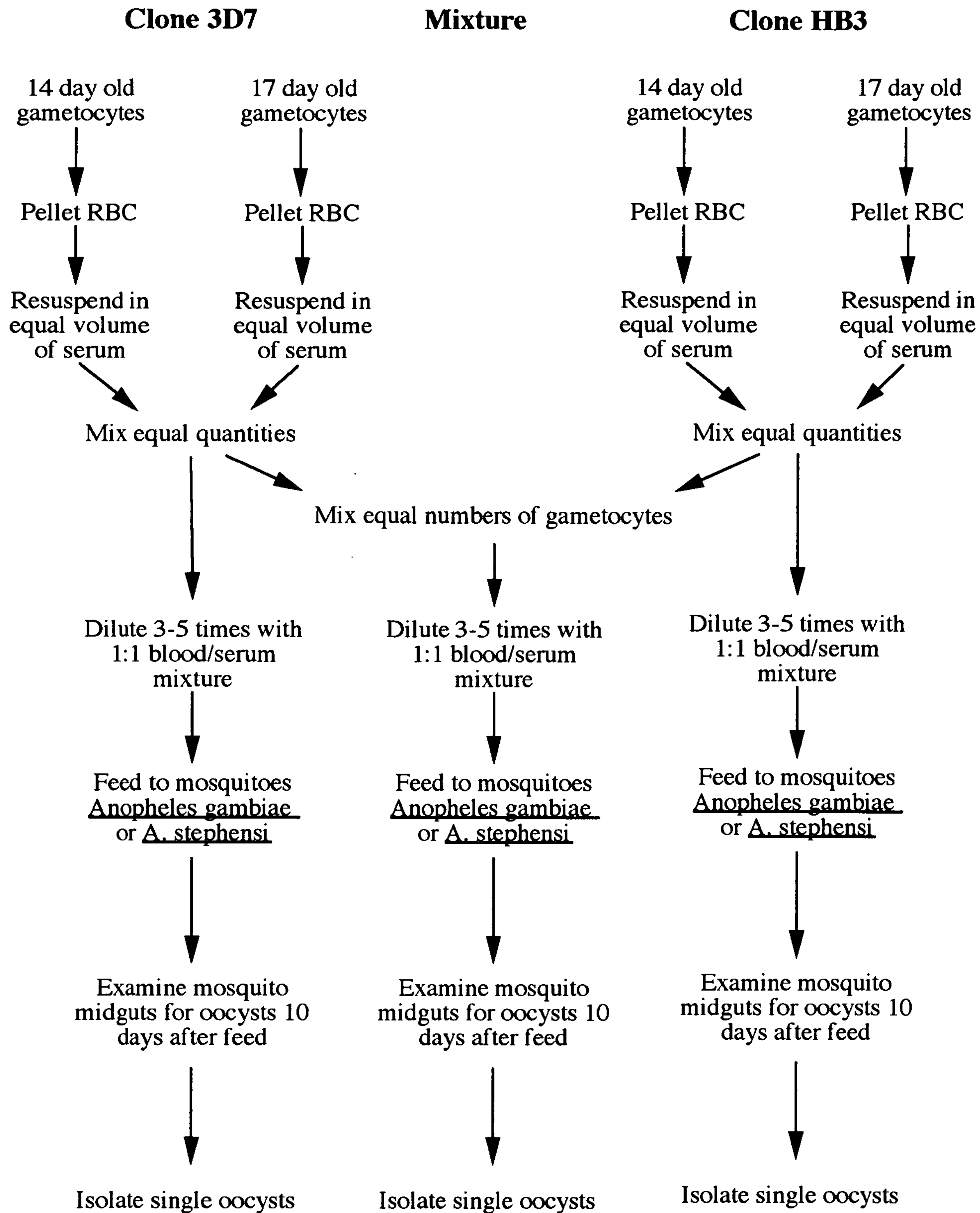


Figure 8: Outline of the procedure for feeding infectious bloodmeals to mosquitoes.

maturity of the gametocytes was assessed according to the classification of Hawking et al. (1971). When a large proportion of stage V (mature) gametocytes were present, the culture was judged to be ready for feeding to mosquitoes. This was usually after 14 days of continuous culture. In the previous crossing experiments [Walliker et al., 1987], 14 day old and 17 day old cultures of each clone were mixed together and used for each crossing experiment, as it is believed that male and female gametocytes mature at different times in a culture. This was repeated in the crossing experiments described here.

The numbers of stage V gametocytes in each culture flask were estimated from the smears and expressed as number of gametocytes per 1000 RBC. To ensure that equal numbers of gametocytes of each clone were mixed during the crossing procedure, the relative amounts of each clone to be added were calculated from the estimates of numbers of gametocytes in each flask from smears taken either on the day before or on the same day as the mosquito feed.

Sex ratio data were also obtained from these smears. The proportions of male and female gametocytes in each culture were estimated and expressed as the number of gametocytes of one sex as a percentage of the total number of mature gametocytes of both sexes [Carter & Miller, 1979].

V.7.ii Membrane feeders.

Glass membrane feeders used for feeding blood to mosquitoes were produced by Thomas P. Young Scientific Glassware, Stirlingshire, from a pattern used at the National Institutes of Health (NIH), Bethesda, Maryland, USA and described in Rutledge *et al.*, 1964 (figure 9). Baudruche membranes from bovine intestines (Long and Long Co., Belleville, New Jersey, USA, obtained from NIH) were moistened in water, stretched over the feeders and secured with elastic bands. The membranes were allowed to dry before feeding.

Water from a circulating waterbath (Grant Instruments (Cambridge) Ltd.) at 39°C was circulated through the feeders to maintain the blood at about 37°C. The maximum number of feeders connected to the waterbath was nine.

V.7.iii Mosquitoes

Adult mosquitoes were collected two days prior to an experimental blood meal, from batches of mosquitoes collected 3 to 5 days after hatching [Vanderberg & Gwadz, 1980]. Female *Anopheles gambiae* or *An. stephensi* were transferred into waxed paper cartons which had a double latex flapped entrance. These cartons had a disc of filter paper on the inside at the bottom to soak up drips of blood, glucose or water, and were securely closed at the top with fine nylon netting fastened with elastic bands and tape. Before the feed, mosquitoes were

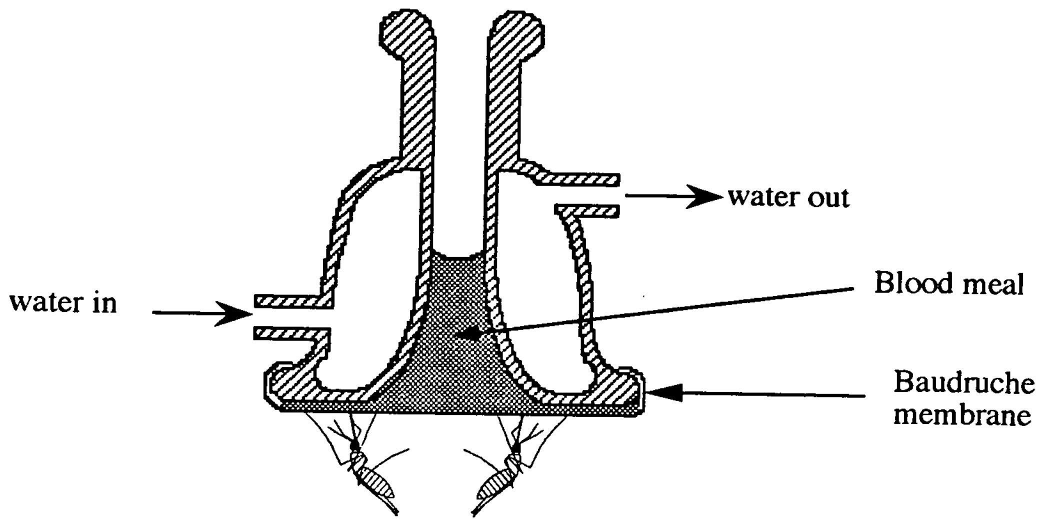


Figure 9 : Mosquito membrane feeder (after Rutledge *et al.*, 1964)

maintained without glucose for 48 hours. During this starvation period water was provided on cotton wool pads to maintain a humid microenvironment within the cartons. Feeding with infectious material took place two days after collection, when the mosquitoes were 5 to 7 days old.

V.7.iv Preparation of infectious bloodmeals.

Fresh (less than 1 week old) whole blood was washed 3 times with incomplete medium as described previously, and was resuspended in heat-inactivated pooled human serum to a haematocrit of 50%. This suspension was warmed to 37°C in a waterbath.

Most of the culture medium was removed from the flasks containing mature gametocytes and the remaining culture was transferred to 10ml centrifuge tubes at 37°C. These were centrifuged at 1500g for 5 minutes to pellet the RBC and the supernatant was removed. The pellet was resuspended in prewarmed heat-inactivated pooled human serum to a haematocrit of 50%. Equal quantities of gametocytes of 3D7 and HB3 were mixed together at this stage for mixed feeding to mosquitoes. The suspension was diluted with 3 to 5 volumes of the 50% RBC/serum suspension. After gentle mixing 1 to 2ml of this bloodmeal was placed into each membrane feeder. For each experiment bloodmeals containing 3D7 gametocytes only, HB3 gametocytes only, and a 1:1 mixture of 3D7 and HB3 gametocytes were made (figure 8). The previously starved

mosquitoes were allowed to engorge on this material for 15 to 25 minutes.

The potential viability of the gametocytes was tested by placing a drop of the bloodmeal on a glass slide and lowering a coverslip over it. The preparation was then examined under 40x Nomarski optics for exflagellation of the male gametocytes.

Following the feed, mosquitoes were maintained at 26°C and 70 to 80% humidity, and fed on a 5% glucose, 0.05% PABA (4-aminobenzoic acid) solution until they were ready for dissection (9-10 days after the infectious feed).

V.8 Dissection of infected mosquitoes.

9 to 10 days after the infectious bloodmeal, mosquitoes of each group, 3D7, HB3, or the mixture, were removed four at a time from the cartons using an aspirator and transferred to a plastic pot containing cotton wool soaked in chloroform. Anaesthetised mosquitoes were removed from this pot, dipped into 70% ethanol and kept moist in a small quantity of RPMI incomplete medium.

Mosquitoes were dissected in batches of four under a 40x magnification stereo microscope. Midguts (figure 10) were removed and examined for the presence of mature oocysts. Preparations were kept moist with incomplete medium. The proportion of mosquitoes with mature oocysts in those mosquitoes which had taken a bloodmeal was

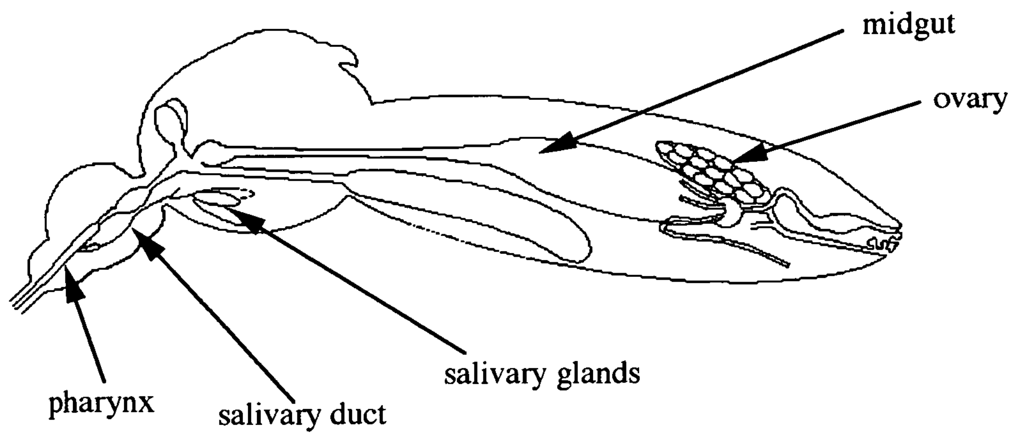


Figure 10 : Schematic diagram of the internal anatomy of a female mosquito.

calculated, and expressed as percentage infectivity.

V.8.i Removal of individual oocysts.

To avoid cross-contamination of DNA between oocysts on a midgut, or between midguts, disposable dissecting needles were necessary. By heating disposable 2μl micropipettes (Camlab) and pulling them out to a fine point, dissecting "microneedles" were obtained that were fine enough for microdissection, transparent and disposable.

Two microneedles were used to tease out a single oocyst from the rest of the midgut. The oocyst was then transferred to a tube containing lysis buffer and the microneedles thrown away. A fresh pair of microneedles were used for the next oocyst et sequentia.

V.9 Preparation of DNA from oocysts.

V.9.i Lysis and proteinase treatment of oocysts.

Single oocysts were transferred into 50μl of oocyst lysis buffer consisting of 100mM NaCl, 25mM EDTA (pH8.0), 10mM Tris-HCl (pH8.8), 0.5% sarkosyl and 1mg ml^{-1} Proteinase K (Boehringer), and incubated at 55°C for 1 hour or at 37°C overnight [Ranford-Cartwright et al., 1991b]

V.9.ii Extraction of DNA from oocysts.

1μg of salmon sperm DNA was added to the lysed oocyst mixture as carrier. The DNA was purified by extracting once with an equal volume of phenol (equilibrated against TE), once with an equal volume of a 1:1 mixture of phenol and chloroform, and once with an equal volume of chloroform [Maniatis et al., 1982]. The DNA was precipitated with propan-2-ol and the pellet washed twice with 70% ethanol before resuspending in 10μl of distilled water and boiling for 7 minutes. Oocyst DNA was stored at -20°C.

V.10 Preparation of DNA from asexual cultures

DNA from asexual cultures was used to determine the appropriate conditions for the Polymerase Chain Reaction (PCR), as well as for controls for the amplification of oocyst DNA.

5ml cultures of the two clones were allowed to reach a parasitaemia of approximately 10%, and centrifuged at 1500g for 10 minutes to pellet the cells. The supernatant was removed and the volume, V, of the pellet was measured. 0.5-1 x V of a 0.15% saponin solution in incomplete RPMI medium was added and mixed gently until lysis of the RBC had occurred. The mixture was centrifuged at 4000rpm for 10 minutes to pellet the parasites and the supernatant was removed. The pellet was transferred to a 1.5ml microfuge tube and an equal volume of buffer A (150mM NaCl, 25mM EDTA) added and mixed. This

was centrifuged in a microfuge (10 000g) for 5 minutes, the supernatant was removed and the pellet washed in buffer A twice more. Finally the pellet was resuspended in 400μl of buffer A, and 10μl of 10% SDS solution and 50μg of Proteinase K (Boehringer) were added. The mixture was incubated at 55°C for 2 hours or at 37°C overnight.

The parasite DNA was extracted once with phenol, three times with a 1:1 mixture of phenol and chloroform, and once with chloroform [Maniatis et al., 1982]. Two volumes of absolute ethanol and 0.3 volumes of 5M sodium acetate solution (pH5.2) were added and the mixture left at -20°C for 30 minutes to precipitate the DNA. The precipitate was washed twice with 70% ethanol, the pellet was dried for 10 minutes in a speedvac (Savant IEC) and was then resuspended in distilled water for 10 minutes at 37°C. The yield of DNA and its purity were estimated by spectrophotometric analysis at 260nm and 280nm. DNA solutions were stored at -20°C.

V.11 Polymerase Chain Reaction (PCR).

PCR is an in vitro method of selectively amplifying specific DNA sequences [Saiki et al., 1985, 1988]. Early studies used the Klenow fragment of Escherichia coli DNA Polymerase I to catalyse extension of annealed primers [Mullis & Faloona, 1987]. The use of a thermostable DNA polymerase, isolated from the thermophilic bacterium Thermus aquaticus simplified the procedure and allowed the reaction to be automated, as well as improving the

specificity, yield, sensitivity and length of targets able to be amplified [Saiki et al., 1988].

PCR amplification requires two oligonucleotide primers flanking the DNA sequence of interest. The procedure involves repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers with DNA polymerase. Each primer hybridises to opposite strands of the target sequence. Their position and orientation allows synthesis of new DNA by the polymerase to proceed across the region between them, thereby effectively doubling the amount of DNA of that segment. Since the extension products are also complementary to and can bind to the primers, each successive cycle can double the amount of DNA synthesised in the previous cycle. Thus there is an exponential accumulation of the specific target sequence by approximately 2^n, where n is the number of cycles performed. PCR is capable of producing selective enrichment of a specific DNA sequence by a factor of at least 10^6.

Analysis of individual diploid cells and haploid human sperm [Li et al., 1988] demonstrated the co-amplification of two genetic loci, and thus allowed the analysis of the genotype of a single diploid cell. In the present study, this technique is applied to the analysis of single P. falciparum oocysts, which contain the meiotic products of single fertilisation events.

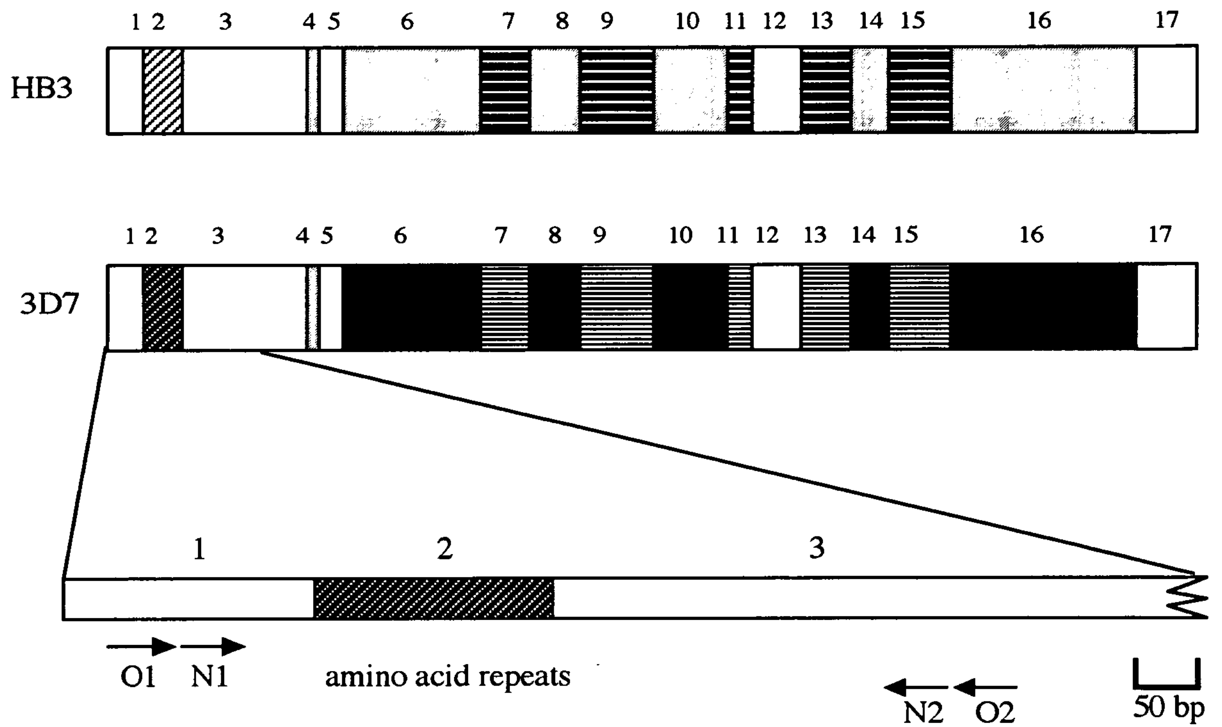
V.11.i Choice of primers

The merozoite surface antigen gene MSP1 was selected as the principal candidate for amplification with PCR. This gene has a variable block (block 2) at the N-terminal end of the molecule, between two highly conserved blocks (1 and 3) [Tanabe et al., 1987]. By selecting primers 5' in block 1 and 3' in block 3, the whole of the polymorphic block 2 may be amplified from all isolates (figure 11). Since block 2 encodes a varying number of tripeptide repeats in different isolates, size polymorphism in the PCR fragment is common. Thus different alleles of the MSP1 gene can be distinguished on the basis of the size of PCR fragments including this block.

A second merozoite surface antigen gene, MSP2, was also selected for PCR analysis. Primers were made to the 5' and 3' ends of the gene, spanning the central variable and repetitive regions (figure 12).

The sensitivity of PCR can be increased dramatically by repeating the amplification using different sets of primers. After a primary PCR amplification with an outer pair of primers, a small proportion of the product can be used as a template for a second round of PCR amplification using a different set of primers nested within the primary pair [Simmonds et al., 1990]. This PCR method has a sensitivity sufficient to detect a single

Figure 11 : Amplification of alleles of the MSP1 gene. The alleles of 3D7 and HB3 are shown as for figure 3. The positions of the outer primers, O1 and O2, and the nested primers, N1 and N2, are shown by the arrows. The exact positions of the primers are : O1: nucleotides 62-87 ; N1: nucleotides 112-131 ; O2 : nucleotides 70-685 (3D7) or 613-592 (HB3) ; N2 : nucleotides 676-659 (3D7) or 583-566 (HB3). The sizes of the PCR product obtained using the outer primer pair (O1 and O2) are 645bp. (3D7) or 552bp. (HB3), and using the nested primer pair (N1 and N2) the sizes are 565bp. (3D7) or 472bp. (HB3).


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
HB3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
3D7
1
2
3
O1
N1
amino acid repeats
N2
O2
50 bp

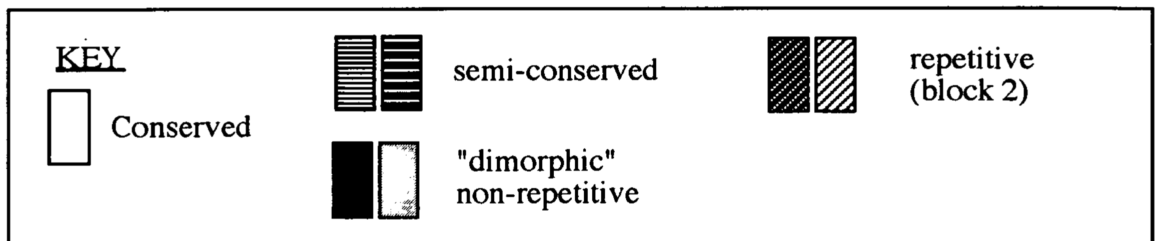
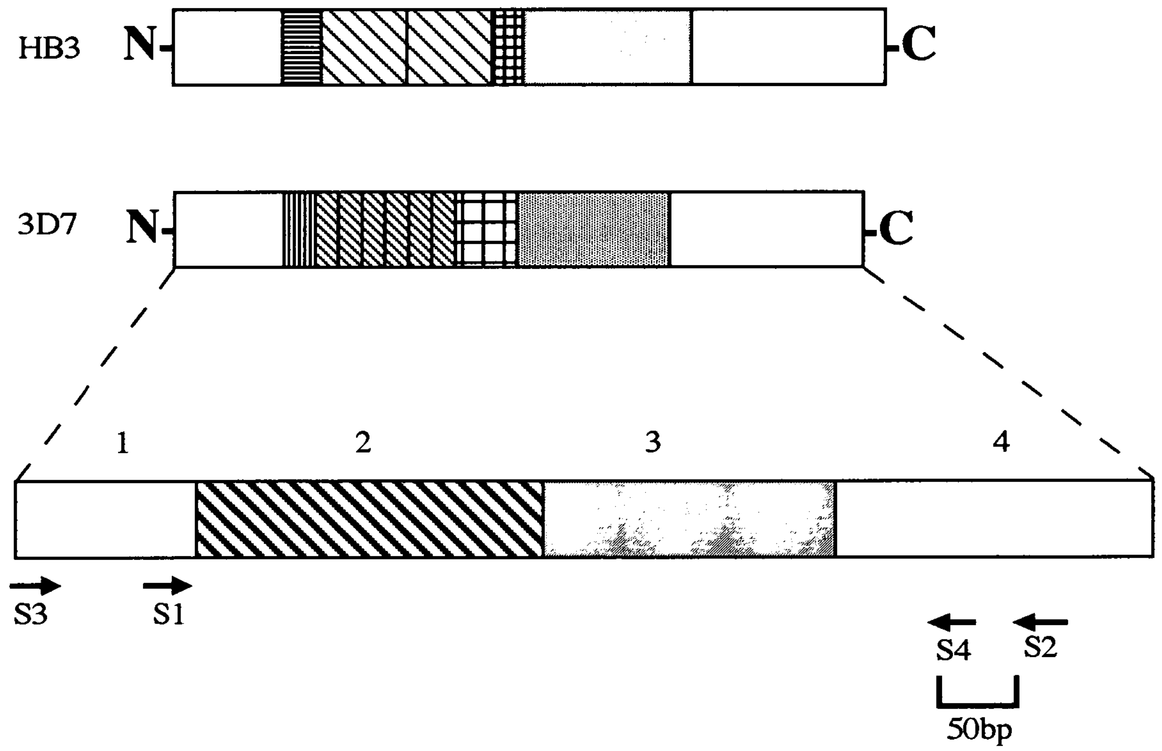
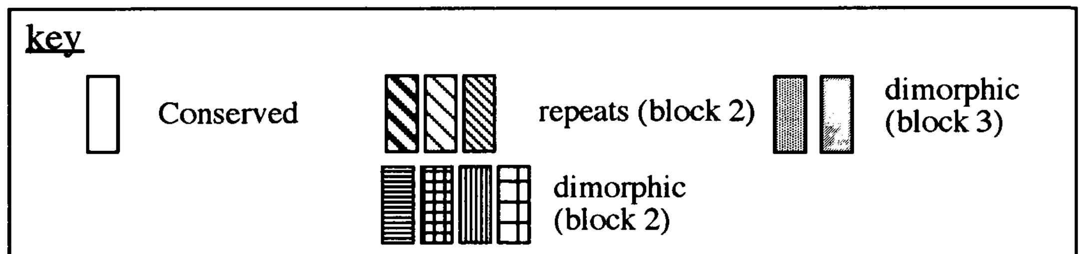

KEY
Conserved
semi-conserved
"dimorphic"
non-repetitive
repetitive
(block 2)

Figure 12 : Amplification of alleles of the MSP2 gene. The alleles of 3D7 and HB3 are shown as in figure 4. The positions of the outer primers, S3 and S2, and the nested primers, S1 and S4, are shown by the arrows. The exact positions of the primers are : S3 : nucleotides 3-23 ; S2 : nucleotides 728-707 (3D7) or 680-659 (HB3) ; S1 : nucleotides 111-129 ; S4 : nucleotides 644-625 (3D7) or 596-577 (HB3). The sizes of the PCR product obtained using the outer primer pair (S3 and S2) are 726bp. (3D7) or 678bp. (HB3), and using the nested primer pair (S1 and S4) the sizes are 534bp. (3D7) or 486bp. (HB3).


HB3
N
C
3D7
N
C
1
2
3
4
S3
S1
S4
S2
50bp


key
Conserved
repeats (block 2)
dimorphic
(block 3)
dimorphic
(block 2)

molecule of target HIV DNA in up to 5μg of human DNA [Simmonds et al., 1990].

The nested primer approach was used for analysis of DNA from individual oocysts, using primers complementary to two single-copy genes, MSP1 and MSP2. These genes were discussed in more detail in sections I.3.ii and I.3.iii.

Oligonucleotide primers were prepared by the Oswel DNA Service, Department of Chemistry, University of Edinburgh. All oligonucleotide primers were purified by HPLC before use.

Two sets of primers were used for each of the two genes amplified. Outer primers to the 5' end of the MSP1 gene are designated O1 and O2, and nested primers to these MSP1 primers are designated N1 and N2. Outer primers to the MSP2 gene are designated S3 and S2 and nested primers to these MSP2 primers are denoted S1 and S4. Further information on the primers used is displayed in table 2 and in figures 11 and 12.

Primer	length	sequence 5' to 3'	Position
O1	26	CACATGAAAGTTATCAAGAACTTGTC	5'-MSP1 Outer
O2	22	GTACGTCTAATTCATTTGCACG	3'-MSP1 Outer
N1	20	GCAGTATTGACAGGTTATGG	5'-MSP1 Inner
N2	18	GATTGAAAGGTATTTGAC	3'-MSP1 Inner
S3	21	GAAGGTAATTAAAACATTGTC	5'-MSP2 Outer
S2	22	GAGGGATGTTGCTGCTCCACAG	3'-MSP2 Outer
S1	19	GAGTATAAGGAGAAGTATG	5'-MSP2 Inner
S4	20	CTAGAACCATGCATATGTCC	3'-MSP2 Inner

Table 2 PCR primers.

All stock solutions of reagents used in DNA amplification were stored at -70°C unless in immediate use, when they were stored at -20°C.

V.11.ii PCR buffer

DNA amplification was carried out in PCR buffer which consisted of 50mM KCl (Analar, BDH), 10mM Tris-HCl

pH8.8 (Aristar, BDH), 2.5mM $MgCl_2$ (AR, Fisons) and 0.02% gelatin. Stocks of the PCR buffer were prepared from solutions which had been autoclaved (KCl and $MgCl_2$) or filtered through a 0.22μM millipore filter (Tris-Cl). Once made the buffer was aliquoted into 1.5ml microfuge tubes. Gelatin (Tissue culture grade, Sigma) was added to the buffer after dilution to a reaction concentration of 0.02%, immediately prior to use.

V.11.iii Deoxynucleotides (dNTPs)

The PCR mixture included the four deoxynucleotides (dGTP,dATP,dCTP and dTTP) at 75μM each dNTP. A 100 x stock (7.5mM) solution of mixed dNTPs was made from 100mM solutions of the dNTPs (BCL).

V.11.iv Oligonucleotide primers.

Oligonucleotide primers from the Oswel DNA Service were diluted to 10μM , aliquoted and stored at -70°C. 100nM of each primer was used per amplification reaction.

V.11.v DNA Polymerase.

The enzyme used initially was native DNA polymerase extracted from *Thermus aquaticus* (Taq Polymerase) by Cetus. The recombinant enzyme, AmpliTaq (Cetus) was used once it became available. 0.5 units of enzyme were used for each PCR.

V.12 PCR on DNA from blood-stage parasites

The most suitable conditions for DNA amplification by the PCR with all sets of oligonucleotide primers were worked out using DNA from blood-stage parasites grown in vitro. PCR was performed using standard conditions [Saiki et al., 1988] in all cases. The automated PCR apparatus used was either a Hybaid Thermal Reactor or a TRIO (Biometra).

V.12.i Reaction Conditions.

DNA was amplified in 20μl reaction volumes in 0.5ml microfuge tubes, each tube containing 100-500ng of DNA from blood-stage parasites. The reaction mix of buffer, gelatin, nucleotides and oligonucleotide primers was overlaid with 50-100μl of sterile mineral oil (Sigma) before the addition of DNA and enzyme. To avoid cross-contamination, special pipettes were kept for PCR reagents which were never used to aliquot solutions containing DNA.

All sets of amplification reactions included a negative control containing 1μg of salmon sperm DNA.

For the MSP1 inner and outer primers the amplification profile was the same :

denaturation at 94°C : 25 seconds
annealing at 50°C : 35 seconds
elongation at 68°C : 2 minutes 30 seconds.

29 cycles of amplification were performed using this profile. The final profile was identical but the elongation step was allowed to continue for 10 minutes.

Amplification profile for the MSP2 outer primers (S3 and S2) was 30 cycles of :

denaturation at $94^{o}C$: 25 seconds
annealing at $42^{o}C$: 1 minute
elongation at $65^{o}C$: 2 minutes,

followed by 10 cycles of amplification with the inner MSP2 primers (S1 and S4) following the profile:

denaturation at $94^{o}C$: 25 seconds
annealing at $50^{o}c$: 1 minute
elongation at $70^{o}C$: 2 minutes

The last cycle in each profile was identical except that the elongation step was allowed to proceed for 10 minutes.

Following amplification the reaction tubes were allowed to return to room temperature before analysis and storage at $-20^{o}C$.

V.13 PCR on oocyst DNA

Oocyst DNA was amplified using the same reaction conditions and primers as for amplification of DNA extracted from blood-stage parasites, with one exception which is discussed below.

V.13.ii Reaction conditions

1-2μl of DNA extracted from a single oocyst (section V.9) was amplified in a 20μl reaction volume, containing the same concentrations of buffer, nucleotides, primers and gelatin as for blood-stage DNA. Amplification was performed using the outer primer pair for each gene (MSP1 and MSP2). 1μl of the PCR product was then transferred to a fresh tube containing 19μl of PCR reaction mixture. The second ('nested') PCR was performed using the inner primer pair for each gene. The reaction conditions were altered as necessary for the second set of primers (section V.12.i).

V.13.ii Use of 7-deaza-2'deoxyguanosine

Oocyst DNA was amplified using the same reaction conditions as for amplification of blood-stage DNA, but 10μM of the dGTP was replaced with the base analogue 7-deaza-2'deoxyguanosine (c^7dGTP) to give a final concentration in the reaction mixture of 10μM and of 65μM dGTP. C^7dGTP was included in the 100 x dNTP stock solution used for PCR on oocyst DNA.

V.14 Preparation of size markers for agarose gel electrophoresis.

Lambda phage DNA (Sigma) was cut with the restriction enzymes *Eco*R1 and *Hin*dIII (Boehringer) using 100ng DNA per μl of digest in the appropriate buffer (Boehringer) at 37°C for 1 hour [Maniatis *et al.*, 1982].

The reaction was stopped with 0.01 x total digest volume of 0.5M EDTA.

This digest was used as an agarose gel size marker with the addition of gel loading buffer (GLB). 500ng of the digest was loaded into 0.5cm wide tracks on the gel.

The sizes of the fragments obtained from this digest are : 21 226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564 and 125 bp. [Maniatis et al., 1982].

V.15 Analysis of PCRs

Agarose gels in 1 x TBE (0.09M Tris-base 0.09M Boric acid, 0.002M EDTA) from 0.8% to 2% were tested for optimum size separation of DNA fragments between 500 and 700 base pairs. 1.5% agarose was found to produce the best separation with a reasonably short running time. Ethidium bromide (3,8-diamino-6-ethyl-5-phenylphenanthridium bromide) at 0.5μg ml^{-1} was included in the gel to stain the DNA [Le Pecq & Paoletti, 1966]. A lambda EcoR1-HindIII digest (section V.14) was also run on the gel as a size marker.

5μl of each PCR was mixed with 0.5μl of 10 x Gel Loading Buffer (0.25% bromophenol blue, 25% Ficoll, 10mM Tris-Cl, 1mM EDTA) and loaded into 0.25mm wells. Gels were run submerged in 1 x TBE at 5V cm^{-1}. The PCR fragments were visualised by shortwave U-V transillumination and photographed from above using a Polaroid system (International Biotechnologies, Inc.)

V.16 Direct sequencing of MSP1 alleles

The alleles of the MSP1 repeat region from the clones 3D7 and HB3 were sequenced directly from PCR products which were purified using Geneclean™ (BIO-101).

V.16.i Purification of PCR-amplified fragments.

DNA from asexual cultures of each of the two clones 3D7 and HB3 was amplified using the outer MSP1 primers O1 and O2 (as described in section V.12) in a total volume of 50μl. 10μl of the PCR product was electrophoresed through a 1.5% agarose gel to confirm that a fragment of the correct size had been amplified.

The remaining 40μl of amplified material was purified to remove unincorporated deoxynucleotides and primers using Geneclean™ (BIO-101).

Initially the oil and aqueous phases were inverted to facilitate the removal of the lower aqueous phase containing the amplified DNA. 250μl of chloroform was added and the tube was vortexed briefly. The aqueous phase (which was now the top phase) was transferred to a fresh tube for genecleaning.

2 volumes of a 6M sodium iodide solution and 5μl of a silica matrix suspension in water (Glassmilk™) were added to the PCR mixture, vortexed briefly and incubated on ice for 5 minutes to allow the binding of the DNA to the silica matrix. The suspension was then centrifuged in a microcentrifuge for 5 seconds and the sodium iodide supernatant removed, taking care not to dislodge the

silica matrix pellet. This pellet was washed twice with 10-50 volumes of ice-cold NEW (NaCl/ethanol/water wash, as supplied by BIO-101), pulse-spinning after each wash and removing the supernatant. Following the last wash, the final traces of the NEW wash were aspirated off using a fine pipette. The DNA was eluted from the Glassmilk™ by resuspension in 10μl of TE (10mM Tris-Cl, 1mM EDTA, pH7.5) and incubating at 45°C for 5 minutes. The suspension was centrifuged for 30 seconds to pellet the Glassmilk™ and the supernatant containing the DNA was transferred to a new tube. A second elution of DNA from the Glassmilk™ was carried out using a further 10μl of TE, and the two supernatants were pooled.

V.16.ii Sequencing reaction.

5-6μl of the DNA solution purified using Geneclean™ was used for each sequencing reaction.

Dideoxy sequencing [Sanger et al., 1977] was performed using Sequenase® Version 2.0 enzyme and sequencing kit (United States Biochemical Corp., Cleveland, Ohio, USA), following a modification of the protocol of Winship (1989), which includes dimethyl sulphoxide (DMSO) at 10% in the sequencing reactions to reduce background and prevent formation of secondary structure when sequencing double-stranded DNA fragments. Both strands of each PCR fragment were sequenced.

Sequencing involves three steps: annealing of the primer and template, elongation and incorporation of

radiolabelled deoxynucleotides, and termination of sequencing using dideoxynucleotides.

(a) Annealing

The final volume of the annealing reaction was 10μl. The reaction conditions were: 40mM Tris-Cl pH7.5, 20mM $MgCl_2$, 50mM NaCl, 10% DMSO, 10ng primer (O1 or O2), 100-200ng DNA (6μl). The mixture was boiled for 3 minutes to denature the template and then immediately snapcooled on ice to minimise renaturation. The annealed template was used within 4 hours.

(b) Elongation and incorporation of radiolabelled deoxynucleotides

To sequence from 20 to 200 bases from the primer, 1μl 0.1M DTT, 1μl [α-^{35}S]dATP (1000Ci mM^{-1}, 10μCi μl^{-1}, Amersham), 1μl 1:15 Labelling Mix (0.5μM each dGTP, dCTP, dTTP) and 1μl (2 units) Sequenase^R (diluted in enzyme dilution buffer of 10mM Tris-Cl pH 7.5, 5mM DTT and 0.5mg ml^{-1} BSA) were added to the cooled annealing mixture and incubated at room temperature for 2 to 5 minutes.

To sequence further away from the primer, 1μl of 1:5 Labelling Mix (1.5μM each of dCTP, dGTP and dTTP) was added instead of the 1:15 Labelling Mix and incubated as before.

(c) Termination

4 tubes of termination mix labelled G,A,T and C were prepared for each fragment of DNA to be sequenced. Each tube contained 2μl of termination mix (80μM dGTP, 80μM

dATP, 80μM dCTP, 80μM dTTP, 50μM NaCl, 10% DMSO, 8μM ddGTP (tube G), 8μM ddATP (tube A), 8μM ddCTP (tube C), 8μM ddTTP (tube T)). Immediately after the labelling/ sequencing incubation, the mixture was divided into four and each quarter added to one of the four termination tubes G,A,T and C. The termination reaction was allowed to continue for 5 minutes at 37°C. 4μl of Stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) were added to each tube and the tubes stored at -20°C and analysed within one week.

V.16.iii Preparation of polyacrylamide sequencing gels

Acrylamide sequencing gels were prepared 2 to 10 hours before use and were stored in a warm room (30°C). Sequencing gels were run on a Baserunner apparatus (International Biotechnologies, Inc.).

(a) Plates

Gel plates were thoroughly cleaned to remove grease and old siliconiser using xylene or acetone, and were then washed twice with 70% ethanol and once with acetone. One of the two glass plates was coated with siliconiser (Sigmacote™, Sigma).

(b) Acrylamide gel

100ml of a 6% denaturing polyacrylamide gel (8M Urea, 6% acrylamide, 0.16% bis-acrylamide, 0.08% ammonium persulphate in 1 x TBE) were used for each gel. The gel

mixture was filtered through a 0.2μM filter into a sidearm flask and degassed. 30μl of TEMED was added to catalyse the polymerisation and the gel was poured onto the plates using the sliding plate method. A shark's tooth comb was put in (upside-down) and the plates were clamped together until the gel had set. Once set, the full apparatus was assembled and the comb removed. Any urea crystals were washed away, the buffer reservoirs were filled with 1 x TBE and the comb was replaced, teeth downwards, to form wells. The gel was pre-run for 15 to 30 minutes at 30 to 40W.

V.16.iv Loading and running.

Just before loading, the four samples G,A,T and C were warmed to 80°C for 2 minutes. 1 to 3μl of each were loaded onto the pre-run, warm gel in the order G-A-T-C. Gels were run at 40W (first run) or 30W (to increase the distance from the primer) for 2.5 to 4 hours, keeping the gel temperature below 60°C. An electrophoresis power pack offering constant voltage operation at 2000V or greater was used (International Biotechnologies, Inc.).

V.16.v Fixing and drying gels

Following electrophoresis the siliconised top plate was removed carefully and the gel which remained stuck to the lower plate was fixed by soaking in 1 litre of 7% acetic acid, 7% methanol for 15 minutes, followed by a second wash in another litre of fixative for 15 minutes.

This washing/fixing diluted the urea to less than 1%, decreasing the stickiness of the gel and abolishing the quenching effect of urea on ^{35}-S.

Following fixing, the gel was drained and a piece of Whatman 3MM filter paper cut to size was lowered over it and smoothed out. The paper and gel were then removed from the glass plate and dried on a vacuum dryer (Hoeffer Scientific Instruments) for 1 to 1.5 hours at 75°C.

V.16.vi Autoradiography

The dried gel was exposed to Agfa Curix film in an autoradiography cassette (Kodak) for 24 to 48 hours and then the film was developed in an automatic Autoradiograph developer (Exograph). Autoradiographs were read on a lightbox (Kodak) and the sequences were entered into a computer (University of Wisconsin Genetics Computer Group, Ercvax, University of Edinburgh) for analysis, comparison with other known sequences on databases (EMBL, Genbank), etc.

VI : Results

Mosquito infectivity studies

VI.1 Gametocyte production

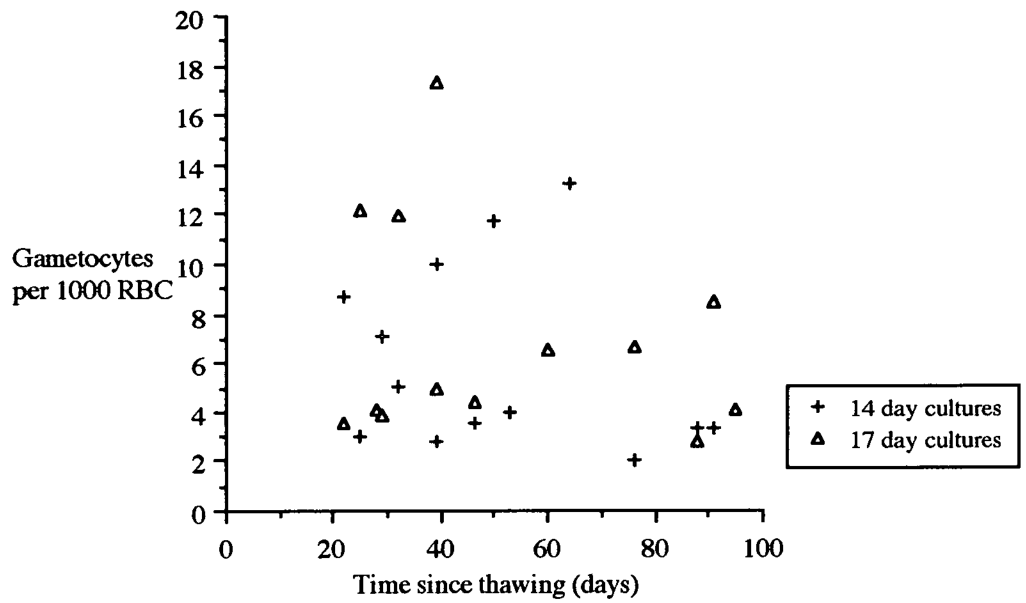
Gametocytes were produced _in vitro_ using medium supplemented with hypoxanthine. No antibiotics, such as gentamycin, were used. Gentamycin has been shown to reduce the incidence of bacterial contamination in cultures, but in our experience affects the infectivity of gametocytes to mosquitoes.

Levels of both 3D7 and HB3 gametocytes produced _in vitro_ were highly variable, but remained at a reasonable level for up to three months after the date that cultures were set up from deep-frozen ampoules. After this time, the number of gametocytes produced in cultures began to fall, especially with HB3 cultures (data not shown). Fresh ampoules were therefore removed from storage in liquid nitrogen to establish new cultures every 3 months, or earlier if gametocyte numbers began to fall off.

VI.2 Sex ratios of gametocytes grown in vitro

The ratio of female to male mature (stage V) gametocytes in cultures fed to mosquitoes was estimated from blood smears taken on the day of the feed (14 or 17 day cultures in most cases). The sex ratio data for gametocytes of both 3D7 and HB3 are shown in Appendix 2, and graphically in this section.

(a)



(b)

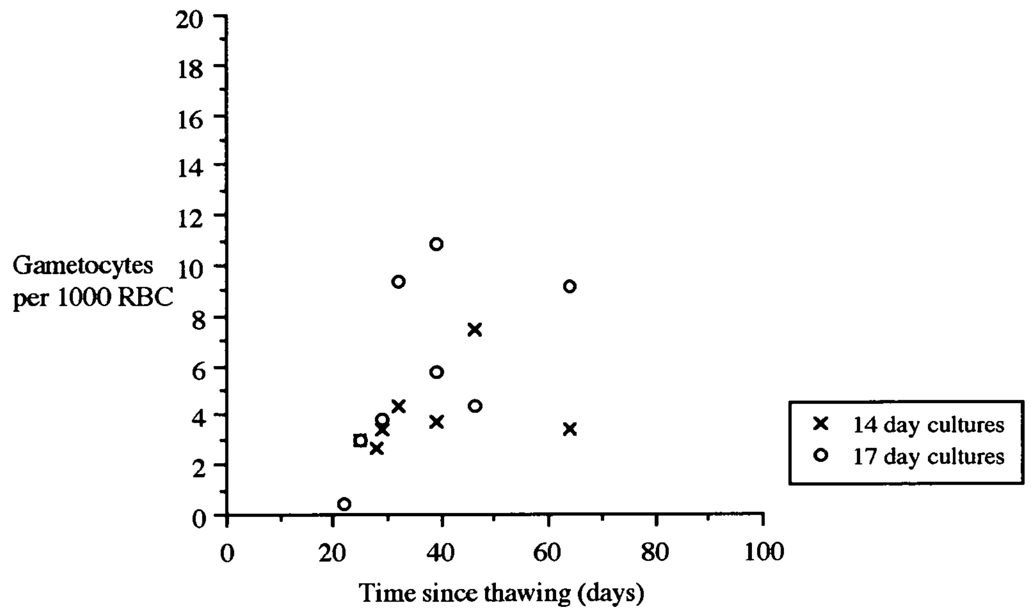


Figure 13

Time since thawing of cultures compared to number of gametocytes per 1000 RBC

(a) for 3D7 cultures
(b) for HB3 cultures

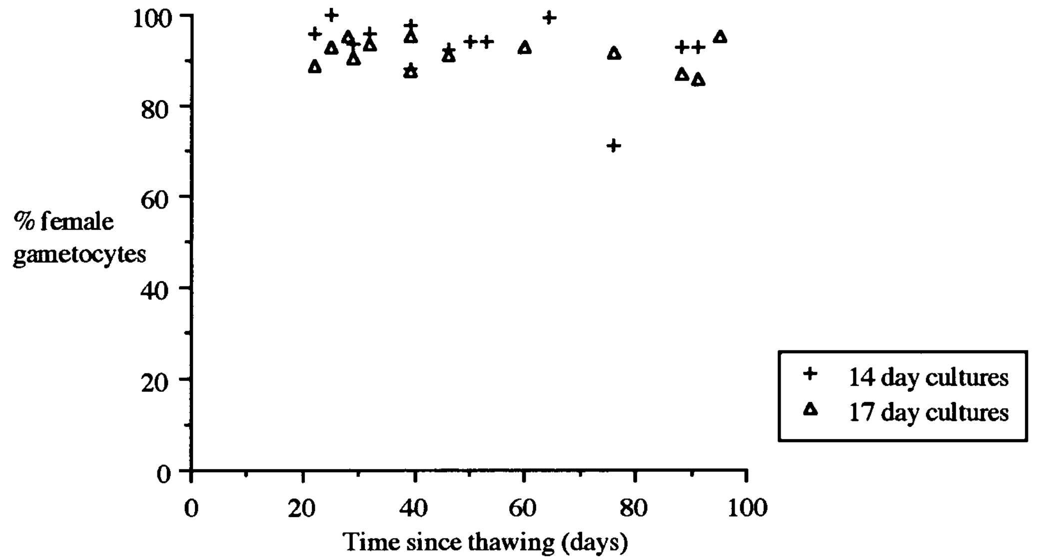
Sex ratios, expressed as the percentage of total mature gametocytes which were female, of both clones, remained fairly constant as the time post-thawing of the stock culture increased (figure 14), although there was perhaps a slight increase in the percentage of gametocytes which were female with increasing time since thawing for HB3 cultures.

		% female g'cytes		female g'cytes /1000 RBC	
Clone	No.of slides	14 day g'cytes	17 day g'cytes	14 day g'cytes	17 day g'cytes
3D7	33	91.6 (7.2)	91.7 (4.7)	5.9 (3.8)	5.6(4.4)
HB3	18	83.4 (6.1)	81.1 (8.8)	3.4 (1.4)	4.7(3.1)

Table 3. Means (and standard deviations in brackets) of percentages of mature female gametocytes, expressed as a % of all mature gametocytes, and number of gametocytes per 1000 RBC of 3D7 and HB3 cultures, 14 and 17 days old.

Smears of 3D7 cultures taken 14 days after the start of a culture exhibited approximately the same sex ratios as in day 17 smears (table 3), suggesting that there was little difference in the time taken for male and female gametocytes to mature fully in this clone. The fraction

(a)



(b)

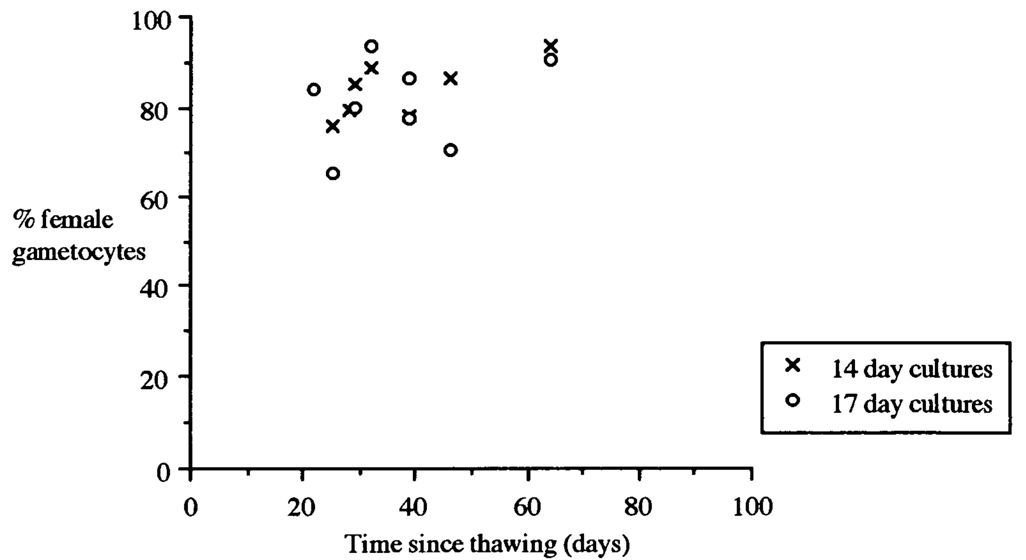


Figure 14

Time since thawing of cultures compared to % mature female gametocytes

(a) for 3D7 cultures
(b) for HB3 cultures

of mature gametocytes that were female decreased slightly from 14 day cultures to 17 day cultures for HB3, although this was not significant. Such a decrease might suggest that male gametocytes take slightly longer to reach maturity than females. The total number of mature gametocytes in a 14 day culture was slightly greater than for a 17 day culture for HB3, but again this was not significant.

Pooled sex ratio data (D14 plus D17) for the two clones used is shown in table 4. Mature 3D7 cultures had a mean of 92% mature female gametocytes, and mature HB3 cultures had a mean of 82% females. The significance of the difference in these two means was tested as follows.

Clone	No. of slides, n	Mean % females, Y	Standard deviation S.D.	Variance s^2
3D7	33	91.7%	± 5.98	35.8
HB3	18	82.1%	± 7.37	54.3

Table 4 : Data on means of percentages of mature female gametocytes (as a proportion of all mature gametocytes) for the two clones used in crossing experiments. Full data are shown in Appendix 2.

In order to compare the two means using a parametric test, such as Student's t-test, the variances in the populations from which the two samples are taken are assumed to be equal. To test this assumption, the significance of the difference in the two variances was tested using the F-distribution. Full workings can be seen in appendix 3. It was concluded that the two sample variances are not significantly different from one another.

Therefore the significance of the difference of the two means can be tested using Student's t-test. Again, the full workings are shown in appendix 3. The mean of the percentage of mature gametocytes which are female for 3D7 cultures was found to differ significantly from that for HB3 cultures. This will be taken into account when calculating allele frequencies in section VI.10.

VI.3 Mosquito infection rates

There was great variation from feed to feed in the proportion of mosquitoes that took a full blood meal when it was offered through a membrane feeder. The proportion feeding could be improved by starving mosquitoes for 48 hours prior to the membrane feed.

The infection rate was the percentage of mosquitoes which, after taking a blood meal, developed oocysts. Mosquitoes were examined for oocysts 11 days after the membrane feed. Infection rates varied from one batch of mosquitoes to another, and it was difficult to account

for this variation. However, the following conclusions could be drawn from the observations made during this study :

(a) There was no correlation between infection rate and the species of mosquito used, *Anopheles stephensi* or *A. gambiae*. Most of the feeds used *A. stephensi*.

(b) There was no relationship between the time since thawing of the stock culture and the infection rate in mosquitoes obtained for 3D7 (figure 15). For HB3 cultures, infection rates dropped to zero after about 65 days post-thawing

(c) There was a correlation between the number of gametocytes per 1000 RBC at the time of the feed and the infection rate for both 3D7 and HB3 cultures (figure 16). This correlation was also noted for the number of female gametocytes per 1000 RBC in the cultures (figure 16).

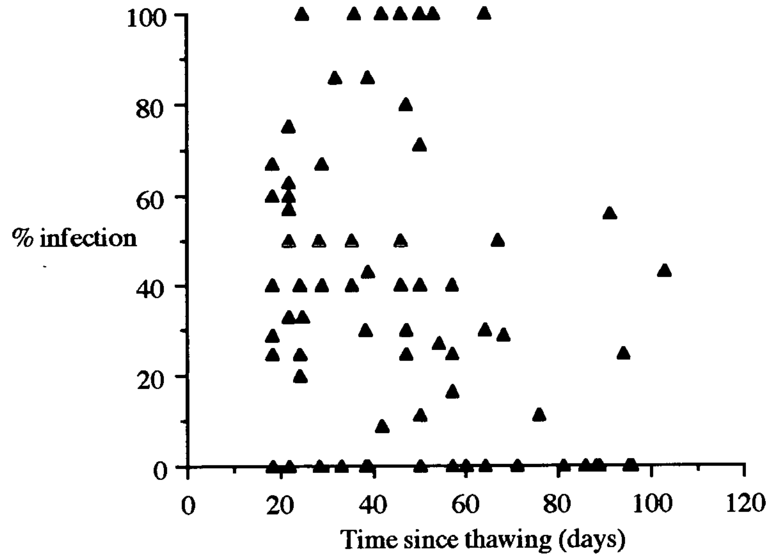
(d) There was no relationship between the percentage of gametocytes which were female in a culture and the infection rate (figure 17). HB3 cultures were less infective to mosquitoes (as determined by infection rates obtained) than 3D7 cultures.

VI.4 Oocyst numbers

Two methods for quantification of oocysts numbers were used. The mean no. of oocysts per infected gut [Burgess, 1960] is calculated as:

$$\frac{\text{Total no. of oocysts}}{\text{Total no. of infected mosquitoes}}$$

(a)



(b)

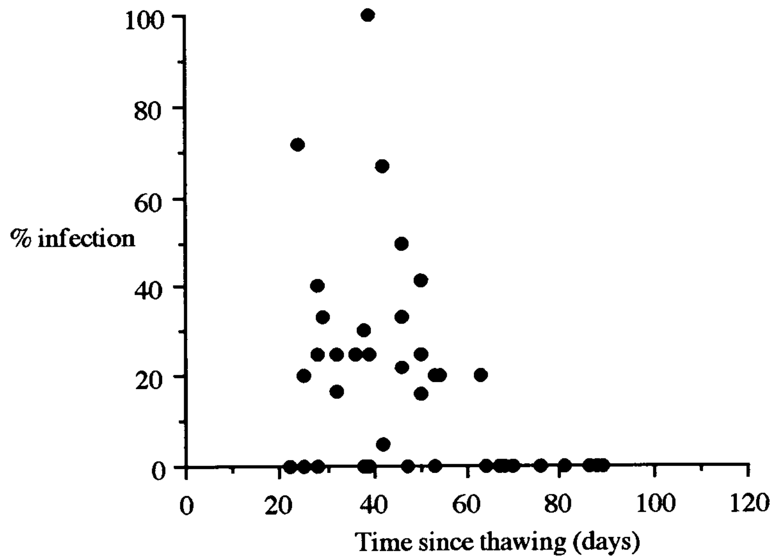
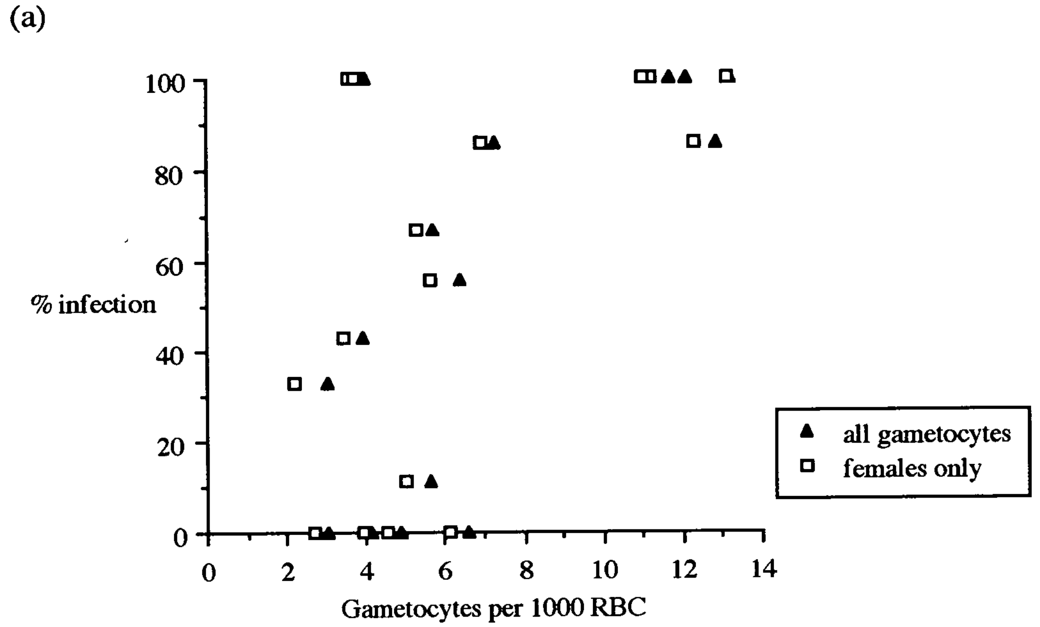


Figure 15

% infection of mosquitoes compared to the age of the culture since thawing
(a) for 3D7 cultures
(b) for HB3 cultures

Each point represents a different mosquito feed.

(a)



(b)

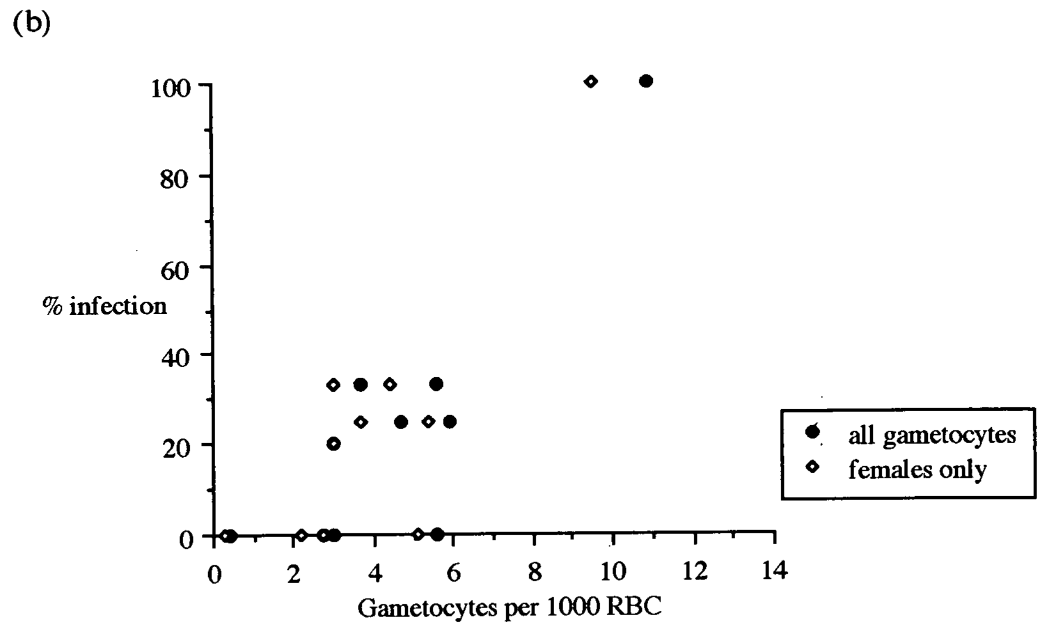
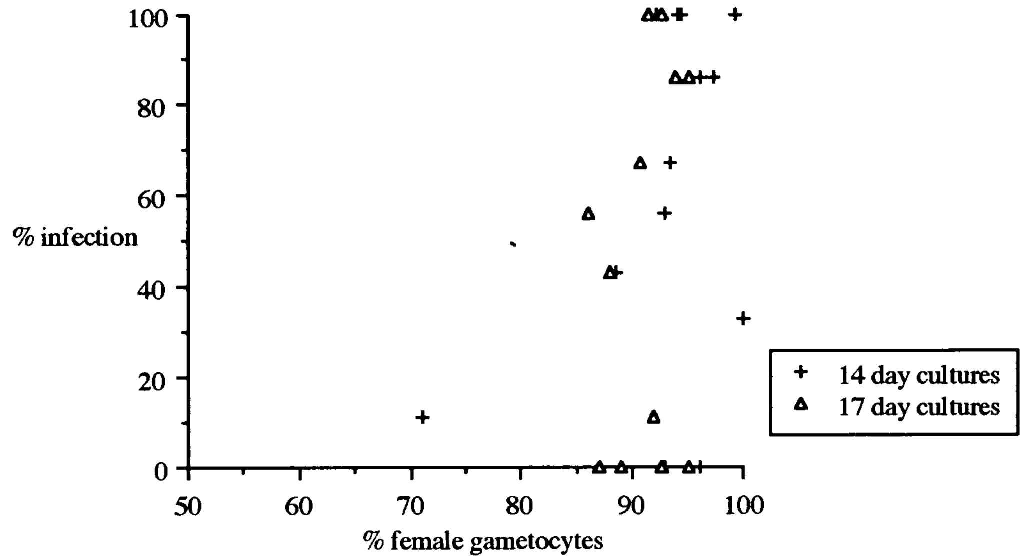
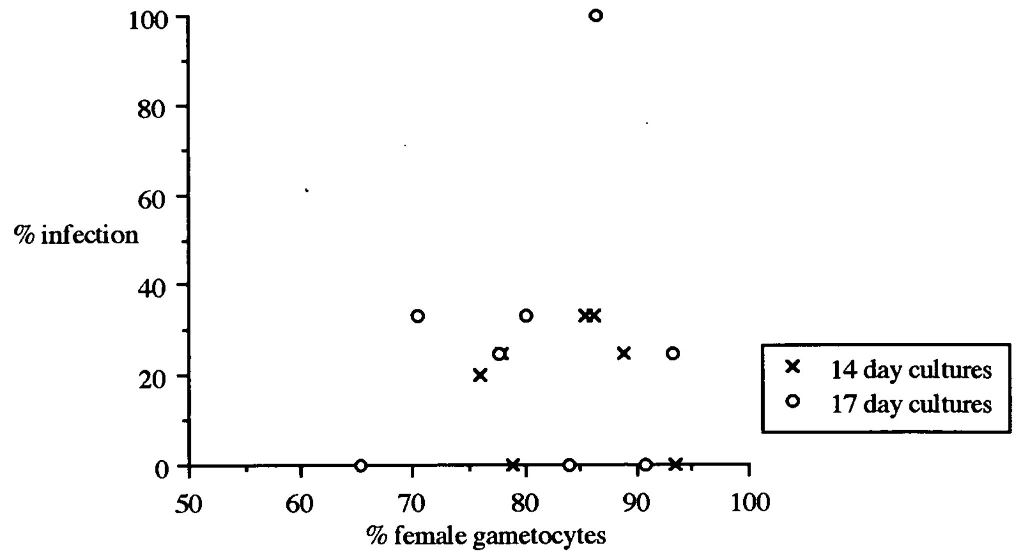


Figure 16
% infection of mosquitoes compared to the number of gametocytes per 1000 RBC in cultures fed to mosquitoes
(a) for 3D7 cultures
(b) for HB3 cultures
Each point represents a different mosquito feed.

(a)



(b)



<u>Figure 17</u>
% infection of mosquitoes compared to the % mature female gametocytes in cultures fed to mosquitoes
(a) for 3D7 cultures
(b) for HB3 cultures

The gut infection index (GII) [Collins, W.E. 1962 ; Collins, W.E. et al., 1977] is calculated as :

$$\frac{\text{Total no. of oocysts}}{\text{Total no. of mosquitoes}} \times 100$$

VI.4.i Mean no. of oocysts per infected gut

Mean oocyst numbers were influenced by the time since thawing of the cultures (figure 18). The highest numbers of oocysts per infected gut were usually obtained with cultures around 40 days post-thaw date. For 3D7 cultures, there was a drop in mean oocyst number after 60 days, although oocysts were still found in lower numbers up to 100 days post-thaw date. Mean oocyst numbers were lower for HB3 cultures than for 3D7. HB3 mean oocyst numbers peaked when cultures around 40 days post-thaw date were used, and declined towards zero in cultures past 65 days post-thaw date.

Mean oocyst numbers did not correlate with infection rates of mosquitoes (figure 19). However, at low infection rates, fewer infected midguts were found from which to take an average oocyst number. These figures are therefore very susceptible to sampling errors.

There was no relationship between mean oocyst numbers and the number of gametocytes per 1000 RBC, or the number of female gametocytes per 1000 RBC, in 3D7 cultures used for membrane feeds (figure 20). HB3 cultures showed a positive correlation between both

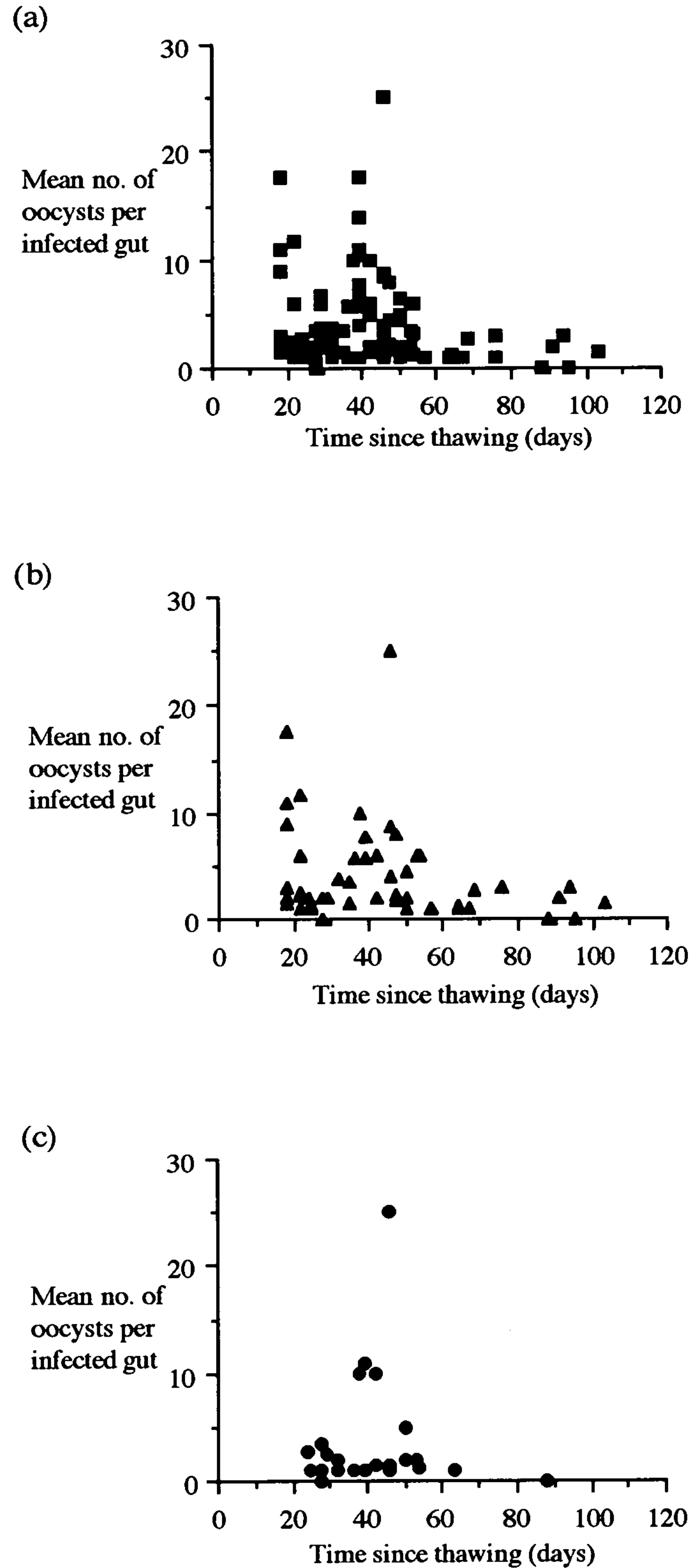


Figure 18

Mean number of oocysts per infected midgut compared to the age of the culture since thawing

(a) for all feeds
(b) for 3D7 feeds only
(c) for HB3 feeds only

Each point represents a different mosquito feed.

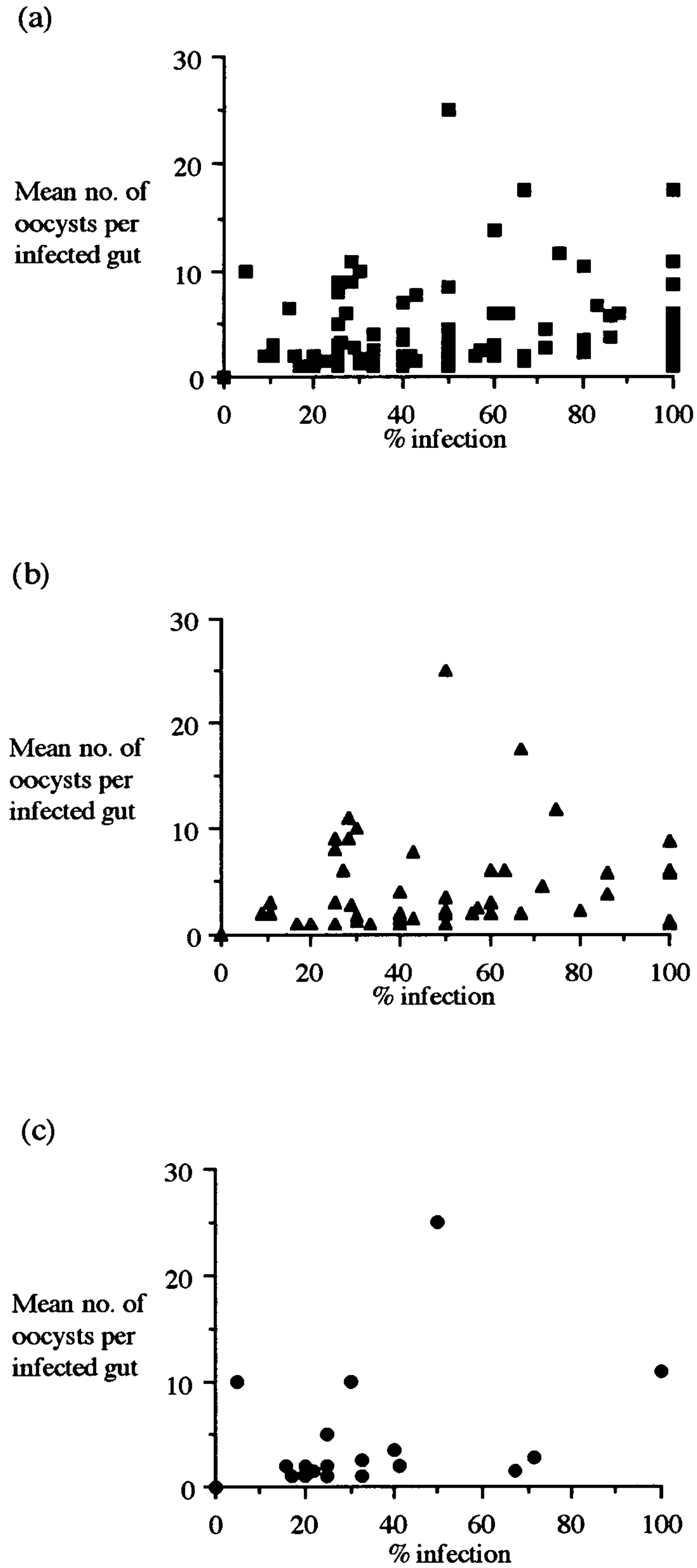


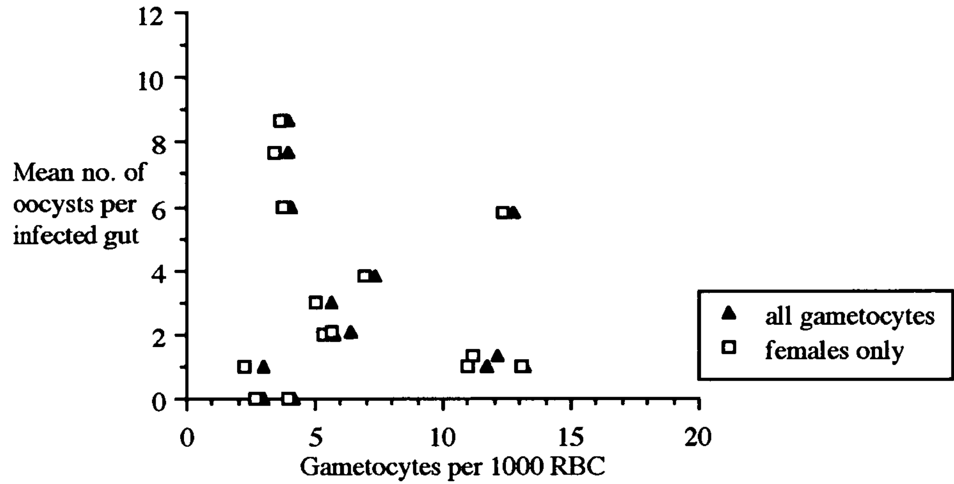
Figure 19

Mean number of oocysts per infected midgut compared to % infection of mosquitoes

(a) for all feeds
(b) for 3D7 feeds only
(c) for HB3 feeds only

Each point represents a different feed

(a)



(b)

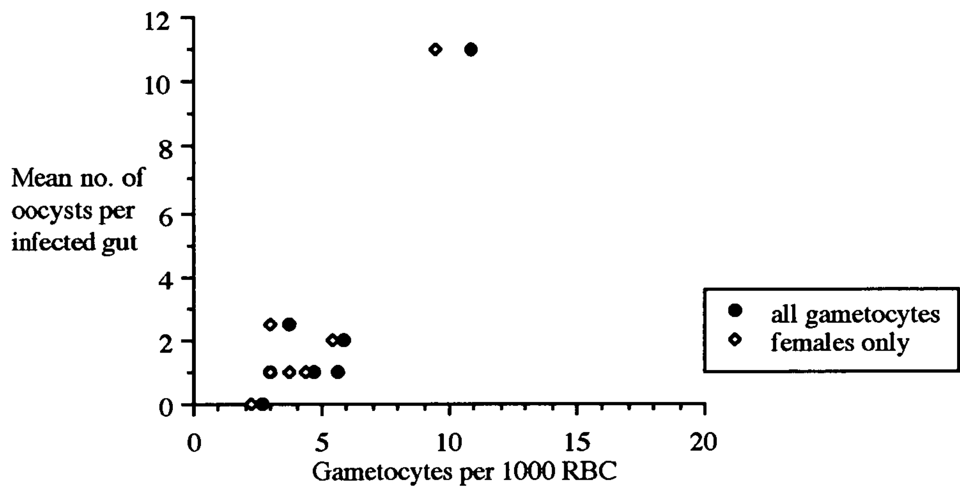


Figure 20

Mean number of oocysts per infected midgut compared to the number of gametocytes per 1000 RBC in cultures fed to mosquitoes

(a) for 3D7 cultures and feeds
(b) for HB3 cultures and feeds

Each point represents a single mosquito feed.

gametocytes per 1000 RBC and female gametocytes per 1000 RBC and mean number of oocysts per positive gut, although the number of data points is small.

Mean oocyst numbers seemed to be slightly influenced by the percentage of female gametocytes in cultures fed to mosquitoes (figure 21), although the difference is not significant. Mean oocyst numbers were lower in the small number of cultures which had lower % female gametocytes.

VI.4.ii Gut infection index (GII)

There was a relationship between gut infection indices and the time since thawing of cultures (figure 22). Highest GII were obtained for cultures most recently thawed, although the range of GII was large for all feeds. These high GII levels are explained by high mean oocyst numbers per infected guts and high infection rates for these cultures (see figures 15 and 18). For HB3 feeds, GII were zero after cultures passed 60 days post-thaw date, reflecting the low or non-infectivity of these cultures (see figure 15). Lower mean oocyst numbers per infected gut could explain the fall in 3D7 GII after 60 days (see figure 18).

GII were higher for 3D7 cultures than for HB3 cultures, again reflecting the higher infection rates and mean oocyst numbers per infected gut for 3D7 cultures.

There was no correlation between GII and the number of gametocytes per 1000 RBC, or the number of female gametocytes per 1000 RBC, in 3D7 cultures fed to

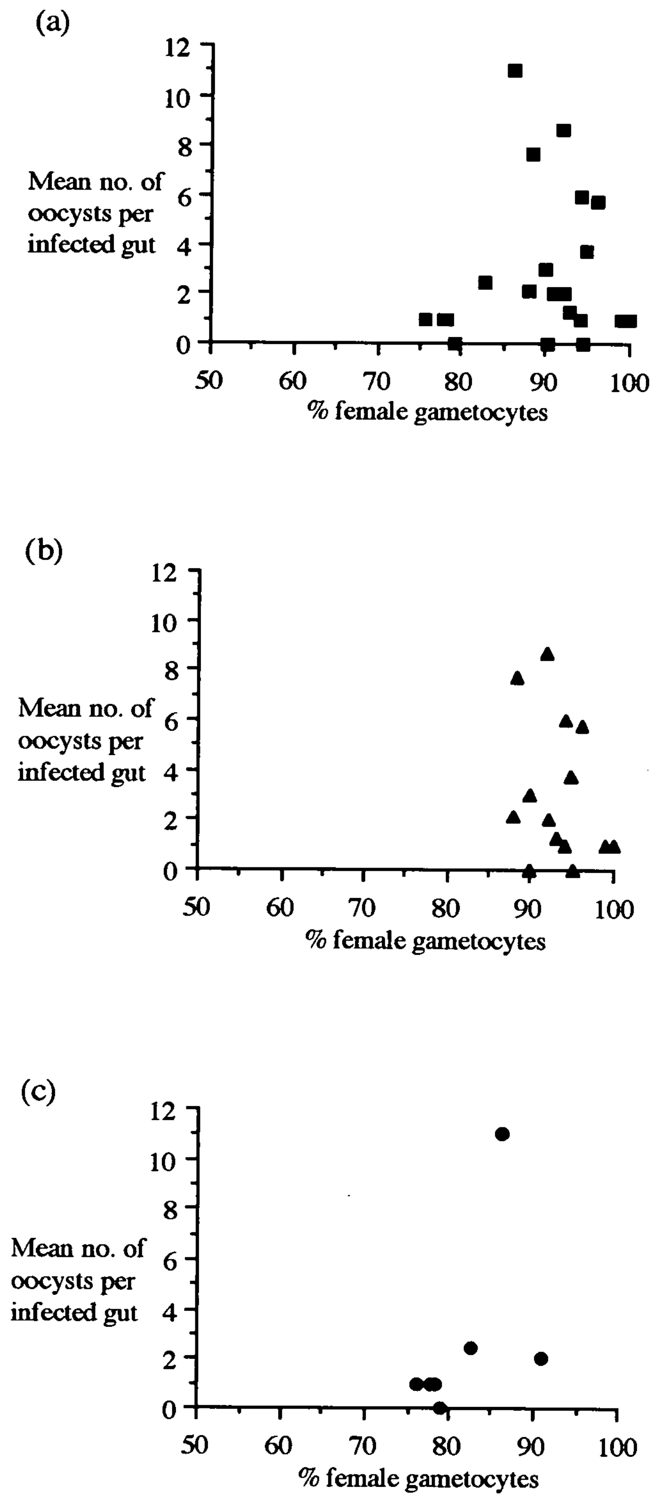


Figure 21

Mean number of oocysts per infected mosquito compared to the % female mature gametocytes in cultures fed to mosquitoes

(a) for all feeds
(b) for 3D7 feeds only
(b) for HB3 feeds only

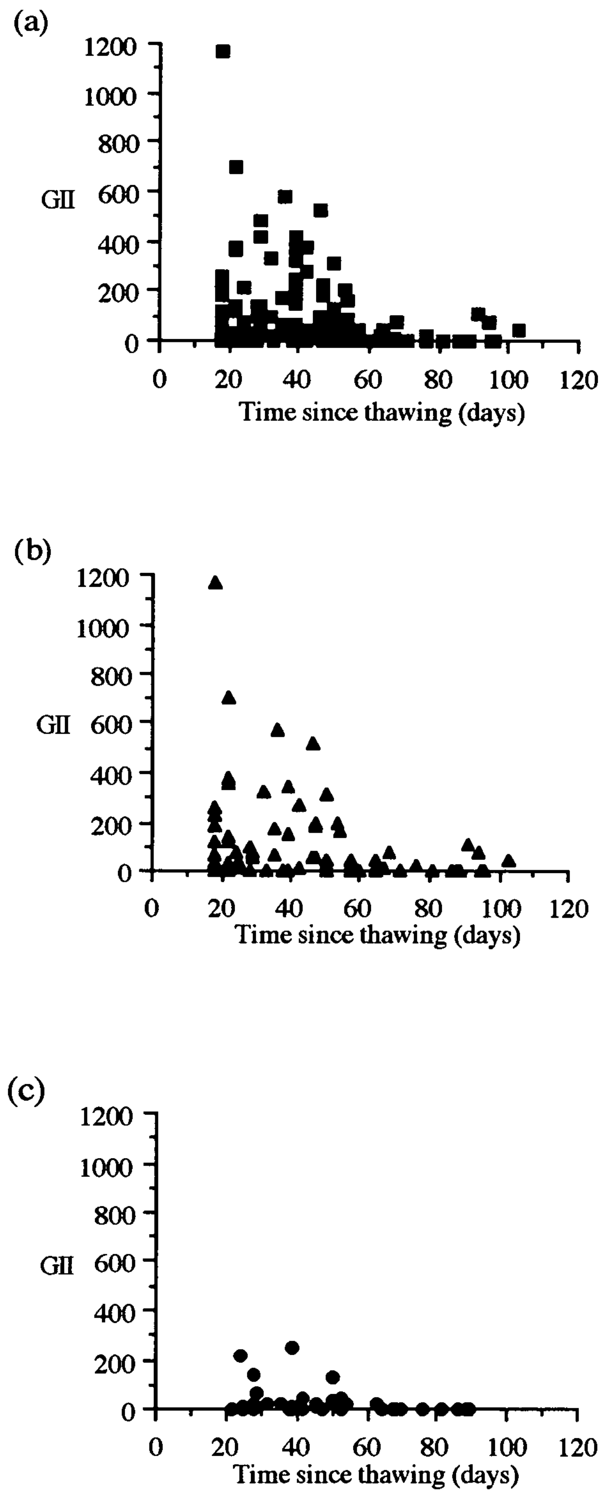


Figure 22

Gut infection index (GII) compared to age of cultures fed to mosquitoes since thawing

(a) for all feeds
(b) for 3D7 feeds
(c) for HB3 feeds

mosquitoes (figure 23). The GII obtained with HB3 cultures increased as the number of gametocytes per 1000 RBC increased.

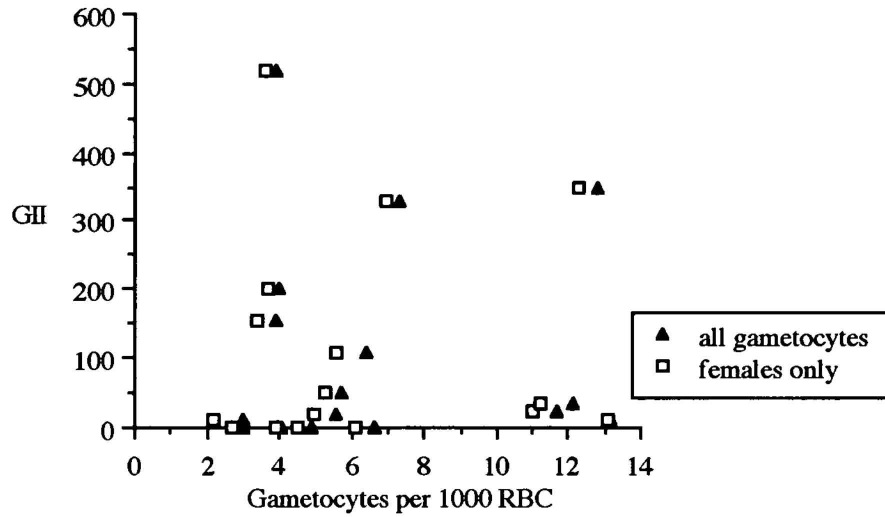
VI.5 Summary of gametocyte production and mosquito infections

Gametocyte production in cultures of HB3 fell after around 60 days post-thaw date. Sex ratios in both 3D7 and HB3 remained constant in all cultures, but the mean sex ratios of the two clones differed significantly from one another.

Infection rates of mosquitoes obtained from different feeds were highly variable, and there was no correlation of infection rate with time post-thaw of the culture or % female gametocytes in the culture. Infection rates were slightly increased with increasing numbers of gametocytes per 1000 RBC in the cultures. HB3 cultures were less infective to mosquitoes than 3D7 cultures. They produced fewer gametocytes per 1000 RBC, and fewer of these gametocytes were female.

Mean oocyst numbers per infected mosquitoes were higher for 3D7 than for HB3. Mean oocyst numbers were highest with cultures of around 40 days post-thaw date. For HB3, oocysts numbers fell to zero past 65 days post-thaw date. There was no relationship between oocyst numbers and infection rates, number of gametocytes, or female gametocytes per 1000 RBC, or with % female gametocytes for 3D7 cultures. HB3 oocyst numbers

(a)



(b)

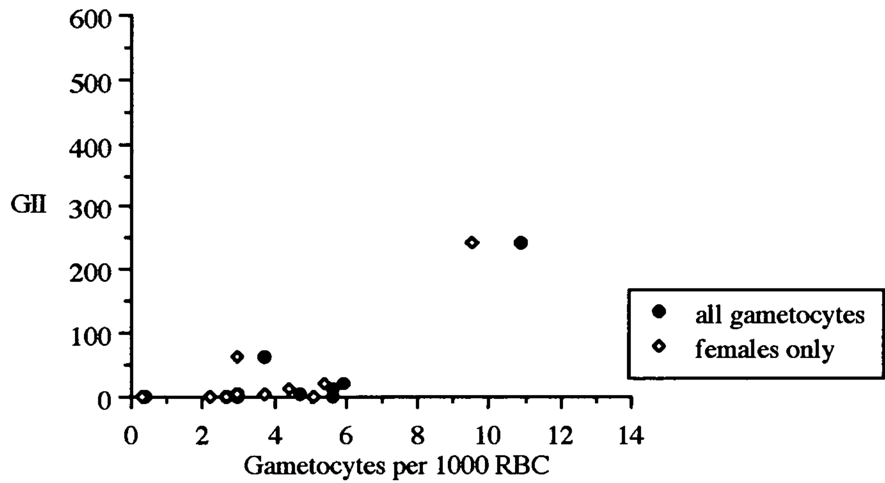


Figure 23

Gut infection index (GII) compared to the number of gametocytes per 1000 RBC in cultures fed to mosquitoes

(a) for 3D7 cultures and feeds
(b) for HB3 cultures and feeds

correlated with the number of gametocytes per 1000 RBC and with the number of female gametocytes per 1000 RBC. Gut infection indices peaked for the lowest times post-thaw of cultures, although the range was large for all times post-thaw. Changes in GII could be accounted for by changes in infection rates and mean oocyst numbers per infected gut for the cultures. GII did not correlate with the number of gametocytes per 1000 RBC for 3D7 cultures, but did correlate for HB3 cultures.

Laboratory crossing studies

VI.6 Polymerase chain reaction on asexual parasite DNA

DNA isolated from both 3D7 and HB3 cultures of asexual parasites was amplified using both outer and nested primers specific for both MSP1 and MSP2 genes.

VI.6.i MSP1 specific primers

The amplified products obtained using the outermost pair of MSP1 primers (O1 and 02) in clone 3D7 and clone HB3 are shown in figure 24. The sizes of the bands were estimated by comparison with the marker fragments (lambda HindIII/EcoRI) to be 650bp. long for 3D7 and 550bp. long for HB3.

The amplified products obtained using the nested primers (N1 and N2) were smaller than those obtained using the outer primer pair, and once again different in

Figure 24

Amplification of genomic DNA isolated from asexual cultures of clones 3D7 and HB3, using primers specific to the MSP1 gene, blocks 1 and 3. Two sets of primers were used : an outer pair, denoted O1 and O2, and a pair internal to the first pair, denoted N1 and N2. 5μl of the amplified product was run on a 1.5% agarose gel in 1xTBE buffer. The gel was stained with ethidium bromide and photographed on a U-V transilluminator.

Lane 1 : lambda DNA marker (digested with HindIII/EcoRI)

Lane 2 : 3D7 DNA, primers O1 and O2

Lane 3 : HB3 DNA, primers O1 and O2

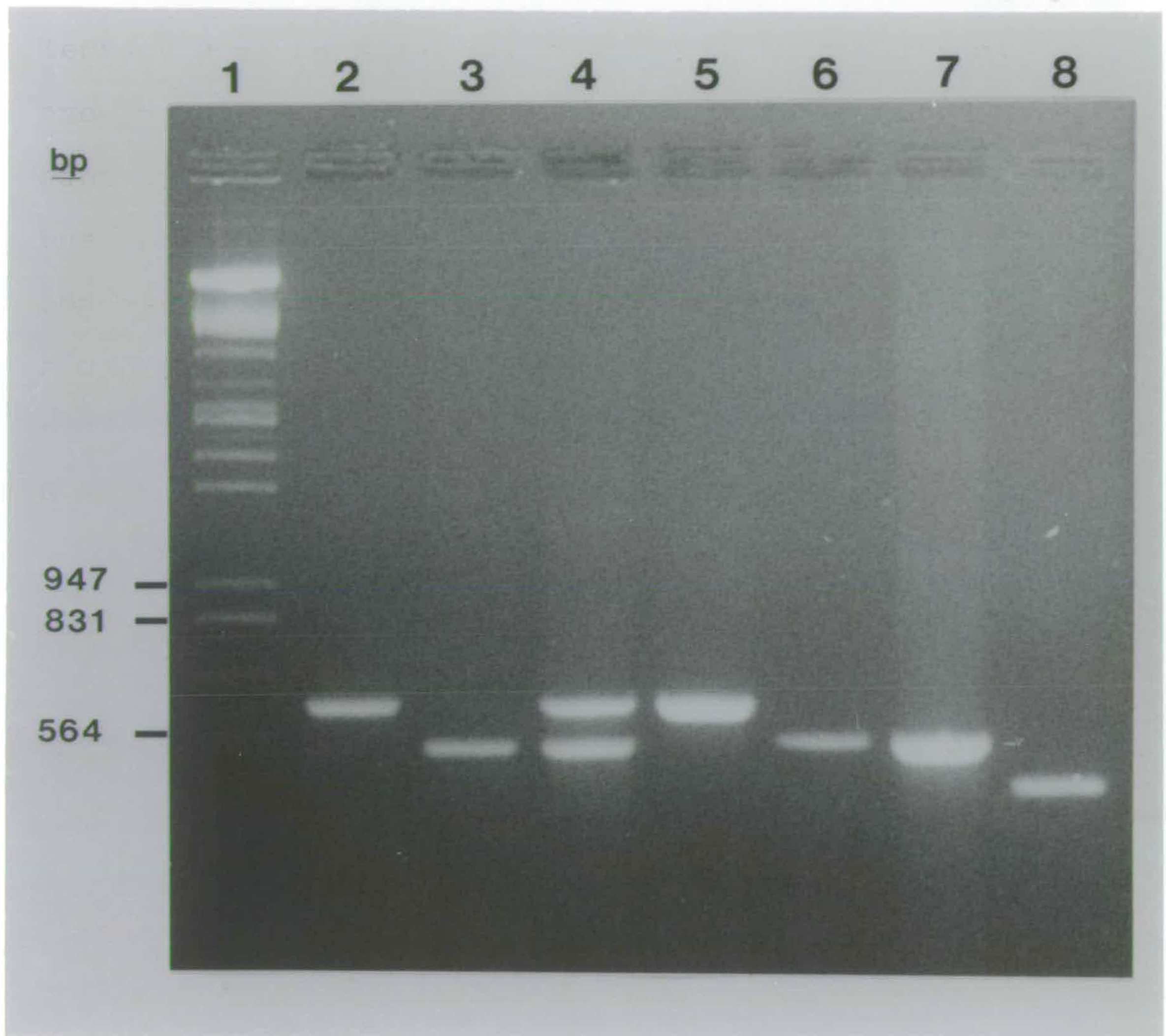
Lane 4 : Mixture of 3D7 and HB3 DNA, primers O1 and O2

Lane 5 : 3D7 DNA, primers O1 and O2

Lane 6 : 3D7 DNA, primers N1 and N2

Lane 7 : HB3 DNA, primers O1 and O2

Lane 8 : HB3 DNA, primers N1 and N2


1
2
3
4
5
6
7
8
bp
947
831
564

size between the two clones. The fragment sizes were estimated to be 560bp. for 3D7 and 470bp. for HB3.

VI.6.ii MSP2 specific primers

Amplified products of MSP2 of different sizes were obtained from asexual cultures of the two clones. The two products obtained from amplification with the outer MSP2 primers (S2 and S3) could be distinguished by a size difference on 1.8% agarose gels following electrophoresis (figure 25). The sizes of the fragments were estimated as 720bp. for 3D7 and 660bp. for HB3.

The amplified products obtained using the nested primer pair (S1 and S4) were approximately 500bp. for 3D7 and 450bp. for HB3.

VI.6.iii Limit of detection of different alleles in mixtures of DNA from two different clones.

By mixing equal amounts of DNA from 3D7 and HB3 before amplification, two products (corresponding to the two different alleles of MSP1 or MSP2 present) could be distinguished after electrophoresis through a 1.5 or 1.8% agarose gel (figure 26).

Dilutions were made of DNA extracted from cultures of the two clones, and mixtures made of different proportions of DNA from this DNA. The DNA mixtures were amplified using the MSP1 outer primer pair and then the nested primer pair (figure 26). Both alleles could be

Figure 25

Amplification of genomic DNA isolated from asexual cultures of clones 3D7 and HB3, using primers specific to the MSP2 gene, blocks 1 and 4. Two sets of primers were used : an outer pair denoted S2 and S3, and a pair internal to the first pair, denoted S1 and S4. 5μl of the amplified product was run on a 1.8% agarose gel in 1xTBE buffer. The gel was stained with ethidium bromide and photographed on a U-V transilluminator.

Lane 1 : lambda DNA marker (digested with HindIII/EcoRI)
Lane 2 : 3D7 DNA, primers S2 and S3
Lane 3 : HB3 DNA, primers S2 and S3
Lane 4 : Mixture of 3D7 and HB3 DNA, primers S2 and S3
Lane 5 : 3D7 DNA, primers S2 and S3
Lane 6 : 3D7 DNA, primers S1 and S4
Lane 7 : HB3 DNA, primers S2 and S3
Lane 8 : HB3 DNA, primers S1 and S4

1 2 3 4 5 6 7 8

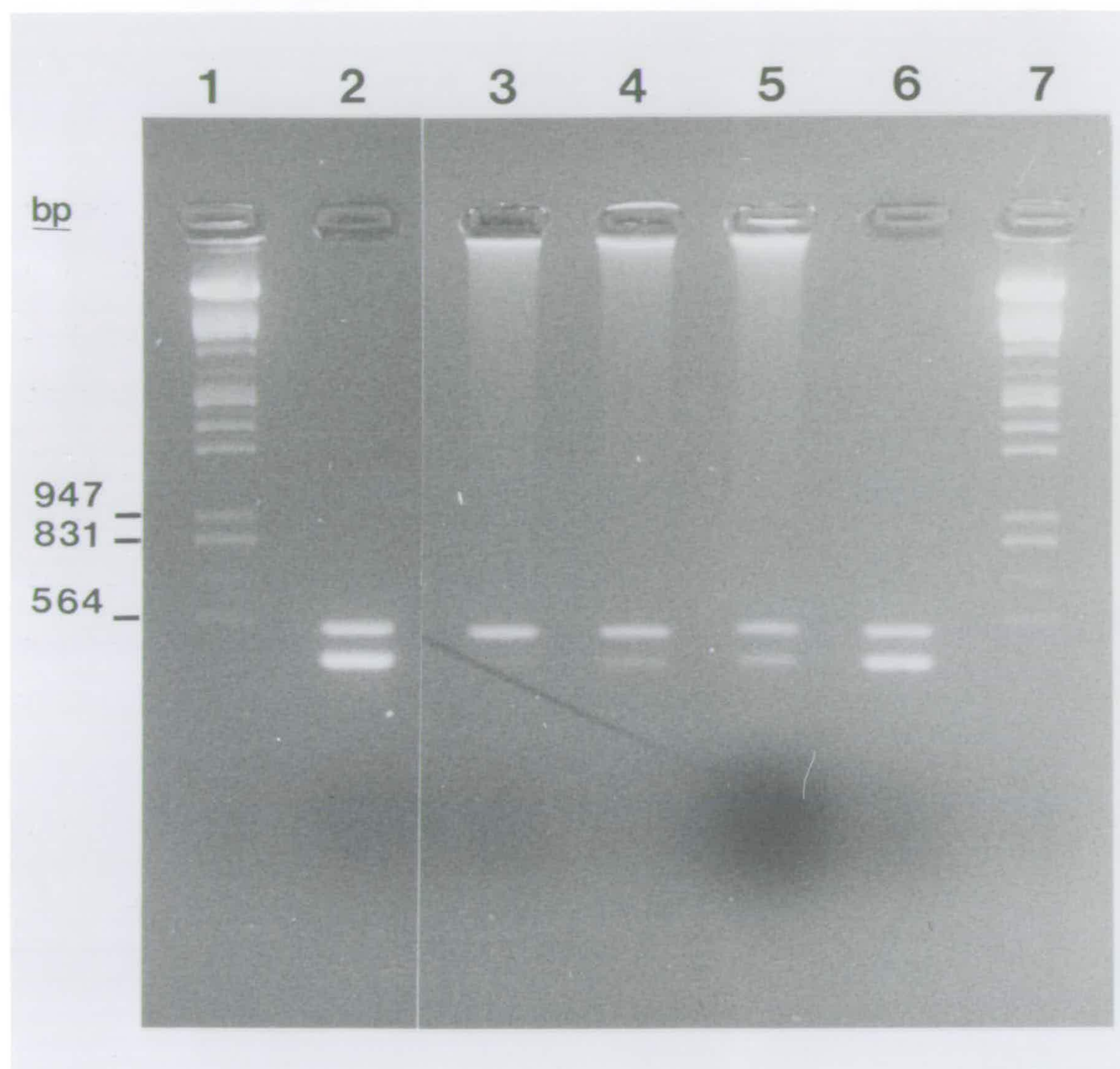
bp

947
831
564

Figure 26

Amplification of part of the MSP1 gene, using primers O1 and O2, followed by nested primers N1 and N2, of different proportions of DNA from asexual cultures of 3D7 and HB3 in mixtures. Dilutions of stock DNA solutions from each clone were made from 10^{-1} to 10^{-3}. The stock solutions and dilutions were mixed as shown below, and amplified using PCR. 5μl of the amplified product was run on a 1.5% agarose gel in 1xTBE buffer. The gel was stained with ethidium bromide and photographed on a U-V transilluminator.

Lane 1	: lambda DNA marker (digested with HindIII/EcoRI)
Lane 2	: 1:1 mixture of 3D7 and HB3 DNA (26ng : 26ng)
Lane 3	: 1 3D7 DNA : 10^{-3} HB3 DNA (26ng : 26pg)
Lane 4	: 1 3D7 DNA : 10^{-2} HB3 DNA (26ng : 260pg)
Lane 5	: 1 3D7 DNA : 10^{-1} HB3 DNA (26ng : 2.6ng)
Lane 6	: 1:1 mixture 3D7 and HB3 DNA
Lane 7	: lambda DNA marker (digested with HindIII/EcoRI)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564

identified in the mixture even when there was 1000 times more of one allele compared to the other. The smallest quantity of DNA amplified was 26pg. Quantities of DNA lower than this were not tested for amplification.

VI.7 Polymerase chain reaction on oocyst DNA

DNA was prepared from individual oocysts as described in section V.9, and was amplified using the optimum conditions for amplifying DNA from asexual parasite DNA with the same primers. No amplified product was detectable. Addition of oocyst DNA to asexual DNA prevented amplification.

The replacement of 10nM of the 75nM of dGTP in the nucleotide mixture with the same amount of the base analogue 7-deaza-2'deoxyguanosine 5'triphosphate (c^7dGTP) allowed amplification of oocyst DNA to proceed [Ranford-Cartwright et al., 1991b] (figure 27).

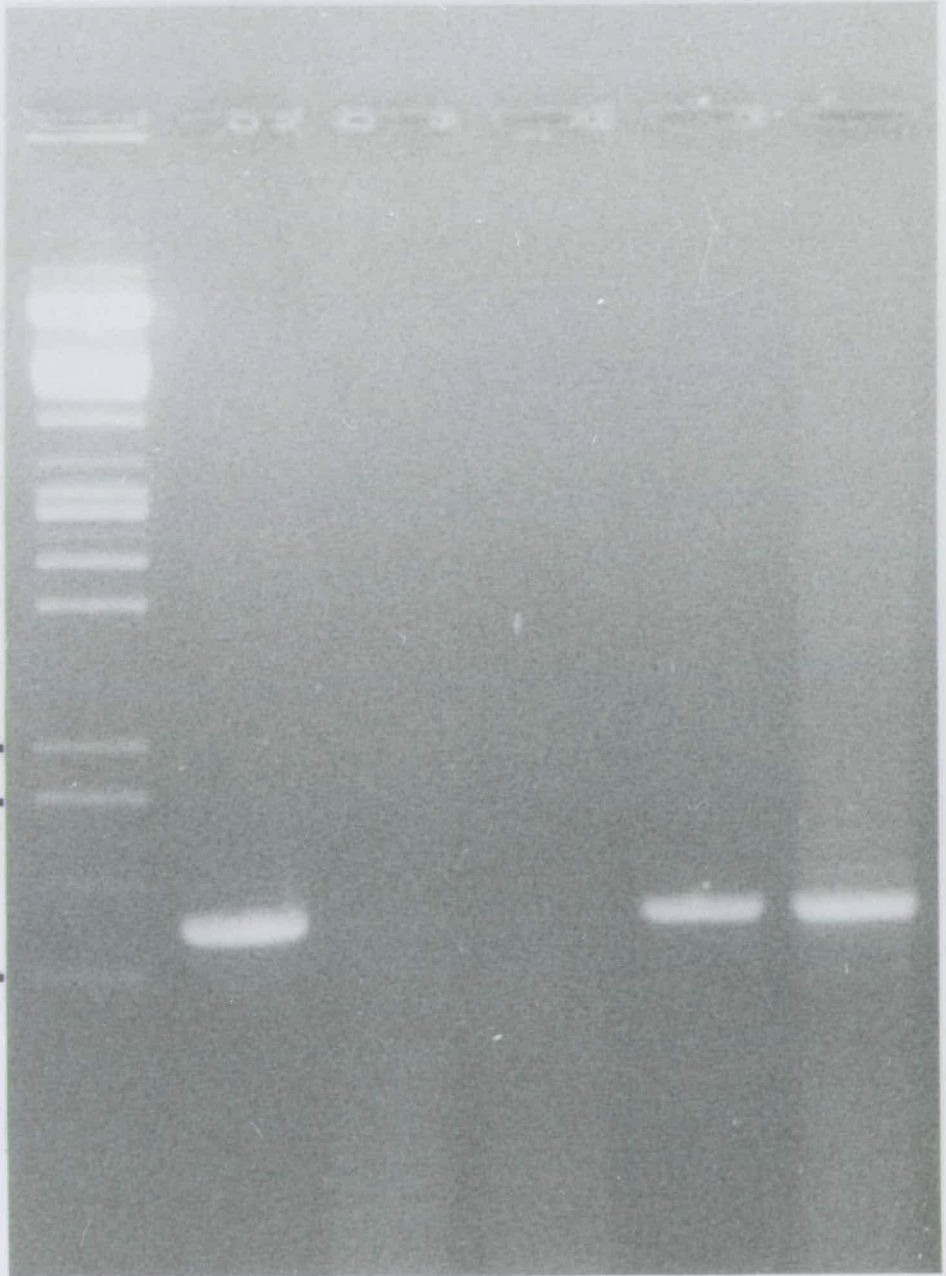
The amplified fragments obtained for all pairs of primers were identical in size to those obtained using DNA from asexual parasites (figure 27). The fragments obtained from amplification reactions including c^7dGTP fluoresce slightly less when stained with ethidium bromide than the same fragments amplified without this base analogue. This is because c^7dGTP does not itself fluoresce with ethidium.

DNA from non-infected mosquito gut tissue did not amplify with any of the primer pairs. DNA extracted from 'sham' cultures, which consist of red blood cells and

Figure 27

Amplification of part of the MSP1 gene, using primers O1 and O2, of DNA extracted from asexual 3D7 cultures and from single oocysts from mosquitoes fed on 3D7 gametocyte cultures. A deoxynucleotide (dNTP) mixture containing the base analogue 7-deaza-2'deoxyguanosine (C^7dGTP) was used in lanes 5 and 6. dGTP only (no c^7dGTP) was used in the remaining lanes. Lane 4 shows the product of mixing asexual and oocyst DNA, before amplifying without c^7dGTP. 5μl of the amplified product was run on a 1.5% agarose gel in 1xTBE buffer. The gel was stained with ethidium bromide and photographed on a U-V transilluminator.

Lane 1	: lambda HindIII/EcoRI marker
Lane 2	: 3D7 asexual DNA, no c^7dGTP
Lane 3	: oocyst DNA (3D7), no c^7dGTP
Lane 4	: oocyst and asexual DNA (3D7), no c^7dGTP
Lane 5	: 3D7 asexual DNA, with c^7dGTP
Lane 6	: oocyst DNA (3D7), with c^7dGTP


1
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6
bp
947
831
564

complete culture medium but no parasites, also did not produce an amplified product with any of the primers.

Each amplification reaction used 1μl of the 10μl of DNA extracted from a single oocyst. 46% of oocyst DNA samples gave a positive PCR product using the outer primer pairs (O1 and O2, or S2 and S3) only. By performing nested PCR the DNA from most (96%) of oocysts could be amplified using either MSP1, MSP2, or both sets of primers.

VI.8 Sequencing

VI.8.i MSP1 alleles

Following PCR amplification of DNA from asexual cultures of 3D7 and HB3, using primers O1 and O2 to the MSP1 gene, the products were sequenced [Ranford-Cartwright et al., 1991a]. Both positive and negative strands of DNA were sequenced for each allele, using the same primers (O1 and O2) as for the PCR. The results are shown in figure 28.

Alignment between the two nucleotide sequences was made using the PILEUP programme on the University of Wisconsin Genetics Computer Group (UWGCG) package (version 7), on the VAX computer, University of Edinburgh Computing Service [Devereux et al., 1984].

The nucleotide sequences were translated using the TRANSLATE programme (UWGCG) and the predicted amino acid sequences were compared to those of other P. falciparum MSP1 alleles already published (figure 29). The alignment

Figure 28

Comparison of the nucleotide sequences from direct sequencing of PCR fragments of MSP1 alleles from clones 3D7 and HB3. The positions of the oligonucleotide primers (oligomers) are marked below the sequences. **R>** and **R<** above the sequences mark the beginning and end of the region encoding the tripeptide repeats.

The sequences were aligned to maximise matched residues using the Pileup programme on the UWGCG sequence analysis package, on the VAX computer, University of Edinburgh.
Dots in the sequence indicate that there are gaps in the sequences at these positions.

```
      1                                                                       60
3D7   ACACATGAAAGTTAT  CAAGAACTTGTCAAA  AAACTAGAAGCTTTA  GAAGATGCAGTATTG
HB3   ACACATGAAAGTTAT  CAAGAACTTGTCAAA  AAACTAGAAGCTTTA  GAAGATGCAGTATTG
       ---------------Oligomer O1-------------->                  ---------Oligo-

      61                                                                     120
3D7   ACAGGTTATGGTTTA  TTTCAAAAGGAAAAA  ATGGTCTTAAATGAA  GAAGAAATTACTACA
HB3   ACAGGTTATGGTTTA  TTTCAAAAGGAAAAA  ATGGTATTAAATGAA  GGAACAAGTGGAACA
       -mer N1------->

      121                                         R>                         180
3D7   AAAGGTGCA...AGT  GCTCAA......AGT  GGTGCA...AGT...  GCTCAAAGTGGTGCA
HB3   GCTGTTACAACTAGT  CACCTGGTTTCAAAG  GGTTCAGTTGCTTCA  GGTGGTTCAGGTGGC

      181                                                                    240
3D7   AGTGCTCAAAGTGGT  GCAAGTGCTCAAAGT  GGTGCAAGTGCTCAA  AGTGGTGCAAGTGCT
HB3   TCAGTTGCTTCAGGT  GGCTCAGTTGCTTCA  GGTGGT.........  ...............

      241                                                                    300
3D7   ACAAGTGCTCAAAGT  GGTCCAAGTGGTACA  AGTGCTCCAAGTGGT  AGTGGTACAAGTCCA
HB3   ............TCA  GGTAAT.........  ...............  ...............

      301        <R                                                          360
3D7   TCATCTCGTTCAAAC  ACTTTACCTCGTTCA  AATACTTCATCTGGT  GCACCTCCAGCTGAT
HB3   TCAAGACGTACAAAT  .........CCTTCA  GATAATTCA......  ...............

      361                                                                    420
3D7   GCAAGCGATTCAGAT  GCTAAATCTTACGCT  GATTTAAAACACAGA  GTACGAAATTACTTG
HB3   ...AGTGATTCAGAT  GCTAAATCTTACGCT  GATTTAAAACACAGA  GTACGAAATTACTTG

      421                                                                    480
3D7   TTCACTATTAAAGAA  CTCAAATATCCCGAA  CTCTTTGATTTAACC  AATCATATGTTAACT
HB3   TTAACTATCAAAGAA  CTCAAATATCCTCAA  CTCTTTGATTTAACT  AATCATATGTTAACT

      481                                                                    540
3D7   TTGTGTGATAATATT  CATGGTTTCAAATAT  TTAATTGATGGATAT  GAAGAAATTAATGAA
HB3   TTGTGTGATAATATT  CATGGTTTCAAATAT  TTAATTGATGGATAT  GAAGAAATTAATGAA

      541                                                                    600
3D7   TTATTATATAAATTA  AACTTTTATTTTGAT  TTATTAAGAGCAAAA  TTAAATGATGTATGT
HB3   TTATTATATAAATTA  AACTTTTATTTTGAT  TTATTAAGAGCAAAA  TTAAATGATGTATGT

      601                                                                    660
3D7   GCTAATGATTATTGT  CAAATACCTTTCAAT  CTTAAAATTCGTGCA  AATGAATTAGACGTA
HB3   GCTAATGATTATTGT  CAAATACCTTTCAAT  CTTAAAATTCGTGCA  AATGAATTAGACGTA
                  <-------------Oligomer N2---------      <------------Oligomer O2------------
```

Figure 29

Comparison of the deduced amino acid sequences of MSP1 alleles. The sequences of CAMP [Weber *et al.*, 1986], K1 [Mackay *et al.*, 1985], MAD20 [Tanabe *et al.*, 1987], Wellcome [Holder *et al.*, 1985] and H1 [Howard *et al.*, 1986] have been published previously. The positions of the 'blocks'[Tanabe *et al.*, 1987] are indicated above the sequences. The sequences were aligned using the Pileup program on UWGCG (VAX). Amino acids are indicated by single letter code. Dots indicate gaps introduced to maximise alignment.

```
                1                                                          50
                <--------------- Block 1 ----------------><----------------
3D7A            THESYQELVK  KLEALEDAVL  TGYGLFQKEK  MVLNEEEITT  KGA.SAQ..S
CAMP            THESYQELVK  KLEALEDAVL  TGYGLFHKEK  MILNEEEITT  KGA.SAQ..S
K1              THESYQELVK  KLEALEDAVL  TGYSLFHKEK  MILNEEEITT  KGA.SAQ..S
MAD20           THESYQELVK  KLEALEDAVL  TGYSLFQKEK  MVLNEGTSGT  AVTTSTPGSS
Wellcome        THESYQELVK  KLEALEDAVL  TGYSLFQKEK  MVLNEGTSGT  AVTTSTPGSS
H1              THESYQELVK  KLEALEDAVL  TGYSLFQKEK  MVLNEGTSGT  AVTTSTPGSS
HB3A            THESYQELVK  KLEALEDAVL  TGYSLFQKEK  MVLNEGTSGT  AVTTSTPGSS

                51                                                        100
                -------------------- Block 2 -----------------------------
3D7A            GAS.A.QSG.  ASAQSGASAQ  SGASAQSGAS  ATSAQSGPSG  TSAPSG....
CAMP            GTS.G.TSG.  TSGTSGTSGT  SGTSAQSGTS  GTSAQSGTSG  TSAQSGTSGT
K1              GTS.G.TSG.  TSGPSGPSGT  ..........  ..........  ..........
MAD20           GSVTSGG...  SVASVASVAS  GG...SGGSV  ASGGSGN...  ..........
Wellcome        GSVASGGSGG  SVASGGSVAS  GGSVASGGSV  ASGGSGN...  ..........
H1              GSVASGGSGG  SVASGGSVAS  GG........  ....SGN...  ..........
HB3A            GSVASGGSGG  SVASGGSVAS  GG........  ....SGN...  ..........

                101                                                       150
                --------------------------------->  <---------------------
3D7A            ...SGTSPSS  RSNTLPRSNT  SSGA.PPADA  SDSDAKSYAD  LKHRVRNYLF
CAMP            SGTSGTSPSS  RSNTLPRSNT  SSGASPPADA  SDSDAKSYAD  LKHRVRNYLF
K1              ......SPSS  RSNTLPRSNT  SSGASPPADA  SDSDAKSYAD  LKHRVRNYLL
MAD20           ........SR  RTN...PSDN  S.........  SDSNTKTYAD  LKHRVQNYLF
Wellcome        ........SR  RTN...PSDN  S.........  SDSDAKSYAD  LKHRVRNYLL
H1              ........SR  RTN...PSDN  S.........  SDSDAKSYAD  LKHRVRNYLL
HB3A            ........SR  RTN...PSDN  S.........  SDSDAKSYAD  LKHRVRNYLL

                151                                                       200
                ------------------------ Block 3 --------------------------
3D7A            TIKELKYPEL  FDLTNHMLTL  CDNIHGFKYL  IDGYEEINEL  LYKLNFYFDL
CAMP            TIKELKYPEL  FDLTNHMLTL  CDNIHGFKYL  IDGYEEINEL  LYKLNFYFDL
K1              TIKELKYPQL  FDLTNHVLTL  CDNIHGFKYL  IDGYEEINEL  LYKLNFYFDL
MAD20           TIKELKYPEL  FDLTNHMLTL  SKNVDGFKYL  IDGYEEINEL  LYKLNFYYDL
Wellcome        TIKELKYPQL  FDLTNHMLTL  CDNIHGFKYL  IDGYEEINEL  LYKLNFYFDL
H1              TIKELKYPQL  FDLTNHMLTL  CDNIHGFKYL  IDGYEEINEL  LYKLNFYFDL
HB3A            TIKELKYPQL  FDLTNHMLTL  CDNIHGFKYL  IDGYEEINEL  LYKLNFYFDL

                201                            230
                --------------------------------->
3D7A            LRAKLNDVCA  NDYCQIPFNL  KIRANELDVL
CAMP            LRAKLNDVCA  NDYCQIPFNL  KIRANELDVL
K1              LRAKLNNVCA  NDYCQIPFNL  KIRANELDVL
MAD20           LRAKLNDACA  NSYCQIPFNL  KIRANELDVL
Wellcome        LRAKLNDVCA  NDYCQIPFNL  KIRANELDVL
H1              LRAKLNDVCA  NDYCQIPFNL  KIRANELDVL
HB3A            LRAKLNDVCA  NDYCQIPFNL  KIRANELDVL
```

was performed using the multiple sequence format programme PILEUP on UWGCG.

The sequence of HB3 was identical to that of its parent isolate H1 [Howard, R.F. et al., 1986]. The amino acid sequence most closely resembles that of the Wellcome strain [Holder et al., 1985]. The tripeptide repeats found in both HB3 and Wellcome are Ser-Val-Ala, Ser-Gly-Gly and Ser-Gly-Asn. The Wellcome allele has 12 such repeats ; the HB3 allele is identical but 4 tripeptides shorter.

The MSP1 allele of the parent isolate NF54, from which 3D7 was cloned, has not been sequenced. The MSP1 allele of 3D7 is most similar to the NF7 allele. The tripeptide repeat region of the NF7 allele is shorter (11 tripeptides) than that of 3D7 (16 tripeptides). Both the 3D7 and NF7 tripeptide repeat regions contain repeats of Ser-Gly-Ala and Ser-Ala-Gln, and also have fewer repeats of Ser-Gly-Pro, Ser-Gly-Ala and Ser-Gly-Thr. The 3D7 allele also contains the tripeptides Ser-Ala-Thr and Ser-Ala-Pro, which have not been reported in any other sequences reported so far. The 3D7 allele is also unique in that it contains a single dipeptide Ser-Gly at the end of the tripeptide repeat region.

Outside the repetitive region, the two alleles follow the dimorphism seen in other sequences. The 3D7 allele falls into the K1 type for the dimorphic part of block 2 ; the HB3 allele falls into the MAD20 type. Blocks 1 and 3 are conserved between all alleles.

The exact sizes of PCR products can be estimated from their nucleotide sequences. The 3D7 PCR product obtained using the outer primers (O1 and O2) is 645bp. long, and the HB3 PCR product is 552bp. long. The nested primer pair (N1 and N2) should amplify a 565bp. fragment from 3D7 DNA and a 472bp. fragment from HB3 DNA.

VI.8.ii MSP2 alleles

The MSP2 allele of clone 3D7 has been sequenced previously [Smythe *et al.*, 1990]. The MSP2 allele from clone HB3 or from its parent clone H1 has not previously been sequenced. Following PCR amplification of DNA from asexual cultures of HB3 the product was sequenced as for MSP1 alleles (section VI.8.i). Both positive and negative strands of DNA were sequenced, using the same primers (S2 and S3) that were used for amplification. The sequence of HB3 obtained is shown in figure 30.

Alignment between the nucleotide sequences of HB3 and 3D7 was made using the PILEUP programme from UWGCG on the VAX (see section VI.8.i) and this is shown in figure 30. The nucleotide sequences were translated and the predicted amino acid sequences were compared to other *P. falciparum* MSP2 sequences already published (figure 31).

The MSP2 allele from 3D7 belongs to the Indochina-1 (IC1) family. Region 2 of the 3D7 allele contains 5 copies of the 4 amino acid repeat Gly-Gly-Ser-Ala, and three complete and one degenerate copies of a 9 base pair repeat. This 9bp. repeat consists of 3 alternative codons

Figure 30

Comparison of the nucleotide sequences from direct sequencing of the PCR fragment of MSP2 alleles from clone HB3 with the previously published nucleotide sequence of clone 3D7 [Smythe et al., 1990]. The positions of the oligonucleotide primers (oligomers) are marked below the sequences. The positions of the 'blocks' are marked above the sequences [Thomas et al., 1990].

The sequences were aligned to maximise matched residues using the Pileup programme on the UWGCG sequence analysis package, on the VAX computer, University of Edinburgh.

Dots in the sequence indicate that there are gaps in the sequences at these positions.

```
     1                                                              60
     <-----------------------------------------------------------------
3D7  ATGAAGGTAATTAAA  ACATTGTCTATTATA  AATTTCTTTATTTTT  GTTACCTTTAATATT
HB3                        <----UNKNOWN  --   TTTATTTTT  GTTACCTTTAATATT
       ------------ Oligomer S3 ------------>

     61                                                            120
     ------------------------------ Block 1 ------------------------------
3D7  AAAAATGAAAGTAAA  TATAGCAACACATTC  ATAAACAATGCTTAT  AATATGAGTATAAGG
HB3  AAAAATGAAAGTAAA  TATAGCAACACATTC  ATAAACAATGCTTAT  AATATGAGTATAAGG
                                                          ----------Oligo-

     121                                                           180
     -------><---------------------------------------------------------
3D7  AGAAGTATGGCAGAA  AGTAAGCCTTCTACT  GGTGCTGGTGGTACT  GCTGGTGGTAGTGCT
HB3  AGAAGTATGGCAAAT  GAAGGTTCTAATACT  AAGAGTGTAGGTGCA  AATGCTCCAAAAGCT
     -mer S1---->

     181                                                           240
     ---------------------------- Block 2 ----------------------------
3D7  GGTGGTAGTGCTGGT  GGTAGTGCTGGTGGT  AGTGCTGGTGGTAGT  GCTGGTTCTGGTGAT
HB3  GATACTATTGCTAGT  GGAAGTCAAAGTAGT  ACAAATAGTGCAAGT  ACTAGTACTACTAAT

     241                                                           300
     ------------------------------------------------------------------
3D7  GGTAATGGTGCAGAT  GCTGAGGGAAGTTCA  AGTACTCCCGCTACT  ACCACAACTACCAAA
HB3  AATGGAGAATCACAA  AATACTACTCCTACC  GCT............  ...............

     301                                                           360
     ------------------------><----------------------------------------
3D7  ACTACCACAACTACC  ACAACTACTAATGAT  GCAGAAGCATCTACC  AGTACCTCTTCAGAA
HB3  ...............  .........GCTGAT  ACCCCTACTGCTACA  GAAAGTAATTCACCT

     361                                                           420
     ------------------------------------------------------------------
3D7  AATCCAAATCATAAA  AATGCCGAAACAAAT  CCAAAAGGTAAAGGA  GAAGTTCAAGAACCA
HB3  TCACCACCCATCACT  ACTACAGAAAGTAAT  TCACCTTCACCACCC  ATCACTACTACAAAA

     421                                                           480
     -------------------------- Block 3 ------------------------------
3D7  AATCAAGCAAATAAA  GAAACTCAAAATAAC  TCAAATGTTCAACAA  GACTCTCAAACTAAA
HB3  AGTAATTCACCTTCA  CCACCCATCACTACT  ACAGAAAGTTCAAGT  TCTGGCAATGCACCA

     481                                                           540
     ------------------------------------------------------------------
3D7  TCAAATGTTCCACCC  ACTCAAGATGCAGAC  ACTAAAAGTCCTACT  GCACAACCTGAACAA
HB3  AATAAAACAGACGGT  AAAGGAGAAGAGAGT  GAAAAACAAAATGAA  TTAAATGAATCAACT

     541                                                           600
     ---------------------------------------------------------------><--
3D7  GCTGAAAATTCTGCT  CCAACAGCCGAACAA  ACTGAATCCCCCGAA  TTACAATCT...GCA
HB3  GAAGAAGGACCCAAA  GCTCCACAAGAACCT  CAAACGGCAGAAAAT  GAAAATCCTGCTGCA
```

```
        601                                                            660
        --------------------------- Block 4 ---------------------------
3D7     CCAGAGAATAAAGGT  ACAGGACAACATGGA  CATATGCATGGTTCT  AGAAATAATCATCCA
HB3     CCAGAGAATAAAGGT  ACAGGACAACATGGA  CATATGCATGGTTCT  AGAAATAATCATCCA
                                   <------------ Oligomer S4 -------------

        661                                                            720
        ----------------------------------------------------------------
3D7     CAAAATACTTCTGAT  AGTCAAAAAGAATGT  ACCGATGGTAACAAG  AAAACTGTGGAGCAGC
HB3     ---UNKNOWN--->
                                                           <------------ Oligo-

        721                                                            780
        ---------------------------------------------------------------->
3D7     AACATCCCTCTTAAA  TAACTCTAGTAATAT  TGCTTCAATAAATAA  ATTTGTTGTTTTAATT
HB3
        -mer S2---------
```

Figure 31

Comparison of the deduced amino acid sequences of MSP2 alleles. The sequences of 3D7 [Smythe et al., 1990], CAMP [Thomas et al., 1990], IC1 [Smythe et al., 1990], NIG60 [Marshall et al., 1991], FC27 [Smythe et al., 1988] and K1 [Smythe et al., 1991] have been published previously. The positions of the 'blocks' [Thomas et al., 1990] are indicated above the sequences.

The sequences were aligned using the Pileup program on UWGCG (VAX). Amino acids are indicated by single letter code. Dots indicate gaps introduced to maximise alignment.

```
         1                                                                50
         <----------------------- Block 1 ------------------><------
3D7A     MKVIKTLSII  NFFIFVTFNI  KNESKYSNTF  INNAYNMSIR  RSMAESKPST
CAMP     MKVIKTLSII  NFFIFVTFNI  KNESKYSNTF  INNAYNMSIR  RSMAESKPST
IC1      MKVIKTLSII  NFFIFVTFNI  KNESKYSNTF  INNAYNMSIR  RSMTESNPST
NIG60    MKVIKTLSII  NFFIFVTFNI  KNESKYSNTF  INNAYNMSIR  RSMKESNPST
FC27     MKVIKTLSII  NFFIFVTFNI  KNESKYSNTF  INNAYNMSIR  RSMANSGSNT
K1       MKVIKTLSII  NFFIFVTFNI  KNESKYSNTF  INNAYNMSIR  RSMANSGSNT
HB3A       <--UNKNOWN  FIFVTFNI  KNESKYSNTF  INNAYNMSIR  RSMANSGSNT

         51                                                              100
         ----------------------- Block 2 -------------------------
3D7A     GAGGTAGGSA  GGSAGGSAGG  SAGGSAG...  ..........  ..........
CAMP     GTGG......  ....SGSAGS  GAGASAG...  ..........  ..........
IC1      GASGSAGGSA  GGSAGGSAGG  SAGGSAGGSA  GGSAGGSAGG  SAGGSAGGSA
NIG60    GAGSGAVASA  GSGAVASAGS  GAVASAGSGA  VASGSQSSTN  SASTSTTNNG
FC27     NSVGANAPNA  ..........  ........DT  IASGSQRSTN  SASTSTTNNG
K1       KSVGANAPNA  ..........  ........DT  IASGSQSSTN  SASTSTTNNG
HB3A     KSVGANAPNA  ..........  ........DT  IASGSQSSTN  SASTSTTNNG

         101                                                             150
         -------------------------------------------><----------
3D7A     .........S  GDGNGADAEG  SSSTPATTTT  TKTTTTTTTT  NDAEASTSTS
CAMP     .........N  GANPGADAER  SPSTPATPAT  PATTTTTTTT  NDAEASTSTS
IC1      GGSAGSGDGN  GANPGADAER  SPSTPA....  ..TTTTTTTT  NDAEASTSTS
NIG60    ESQTTTPTA.  ..........  ..........  ..........  ..........
FC27     ESQTTTPTA.  ..........  ..........  ..........  ADTIASGSQR
K1       ESQTTTPTA.  ..........  ..........  ..........  ..........
HB3A     ESQNTTPTA.  ..........  ..........  ..........  ..........

         151                                                             200
         ----------------------- Block 3 -------------------------
3D7A     SENPNHKNAE  TNPKGKGEVQ  EPNQANKETQ  NNSNVQQDSQ  TKSNVPPTQD
CAMP     SENPNHKNAE  TNPKGKGEVQ  KPNQANKETQ  NNSNVQQDSQ  TKSNVPPTQD
IC1      SENPNHNNAE  T.........  ..NQANKETQ  NNSNVQQDSQ  TKSNVPPTQD
NIG60    ..........  ..........  ..ADTPTAT.  ..........  .KSNSPSPPI
FC27     STNSASTSTT  NNGESQTTTP  TAADTPTATE  SISPSPPITT  ..........
K1       ..........  ..........  ..ADTPTATE  SNSRSPPITT  TESNSRSPPI
HB3A     ..........  ..........  ..ADTPTATE  SNSPSPPITT  TESNSPSPPI

         201                                                             250
         ---------------------------------------------------------
3D7A     ADTKSPTAQP  EQAENSAPTA  EQTESPELQS  A.........  ..........
CAMP     ADTKSPTAQP  EQAENSAPTA  EQTESPELQS  A.........  ..........
IC1      ADTRSPTAQP  EQAENSAPTA  EQTESPELQS  A.........  ..........
NIG60    TTTKSNSPSP  PITT......  ..........  ........TE  SSSSGNAPNK
FC27     ..........  ..........  ..........  ........TE  SSKFWQCTNK
K1       TTTESNSRSP  PITTTESNSR  SPPITTTESN  SRSPPITTTE  SSSSGNAPNK
HB3A     TTTKSNSPSP  PITT......  ..........  ........TE  SSSSGNAPNK

         251                                                             300
         ----------------------------------------><-----------
3D7A     ..........  ..........  ..........  ........PE  NKGTGQHGH
CAMP     ..........  ..........  ..........  ........PE  NKGTGQHGH
IC1      ..........  ..........  ..........  ........PE  NKGTGQHGH
NIG60    TDGKGEESEK  QNELNESTEE  GPKAPQEPQT  AENENPAAPE  NKGTGQHGH
FC27     TDGKGEESEK  QNELNESTEE  GPKAPQEPQT  AENENPAAPE  NKGTGQHGH
K1       TDGKGEESEK  QNELNESTEE  GPKAPQEPQT  AENENPAAPE  NKGTGQHGH
HB3A     TDGKGEESEK  QNELNESTEE  GPKAPQEPQT  AENENPAAPE  NKGTGQHGH
```

```
        301                                                        350
        ------------------------ Block 4 -------------------------
3D7A    HGSRNNHPQN  TSDSQKECTD  GNKENCGAAT  SLLNNSSNIA  SINKFVVLIS
CAMP    HGSRNNHPQN  TSDSQKECTD  GNKENCGAAT  SLLNNSSNIA  SINKFVVLIS
IC1     HGSRNNHPQN  TSDSQKECTD  GNKENCGAAT  SLLNNSSNIA  SINKFVVLIS
NIG60   HGSRNNHPQN  TSDSQKECTD  GNKENCGAAT  SLLNNSSNIA  SINKFVVLIS
FC27    HGSRNNHPQN  TSDSQKECTD  GNKENCGAAT  SLLNNSSNIA  SINKFVVLIS
K1      HGSRNNHPQN  TSDSQKECTD  GNKENCGAAT  SLLNNSSNIA  SINKFVVLIS
HB3A    HGSRNNHP    --UNKNOWN-->

        351         361
        ------------>
3D7A    ATLVLSFAIF  I
CAMP    ATLVLSFAIF  I
IC1     ATLVLSFAIF  I
NIG60   ATLVLSFAIF  I
FC27    ATLVLSFAIF  I
K1      ATLVLSFAIF  I
HB3A
```

for the amino acid threonine [figures 30 and 31]. Region 3 of 3D7 is identical to the CAMP allele [Thomas et al., 1990], and similar to that of the IC1 allele [Smythe et al., 1990].

The HB3 allele belongs to the FC27 family, and is most similar to the K1 allele [Smythe et al., 1991]. In block 2 both K1 and HB3 have one copy of a 32 amino acid repeat, similar to that found in FC27. The HB3 repeat differs in one amino acid (residue 104 in figure 31), substituting asparagine for threonine. Both K1 and HB3 have tandem repeats of a 12 amino acid sequence in block 3. The K1 allele has 5 tandem repeats of the amino acids ESNSRSPPITTT. The HB3 allele has two copies of a similar repeat ESNSPSPPITTT (with one substitution, P for R, at the fifth residue of the repeat), and one copy of the repeat KSNSPSPPITTT. An identical 12 amino acid repeat is present in the MSP2 allele of the isolate NIG60 [Marshall et al., 1991]. Blocks 1 and 4 are identical for all alleles.

The exact sizes of the PCR products can be estimated from their nucleotide sequences. The PCR products obtained using the outer primer pair (S2 and S3) should be 726bp. for 3D7 and 678bp. for HB3. The nested primer pair (S1 and S4) should amplify a 534bp. product from 3D7 and a 486bp. product from HB3.

VI.9 Frequencies of hybrid and parental oocysts

DNA from each oocyst was amplified with outer and nested primers specific for the MSP1 gene and with outer and nested primers specific to the MSP2 gene. 4μl of the amplified product was subjected to electrophoresis through 1.5% (MSP1) or 1.8% (MSP2) agarose gels (section V.15). After staining with ethidium bromide, the amplified products were classified as containing either of the two parental alleles, or both of the parental alleles, by the presence of one or two bands of the appropriate size on these gels. The results are listed fully in appendix 4 and summarised below (table 5).

Locus	No.oocysts tested	No. with positive PCR	% success	oocyst type 3D7	HB3	Hybrid
MSP1	128	98	76.6%	30	14	54
MSP2	108	87	80.6%	33	8	46
TOTAL	128	124	96.9%	43	16	65

Table 5 : Summary of PCR results of 128 oocysts (full data are in appendix 4).

All 128 oocyst DNA samples listed in appendix 4, from 9 different mosquito feeds, were tested for amplification with the MSP1 outer and nested primers. 76.6% of all the oocyst DNA samples gave a PCR product with these primers.

The same oocyst DNA samples were amplified using primers to the MSP2 gene. For twenty samples there was no DNA left after MSP1 amplification to test with the MSP2 primers, and thus 108 of the original 128 oocyst DNA samples were tested. 81% of these oocysts gave a PCR product with the MSP2 primers. Some samples that did not amplify using the MSP1 primers did amplify using the MSP2 primers.

Overall, using both sets of primers (outer and nested) for each gene (MSP1 and MSP2) 96.9% of all the oocyst DNA samples could be amplified using primers specific to one or both genes.

VI.10 Calculation of allele frequencies and expected numbers of hybrid and parental oocysts

The frequencies of the two alleles of each of the genes in the oocysts can be calculated from the data given in table 5. If mating is random, the frequencies of alleles in a population should remain constant from one generation to the next, provided there is no selection, according to Hardy-Weinberg equilibrium law.

The number of each type of zygote (the heterozygote and the two homozygotes) for each gene that would be expected if mating is random between gametes can be calculated and compared to the observed figures. The comparison is traditionally done using the so-called Chi-square (X^2) test. A closer approximation of actual Chi-square can be obtained using G-tests. Both methods of

comparison will be applied to the data. Examples of the calculations are given in appendix 3.

VI.10.i MSP1 alleles

The frequency of the 3D7 MSP1 allele (p) is:

$$\frac{(2 \times 30) + 54}{(2 \times 98)} = \frac{114}{196} = 0.582$$

The frequency of the HB3 MSP1 allele (q) is:

$$\frac{(2 \times 14) + 54}{(2 \times 98)} = \frac{82}{196} = 0.418$$

Assuming random mating between equal numbers of gametes of both clones, the expected numbers of heterozygotes and homozygotes are as follows:

3D7 homozygotes = $p^2 = (0.582)^2 \times 98 = 33.2$

HB3 homozygotes = $q^2 = (0.418)^2 \times 98 = 17.1$

heterozygotes = $2pq = (2 \times 0.582 \times 0.418) \times 98 = 47.7$

98

Constructing a table for comparison :

	observed	expected
3D7 homozygote	30	33.2
HB3 homozygote	14	17.1
Heterozygote	54	47.7
	98	98

$X^2_{2d.f.} = 1.70 \quad 0.5>p>0.3$

$G_{2d.f.} = 1.72 \quad 0.5>p>0.3$

With two degrees of freedom, 30% to 50% of samples taken at random from a population would have given differences as great or greater than those observed. Thus the observed numbers do not differ significantly from those expected if mating between gametes is random.

VI.10.ii MSP2 alleles

The frequency of the 3D7 MSP2 allele, p, is:

$$\frac{(2 \times 33) + 46}{(2 \times 87)} = \frac{112}{174} = 0.644$$

The frequency of the HB3 MSP2 allele, q, is:

$$\frac{(2 \times 8) + 46}{(2 \times 87)} = \frac{62}{174} = 0.356$$

The expected number of heterozygotes and homozygotes obtained if mating is random is:

3D7 homozygotes = $p^2 \times 87$ = 36.1

HB3 homozygotes = $q^2 \times 87$ = 11

Heterozygotes = $2pq \times 87$ = 39.9

87

Constructing a table for comparison:

	observed	expected
3D7 homozygotes	33	36.1
HB3 homozygotes	8	11
heterozygotes	46	39.9
	87	87

$X^2_{2d.f.} = 2.02 \quad 0.5>p>0.3$

$G_{2d.f.} = 2.1 \quad 0.5>p>0.3$

There is no significant difference between the observed frequencies of homozygotes and heterozygotes and the frequencies that would be expected if mating between the gametes is random.

Thus the data for both MSP1 and MSP2 allele frequencies provide no evidence of deviation from Hardy-Weinberg equilibrium. However Hardy-Weinberg equilibrium can only be applied in this form if the frequencies of the alleles are identical in male and female gametes. From section VI.2, the sex ratios of the two clones are significantly different.

It is possible to include this extra information and recalculate the allele frequencies found in male and female gametes of both clones. From this a more accurate estimation can be made of the expected frequencies of homozygotes and heterozygotes after mating. These have

been calculated for both MSP1 and MSP2 data. Full calculations are shown in appendix 3.

The new calculations, involving different allele frequencies between the male and female gametes of each clone, make the following assumptions :

(i) that the number of male gametes produced from a male gametocyte of 3D7 is the same as the number produced from a male gametocyte of HB3 (estimated to be 8),

(ii) that all gametes are equally viable.

Allowing random mating between all gametes, the expected frequencies of MSP1 homozygotes and heterozygotes are shown in the table of comparison below.

	observed	expected
3D7 homozygotes	30	32.1
HB3 homozygotes	14	15.8
Heterozygotes	54	50.2
	98	98

X^2_{2df} = 0.63 $0.8>p>0.7$

G_{2df} = 0.44 $0.8>p>0.7$

For MSP2 alleles, the expected values are:

	observed	expected
3D7 homozygotes	33	34.4
HB3 homozygotes	8	10.3
Heterozygotes	46	42.3
	87	87

$X^2_{2df} = 0.89$ $0.7>p>0.5$

$G_{2df} = 0.84$ $0.7>p>0.5$

After the difference in gene frequency between male and female gametes has been taken into account, the observed data fits that expected if mating is random even more closely.

(c) Field studies

VI.11 Methods

A natural progression from the laboratory studies on the frequency of cross-fertilisation was to the analysis of oocysts taken from wild-caught mosquitoes. Amplification of oocyst DNA from wild-caught mosquitoes had never been attempted before.

Dr. Peter Billingsley, Imperial College of Science, Technology and Medicine, London, kindly agreed to dissect a small number of mosquitoes caught in the Tanzanian

villages of Michenga and Namawala, during a field trip to the Swiss Tropical Institute Field Laboratory at Ifakara, Tanzania.

Mosquitoes were collected by resting catch inside houses, and then kept in an insectory for 6-7 days. The mosquitoes were dissected and single oocysts were removed from the mosquito mid-guts. The oocysts were incubated in oocyst lysis buffer containing proteinase K, as in section V.9, and stored at -20°C, before being brought back to the UK on dry ice.

Extraction and amplification of the DNA from these oocysts was performed in Edinburgh.

VII.12 Results

DNA was extracted from seven oocysts, which had been collected as detailed in table 6 below.

Oocyst number	Village	date collected	no. of oocysts	mosquito species
wa1	Michenga	13/4/91	1	*An. gambiae*
wa2	Namawala	16/4/91	1	*An. gambiae*
wa3	Namawala	16/4/91	1	*An. funestus*
wa4	Namawala	16/4/91	1	*An. funestus*
wa5	Namawala	16/4/91	1	*An. gambiae*
wa6	Namawala	16/4/91	1	from *An. gambiae* with 4 oocysts
wa7	Namawala	16/4/91	1	

Table 6. Details of oocysts collected from Tanzania

Amplification of oocyst DNA was performed using the MSP1 outer primers (O1 and O2) and the nested primer pair (N1 and N2). The results are shown in figure 32.

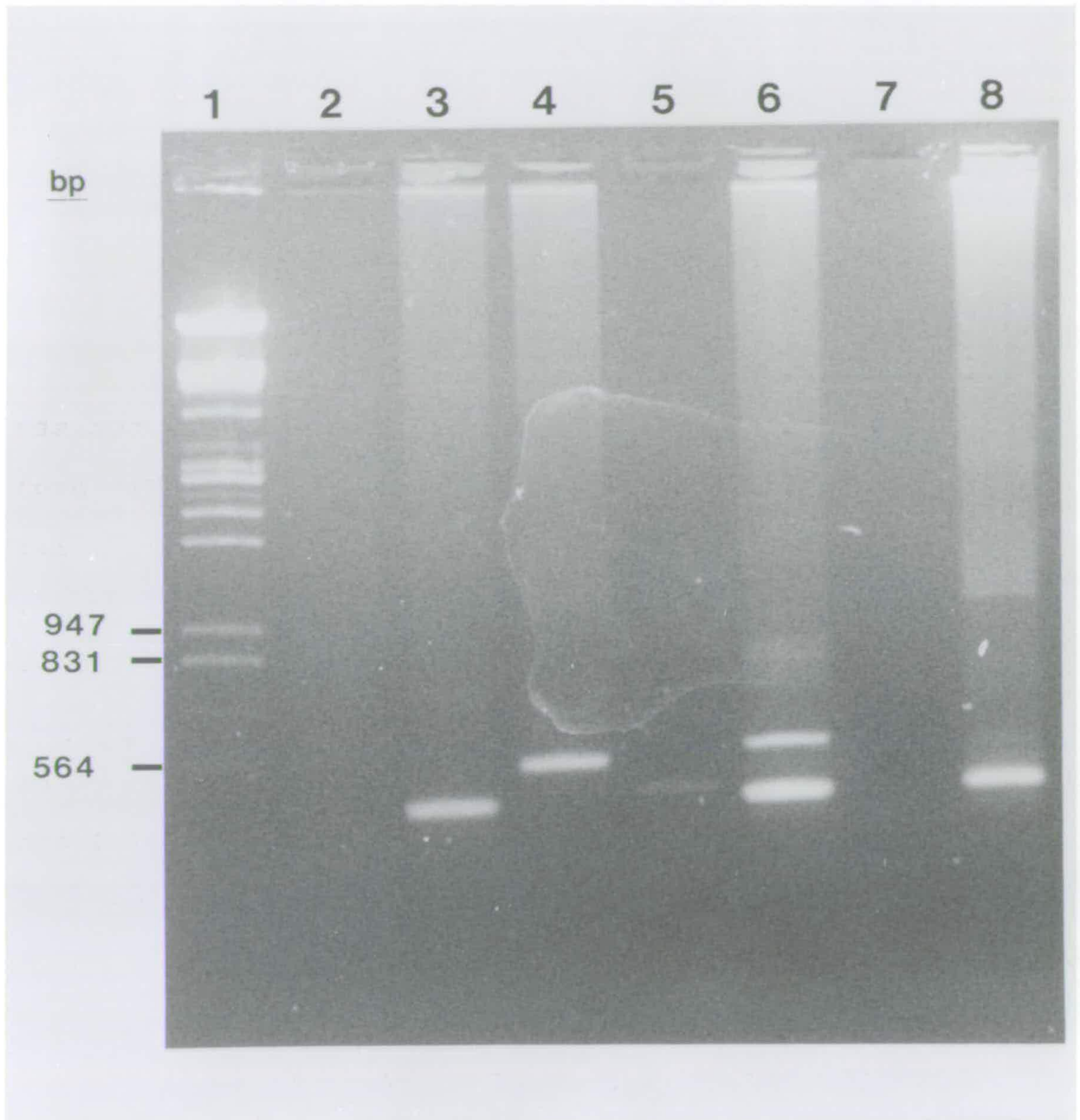
Positive amplification was obtained with oocysts wa2, wa3, wa4, wa5 and wa7. There were two sizes of the MSP1 allele, estimated to be 440bp. and 550bp.. DNA from five out of the seven oocysts collected could be amplified. DNA from one of the oocysts (wa5) amplified fragments of two different sizes, indicating that this was a hybrid oocyst, arising from cross-fertilisation between two gametes possessing different alleles of MSP1.

The techniques for extraction and amplification of oocyst DNA developed in the laboratory were successfully applied to material collected in the field. A similar percentage of the oocysts collected gave a positive result when their DNA was amplified by the PCR, although the numbers collected from the field are small, making comparisons in success between laboratory and field difficult.

Interestingly one of the oocysts was a hybrid, from a cross-fertilisation event in the wild. This is the first direct evidence that cross-fertilisation occurs between unlike gametes in natural populations of *P. falciparum*. It is possible that there may be more than one hybrid oocyst, if the different alleles present in this local population are not variable in size, but differ only in sequence. Such alleles may not produce a

Figure 32

Amplification of DNA extracted from oocysts dissected from wild-caught mosquitoes in Michenga and Namawala villages, Tanzania. Lane 1 is a marker track of lambda DNA digested with HindIII and EcoRI. Lanes 2-8 are the PCR products of the oocyst DNA. DNA was amplified using primers specific to the MSP1 gene. Following an initial 30 cycles of amplification using the outer primer pair (O1 and O2), 1μl of the amplified material was used as source DNA for a second round of amplification using primers internal to the first pair (N1 and N2). 5μl of the amplified product was run on a 1.5% agarose gel in 1xTBE buffer. The gel was stained with ethidium bromide and photographed on a U-V transilluminator.



1
2
3
4
5
6
7
8
bp
947
831
564

doublet on analysis of the amplification products. However, sequence differences between alleles could be detected by Southern blotting of the gel and hybridisation with allele-specific probes.

The field studies initiated with the help of Dr. Billingsley have been expanded and further developed by Prof. Walliker and Mr. Hamza Babiker, Edinburgh University, on a field trip to Ifakara, Tanzania.

VII General discussion

The principle new subject of this work has been the investigation of crossing between parasite clones in the laboratory. The PCR technique has been applied for the first time to the mosquito stages of the parasite life-cycle, to investigate the frequency of cross-fertilisation. These results will be discussed in this section, along with their implications with regard to field populations of malaria parasites.

VII.1 Meiosis and genetic recombination.

Genetic diversity in a population of any species arises by three principle mechanisms : gene mutation, recombination and gene flow from other populations.

(i) Gene mutation : In organisms that can only reproduce by asexual division, mutation is the only way in which genetic changes may come about. In a bacterial species, where there is rapid asexual division, even a low mutation rate can provide sufficient variability for the organism to survive in a slowly changing environment [Mayr, 1963].

(ii) Genetic recombination: Genetic recombination is the process by which new combinations of genes arise in the progeny of crosses between two parent organisms. Recombination occurs principally at meiosis ; more rarely at mitosis. In most higher organisms, meiosis usually occurs immediately prior to gamete formation. In lower

eukaryotes however, it may take place in the first divisions of the zygote, resulting in a lifecycle that is mainly haploid. Meiosis in malaria parasites occurs shortly after zygote formation in the mosquito vector.

Meiosis is a two step cell division resulting in a halving of the diploid chromosome number. Individual members of a chromosome pair segregate from one another independently of members of other pairs, thus permitting reassortment of genes located on different chromosomes. Crossing-over between genes on homologous chromosomes may also occur during the first meiotic division. Recombination includes both reassortment and crossing-over. Mayr (1963) considered that "through recombination, a population can generate ample genotypic variability for many generations without any genetic input (by mutation or gene flow) whatsoever".

Mosquito transmission of malaria parasites provides a highly efficient means of generating parasites with novel genotypes, both by independent assortment of unlinked genes and by crossing-over events. The capacity of *P. falciparum* to undergo genetic recombination is considerable, and needs to be taken into account for control strategies either by chemotherapy or vaccination, especially since natural infections in man are frequently mixtures of parasites of different genotypes [Graves *et al.*, 1984 ; Thaithong *et al.*, 1984 ; Webster *et al.*, 1985; Conway *et al.*, 1991].

VII.2 Development of the PCR technique

Studies using the murine malaria parasite P. yoelii nigeriensis failed to detect chromosome banding patterns in oocyst material, presumably because of the very small quantities of DNA contained in a single oocyst.

Amplification of murine parasite DNA using the polymerase chain reaction (PCR) was not possible because of a lack of sequence data. The human malaria parasite P. falciparum was selected for this work because (i) sequence information is available for a number of genes, including some which exhibit allelic diversity and (ii) crossing experiments had previously been performed using this species [Walliker et al., 1987]. The two clones chosen for in vitro culture of gametocytes to feed to mosquitoes were the same clones used in previous crossing experiments.

As has been previously reported [Ponnudurai et al., 1989] infection rates of mosquitoes vary greatly from feed to feed. It has been suggested that there is much variability in the mosquito population which would account for differences seen in feeding experiments. HB3 gametocytes regularly infected a lower proportion of mosquitoes, and oocyst numbers in these mosquitoes were lower, than 3D7 cultures. Variation in the infectivity of clones to mosquitoes has been established by other workers [Ponnudurai et al., 1982b, 1989]

VII.2.i Amplification of asexual parasite DNA

The primers which were designed and synthesised for this work amplified products of different sizes for DNA of both 3D7 and HB3, for both genes. Differentiation of the two clones was simple and easy following electrophoresis.

It was possible to distinguish the presence of a mixture of DNA from both clones, even when there was a thousand-fold difference in the amounts of each allele added for amplification. Thus, if both alleles were not present in equal amounts in an oocyst both would still be amplified. Very small quantities of DNA (as low as 26pg) could be detected by the PCR.

VII.2.ii Amplification of oocyst DNA

The oocyst lysis buffer used was based on a sperm lysis buffer for DNA extraction for amplification from single sperm [Li *et al.*, 1988]. DNA extraction procedures using phenol and chloroform had to be used, because other methods did not remove a PCR-inhibitory factor present in the oocysts. It is possible that a haem-type molecule, perhaps related to the parasite pigment haemozoin (produced from parasitic digestion of haemoglobin in the red blood cell), was responsible for the inhibition of Taq polymerase. Haem is known to be a potent inhibitor of this polymerase [Higuchi, 1989].

Incorporation of a proportion of the base analogue 7-deaza-2'-deoxyguanosine triphosphate (C^7dGTP) into the

amplification reaction was essential for successful amplification of oocyst DNA. Some amplification occurred without the base analogue, but this was irregular and unpredictable.

c^7dGTP breaks down secondary structure in double-stranded DNA and its inclusion in amplification reactions has been recommended for DNA where secondary structure is thought to be preventing efficient amplification [McConlogue et al., 1988]. The N-7 position of the guanine ring in dGTP is replaced with a methine moiety [Barr et al., 1986] in c^7dGTP. By incorporation into DNA fragments c^7dGTP abolishes Hoogsteen bond formation (stacking) but allows Watson-Crick pairing to continue, thereby breaking down structures such as hairpin loops and base compressions.

There is no evidence that the parasite genome in an oocyst has different secondary structure to the genome in blood-stage parasites, yet DNA extracted by the same methods from both stages of the parasite does not behave similarly under PCR conditions. It has been suggested that oocyst DNA may be in a more 'active' form, perhaps because of the intense mitotic activity in the developing oocyst [J.G. Scaife, personal communication]. The folding or protein-binding qualities of this 'active' DNA may decrease the efficiency of binding of the primers used for amplification, or slow down amplification by some other means. Successful amplification may occur if the DNA extraction procedure has been slightly more

efficient, so that the relative amount of DNA added to the PCR is greater. Addition of c^7dGTP allows a high proportion of oocyst DNA samples to be amplified successfully. Amplification with all primers was parasite-specific.

The design and use of nested PCR primers [Simmonds et al., 1990] increased the number of oocyst DNA samples that could be amplified successfully. 30 cycles of amplification may produce insufficient product for visualisation with ethidium bromide. A further 10 to 30 cycles using nested primers internal to the first pair on a small fraction of the first PCR product increases the overall efficiency of the reaction. Almost all of the template used in the nested PCR will be PCR fragments, with little or no secondary structure. 96% of DNA samples from single oocysts could be amplified using this method.

VII.3 Genetic diversity

The two genes studied in this work encode the antigens MSP1 and MSP2, both of which contain repetitive regions. Many of the malaria antigens which have been cloned and sequenced contain repetitive regions which are often immunodominant. There is some evidence that repetitive structures in malaria antigens may be involved in immune evasion mechanisms. Much of the antibody response induced by the parasite infection in man is directed against epitopes encoded by repeat sequences. These immunodominant structures may act as a

'smokescreen' by preventing the development of effective antibody responses to more relevant epitopes. Anders [1986] suggested that the development of effective immunity may be prevented or delayed as a result of interference by the repeats in the normal maturation of high affinity antibody responses, resulting in continued proliferation of B-lymphocytes which produce low affinity antibodies.

MSP1 and MSP2 polymorphism is allelic polymorphism at a single locus for each gene. Repeats in the MSP1 and MSP2 gene products are generally conserved within the molecule, although they can vary from isolate to isolate. There is considerable diversity in the number and sequence of the repeats in alleles of both MSP1 and MSP2 genes.

The two MSP1 alleles sequenced and presented in this study are different from other alleles previously sequenced, except for the HB3 allele of MSP1, which is identical to the allele of the parent isolate H1. The MSP1 3D7 allele encodes new tripeptides not previously seen in other alleles. The HB3 allele of MSP2 is also different from any allele sequence published elsewhere.

The new sequences presented here emphasize the great diversity present in repetitive regions of these genes, and perhaps lend support to the 'smokescreen' theory for the function of repetitive structures.

VII.4 Generation of diversity

The diversity in repeat regions, both in sequence and length, can be explained using mutation and recombination involving crossing-over. A mutation, deletion or insertion in a single repeat unit may spread throughout the repeat array by unequal crossing-over or gene conversion. Replication slippage has been postulated for the evolution of alleles of minisatellite DNA [Jeffreys, Newmann & Wilson, 1990], and has been studied extensively in the transcriptionally inert repetitive satellite DNA in eukaryotes [Smith, 1976]. An analogy to the minisatellite evolution is further suggested by the presence in the MSP2 repeats of Chi and Chi-like sequences, thought to be involved in DNA recombination [Jeffreys, Wilson & Thein, 1985].

Such mechanisms for the spread of mutations in a repeat array could result in parasite populations with antigens which differ in sequence and antigenic properties, many of which can coexist at any one time and within a restricted geographical area.

VII.5 Frequency of cross-fertilisation

This work has produced, for the first time, direct evidence of cross-fertilisation of genetically different malaria parasites in the mosquito vector. Direct examination of the products of fertilisation in the mosquito obviates the need for chimpanzees and for time-consuming cloning of progeny previously necessary in

crossing experiments involving human parasites [Walliker *et al.*, 1987]. The latter experiments suggested that a higher number of recombinant progeny clones were produced, following transmission of a mixture of gametocytes, than might be expected if mating between gametes was random. Recombinant progeny may be at a selective advantage during multiplication in the chimpanzee host, or during adaptation to *in vitro* culture and cloning. Alternatively there may be excess production of recombinant parasites in the mosquito, because of a favouring of cross-fertilisation (between gametes of different clones) over self-fertilisation (between gametes of the same clone) in the mosquito. Mechanisms to allow the favouring of cross-fertilisation are known in other protozoa. For example, mating types exist in the ciliated protozoan *Paramecium aurelia*. Individuals with the same mating type cannot conjugate [Sonneborn, 1957]. In *Euplotes* mating types are determined by multiple alleles at a single locus, with each allele determining a single mating type. In other protozoa, there is no direct relationship between a gene and a mating type, although a complicated underlying genetic control exists. Mating types have been found in some but by no means all protozoa [see Preer, 1968, for a review of protozoan genetics].

Direct examination of the parasite stages in the mosquito avoids possible distortion by selection acting to favour recombinant progeny in the chimpanzee and during *in vitro* culture.

There could still be selection acting immediately after zygote formation, for example to favour the development of oocysts from heterozygotes over oocysts from homozygotes.

In the analysis of 128 oocysts presented in this work there is no evidence for over-representation of oocysts developing from heterozygotes compared to oocysts developing from homozygotes. Thus, cross-fertilisation does not appear to be favoured over self-fertilisation.

Therefore the excessive number of recombinants found in the progeny cloned after transmission through chimpanzees [Walliker et al., 1987] cannot be explained by favoured cross-fertilisation. It seems likely that selection acting in the chimpanzee or during in vitro culturing and cloning was responsible for the high numbers of recombinants amongst the progeny clones. It is interesting to speculate on why recombinants may have a selective advantage. For example, recombinant progeny may be selected because they contain combinations of genes favouring rapid asexual reproduction which are not found in either parent. Hybrid vigour in plants such as maize, and heterozygote advantage in animals are well known phenomena where a heterozygote in some way has a selective advantage over homozygotes.

The techniques described in this thesis for the amplification of DNA from a single oocyst allow this stage of the parasite life-cycle to be examined in detail

for the first time. The frequency of cross-fertilisation in a laboratory cross has been determined, to investigate if a favouring of cross-fertilisation could explain the high numbers of recombinants in the progeny of a previous laboratory cross. The results presented here show that there was no favouring of cross-fertilisation i.e. that mating was random.

The technique has also been tested in field conditions, albeit on a small scale, but seems promising. Work is currently underway to establish the frequency of hybrid oocysts (developing from cross-fertilisation events) in natural populations in Tanzania, Sudan and Papua New Guinea.

It is feasible that the frequencies of different alleles of the two genes could be examined in natural populations without examining patient blood. These frequencies would reflect the allele frequencies present in the gametocyte population, which is rarely if ever considered in studies of diversity in natural populations.

VII.6 The clonality debate

It has been postulated that parasitic protozoa reproduce clonally [Tibayrenc et al., 1990, 1991], i.e. that genetic recombination in natural populations is rare or absent. While this may be true of other parasitic protozoa, such as Trypanosoma cruzi, there is good evidence that populations of Plasmodium are panmictic.

Carter and Voller [1975] analysed allelic forms of two enzymes, lactose dehydrogenase (LDH) and glucose-6-phosphate isomerase (GPI), in isolates of P. falciparum from The Gambia and Tanzania, and concluded that the proportions of possible genotypes in the population did not differ significantly from those expected if the population is randomly interbreeding. Similar conclusions were drawn from an analysis of isoenzymes in P. vivax from India [Joshi et al., 1989].

Analysis of isoenzymes of P. chabaudi collected from thicket rats (Thamnomys rutilans) from Central African Republic also suggested that the natural population was randomly interbreeding [Beale, Carter & Walliker, 1978].

In a study of allelic variation at 20 loci in 20 isolates of P. falciparum from Brazil, Thailand and Zimbabwe [Creasey et al., 1990], no two isolates were identical, and allele frequencies varied between the three countries. A detailed study of three blood form antigens in a P. falciparum population in The Gambia [Conway & McBride, 1991] showed a large number of different alleles at each locus. The number of different genotypes (different combinations of alleles at each locus) expected assuming free recombination, calculated from the frequencies of the individual alleles, did not differ from the observed genotype diversity. The authors concluded that recombination between unlinked loci was a common consequence of sexual reproduction in P. falciparum.

Markedly seasonal malaria transmission in a small, relatively isolated, remote village might be expected to result in clonal-type infections, if only a few genotypes survive the prolonged dry season. In such a village in Eastern Sudan, 29 isolates were characterised with respect to 18 different, genetically controlled markers [Babiker et al., 1991a]. No two isolates were genotypically identical, even among isolates taken from members of the same household. Analysis of certain 2D-PAGE proteins showed that all possible combinations of alleles involved were present in the natural population and, although the number of isolates was small, there was no evidence of linkage disequilibrium. This suggests that even during the dry season, or immediately after the rains begin, parasite populations are diverse and not clonal.

It can be concluded from samples taken from natural populations that there is a large diversity of different, distinct genotypes ('clones'). There is no evidence for over-representation of particular genotypes, which could be indicative of clonality, and there is evidence of random mating and of free recombination between unlinked loci.

Mixed infections (of more than one genetically distinct parasite type) are common in nature ; thus there is ample opportunity for uptake of genotypically distinct gametocytes from an infected individual during mosquito feeding, allowing cross-fertilisation (as well as self-

fertilisation) to occur. Cross-fertilisation may also occur following interrupted feeding by a mosquito on more than one infected person, each of whom was infected with different parasites. Recombination, leading to the production of novel genotypes, will almost certainly occur following cross-fertilisation.

VII.7 Importance of cross-fertilisation

Without cross-fertilisation there will be no recombination (mitotic recombination is extremely rare). On a practical level, the frequency of cross-fertilisation, and thus recombination, will affect, for example, the rate of spread of drug resistance in natural populations. Curtis and Otoo [1986] put forward a model to compare the rates of increase in the frequencies of two, hypothetical, unlinked, drug-resistance genes in a population where the two drugs are used either in a mixture or separately. With random mating and free recombination in the parasite population, where only a small fraction of the human host population is taking the drugs, and where initially both parasite resistance genes are rare, the build-up of resistance to the drug mixture is delayed compared to a hypothetical parasite population where there is no recombination (selection pressure was held constant). The authors concluded that if both resistance genes are rare initially, and a large proportion of the parasite population is not exposed to the drugs, administering a mixture of drugs would be

preferable to administering the drugs in sequence, provided that there is random mating and free recombination between the loci.

More recently, Dye [1991] proposed a stochastic population model and tested the effect of drug mixtures on the rise in frequency of drug resistance genes. He suggested that the frequency of outcrossing and effective selection pressure are inevitably linked. A double-resistance genotype will obviously be selected for if transmitted to a host taking the drugs, but the proportion of such hosts in the population is small. More cross-fertilisation will increase the probability of the double-resistance genotype arising (as a result of recombination between two gametes each possessing a single, different resistance gene). However recombination will tend to separate resistance genes in already an already double-resistant gamete when mating occurs with a drug-sensitive gamete. The outcome (whether the double resistance genotype increases in the population) will depend on the initial frequencies of the two resistance genes, and on the balance between genetic recombination and the intensity of selection. A higher rate of cross-fertilisation may increase the frequency of the double-resistance genotype if selection is very strong. Mixed infections in a host are common, as has been shown in natural populations, and parasite genotypes within one host are different to those found in another host. Assuming that mosquitoes feed at random on members of a

population the transmission dynamics in a hyperendemic area are probably closer to the Curtis and Otoo model ; that is, mosquitoes take parasites from the entire local parasite population, instead of from discrete subpopulations. With low drug usage in the population, build-up of resistance to two drugs, when used in a mixture, will be delayed, compared to build-up of resistance if the two drugs are used in sequence.

VII.8 Sex allocation theory

Dye also suggests that the female-biased sex ratios that are usually found amongst P. falciparum gametocytes can be explained as the evolutionary consequence of non-random mating [Hamilton, 1967]. Modern evolutionary theory predicts that natural selection acting on individuals will result in an equilibrium sex ratio, usually 1:1. In a study of naturally occurring lizard malarias, stable sex ratios varied from 36% to 50% microgametocytes ("males")[Schall, 1989]. There was significant variation in gametocyte sex ratios among species, among infections within a species, and sometimes within infections over time. In most infections, macrogametocytes were more abundant, although occasionally microgametocytes predominated. In human malaria species, reported gametocyte sex ratios vary greatly, both between species and during infections. In two P. ovale infections the proportion of microgametocytes varied from 11% to 37% and from 11% to

38% over four days [James, Nicol & Shute, 1932]. In a *P. falciparum* infection observed over 13 days there was a gradual increase in the proportion of microgametocytes from 24% to 50% [James, 1931]. However, most published reports simply state that macrogametocytes are usually more abundant than microgametocytes, and there is a paucity of longitudinal counts during infections. It is interesting that in the reports that are published, the proportion of males increases from an initially low level to a higher level over the course of the infection, occasionally reaching the equal ratio predicted by evolutionary theory. *In vitro* culturing of gametocytes of the two clones in this work revealed a significant difference in the mean sex ratio obtained, although there was variation from culture to culture. The sex ratios of both clones were strongly female-biased.

A biased sex ratio (away from 1:1) may occur where there is parent-offspring conflict, local mate competition (among males) or local resource competition (among females). Hamilton's theory of local mate competition predicts a female-biased sex ratio in species in which the sons of a parent must compete with one another for mates [Hamilton, 1967]. In a clonal *Plasmodium* infection, all males will be identical. There will be an optimum investment of resources if there are just enough male gametes to fertilise all female gametes. This type of economy in the production of males has been reported in certain small arthropods, wherever

reproduction is quite regularly by brother-sister mating (see Hamilton, 1967 for review and references). Data on gametocyte sex ratios collected from Papua New Guinea show the female-bias previously reported, and the authors suggest that this could be indicative of a low rate of outcrossing (estimated as 38%) [Read *et al.*, 1992].

Work on parasitoid wasps such as *Nasonia* and *Caraphractus* suggests that first-comers to a host may use a sex ratio less female biased than the extreme, to allow for the chance of double parasitism. The second-comers best sex ratio, assuming that they can detect previous parasitism, will be based only on the expected sex ratio of the first-comers.

Applying this hypothesis to *Plasmodium*, it is possible that the first (clonal) infection of an uninfected human host could produce the female biased sex ratios seen in many reported cases. As the result of super-infection with another clone, the primary clone itself may be triggered to alter the sex ratio in its gametocytes closer to 1:1, or the secondary clone may bias its sex ratio in the opposite direction (towards an excess of males) ; the overall sex ratio of gametocytes would then shift towards 1:1.

Alternatively there may not be a 'trigger' of super-infection to changing the sex ratio of the primary infection. Instead, a clone may produce a female biased sex ratio initially, and then as the period of infection increases (and with it the probability of super-

infection) the sex ratio may shift gradually towards 1:1, regardless of whether super-infection actually occurs.

There is, however, no evidence that malaria parasites have adaptive sex ratios. The timing of gametocyte production in the lizard malaria P. mexicanum does not seem to be adaptive for a seasonable environment [Bromwich & Schall, 1986]. The observed variation in sex ratios among infections may be misleading, as sex ratios may be more similar among infections if only viable gametocytes had been counted. It is possible, although perhaps unlikely, that there may be a differential mortality between the sexes, with female gametocytes being less long-lived. A similar differentiation between the sexes in the length of time that a gametocyte remains fertile i.e. infective to mosquitoes, may also complicate the estimation of actual sex ratios of mature, fertile gametocytes. If male gametocytes are indeed fertile for longer than females, this may account for the gradual increase in the proportion of microgametocytes observed in natural P.falciparum and P. ovale infections.

In conclusion, evidence from isoenzyme studies and from the analysis of other allelic variation strongly suggests that natural populations of malaria parasites in endemic areas are randomly interbreeding. No evidence has been provided for linkage disequilibrium, even in parasite populations from an area of markedly seasonal transmission. Recombination between unlinked loci has

been reported as a common consequence of sexual reproduction in P.falciparum.

The female-biased gametocyte sex ratios from peripheral blood smears from natural infections seem to suggest that self-fertilisation is more common than outcrossing. However, there is no study in which both gametocyte sex ratios and the diversity of these gametocytes has been analysed. It is possible that in clonal infections of a single host, or in populations where there is a restricted diversity of genotypes, female-biased gametocyte sex ratios may be more common, since they represent an optimum allocation of resources if outcrossing is rare. In endemic areas where there is a huge diversity of parasite genotypes, even in relatively small and isolated populations, sex ratios closer to the 1:1 ratio may be more common, reflecting the higher degree of outcrossing.

This type of study is obviously crucial to a better understanding of the role, if any, that sex ratio data can play in estimating the extent of cross-fertilisation in a parasite population. The methods described in this thesis provide a direct means of measuring the (minimum) amount of cross-fertilisation taking place, by collecting and examining the mosquito stages of the parasite in natural populations. Sex ratio studies should be complimentary to these studies.

It would be important to examine only the gametocyte genotypes present in an infected host, since a clonal

sexual population will not necessarily be reflected in the asexual population of a given host. Conversely, an infected host may have a clonal population of asexual parasites but gametocytes of different genotypes. Direct examination of gametocyte genotype diversity has previously been impossible because of a lack of gametocyte-specific monoclonal antibodies. New DNA amplification techniques, coupled with improved separation techniques, could be applied to this sort of work.

Only when these data are available can the issue of the relevance of sex ratio data to outcrossing in malaria parasite populations be addressed. To date there is no evidence at all that Plasmodium gametocyte sex ratios can be altered by different environmental conditions. If Plasmodium gametocyte sex ratios are not adaptive, then theories of sex allocation cannot be applied.

VIII Final conclusions

The work presented herein describes the development of the polymerase chain reaction for amplification of malaria parasite DNA extracted from mosquito stages of the life-cycle. Allelic size polymorphism in two genes was utilised to distinguish oocysts arising from heterozygotes following cross-fertilisation from oocysts developing from homozygotes following self-fertilisation, in the mosquito midgut. The frequency of cross-fertilisation was determined between two clones previously used in crossing experiments. The frequency of interbreeding between the two clones did not differ from that expected if mating between gametes of the two clones was random.

Thus there is no evidence that cross-fertilisation is favoured over self-fertilisation. This was proposed as a mechanism to explain high numbers of recombinant progeny clones obtained in previous crossing experiments with human malaria parasites. It now seems likely that these high numbers of recombinants arose because of selective pressure in the vertebrate host or during *in vitro* culturing.

This technique has also been successfully used to isolate and amplify DNA from oocysts isolated from wild-caught mosquitoes. Examination of the rates of cross-fertilisation in natural population can now be made directly on mosquito populations.

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Appendix 1

The composition of buffers,solutions and media

All buffers, solutions and media were prepared using double-distilled water and reagents of the best grade available.

Buffers and solutions

Reagents were autoclaved or filter-sterilised before mixing to give sterile solutions.

Buffer A
50mM NaCl
25mM EDTA

Deep freeze solution
28% glycerol
3% sorbitol
0.65% NaCl
Sterilised by filtration

50 x Denhardt's soln.
1% Ficoll
1% polyvinylpyrrolidone
1% BSA (pentax fraction V)
Sterilised by filtration

Gel loading buffer
0.25% bromophenol blue
25% ficoll
10mM Tris-Cl
1mM EDTA
Not autoclaved or filtered. Stored at room temperature.

Hybridisation soln.
6 x SSC
2% SDS
5 x Denhardt's soln
0.01M EDTA
200μg ml^{-1} salmon sperm DNA
Not autoclaved or filtered.

Tris-borate-EDTA (TBE)
0.09M boric acid
0.09M Tris
0.002M EDTA

10:1 Tris-EDTA (TE)
10mM Tris-HCl
1mM EDTA
Used at the required pH within the range pH 7.4-8.0

50:1 Tris-EDTA (TE)
50mM Tris-HCl
1mM EDTA
Used at the required pH within the range pH 7.4-8.0

Lysis buffer B
- 50mM NaCl
- 50mM EDTA
- 10mM Tris-Cl pH8.0
- 2% sarkosyl

Oocyst lysis buffer
- 100mM NaCl
- 25mM EDTA (pH8.0)
- 10mM Tris-Cl (pH8.8)
- 0.5% Sarkosyl

20 x saline-sodium citrate buffer (SSC)
- 3M NaCl
- 0.3M sodium citrate.$2H_2O$

PCR Reagents

Solutions for making PCR reagents were autoclaved or filter-sterilised before mixing to give sterile solutions.

PCR Buffer
50mM KCl (Analar, BDH)
10mM Tris-Cl(pH8.8) (Aristar, BDH)
2.5mM $MgCl_2$ (AR, Fisons)
0.02% gelatin (tissue culture grade, Sigma)

PCR dNTP solution
75μM dGTP (BCL)
75μM dTTP (BCL)
75μM dATP (BCL)
75μM dCTP (BCL)
Not autoclaved or filtered.

PCR c^7dNTP solution
65μM dGTP (BCL)
10μM c^7dGTP (BCL)
75μM dTTP (BCL)
75μM dATP (BCL)
75mM dCTP (BCL)
Not autoclaved or filtered.

Sequencing reagents

Polyacrylamide sequencing gel
- 8M Urea
- 6% acrylamide
- 0.16% bis-acrylamide
- 0.08% ammonium persulphate
- 0.03% TEMED

in 1 x TBE

Fixing solution
- 7% acetic acid
- 7% methanol

The following reagents were included in the Sequencing kit obtained from United States Biochemicals.

RX reaction buffer
- 40mM Tris-Cl(pH7.5)
- 20mM $MgCl_2$
- 50mM NaCl
- 10% DMSO

Labelling Mix
- 7.5μM dGTP
- 7.5μM dATP
- 7.5μM dTTP
- 7.5μM dCTP

Sequenase[R] enzyme dilution buffer
- 10mM Tris-Cl(pH7.5)
- 5mM DTT
- 0.5mg ml^{-1} BSA

Stop solution
- 95% formamide
- 20mM EDTA
- 0.05% bromophenol blue
- 0.05% xylene cyanol FF

Termination mixes

80μM dGTP	and	8μM ddGTP	(G-mix)
80μM dATP	or	8μM ddATP	(A-mix)
80μM dCTP	or	8μM ddCTP	(C-mix)
80μM dTTP	or	8μM ddTTP	(T-mix)

50μM NaCl
10% DMSO

Appendix 2 : Mosquito infectivity data

Spaces or nd (not done) indicate that the particular data are not available.

Date of feed	Clone	Time since thawing	% infectn.	GII	Mean no oocysts/ +ve gut	G'cytes/1000 rbcs			% ♀ gametocytes			♀ gametocytes/1000 rbc		
						D14	D17	All	D14	D17	All	D14	D17	All
25/11/88	3D7	46	50	nd	25									
	HB3	46	50	nd	25									
	MIX		50	nd	25									
23/1/89	3D7	18	0	0	0									
	3D7	18	29	32	11.0									
	3D7	18	60	120	2.0									
	3D7	18	40	60	1.5									
	3D7	18	25	225	9.0									
	3D7	18	29	257	9.0									
	3D7	18	60	180	3.0									
27/1/89	3D7	22	0	0	0									
	3D7	22	0	0	0									
	3D7	22	0	0	0									
	3D7	22	0	0	0									
	3D7	22	0	0	0									
	3D7	22	33	33	1.0									
27/1/89	3D7	22	63	375	6.0									
(Tipper)	3D7	22	50	117	2.3									
	3D7	22	60	360	6.0									
	3D7	22	57	143	2.5									

Date of feed	Clone	Time since thawing	% infectn.	GII	Mean no oocysts/ +ve gut	G'cytes/1000 rbcs			% ♀ gametocytes			♀ gametocytes/1000 rbc		
						D14	D17	All	D14	D17	All	D14	D17	All
21/2/89	3D7	47	25	200	8.0									
(Tipper)	3D7	47	30	50	1.7									
	3D7	47	80	180	2.2									
	HB3	47	0	0	0									
	MIX		50	225	4.5									
	MIX		0	0	0									
24/2/89	3D7	50	40	40	1.0									
(Tipper)	3D7	50	0	0	0									
	HB3	50	25	125	5.0									
	HB3	50	41	29	2.0									
	MIX		40	40	1.0									
	MIX		0	0	0									
24/2/89	3D7	50	0	0	0									
	3D7	50	71	314	4.4									
3/3/89	3D7	57	40	40	1.0									
	3D7	57	0	0	0									
	3D7	57	25	25	1.0									
	3D7	57	17	17	1.0									
28/5/89	3D7	24	40	80	2.0									
	HB3	24	71	214	2.8									
1/6/89	3D7	28	50	100	2.0									
	3D7	28	0	0	0									
	HB3	28	25	25	1.0									
	MIX		nd	nd	nd									

Date of feed	Clone	Time since thawing	% infectn.	GII	Mean no oocysts/ +ve gut	G'cytes/1000 rbcs			% ♀ gametocytes			♀ gametocytes/1000 rbc		
						D14	D17	All	D14	D17	All	D14	D17	All
2/6/89	3D7	29	40	80	2.0									
	HB3	28	40	140	3.4									
6/6/89	3D7	33	0	0	0									
	HB3	32	17	17	1.0									
	MIX		0	0	0									
27/6/89	HB3	53	20	40	2.0									
7/7/89	3D7	64	30	40	1.3									
	3D7	64	0	0	0									
	HB3	63	20	20	1.0									
	MIX		40	40	1.0									
	MIX		0	0	0									
14/7/89	3D7	71	0	0	0									
	3D7	71	0	0	0									
	HB3	70	0	0	0									
	HB3	70	0	0	0									
	MIX		0	0	0									
	MIX		0	0	0									
1/9/89	3D7	35	50	175	3.5									
	3D7	35	40	60	1.5									
30/10/89	3D7	94	25	75	3.0									
6/11/89	3D7	103	43	43	1.5									
10/11/89	3D7	18	67	1167	17.5									

Date of feed	Clone	Time since thawing	% infectn.	GII	Mean no oocysts/ +ve gut	G'cytes/1000 rbcs			% ♀ gametocytes			♀ gametocytes/1000 rbc		
						D14	D17	All	D14	D17	All	D14	D17	All
9/2/90	3D7	36	100	575	5.8									
	HB3	36	25	25	1.0									
	MIX		25	25	1.0									
23/3/90	3D7	24	20	20	1.0									
	3D7	24	25	25	1.0									
6/4/90	3D7	38	30	nd	10									
	HB3	38	30	nd	10									
	MIX		30	nd	10									
7/6/90	3D7	50	11	22	2.0									
	HB3	50	16	32	2.0									
	MIX		14	93	6.5									
11/6/90	3D7	54	27	160	6.0									
	HB3	54	20	27	1.3									
	MIX		26	83	3.2									
25/6/90	3D7	68	29	79	2.8									
	HB3	68	0	0	0									
	MIX		0	0	0									
13/7/90	3D7	86	0	0	0									
	HB3	86	0	0	0									
16/7/90	3D7	89	0	0	0									
	HB3	89	0	0	0									

Date of feed	Clone	Time since thawing	% infectn.	GII	Mean no oocysts/ +ve gut	G'cytes/1000 rbcs D14	D17	All	% ♀ gametocytes D14	D17	All	♀ gametocytes/1000 rbc D14	D17	All
20/7/90	3D7	96	0	0	0									
17/8/90	3D7	38	0	0	0									
	HB3	38	0	0	0									
12/10/90	3D7	39	0	0	0									
	HB3	39	0	0	0									
15/10/90	3D7	42	100	275	6.0									
	HB3	42	67	38	1.5									
	MIX		100	375	5.0									
19/10/90	3D7	46	40	57	4.0									
	HB3	46	22	21	1.5									
	MIX		33	44	2.0									
	MIX		60	100	2.7									
	MIX		50	78	8.5									
9/11/90	3D7	67	50	6	1.0									
	HB3	67	0	0	0									
19/11/90	3D7	76	11	18	3.0	2.0	6.7	5.6	71	92	92	1.4	6.2	5.0
	HB3	76	0	0	0									
	MIX		25	13	1.0									
23/11/90	3D7	81	0	0	0									
	HB3	81	0	0	0									
26/11/90	3D7	84	20	20	1	9.2	3.5	5.9	77	83		7.1	2.9	4.7
	HB3	84	0	0	0	2.5	3.2	2.5	80	77	78	2.0	2.9	2.5
30/11/90	3D7	88	0	0	0	3.3	2.8	3.0	93	87	90	3.0	2.4	2.7
	HB3	88	0	0	0									

Appendix 3

(a) Sex ratio data

From the pooled sex ratio data shown in Table 4, section VI.2, the mean of the percentage female gametocytes in the cultures of 3D7 was different to that in HB3 cultures. A parametric test, such as student's t-test, can be applied to test this hypothesis. Such a test assumes that the variances in the populations from which the two samples were taken are equal. The variances, given in table 4, are 35.8 for 3D7 cultures, and 54.3 for HB3 cultures.

The significance of the difference in these two variances can be tested using the F-distribution.[1]

1. Test of significance of difference in two variances

The null hypothesis is that the two variances are equal, i.e. that $H_0 : \sigma_1^2 = \sigma_2^2$. The alternative hypothesis is that the two variances are not equal, i.e. $H_1 : \sigma_1^2 \neq \sigma_2^2$. In view of the alternative hypothesis this is a two-tailed test.

$$F_S = \frac{s_1^2}{s_2^2}$$

Thus, $$F_S = \frac{54.3}{35.8} = 1.52$$

For a two-tailed test, the critical value is $F_{\alpha/2[17,32]}$

$F_{0.025\ [17,32]} = 2.26$

$F_{0.05\ [17,32]} = 1.98$

[1] Percentages are often transformed by arcsin to improve the normality of the distribution. This was tested for this data set : the Fmax test on the raw data gave a value of 1.554 (not significant) ; the Fmax test on the arcsin transformed data gave a result of 1.506 (also not significant). Thus, from the first result we may conclude that no real need for the transform exists, and from the second, that when the transform is performed there is no real improvement in the homogeneity of the data. Given these results, and the high significance of the other tests used, it was decided that transformation was not appropriate for this data set.

Since this is a two-tailed test, these probabilities are doubled. Thus, the F_{value} of 1.98 represents a probability of α=0.10, since the right-hand tail area of α=0.05 is matched by a similar left-hand area to the left of

$$F_{0.95[17,32]} = \frac{1}{F_{0.05[17.32]}} = 0.51$$

$$[F_s > F_{0.05[17,32]} \text{ is significant}]$$

Therefore, assuming the null hypothesis is true, the probability of observing an F_{value} greater than 1.52 and smaller than 1/1.52 = 0.66 is P>0.1.
It can be concluded that the two sample variances are not significantly different from one another.

Since the two sample variances are not significantly different from one another, the significance of the difference of the two means can be tested using Student's t-test. From table 4, the mean % female gametocytes for 3D7 cultures is 91.7%, and for HB3 cultures is 82.1%.

<u>2.Test of significance of difference of two means (Student's t-test)</u>.

The null hypothesis H_o is that the two sample means come from a population with equal mean (μ),

i.e. $H_o : \mu_1 = \mu_2$.

The alternative hypothesis H_1 is that $\mu_1 \neq \mu_2$.

The statistic t_s is calculated :

$$t_s = \frac{(Y_1 - Y_2) - (\mu_1 - \mu_2)}{\sqrt{\left(\frac{(n_1-1)s_1^2 + (n_2-1)s_2^2}{n_1 + n_2 - 2}\right)\left(\frac{n_1 + n_2}{n_1 n_2}\right)}}$$

Since we are testing if $\mu_1=\mu_2$, $\mu_1-\mu_2=0$.

$$t_s = \frac{91.7-82.1}{\sqrt{\left(\frac{32(35.8) + 17(54.3)}{(33 + 18 - 2)}\right)\left(\frac{33 + 18}{33 \times 18}\right)}} = \frac{9.6}{\frac{2068.7(0.086)}{49}}$$

$$= \frac{9.6}{\sqrt{(42.22 \times 0.086)}} = \underline{5.04}$$

$[d.f. = n_1 + n_2 - 2 = 49]$

Critical t_{value} $t_{0.025[49]} = 2.0$

$t_{0.005[49]} = 2.7$

$[t_s > t_{0.05[49]}$ is significant$]$

Since this is a 2-tailed test, a t_{value} of 2.7 represents a probability of $\alpha=0.01$, since the right-hand tail area of $\alpha=0.005$ is matched by a similar left-hand tail area to the left of 1/2.7 = 0.37.

The null hypothesis is rejected if $|t| \geq t_{0.025[49]}$ = 2.0. Since the test t_s = 5.04, the null hypothesis is rejected i.e. difference in the two means is significant.

Confidence limits of the difference between the two means can be calculated :

$$L_1 = (Y_1 - Y_2) - t_{\alpha[v]}S_{Y1-Y2}$$

$$L_2 = (Y_1 - Y_2) + t_{\alpha[v]}S_{Y1-Y2}$$

($Y_1 - Y_2 = 9.6$, $t_{0.025[49]} = 2.0$, $S_{Y1} - S_{Y2} = 1.9$ (denominator of t_s))

Thus,

$$L_1 = 9.6 - 2(1.9) = 5.8$$

$$L_2 = 9.6 + 2(1.9) = 13.4$$

The 95% confidence limits do not contain the zero point (= no difference). This was to be expected, as the difference Y_1-Y_2 was found to be significant.

In summary, the mean of the percentage of mature gametocytes which are female for 3D7 cultures differs significantly from that for HB3 cultures.

(b) Allele frequencies

1. Calculation of allele frequencies from oocysts

Hardy-Weinberg equilibrium law states that if the gene frequencies of two alleles among the parents are p and q, then the genotype frequencies among the progeny will be p^2 (homozygote), q^2 (homozygote) and 2pq (heterozygote). This relationship holds if there is

random mating, no selection for the genotypes under consideration, if genes segregate normally in gametogenesis and if the gene frequencies are the same in males and females.

The observed frequencies of the two alleles of MSP1 and MSP2 can be tested for agreement with a population in Hardy-Weinberg equilibrium. If the population is in equilibrium, then the gene frequency is the same in parents and progeny. Thus the gene frequency observed in the progeny (oocysts) can be used as if it were the parental gene frequency to calculate the genotype frequencies expected to arise in the progeny by Hardy-Weinberg law.

(i) MSP1 alleles

The allele frequencies in the progeny can be calculated from the number of self-fertilised (homozygous) oocysts and hybrid oocysts.

For the MSP1 allele, the observed numbers from table 5 are:

3D7 selfers	30
HB3 selfers	14
Hybrids	54
Total	98

Thus p, the frequency of the 3D7 allele, is :

$$\frac{2 \times \text{No. of 3D7 selfers} + 1 \times \text{No. of hybrids}}{2 \times \text{total}}$$

$$p = \frac{(2 \times 30) + 54}{(2 \times 54)} = \underline{0.582}$$

q, the frequency of the HB3 MSP1 allele, is :

$$\frac{2 \times \text{No. of HB3 selfers} + 1 \times \text{No. of hybrids}}{2 \times \text{total}}$$

$$q = \frac{(2 \times 14) + 54}{(2 \times 98)} = \underline{0.418}$$

The expected frequency of 3D7 self-fertilisation (from Hardy-Weinberg equilibrium law) is p^2, of HB3 self-fertilisation is q^2, and of cross-fertilisation is 2pq.

$p^2 = (0.582)^2 = 0.339$

$q^2 = (0.418)^2 = 0.175$

$2pq = (2 \times 0.582 \times 0.418) = 0.487$

The expected number of each type of oocyst can be calculated by multiplying the frequencies and the total number of oocysts (98) ; thus:

3D7 selfers	=	0.339 x 98	= 33.2
HB3 selfers	=	0.175 x 98	= 17.1
Hybrids	=	0.487 x 98	= 47.7
Total			98

Constructing a table for comparison,

	observed	expected
3D7 selfers	30	33.2
HB3 selfers	14	17.1
Hybrids	54	47.7
total	98	98

Performing a X^2 test for goodness of fit,

$$X^2_{[2]} = \sum \frac{(\text{observed} - \text{expected})^2}{\text{expected}}$$

$$X^2_{[2]} = \frac{(30 - 33.2)^2}{33.2} + \frac{(14 - 17.1)^2}{17.1} + \frac{(54 - 47.7)^2}{47.7}$$

$$X^2_{[2]} = 0.308 + 0.562 + 0.832$$

$$\underline{X^2_{[2]} = 1.702}$$

There are two degrees of freedom. The probability of $X^2_{[2]} > 1.702$ lies between 50% and 30%. There is therefore no reason to reject the null hypothesis i.e. there is no significant difference between the frequencies observed and those expected to occur in a randomly mating population.

The X^2 test for goodness of fit calculated above gives a statistic, called X^2, the sampling distribution of which approximates that of a chi-square distribution. An alternative method of testing the goodness of fit, the sample distribution of which also approximates as chi-square, is the G-statistic [Sokal & Rohlf, 1984].

$$G = 2 \sum^{a} f_i \ln (f_i/\hat{f}_i)$$

The G-statistic is the sum of the independent contributions of departures from expectation ($\ln (f_i/\hat{f}_i)$) weighted by the frequency of the particular class (f_i).

The advantages of the G-test over X^2 are that G is easier to compute, and that G follows the chi-square distribution more closely than X^2 for goodness of fit

does. In general however, G will be numerically similar to X^2.

For the MSP1 allele data, G can be calculated as :

$$G = 2 \times [30 \ln(30/33.2) + 14 \ln(14/17.1) + 54 \ln(54/47.7)]$$

$$= 2 \times [-3.041 + -2.800 + 6.699]$$

$$= 2 \times 0.858$$

$$G_{[2]} = \underline{1.72} \qquad [0.5 > p > 0.3]$$

Therefore for the MSP1 data, G and X^2 are similar.

<u>(ii) MSP2 alleles</u>

Repeating the calculations for MSP1 allele data in (i) above for the MSP2 data :

$$p = \frac{(2 \times 33) + 46}{(2 \times 87)} = \underline{0.644}$$

$$q = \frac{(2 \times 8) + 46}{(2 \times 87)} = \underline{0.356}$$

The frequencies of the homozygotes, p^2 and q^2, and the heterozygote, 2pq, can be calculated :

$$p^2 = (0.644)^2 = 0.415$$

$$q^2 = (0.356)^2 = 0.127$$

$$2pq = (2 \times 0.644 \times 0.356) = 0.458$$

The observed frequencies can be compared to those expected :

	observed	expected
3D7 selfers	33	36.1
HB3 selfers	8	11
Hybrids	46	39.9
total	87	87

Testing the significance of the differences between the expected and observed numbers of each type of oocyst :

$$X^2_{[2]} = \frac{(33 - 36.1)^2}{36.1} + \frac{(8 \times 11)^2}{11} + \frac{(46 - 39.9)^2}{39.9}$$

$$X^2_{[2]} = 2.017$$

$$X^2_{[2]} = \underline{2.02} \qquad (0.5 > p > 0.3)$$

The G-statistic calculated for the same data is 2.07. Thus the differences between the observed numbers and those expected if mating between gametes is random are not significant.

(iii) Estimation of allele frequencies in male and female gametes of each clone

One of the assumptions of Hardy-Weinberg equilibrium law is that the allele frequencies do not vary between males and females of each of the parents. In the laboratory crossing experiments, equal numbers of gametocytes of each clone were mixed before feeding to mosquitoes. However the proportion of female to male

gametocytes (and therefore gametes) in 3D7 cultures is different to the proportion in HB3 cultures.

91.7% of 3D7 gametocytes and 82.1% of HB3 gametocytes are female. Assuming that all gametocytes and gametes are equally viable, and that the numbers of male gametes produced by a single male gametocyte is the same for the two clones (i.e. 8), the allele frequencies can be calculated for each type of gamete.

For MSP1, the allele frequencies overall are:

3D7 allele, p = 0.582

HB3 allele, q = 0.418

0.917 of all gametocytes in 3D7 cultures are female and 0.083 are male. Following exflagellation, 0.58 of 3D7 gametes are female and 0.42 of gametes are male.

0.821 of all gametocytes in HB3 cultures are female and 0.179 are male. Following exflagellation, 0.36 of HB3 gametes are female and 0.64 are male.

In a mixture of gametocytes of both clones, (0.58 x 0.582 =) 0.34 will be female gametes of 3D7, and (0.36 x 0.418 =) 0.15 will be female gametes of HB3. Of male gametes (0.42 x 0.582 =) 0.24 will be 3D7 type and (0.64 x 0.418 =) 0.27 will be HB3.

Thus in a mixture of gametocytes, the male gametes of HB3 will outnumber the female gametes of HB3 and the male gametes of 3D7. The female gametes of 3D7

will outnumber the male gametes of 3D7 and the female gametes of HB3.

If mating between all gametes is random, then the expected frequencies of the zygotes can be calculated.

$3D7_{male} \times 3D7_{female} \quad \frac{0.24}{(0.24 + 0.27)} \times \frac{0.34}{(0.34 + 0.15)} = \underline{0.327}$

$3D7_{male} \times HB3_{female} \quad \frac{0.24}{(0.24 + 0.27)} \times \frac{0.15}{(0.34 + 0.15)} = \underline{0.144}$

$HB3_{male} \times 3D7_{female} \quad \frac{0.27}{(0.24 + 0.27)} \times \frac{0.34}{(0.34 + 0.15)} = \underline{0.367}$

$HB3_{male} \times HB3_{female} \quad \frac{0.27}{(0.24 + 0.27)} \times \frac{0.15}{(0.34 + 0.15)} = \underline{0.162}$

The observed and expected numbers of each type of oocyst are :

	observed	expected
3D7 selfer	30	32
HB3 selfer	14	15.9
Hybrid	54	50.1
Total	98	98

The difference between the observed and expected values is not significant ($X^2_{[2]} = 0.66$; $G_{[2]} = 0.66$)

The above calculations can be repeated for the data for alleles of MSP2 (p = 0.644 and q = 0.356), to allow for the diiferent proportions of male and female gametocytes in cultures of the two clones.

Thus in a mixture of gametocytes of the two clones, following exflagellation (0.58 x 0.644 =) 0.374 will be 3D7 female gametes, (0.42 x 0.644 =) 0.27 will be 3D7 male gametes, (0.36 x 0.356 =) 0.128 will be HB3 female gametes and (0.64 x 0.356 =) 0.228 will be HB3 male gametes.

The frequencies of the zygote types following random mating between all gametes can be calculated :

$$3D7_{male} \times 3D7_{female} \quad \frac{0.27}{(0.27 + 0.228)} \times \frac{0.374}{(0.374 + 0.128)} = \underline{0.404}$$

$$3D7_{male} \times HB3_{female} \quad \frac{0.27}{(0.27 + 0.228)} \times \frac{0.128}{(0.374 + 0.128)} = \underline{0.138}$$

$$HB3_{male} \times 3D7_{female} \quad \frac{0.228}{(0.27 + 0.228)} \times \frac{0.374}{(0.374 + 0.128)} = \underline{0.341}$$

$$HB3_{male} \times HB3_{female} \quad \frac{0.228}{(0.27 + 0.228)} \times \frac{0.128}{(0.374 + 0.128)} = \underline{0.117}$$

The observed and expected numbers of each type of oocyst are :

	observed	expected
3D7 selfer	33	35.1
HB3 selfer	8	10.2
Hybrid	46	41.7
Total	87	87

The difference between the observed and expected values is not significant ($X^2_{[2]} = 1.04$ [0.7>p>0.5]; $G_{[2]} = 1.07$ [0.7>p>0.5])

Appendix 4 : Oocyst data

The number of oocysts dissected from mosquitoes of each feed is shown in column 2. The results of PCR analysis of each oocyst are shown in the remaining columns. '3D7' and 'HB3' denote oocysts containing only 3D7 or HB3 alleles of the gene amplified. 'Hyb' denotes an oocyst containing both 3D7 and HB3 alleles of the gene amplified. '0' denotes that DNA from this oocyst did not amplify. n.d. = not done.

		PCR RESULTS											
Date	No.of	MSP1				MSP2				Combined			
	ooc.	3D7	HB3	HYB	0	3D7	HB3	HYB	0	3D7	HB3	HYB	0
17/6/90	7	1	1	2	3	2	0	3	2	2	1	3	1
22/6/90	15	5	2	5	3	6	0	5	4	7	2	6	0
16/8/90	9	5	1	2	1	5	0	1	3	6	1	2	0
25/10/90	14	4	1	5	4	6	2	5	1	6	2	6	0
29/10/90	4	0	0	3	1	0	0	4	0	0	0	4	0
	6	0	3	0	3	3	3	0	0	3	3	0	0
	5	0	0	2	3	0	1	3	1	0	1	3	1
total	15	0	3	5	7	3	4	7	1	3	4	7	1
11/2/91	10	1	2	5	2	2	1	4	3	2	2	6	0
	11	2	0	5	4	4	0	7	0	4	0	7	0
	7	2	0	5	0	1	0	5	1	2	0	5	0
total	28	5	2	15	6	7	1	16	4	8	2	18	0
13/2/91	6	2	1	0	3	1	0	2	3	2	1	2	1
20/1/91	8	1	1	5	1	0	1	4	3	1	1	6	0
	8	2	1	5	0	nd	nd	nd	nd	2	1	5	0
	12	3	1	7	1	nd	nd	nd	nd	3	1	7	1
total	28	6	3	17	2	0	1	4	3	6	3	18	1
20/5/91	6	2	0	3	1	3	0	3	0	3	0	3	0
TOTAL	128	30	14	54	30	33	8	46	21	43	16	65	4

Publications arising from this study

Ranford-Cartwright, L.C., Balfe, P., Carter, R. & Walliker, D. (1991a). Direct sequencing of enzymatically amplified DNA of alleles of the merozoite surface antigen MSA-1 gene from the malaria parasite *Plasmodium falciparum*. Mol. Biochem. Parasitol. 46, 185-188.

Ranford-Cartwright, L.C., Balfe, P., Carter, R. & Walliker, D. (1991b). Genetic hybrids of *Plasmodium falciparum* identified by amplification of genomic DNA from single oocysts. Mol. Biochem. Parasitol. 49, 239-244.

Conference abstracts

VII International Congress of Parasitology.

Paris, August 20-24, 1990

Oral and poster presentation : 'Frequency of cross-fertilisation between two clones of P. falciparum determined by polymerase chain reaction analysis on single oocysts.' L.C. Ranford-Cartwright, P. Balfe, R. Carter & D. Walliker

British Society for Parasitology 3rd Malaria Meeting.

Edinburgh, February 13-16, 1991

Poster presentation : 'Cross-fertilisation between clones of Plasmodium falciparum '. L.C. Ranford-Cartwright, P. Balfe, R. Carter & D. Walliker

British Society for Parasitology 4th Malaria Meeting

London, February 13-14, 1992

Oral presentation: 'Frequency of cross-fertilisation in Plasmodium falciparum'. L.C. Ranford-Cartwright, R. Carter, P. Balfe & D. Walliker.

Oral Presentation: 'Prevalence of cross-fertilisation in P. falciparum in Tanzania'. H.A. Babiker, L.C. Ranford-Cartwright, D. Walliker, T. Teuscher & D. Charlwood.

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Short communication

Direct sequencing of enzymatically amplified DNA of alleles of the merozoite surface antigen MSA-1 gene from the malaria parasite *Plasmodium falciparum*

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(Received 30 October 1990; accepted 3 January 1991)

Key words: *Plasmodium falciparum*; Merozoite surface antigen MSA-1 gene; Polymerase chain reaction

Merozoites of the malaria parasite *Plasmodium falciparum* carry surface proteins processed from a molecule denoted the precursor to the major merozoite surface antigen or MSA-1. This protein is a candidate for a malaria vaccine, because monkeys show some degree of protection against challenge following immunisation with the antigen [1–4].

The protein varies antigenically between parasites from different parts of the world [5]. DNA sequence analysis of the MSA-1 gene from a number of isolates [6–8] has revealed that the gene can be divided into blocks, some of which are conserved and others of which vary between isolates. The first variable block (block 2) near to the N-terminus of the gene is especially variable, encoding a polymorphic region of tandemly repeated amino acids, the tripeptide repeats. In different isolates this region contains varying numbers of the amino acid repeat S-X-X. However there are variants such as RO-33 [9] and CSL-2 [10] lacking repeats in this block.

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Note: Nucleotide sequence data reported in this paper have been submitted to the EMBL database with the accession numbers X52962 (HB3A) and X52963 (3D7A).

Abbreviations: MSA-1, precursor to the major merozoite surface antigen; PCR, polymerase chain reaction.

We report here sequence data on the region around and including the tripeptide repeat region of the MSA-1 gene of two parasite clones of *P. falciparum*, HB3A and 3D7A, used in previous genetic crossing experiments [11].

The region to be sequenced was amplified from genomic DNA using the polymerase chain reaction (PCR) [12]. The PCR product begins 62 nucleotides after the start of the MSA-1 coding region and ends at position 614 (HB3A) or 707 (3D7A). The resulting double-stranded amplified DNA fragments were purified from agarose gels using Geneclean (Bio-101) and sequenced directly by double stranded sequencing according to the protocol of Winship [13,14]. The sequences were determined on both strands for each of the amplified fragments (Fig. 1).

The HB3A tripeptide repeat region is identical to that of its parent isolate H1 [15]. It is similar to that of the Wellcome strain [16], containing repeats of S-V-A or S-G-N (Fig. 2). The Wellcome repeat region contains 13 tripeptides but the HB3A repeat region is shorter, being only 9 tripeptides long.

The 3D7A tripeptide repeat is most similar to that of the NF7 isolate [6] although the NF7 repeat region is shorter, containing 11 tripeptides instead of the 16 tripeptides seen in 3D7A. The tripeptides are most commonly S-A-Q and S-G-A, with S-G-T and S-G-P also present. 3D7A also has tripeptides S-A-T and S-A-P which are not found in any other sequences reported so far. 3D7A is also unusual in

```
      1                                                               60
3D7   ACACATGAAAGTTAT CAAGAACTTGTCAAA AAACTAGAAGCTTTA GAAGATGCAGTATTG
HB3   ACACATGAAAGTTAT CAAGAACTTGTCAAA AAACTAGAAGCTTTA GAAGATGCAGTATTG

      61                                                             120
3D7   ACAGGTTATGGTTTA TTTCAAAAGGAAAAA ATGGTCTTAAATGAA GAAGAAATTACTACA
HB3   ACAGGTTATAGTTTA TTTCAAAAGGAAAAA ATGGTATTAAATGAA GGAACAAGTGGAACA

      121                                     ---->                  180
3D7   AAAGGTGCA...AGT GCTCAA......AGT GGTGCA...AGT... GCTCAAAGTGGTGCA
HB3   GCTGTTACAACTAGT ACACCTGGTTCAAAG GGTTCAGTTGCTTCA GGTGGTTCAGGTGGC

      181                                                            240
3D7   AGTGCTCAAAGTGGT GCAAGTGCTCAAAGT GGTGCAAGTGCTCAA AGTGGTGCAAGTGCT
HB3   TCAGTTGCTTCAGGT GGCTCAGTTGCTTCA GGTGGTTCAGGTAAT ...............

      241                                                            300
3D7   ACAAGTGCTCAAAGT GGTCCAAGTGGTACA AGTGCTCCAAGTGGT AGTGGTACAAGTCCA
HB3   ............... ............... ............... ...............

      301    <----                                                   360
3D7   TCATCTCGTTCAAAC ACTTTACCTCGTTCA AATACTTCATCTGGT GCACCTCCAGCTGAT
HB3   TCAAGACGTACAAAT .........CCTTCA GATAATTCA...... ...............

      361                                                            420
3D7   GCAAGCGATTCAGAT GCTAAATCTTACGCT GATTTAAAACACAGA GTACGAAATTACTTG
HB3   ...AGTGATTCAGAT GCTAAATCTTACGCT GATTTAAAACACAGA GTACGAAATTACTTG

      421                                                            480
3D7   TTCACTATTAAAGAA CTCAAATATCCCGAA CTCTTTGATTTAACC AATCATATGTTAACT
HB3   TTAACTATCAAAGAA CTCAAATATCCTCAA CTCTTTGATTTAACT AATCATATGTTAACT

      481                                                            540
3D7   TTGTGTGATAATATT CATGGTTTCAAATAT TTAATTGATGGATAT GAAGAAATTAATGAA
HB3   TTGTGTGATAATATT CATGGTTTCAAATAT TTAATTGATGGATAT GAAGAAATTAATGAA

      541                                                            600
3D7   TTATTATATAAATTA AACTTTTATTTTGAT TTATTAAGAGCAAAA TTAAATGATGTATGT
HB3   TTATTATATAAATTA AACTTTTATTTTGAT TTATTAAGAGCAAAA TTAAATGATGTATGT

      601                                                            660
3D7   GCTAATGATTATTGT CAAATACCTTTCAAT CTTAAAATTCGTGCA AATGAATTAGACGTA
HB3   GCTAATGATTATTGT CAAATACCTTTCAAT CTTAAAATTCGTGCA AATGAATTAGACGTA
```

Fig. 1. Nucleotide sequences of part of the MSA-1 gene of HB3A and 3D7A, shown 5′ to 3′. The arrows above the sequence mark the start and end of the tripeptide repeat region.

```
                  9        18        27        36        45        54        63
    3D7A  SAQSGASAQ SGASAQSGA SAQSGASAQ SGASATSAQ SGPSGTSAP SGSGT.... ...SPSSRS
     NF7  SAQSGASAQ SGASAQSGA SAQSGTSGP SGPSGT... ......... ......... ...SPSSRS
    CAMP  SAQSGTSGT SGTSGTSGT SGTSGTSAQ SGTSGTSAQ SGTSGTSAQ SGTSGTSGT SGTSPSSRS
      K1  SAQSGTSGT SGTSGPSGP SGT...... ......... ......... ......... ...SPSSRS
   MAD20  SSGSVTSGG ...SVASVA SVASGG... SGGSVASGG SGNSRR
Wellcome  SKGSVASGG SGGSVASGG SVASGGSVA SGGSVASGG SGNSRR
    HB3A  SKGSVASGG SGGSVASGG SVASGG... ......... SGNSRR
```

Fig. 2. Variation in the tripeptide repeat region. Predicted amino acid sequences of the tripeptide repeat regions of the HB3A, Wellcome [16], K1 [18], MAD20 [7], 3D7A, CAMP [17] and NF7 [6] parasites.

that it contains one dipeptide S-G towards the end of this region.

This work emphasises the remarkable diversity seen in the repeat region of alleles of the MSA-1 gene. Each isolate examined so far differs either in numbers of repeats or in the repeats themselves.

Acknowledgements

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Genetic hybrids of *Plasmodium falciparum* identified by amplification of genomic DNA from single oocysts

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Individual oocysts from *Plasmodium falciparum*-infected *Anopheles gambiae* and *Anopheles stephensi* mosquitoes have been examined by the PCR technique, after their removal from the midgut. The DNA obtained from these oocysts has been amplified using oligonucleotide primers specific for part of the merozoite surface antigen MSA-1 gene. This technique distinguishes oocysts which are the products of self-fertilisation events from those which are the products of cross-fertilisation between different parasite clones.

Key words: *Plasmodium falciparum*; MSA-1 gene; Mosquito; Oocyst; Polymerase chain reaction

Introduction

Important genetic events take place during the mosquito phase of the life-cycle of malaria parasites. Fertilisation of gametes in the lumen of the midgut results in diploid zygotes (ookinetes), and meiosis occurs within a few hours of zygote formation [1]. The developing oocyst, therefore, contains the haploid products of meiosis. Genetic crossing experiments, in which mixtures of gametocytes of 2 clones are fed to mosquitoes, have demonstrated that recombination occurs at this stage [2]. Furthermore, the numbers of recombinant forms obtained in such crosses is greater than would be predicted if random fertilisation events were to occur [3]. In order to understand the genetic processes involved in recombination, there is an obvious need to develop methods for examining the genome of these mosquito stages. However, techniques for such work have not been available until now because of the difficulty in obtaining adequate quantities of DNA from these forms.

We describe here the use of the polymerase chain reaction (PCR) [4] to examine the oocyst genome. We show that amplified fragments of a *Plasmodium falciparum* antigen gene (MSA-1) can be obtained by this technique. By making use of allelic variants of this antigen differing in the size of these fragments, we identify hybrid oocysts in mosquitoes fed on mixtures of gametocytes of 2 cloned lines.

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Abbreviations: PCR, polymerase chain reaction; MSA-1, merozoite surface antigen 1; RBC, red blood cell; PABA, para-aminobenzoic acid (4-aminobenzoic acid); c^{7}dGTP, 7-deaza-2′-deoxyguanosine.

Materials and Methods

Parasites and culture methods. The parasites used in this work were 3D7A, cloned from the isolate NF54 from the Netherlands by limiting dilution, and HB3A, cloned by microscopic selection from isolate H1 from Honduras [2]. Parasites were cultured in vitro under conditions permitting the development of gametocytes infective to mosquitoes [5,6]. Routine

cultures of asexual blood forms [7,8] were also set up, for the preparation of parasite DNA. These were grown to an 8–10% parasitaemia and their DNA was then extracted [9].

Infection of mosquitoes. Mosquitoes (*Anopheles gambiae* or *Anopheles stephensi*) were infected with gametocytes of clones 3D7A and HB3A separately, and with a 1:1 mixture of gametocytes of both clones. Cultures containing mature gametocytes were centrifuged and the pellet resuspended in 3 vols. of uninfected human RBCs with sufficient human serum to give a 50% haematocrit [7]. This suspension was fed to mosquitoes, previously starved for 48 h, through a membrane feeder [10]. The mosquitoes were then maintained at 26°C and 70–80% relative humidity, and fed on a 5% glucose/0.05% PABA (4-aminobenzoic acid) mixture [11].

Removal of oocysts from mosquitoes. Mosquitoes were dissected 9–10 days after the infective blood meal and their midguts examined for the presence of oocysts. Individual oocysts were dissected off the wall of the insect midgut using microcapillary tubes which had been heated and pulled out to a very fine point. Fresh microcapillary tubes were used for each oocyst to minimise the possibility of cross-contamination of DNA from other oocysts.

Preparation of oocyst DNA for polymerase chain reaction. Single oocysts were transferred to 0.5 ml microcentrifuge tubes containing 50 μl of oocyst lysis buffer (100 mM NaCl/25 mM EDTA pH 8.0/10 mM Tris-HCl pH 8.8/ 0.5% Sarkosyl/1 mg ml^{-1} proteinase K) and incubated at 55°C for 1 h, before the addition of salmon sperm DNA (1 μg) as a carrier. The DNA was purified by extracting once with phenol, once with a 1:1 mixture of phenol and chloroform, and once with chloroform, and then precipitated with isopropanol. The pellet was washed twice with 70% ethanol before resuspending in 10 μl of distilled water [9].

Design of polymerase chain reaction primers to MSA-1. MSA-1 is a merozoite surface antigen of *P. falciparum*, also known as p190 [12], PMMSA [13], PSA [14] and gp195 [15]. In the present work, PCR was carried out using oligonucleotide primers, prepared by the Oswel DNA Service, Edinburgh, from the conserved regions (blocks 1 and 3) surrounding the first variable region (block 2) of the MSA-1 gene of 3D7A and HB3A. The primers used were: P1, 5′-CACATGAAAGTTATCAAGAACTTGTC-3′; and P2, 5′-GTACGTCTAATTCATTTGCACG-3′ [16]. PCR on this region has been reported previously [17], but the fragment amplified in our work is longer and both primers are different. The sequence of these 3D7 and HB3 fragments is described elsewhere [16].

Polymerase chain reaction technique. DNA extracted from *P. falciparum* asexual cultures was amplified using AmpliTaq DNA polymerase (Cetus) in 20 μl reaction mixes containing 1 × *Taq* Buffer (50 mM KCl/10 mM Tris-HCl (pH 8.8)/2.5 mM $MgCl_2$/0.02% gelatin), 100 nM of each primer, 75 μM each of dATP, dTTP, dCTP, dGTP and 0.5 units of the enzyme. The samples were overlaid with mineral oil and subjected to 30 cycles of amplification in an automated heat block (Hybaid) as follows: 94°C, 25 s; 50°C, 35 s; 68°C, 150 s.

Oocyst DNA was amplified using the same reaction conditions, but replacing 10 μM of the dGTP with the base analogue 7-deaza-2′-deoxyguanosine (c^7dGTP). 2 μl of oocyst DNA was used for each reaction.

Results

The oligonucleotide primers selected from the 5′-end of the MSA-1 gene produced efficient amplification of specific sequences from DNA obtained from asexual cultures of *P. falciparum*. Fig. 1 shows the amplification of asexual and oocyst DNA from the clone 3D7A, both in the presence and absence of 7-deaza-2′-deoxyguanosine (c^7dGTP). Oocyst DNA was only amplified efficiently when c^7dGTP was present.

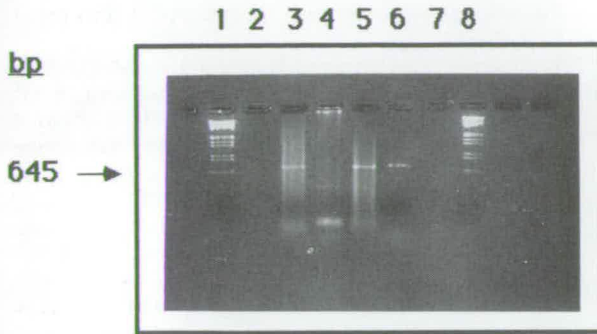

Fig. 1. Amplification of *P. falciparum* DNA from asexual cultures (lanes 3 and 5) and from single oocysts (lanes 4 and 6), using oligonucleotide primers specific to the 5′-end of the MSA-1 gene. Lanes 1 and 8: λ DNA cut with *Eco*RI and *Hin*dIII; lanes 3 and 4: DNA amplified in the absence of c^7dGTP; lanes 5 and 6: DNA amplified in the presence of c^7dGTP; lanes 2 and 7: blank. Amplified DNA in lanes 3 and 5 was from 100 ng from an asexual 3D7A culture; in lanes 4 and 6 from 2 μl of 3D7A oocyst DNA. 10 μl of each PCR was resolved on a 1.5% agarose gel in Tris-borate buffer and stained with ethidium bromide.

Amplification of DNA from asexual cultures of the two clones 3D7A and HB3A produced fragments of different lengths, as shown in Fig. 2. The fragments amplified from 3D7A and from HB3A were 645 and 552 bp long, respectively. Fig. 2 also shows the products of amplification of DNA from individual oocysts. The PCR fragments obtained from oocyst DNA from mosquitoes fed on one or the other of the 2 clones 3D7A and HB3A are of the same length as those obtained from amplification of DNA from blood forms of these clones. Amplification of DNA from individual oocysts from mosquitoes fed on a mixture of the 2 clones resulted in some producing fragments of 645 bp, some in fragments of 552 bp, and some in fragments of both 552 bp and 645 bp. Five out of 10 oocysts from one mosquito produced both fragments, 4 produced fragments of 645 bp only, and one produced a fragment of 552 bp only (full data not shown in Fig. 2).

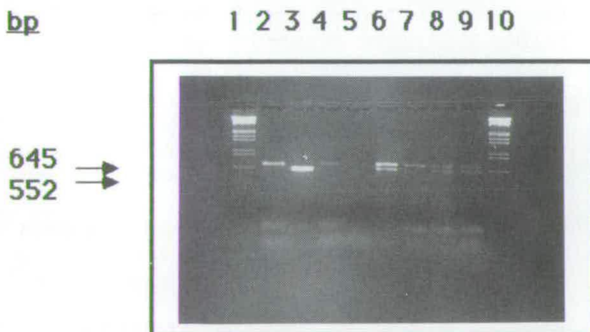

Fig. 2. Amplification of *P. falciparum* DNA from asexual cultures and from single oocysts using oligonucleotide primers specific to the 5′-end of the MSA-1 gene. In each lane DNA was amplified in the presence of c^7dGTP. Lanes 1 and 10, λ DNA cut with *Eco*RI and *Hin*dIII; lane 2, asexual 3D7A DNA; lane 3, asexual HB3A DNA; lane 4, 3D7A oocyst DNA; lane 5, blank; lanes 6–9, DNA from single oocysts from mixed feed.

Discussion

The oligonucleotide primers used in this work produced efficient amplification of DNA from asexual culture of *P. falciparum*. The amplified fragments from the 2 clones 3D7A and HB3A were different in length (Fig. 2), due to sequence variation in the repeat region of the area amplified [16]. The size difference between the two clones allowed these alleles of the MSA-1 gene to be distinguished.

Efficient and reliable amplification of oocyst DNA was not possible using the same reaction conditions as for blood form DNA (Fig. 1). The incorporation of 7-deaza-2′-deoxyguanosine into the reaction allowed amplification to proceed. This base analogue significantly increases the specificity of the PCR with nucleic acid residues that contain stable secondary structures and/or have compressed regions, without affecting the fidelity of the PCR [18]. However, since PCR products containing c^7dGTP do not stain as efficiently with ethidium bromide, only a proportion of the total dGTP is replaced with c^7dGTP [19]. We do not know why amplification of oocyst DNA requires c^7dGTP. It is possible that the DNA in the oocyst is in a different conformational state from that found in blood stages.

Amplification of oocyst DNA from mosquitoes fed on HB3A alone, on 3D7A alone, or on a mixture of the 2 clones produced fragments of the same size as those obtained from amplification of asexual forms of these clones (Fig. 2).

Mosquitoes fed on a 1:1 mixture of the 2 clones are expected to contain some selfed oocysts, the products of fertilisation events between gametes of the same parental clone, as well as hybrid oocysts, produced from cross-fertilisation events between different parental clones. Selfed oocysts are expected to possess only 3D7A alleles or only HB3A alleles. Hybrid oocysts, on the other hand, are expected to contain both 3D7A and HB3A alleles. In this study, amplification of DNA from individual oocysts in mosquitoes fed on mixed gametocytes showed that some possessed both parental alleles of the MSA-1 gene, while others exhibited alleles representing only a single parental form (Fig. 2). From a single mixed feed, 5 of the oocysts obtained were hybrid, 4 possessed only the 3D7A allele, and 1 only the HB3A allele. Thus, the amplification of oocyst DNA can distinguish between selfed oocysts and hybrid oocysts.

Analysis of single oocysts has been difficult because of the very small amounts of DNA present. A single oocyst when mature contains not more than 10000 haploid sporozoites [20]. PCR amplification of the DNA will allow further analysis of these stages in the *Plasmodium* lifecycle, including an investigation of the frequency of cross-fertilisation in the mosquito. The technique should be valuable for examining the nature of infection in wild-caught mosquitoes, and in experimental studies for examining the inheritance and expression of different parental characters in the zygote and oocyst stages.

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