J. Genet., Vol. 73, Number 2–3, August and December 1994, pp. 91–98. © Printed in India.

Diversity in two candidate malaria vaccine antigens in different Indian strains of *Plasmodium falciparum*

CHERYL A. LOBO, RADIYA F. PACHA and SHOBHONA SHARMA*
Molecular Biology Unit, Tata Institute of Fundamental Research, Homi Bhabha Road, Bombay 400 005, India

MS received 18 October 1994

Abstract. Polymorphism in two malarial antigens, merozoite surface antigen-1 (MSA-1) and ring erythrocyte surface antigen (RESA), has been characterized in four different Indian strains of *Plasmodium falciparum*. The Indian strains were obtained from two malaria endemic regions of India, viz. Surat (Gujarat) and Delhi, and established in culture. Monoclonal and polyclonal antibodies raised against different domains of these antigens were used in the study. In MSA-1 a novel intragenic crossover was detected in the central conserved domain in two of the Indian strains. The repeat domain of RESA was found to be absent in the two strains of *P. falciparum* isolated from Surat. These differences in immunoreactivity have been extended to the DNA level by appropriate PCR studies. MSA-1 and RESA are candidate vaccine antigens and these diversities will have an important bearing on the design of a suitable malaria vaccine.

Keywords. Malaria; antigens; diversity; strain; *Plasmodium*.

1. Introduction

Antigenic diversity is a common strategy adopted by many infectious disease organisms to evade host immune response. Several stage-specific candidate vaccine antigens have been identified in the human malarial parasite *Plasmodium falciparum* and diversity has been documented in many of these antigens (Kemp *et al.* 1987; Howard and Pasloske 1993). Of particular importance is the glycoprotein merozoite surface protein-1 (MSA-1), which is the most studied antigen of *P. falciparum*. MSA-1 may play a role in erythrocyte invasion, since antibodies raised against MSA-1 can inhibit this process (Cooper *et al.* 1992). Passive transfer of antibodies against MSA-1 also confers protection *in vivo* (Lew *et al.* 1989). Comparisons of MSA-1 sequences of several strains of *P. falciparum* have shown that there are conserved and variable blocks and that there are only two groups of alleles for the variable blocks. This led to the dimorphic model of MSA-1 (Tanabe *et al.* 1987; Cooper 1993). Several intragenic crossovers have been predicted and documented (Peterson *et al.* 1988; Jongwutiwes *et al.* 1992), but they have been found to be concentrated in the 5′-region of the gene. Portions of both the variable N-terminal region and the conserved C-terminal region are thought to be important for the functioning of the protein (Cooper 1993).

Even though very little is understood about the possible function of the RESA molecule, it is a potential vaccine candidate (Howard and Pasloske 1993). The

*For correspondence

relevant B-cell and T-cell epitopes for this protein have been mapped to the repetitive regions EENVEHDA and EENV (Sjolander *et al.* 1990). It has also been reported in every natural strain of *P. falciparum* studied (Perlmann *et al.* 1987).

In this paper we report an analysis of the structure of these two proteins in four Indian strains of *P. falciparum.*

2. Materials and methods

2.1 *Parasite strains*

Plasmodium falciparum strains were obtained from malaria patients from two endemic areas of India. The two strains obtained from Surat (Gujarat, West India) were called S-24 and S-45, while the two from Delhi (North India) were named DU-1 and DU-2. The Gambian strain FCR3 FMG was also used in this study. The parasites were cultured in human RBC (O Rh+; 8% haematocrit) in RPMI-1640 medium supplemented with 10% human serum, in candle jars (Trager and Jensen 1976).

2.2 *Antigen preparation*

Parasitized erythrocytes were harvested at day 6 after subculture (parasitaemia of 10–15%). The erythrocytes were lysed with saponin and the parasites were pelleted at 10,000 *g* at 4°C. The parasite pellets were resuspended in phosphate buffered saline (PBS), pH 7·4, and sonicated for 3 min on ice.

2.3 *ELISA*

Two hundred µl of a 5 µg/ml solution of a sonicated parasite extract of asynchronous cultures of *P. falciparum* was added to wells of a polyvinylchloride microtitre plate. The plate was incubated overnight at 4°C. The antigen was removed and the plate washed four times with 0·05% Tween 20–PBS (PBS-T) for 5 min each. All further reactions were carried out at 37°C. Each well was saturated with 200 µl of 7% sheep serum in PBS-T for 1 h. This was followed by incubation for 2 h with the respective serum samples, diluted 1 : 100 in PBS-T. After extensive washing, 200 µl of HRP-conjugated anti-IgG was added and incubation was continued for 1 h. After washing off unbound antibody, the substrate 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid) (ABTS) was added, and an ELISA reader was used to read the absorbances at 405 nm. The absorbance values obtained for the nonspecific sera (0·05–0.09) were subtracted to normalize the readings obtained. Readings were taken for four wells in each case and standard error was calculated.

Monoclonal antibodies (MAbs) against five different epitopes of the MSA-1 antigen, shown in figure 1, were used as primary antibodies. The antibody used to detect RESA was a polyclonal antiserum (R27) raised in a rabbit against a fusion protein of the tetramer of the octapeptide repeat (EENVEHDA) of RESA and a divalent synthetic IgG-binding domain derived from staphylococcal protein A (Sjolander *et al.* 1990).

2.4 *Western blot analysis*

Parasite antigens (60 µg per lane) were separated on a 7.5% SDS polyacrylamide

gel and blot-transferred to nitrocellulose paper after electrophoresis (Towbin *et al.* 1979). The blots were subjected to immunoanalysis by the same sequence of steps as in ELISA.

2.5 *PCR analysis*

DNA of the different parasite strains was prepared by the Chelex method (Wooden *et al.* 1992). Briefly: Five hundred µl of *P. falciparum* culture at parasitaemia greater than 1% was lysed in PBS with 1% saponin for 5 min on ice. The samples were spun for 2 min in a microfuge. To the pellet, 150 µl of water and 50 µl Chelex-100 (Biorad, USA; made to 20% w/v in water adjusted to pH 9·5 with 1 M NaOH) were added, and the samples boiled for 7 min. Ten µl of the supernatant was used in each polymerase chain reaction (PCR).

Standard reagents were used for PCR, with final concentrations of 200 µM each dNTP, 0·5 µM primer, 1× Vent buffer and 1 unit Vent DNA polymerase (New England Biolabs, USA). Amplification was carried out with the following conditions: one cycle, denature at 95°C for 5 min, anneal at 48°C for 2 min; extend at 72°C for 4 min, followed by 25 cycles; denature at 95°C for 1 min; anneal at 48°C for 2 min; extend at 72°C for 4 min.

For MSA-1, K1 and MAD-20-specific oligonucleotides (Tanabe *et al.* 1987) were used to amplify regions b and d:

For region b (figure 1),
Forward primer from block 12 (conserved): 5′-AATGGTGAATCATCTCCATT
Reverse primer block 16 (K1 strain): 5′-TATCCGTATCTATTGAATCC
Reverse primer block 16 (MAD-20 strain): 5′-CTTATACCTTCCTGTGCAA
For region d (figure 1),
Forward primer block 8 (K1 strain): 5′-TCAATCGGATAATTCGGAAC
Reverse primer block 10 (K1 strain): 5′-TTCATTTGCACTTACTTCGGG
Forward primer block 8 (MAD-20 strain): 5′-CTGAAGATGGGGGTCACTC
Reverse primer block 10 (MAD-20 strain): 5′-ACTGTTAAGGGATCATGACT

For RESA, the region amplified by PCR was the sequence encoding the entire repetitive region at the C-terminal end of the RESA molecule (Favoloro *et al.* 1986):

Forward primer, bp no. 3612 to 3632: 5′-TTAGCACAATTAATACAGAA
Reverse primer, bp no. 4202 to 4222: 5′-TCATCATATTCTTCATTGTG.

3. Results and discussion

Monoclonal antibodies raised against different domains of MSA-1 were used to detect specific antigenic diversity among the strains. No significant polymorphism in the size of the protein was observed for MSA-1 among the five lines tested (figure 1). However, minor differences cannot be ruled out since it is a high-molecular-weight protein (190 kDa) and small differences may be difficult to detect. Distinct differences were detected when MAbs for specific regions of the MSA-1 protein were used. It was found that strains S-24 and DU-2 lacked the epitope for region d (table 1). Western blots of these parasite extracts probed with the MAbs separately showed the same result as ELISA (data not shown).

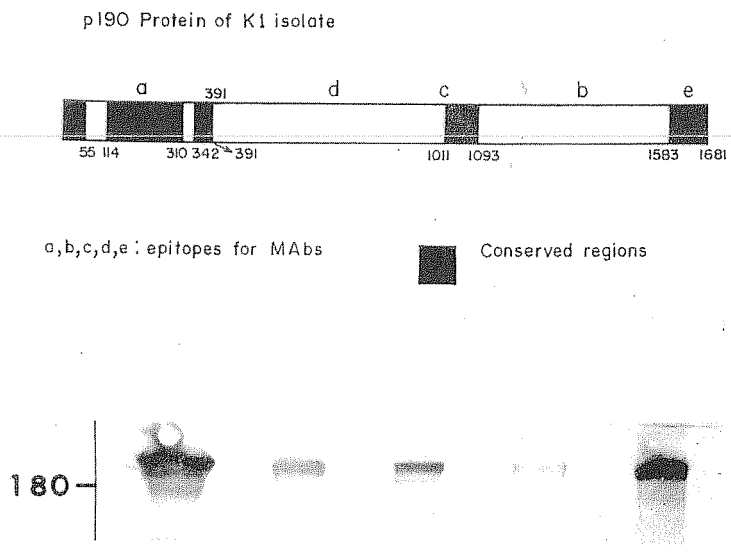

Figure 1. Top panel shows antigen map of MSA-1, also called p190. Epitopes a, b, c, d and e are expressed proteins F2, F9, F7, F5 and F10, corresponding to amino acid positions 106–321, 1194–1439, 915–1100, 671–833 and 1412–1608 respectively (Muller *et al.* 1989). The conserved regions, shaded black, are based on Tanabe *et al.* (1987). Lower panel shows a Western blot of proteins of asynchronous cultures of the five strains of *P. falciparum* blotted and probed with a pool of the MAbs raised against a, b, c, d, e of MSA-1. Lanes from left to right are: DU-2, DU-1, S-45, S-24, FCR3.

The MAbs used in our study were raised against expressed protein domains of the K1 strain (Muller *et al.* 1989) and, therefore, the two Indian strains S-24 and DU-2 do not possess the K1-specific epitope in region d. The region d of the K1 strain covers the variable blocks 8 and 9 (Tanabe *et al.* 1987). Regions a, c and e mainly cover the conserved regions of MSA-1, and therefore it is not surprising that all the isolates react with antibodies against these regions. The observation that MAb against region d of K1 does not react with S-24 and DU-2 implies that these two strains may belong to the MAD-20 strain type. Region b covers blocks 14, 15 and 16, of which 14 and 16 are variable and 15 is a semiconserved region. The observation that MAb against region b reacts with every line tested implies that either there is a crossover in the conserved region c in the two lines S-24 and DU-2, giving rise to chimaeras of the two strains for b and d regions, or the antibody against b is against a semiconserved epitope and hence it reacts with both the K1 and MAD-20 domains.

MSA-1 has been reported to be largely dimorphic (Tanabe *et al.* 1987), except for the highly variable repeat region (Peterson *et al.* 1988). Recombination events have been observed between the conserved regions in the amino-terminal portion that result in different permutations and combinations of the variable regions in the amino-terminal domain (Peterson *et al.* 1988; Jongwutiwes *et al.* 1992). However, so far no strain has been documented with a recombination event in the central conserved region, c or block 12.

To test whether the nonreactivity of S-24 and DU-2 with MAb against region d

Table 1. ELISA reactivities of the five *P. falciparum* isolates with MAbs against various regions of MSA-1.

MAb	FCR3	S-24	S-45	DU-1	DU-2
a	0·67 ± 0·02	0·59 ± 0·02	0·71 ± 0·01	0·38 ± 0·01	0·60 ± 0·06
b	0·76 ± 0·04	0·73 ± 0·03	0·82 ± 0·08	0·69 ± 0·01	0·70 ± 0·02
c	0·52 ± 0·01	0·62 ± 0·01	0·68 ± 0·03	0·52 ± 0·03	0·61 ± 0·03
d	0·71 ± 0·01	0·20 ± 0·02	0·57 ± 0·05	0·54 ± 0·07	0·17 ± 0·06
e	0·75 ± 0·06	0·71 ± 0·01	0·80 ± 0·01	0·65 ± 0·01	0·69 ± 0·09
Pool	0·93 ± 0·08	0·94 ± 0·02	0·87 ± 0·01	0·85 ± 0·03	0·79 ± 0·01

Details about the monoclonal antibodies are given in Materials and methods and in figure 1. The absorbance values shown here are normalized for the blank readings obtained with nonspecific sera. Numbers are means of four readings and standard errors.

of K1 is due to polymorphism, a PCR was carried out using oligonucleotides specific for the two strain types. For S-24, it was found that region d was amplified only with primers specific for strain MAD-20, while region b was amplified exclusively with primers for strain K1 (figure 2). With primers for region b, other than the expected band, smaller, more intense bands were also visible. The origin of these bands is not clear. They may be nonspecific amplifications, as they were detected in the other K1-type strains as well. However, PCR-amplified region d had no such ambiguity and belonged clearly to the MAD-20 type. Thus strain S-24 indeed has DNA that has resulted from a crossover between the two forms in the conserved region c situated in the centre of the molecule, giving rise to a chimaera of region b and d of the two allelic types.

The result of an ELISA study that tested for the presence of the octapeptide repeat region of the RESA molecule among the five lines is shown in table 2. A polyclonal rabbit serum raised against a tetramer of the synthetic octapeptide EENVEHDA coupled to a divalent synthetic IgG-binding domain derived from staphylococcal protein A (Sjolander *et al.* 1990) was used for this assay. This rabbit antiserum was specific for the octapeptide repeat, since all the reactivity could be inhibited by the use of the octapeptide. Strains S-24 and S-45 failed to react with this antibody, indicating the absence of this epitope in these two strains. The antigen preparation gave a normal reaction for the presence of MSA-1, tested with the pooled MAbs (table 2). The residual absorbance readings of 0·65 and 0·82 are likely to be due to cross-reactivity of the antibody with other parasite antigens, as observed earlier (Sjolander *et al.* 1990). Indeed, when the reactivity of the RESA antigen from the Indian isolates was examined on Western blots, the 155-kDa band was missing while other faint reactivities at 200 kDa and at lower molecular weights could be seen (figure 3). The antibody used has no cross-reactivity with the tetrapeptide repeat EENV of RESA (Sjolander *et al.* 1990), and hence it is not yet known whether this set of repeats is expressed in the Indian lines. RESA contains two extensive blocks of tandemly repeated amino-acid sequences, one in the middle of the molecule and one in the carboxy-terminal region (Cowman *et al.* 1984). The C-terminal repeat region, which is the immunodominant B-cell epitope, contains the octapeptide EENVEHDA tandemly repeated five times. This epitope is reported to be conserved in different *P. falciparum* strains (Perlmann *et*

al. 1987). The absence of reactivity with antibody raised against this epitope in two of the four Indian strains tested questions the ubiquity of this epitope. However, these lines from India have been maintained in culture for four years, and it is possible for both of them to have been selected for loss of RESA, as found earlier with a cultured line that allowed a large deletion and an inversion of the signal-encoding exon in the RESA gene (Cappai *et al.* 1989; Pologe *et al.* 1990).

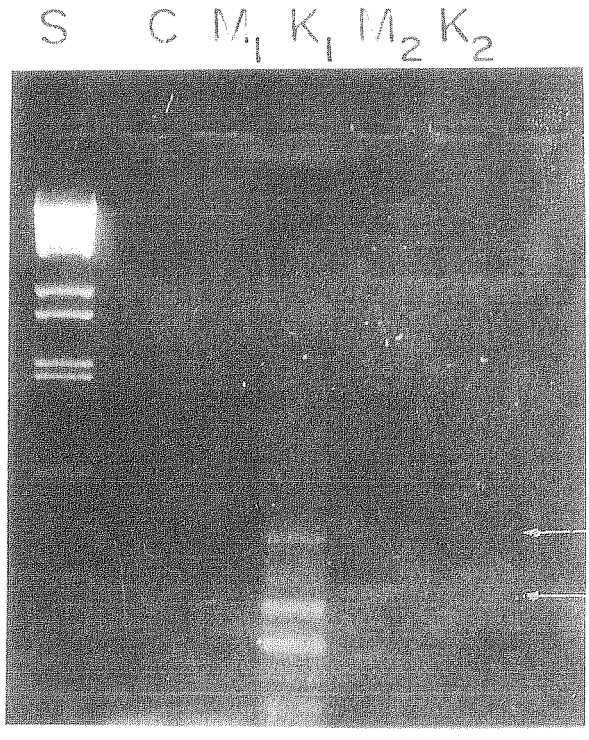

Figure 2. PCR-amplified fragments of MSA-1 gene from the strain S-24, using K1 and MAD-20 strain-specific oligonucleotides, as described in Materials and methods. Samples were run on a 2% agarose gel. Lanes: S, phage lambda DNA restricted with *Bst*EII; C, PCR using both sets of primer with a control plasmid DNA; M_1, reaction using primers of MAD-20 strain for region b (blocks 12–16); K_1, reaction using primers of K1 strain for region b; M_2, reaction using primers of MAD-20 strain for region d (blocks 8–10); K_2, reaction using primers of K1 strain for region d. Arrows show the expected fragments for each region, b and d.

Table 2. ELISA reactivities of the five isolates with a polyclonal antibody against the octapeptide repeat of RESA.

Antibody	FCR3	S-24	S-45	DU-1	DU-2
Anti-RESA[a]	1·98[b] ± 0·03	0·65 ± 0·05	0·82 ± 0·10	2·14 ± 0·06	2·64 ± 0·08
Anti-MSA-1 (pool)[c]	2·09 ± 0·01	2·13 ± 0·03	1·86 ± 0·03	1·98 ± 0·02	2·46 ± 0·04

[a]R27 serum against RESA octapeptide
[b]A_{405} values were corrected by subtracting the reactivity of preimmune serum (A_{405}, 0·09)
[c]Pool of MAbs against MSA-1 (see text).

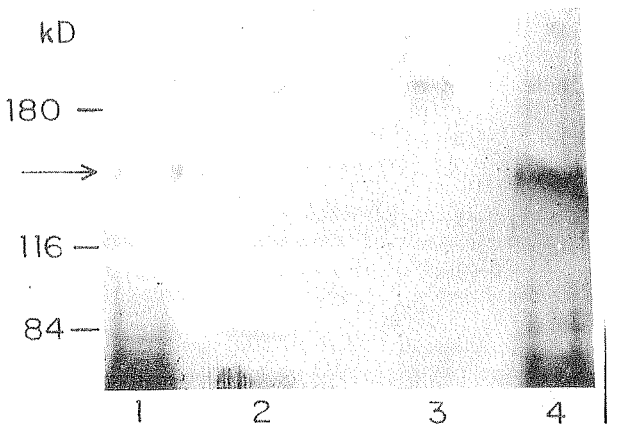

Figure 3. Proteins of asynchronous cultures of the five isolates blotted and probed with a polyclonal antiserum raised against RESA repetitive region (Sjolander *et al.* 1990). Lanes: 1, DU-2; 2, S-45; 3, S-24; 4, FCR3.

To test this we performed a PCR using primers flanking the repeat regions, and it was observed that each of the strains had identical DNA fragments amplified, indicating that there is no gross deletion or rearrangement in the DNA of these two lines. Further analysis of the transcripts will show whether the gene is transcribed at all, or if the problem is with translation. The repetitive regions of most surface antigens of the malarial parasites are known to be the more unstable regions (Schofield 1991), and it is possible that the repeat units have diversified and hence the new epitopes do not cross-react with the rabbit antiserum. MSA-1 and RESA are prime candidates for a malaria vaccine (Mitchell 1989). Polymorphisms of epitopes of these antigens among parasite isolates from different malaria endemic areas must therefore be assessed very rigorously before inclusion of the antigens in a subunit malaria vaccine.

Acknowledgements

We are indebted to Klaus Fruh for the monoclonal antibodies against various domains of MSA-1 and to Marita Troye-Blomberg for the anti-RESA antibody. We also thank Dr N. Patel and Dr D. S. Chowdhury for their help with collection of patient blood for the parasite isolates. This work was supported by a grant from Department of Science and Technology, India, No. SP/SO/D-72/89.

References

Cappai R., Van Schravendijk M., Anders R. F., Peterson M. G., Thomas L. M., Cowman A. F. and Kemp D. J. 1989 Expression of the RESA gene is prevented by a subtelomeric deletion. *Mol. Cell. Biol.* 9: 3584–3587

Cowman A. F., Coppel R. L., Saint R. B., Favalaro J., Crewther P. E., Stahl H. D., Bianco A. E., Brown G. V., Anders R. F. and Kemp D. J. 1984 The ring-infected erythrocyte surface antigen (RESA) polypeptide of *Plasmodium. falciparum* contains two separate blocks of tandem repeats

encoding antigenic epitopes that are naturally immunogenic in man. *Mol. Biol. Med.* 2: 207–221

Cooper J. A. 1993 Merozoite surface antigen-1 of *Plasmodium*. *Parasitol. Today* 9: 50–54

Cooper J. A., Cooper, L. T. and Saul A. J. 1992 Mapping of the region predominantly recognized by antibodies to the *Plasmodium falciparum* merozoite surface antigen MSA-1. *Mol. Biochem. Parasitol.* 51: 301–312

Favoloro J. M., Coppel R. L., Corcoran L. M., Foote S. J., Brown G. V., Anders R. F. and Kemp D. J. 1986 Structure of the RESA gene of *Plasmodium falciparum*. *Nucl. Acids Res.* 14: 8265–8277

Howard R. J. and Pasloske B. L. 1993 Target antigens for asexual malaria vaccine development. *Parasitol. Today* 9: 369–372

Jongwutiwes S., Tanabe K., Nakazawa S., Yanagi T. and Kanbara H. 1992 Sequence variation in the tripeptide repeats and T-cell epitopes in P190 (MSA-1) of *Plasmodium falciparum* from field isolates. *Mol. Biochem. Parasitol.* 51: 81–90

Kemp D. J., Coppel R. L. and Anders R. F. 1987 Repetitive proteins and genes of malaria. *Annu. Rev. Microbiol.* 41: 181–208

Lew A. M., Langford C. L., Anders R. F., Saul A., Fardoulys C., Geysen M. and Sheppard M. 1989 A protective monoclonal antibody recognizes a linear epitope in the precursor to the major merozoite antigens of *Plasmodium chabaudi adami*. *Proc. Natl. Acad. Sci. USA* 86: 3768–3772

Mitchell G. H. 1989 An update on candidate malaria vaccines. *Parasitology* 98: S29–S47

Muller H.-M., Fruh K., Von Brunn A., Eposito F., Lombardi S., Crisanti A. and Bujard H. 1989 Development of the human immune response against the major surface protein (gp190) of *Plasmodium falciparum*. *Infect. Immun.* 57: 3765–3769

Perlmann H., Berzins K., Wahlin B., Udomsangpetch R., Ruangjirachuporn W., Wahlgren M. and Perlmann P. 1987 Absence of antigenic diversity in Pf155, a major parasite antigen in membranes of erythrocytes infected with *Plasmodium falciparum*. *J. Clin. Microbiol.* 25: 2347–2354

Peterson M. G., Coppel R. L., McIntyre P., Langford C. J., Woodrow G., Brown G. V., Anders R. F. and Kemp D. J. 1988 Variation in the precursor to the major merozoite surface antigens of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 27: 291–302

Pologe L. G., De Bruin D. and Ravetch J. V. 1990 A and T homopolymeric stretches mediate a DNA inversion in *Plasmodium falciparum* which results in loss of gene expression. *Mol. Cell. Biol.* 10: 3243–3246

Schofield L. 1991 On the function of repetitive domains in protein antigens of *Plasmodium* and other eukaryotic parasites. *Parasitol. Today* 7: 99–105

Sjolander A., Stahl S., Nygren P., Aslund L., Ahlborg N., Wahlin B., Scherf, A., Berzins K., Uhlen M. and Perlmann P. 1990 Immunogenicity and antigenicity in rabbits of a repeated sequence of *Plasmodium falciparum* antigen Pf155/RESA fused to two immunoglobulin G-binding domains of staphylococcal protein A. *Infect. Immun.* 58: 854–859

Tanabe K., Mackay M., Goman M. and Scaife J. G. 1987 Allelic dimorphism in a surface antigen gene of the malaria parasite *Plasmodium falciparum*. *J. Mol. Biol.* 195: 273–287

Towbin H., Stahelin T. and Gordon J. 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76: 4350–4354

Trager W. and Jensen J. B. 1976 Human malaria parasites in continuous culture. *Science* 193: 673–675

Wooden J., Gould E. E., Payll A. T. and Sibley C. H. 1992 *Plasmodium falciparum*: A simple polymerase chain reaction method for differentiating strains. *Exp. Parasitol.* 75: 207–212