

Genetic complexity and transmissibility of *Plasmodium falciparum* parasites causing severe malaria in central-east coast India

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Abstract. Heterogeneity in parasite virulence has been proposed to be one of the several factors that contribute to the wide spectrum of disease severity in *Plasmodium falciparum* malaria. In the present study, we have attempted to determine the association of multiple genotype infection (MGI) and /or any specific genotype with the severity of *P. falciparum* malaria. Analysis of two highly polymorphic regions of the merozoite surface protein (MSP1 and MSP2) and three microsatellite loci (MS) have shown a strong association between multiple genotype infection (MGI) and severe *P. falciparum* malaria. Evidence for stratification of the parasite population in to differing virulence has been found. The evolution of such parasite virulence has been discussed in the light of kin and group selection model.

INTRODUCTION

Plasmodium falciparum, which causes both “mild” and “severe” form of clinical malaria, kills up to 3 million people every year all over the world (Breman *et al.*, 2001). Severe malaria is a complicated syndrome characterized by high fever, severe anaemia, cerebral malaria, respiratory distress, metabolic acidosis and multiorgan dysfunction. If the patient is not treated appropriately, these conditions may lead to death. The important factors of parasite virulence are (i) capacity to multiply rapidly (Chotivanich *et al.*, 2000) and, (ii) ability to induce the parasitised RBCs (pRBCs) to bind with vascular endothelium (cytoadherence), with non-infected RBCs (rosetting) and with other pRBCs (autoagglutination) (Dondrop *et al.*, 2004). Hence, a pRBC that binds to multiple erythrocytes and endothelial receptors may cause more obstruction than which adhere with single-receptor in the microvasculature. But, whether multiadhesion of the pRBCs

found in the patients with severe malaria is due to coexpression of multiple binding adhesins in a single clonal population of parasites or due to multiclonal infection, where each clone adheres with a distinct receptor specificity (without excluding the possibility that individual clones are multiple adhesive) is yet to be established. Cross-sectional and longitudinal genetic epidemiological studies in areas with high perennial transmission have persistently showed concomitant multiple infections with different strains of *P. falciparum* at the individual level (Branch *et al.*, 2001). Such genetic heterogeneity within the parasite population may influence the ultimate clinical manifestations of the disease. At least three studies provide evidence of an association between genetic diversity in an infection and disease severity, but it is less diverse infections that are more virulent. Only two studies show evidence that symptomatic infections—those detected when sick people report to clinic—contain more genotypes than infections discovered by

random sampling of asymptomatic people (Read *et al.*, 2002). The present study reports the effect of mixed genotype infections of *P. falciparum* infections on clinical outcome and rate of gametocyte production in central east coast India.

MATERIALS AND METHODS

Study Area

The state of Orissa is situated along the Bay of Bengal and extends from 17°49'N to 22°34' N latitude and 81°28'E to 87°29' E longitude with a land area of about 155,707 sq Kms. The total population of the state is 36.8 million, 22.21% of them being tribal (aboriginal) (Census, 2001). The state is considered as hyperendemic for malaria and the transmission is perennial with a seasonal peak (July to October). With 3.5% of the country's population, Orissa contributes 25% of a total of 1.5-2 million reported annual malaria cases, 39.5% of 0.89 million *P. falciparum* malaria and 30% of >1000 deaths caused due to malaria in India (Kumar *et al.*, 2007). About 3/4th of the state is mountainous and covered with forest. *Anopheles culicifacies* and *Anopheles fluviatilis* are the main vectors of the state. The entomological inoculation rate in Orissa has not been measured.

Patients

The severe cases were recruited from in-patients admitted in SCB Medical College and Hospital, Cuttack between July 2005 to October 2007. The mild malaria patients were recruited from subjects attending the outpatient clinic at primary health centres (PHC). The severe cases were referred to this tertiary care centre when severe complications developed after treatment with chloroquine (10 mg/kg body weight on days 0 and 1, and 5 mg/kg body weight on day 2) at the rural health care centres. Malaria was diagnosed using the thick-film method and subsequently confirmed by PCR. The inclusion criteria for the mild cases were body temperature >37.5°C, parasitaemia >1000/µl with *P. falciparum* mono infection and no intestinal geohelminthic infections

and severe cases were body temperature >37.5°C, parasitaemia >1000/µl with *P. falciparum* mono infection, no intestinal geohelminthic infections and fulfilling the defining criteria of WHO (2000) for cerebral malaria (unrousable coma persisting for at least 30 minutes after a generalised convulsion and not attributable to any other cause). The exclusion criteria were symptoms of mild or severe malaria with other acute infections and prior hospitalisation for any other reason, intake of any antimalarial drugs within the preceding week, pregnant women and having other chronic diseases, like tuberculosis, leprosy and malnutrition. About 0.5ml of venous blood was collected in EDTA vials from enrolled (mild as well as severe) patients belonging to same geographical locality and same ethnic group. Each patient was treated according to local guide lines and care was provided until discharge from the respective hospitals. The Ethical Committee of the Regional Medical Research Centre, Bhubaneswar had approved the study.

Isolation and amplification of DNA for *P. falciparum* genotyping

DNA was isolated from 100ul of blood following the standard protocol (Sambrook & Russel, 2001). In brief, erythrocytes were lysed with lysis buffer (10mM Tris-HCl pH-8.0, 0.1M EDTA pH 8.0, RNase 20ug/ml, 0.5% SDS and proteinase K 100ug/ml) at 55C for 16 hrs. DNA was obtained by phenol extraction /ethanol precipitation and resuspended in 50ul of DNase free water. The genetic diversity of *P. falciparum* isolates collected from each patient was studied by PCR amplification of highly polymorphic regions of the merozoite surface protein 1 and 2 (MSP1 and MSP2) and three microsatellite markers, C1M4, C3M27 and C4M69 located on chromosomes 1, 3 and 4, respectively. The various allelic forms of MSP1 block 2 and MSP2 block 3 were identified by size polymorphism as described by Snounou *et al.* (1999). Microsatellite typing was done using the locus-specific primers as described by Su *et al.* (1999). All PCR amplifications contained

a positive control (standardised laboratory strain of 3D7) and a negative control (containing no target DNA). The amplicons were separated by 2%Neusive/ 3%Metaphor agarose gel electrophoresis and visualized by UV transillumination after staining with EtBr. The single genotype infection (SGI) was defined as those which contains only one allele for each of the 5 loci, while mixed genotype infection (MGI) were defined as those in which at least one of the 5 loci contained more than one allele.

Detection of sub – patent gametocytes

The *pfg 337* messenger RNA was amplified by reverse transcriptase polymerase chain reaction (RT - PCR) to examine the presence of gametocytes in the blood, since this gene is expressed only in *Plasmodium falciparum* gametocytes (Alano *et al.*, 1991). Total RNA was first isolated using High Pure RNA isolation kit (Roche, UK). RT-PCR of *pfg 337* was performed in a two step reaction protocol using GenAmp RNA PCR core kit (Roche, UK). The PCR thermal conditions and primers used here was as described by Menegon *et al.* (2000). The presence of *pfg 337* in the blood samples was also examined by PCR of genomic DNA. The absence of mRNA indicates that the infection consists of only asexual forms.

Statistical analysis

Data were analysed using the Yates corrected χ^2 or Fisher's exact test by Epi-Info 6.1 (CDC, Atlanta, GA, USA).

RESULTS

Characteristics of malaria Patients

The study population included 96 malaria patients (48: mild, 48: severe) symptomatic with *P. falciparum* infection and ranging in age from 16 to 47 years. The mild malaria group consisted of 36 males and 12 females with a median age of 34, while severe group consisted of 36 males and 12 females with a median age of 31. Mean parasite density was significantly high ($P < 0.05$) among the severe malaria patients (45601.7/ μ l) compared to uncomplicated malaria patients (3827.6/ μ l).

In the severe group, all were cases of cerebral malaria (Glasgow coma score < 10 at least 1 hour after epileptic fit). Of these, 4 (8.3%) had hyperparasitaemia (> 25000 parasites/ μ l), 21 (43.8%) had scizontaemia, 19 (39.6%) had generalized convulsions, 7 (14.6%) had severe anaemia (Hb < 5 g/dl) and 5 (10.4%) had hypotension.

Genetic characteristics of *P. falciparum* isolates

Nested PCR detected all the three allelic families of MSP1 (block2) (K1, MAD20 and RO33) in mild as well as severe malaria cases. The overall frequency of K1 allele was 49.5%, MAD20 was 29.4% and RO33 was 21.1%. In case of MSP2 (block3), both FC27 and 3D7 family specific alleles were detected in mild and severe malaria cases. The overall frequency of FC27 allele was 52.6%, while 3D7 allele was 47.4%. The overall mean clone number of MSP1 / MSP2 was 1.34 / 2.5 in mild cases and 1.40 / 3.1 in severe cases. On statistical comparison, the mean clone number of 3D7 allele of MSP2 was found to be significantly high in severe cases ($P = 0.003$) than mild cases but no significant difference was observed on the distribution of the frequency of family specific alleles between mild and severe malaria patients (Table 1).

Amongst the three (MS) microsatellite loci C3M27 was monomorphic, for which a single 150bp (B) product was observed for all isolates. In case of C1M4 loci total three different products (A1: 130, A2: 150 and A3: 160bp) were amplified in isolates present both in mild and severe group of patients. Out of four distinct size products C1 (300bp), C2 (380bp), C3 (400bp) and C4 (450bp) detected for C4M69 the frequency of 450bp product was found to be significantly high ($P = 0.0001$, Fisher's exact test) among the isolates associated with severe malaria cases compared to isolates associated with mild malaria cases (Fig. 1).

MSP1, MSP2 and MS fragments that could be assigned to specific allelic families were used to characterise parasite sub-population. Of the 96 *P. falciparum* isolates, 8 (8.3%) comprised of three genotypes, 63 (65.6%) with two genotypes and the

Table 1. The MSP1 and MSP2 allelic families found in *P. falciparum* isolates associated with mild and severe malaria cases

	<i>MSP1 gene</i>			<i>MSP2 gene</i>	
	K1	MAD20	RO33	FC27	3D7
Allelic Frequency (%)	M:53.4, S:46.9	M:25.6, S:31.8	M:20.9, S:21.2	M:57.5, S:50.0	M:42.5, S:50.0
Mean no. of clones (P=0.501)	M:0.71, S:0.64 (P=0.501)	M:0.34, S:0.43 (P=0.408)	M:0.28, S:0.29 (P=0.920)	M:1.4, S:1.54 (P=0.446)	M:1.1, S:1.54 (P=0.003)
Length polymorphism (product size range, bp)	150-300	150-300	150	320-800	400-750

M: mild malaria , S: severe malaria

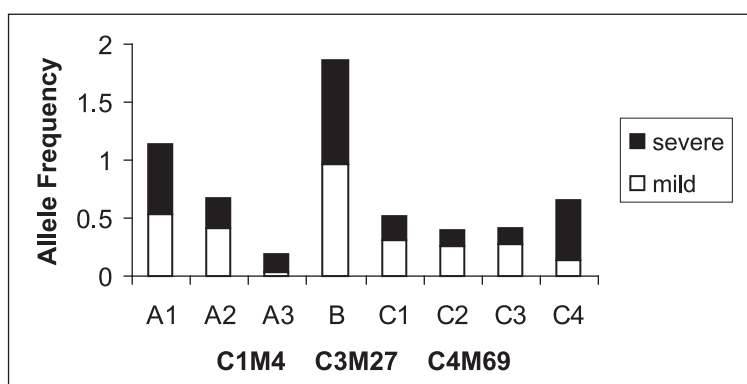


Fig. 1. Frequency distribution of the alleles of microsatellites (C1M4, C3M27 and C4M69) associated with mild and severe malaria.

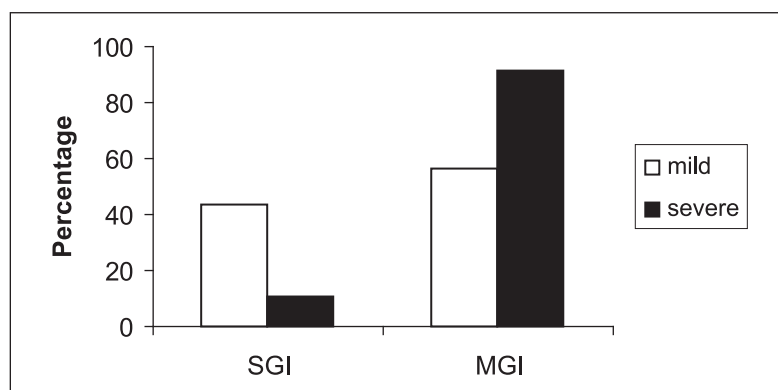


Fig. 2. Prevalence of single genotype (SGI) and mixed genotype infections (MGI) in mild and severe malaria cases.

remaining 25(26.1%) with a single genotype. In the mild group out of total 48 patients 21(43.7%) patients were carrying single genotype infection (SGI) and 27(56.3%) patients mixed genotype infections (MGI).

In the severe group, only five (10.4%) patients were carrying SGI and rest 43(89.6%) were having MGI (Fig. 2). When compared, the multiple genotype infections (MGI) were found to be more prevalent among severe

malaria patients than the mild ones (Odds ratio: 4.60, 95%CI: 1.784-11.86, P=0.002). However, no significant difference was observed for any of the allelic form in mild and severe cases in SGI group.

Prevalence of gametocyte among mild and severe malaria patients

The RT-PCR revealed a higher gametocytaemia rate among severe malaria cases (81.3%) compared to mild malaria cases (56.3%) (P= 0.0147, Fisher's exact test).

DISCUSSION

Our study has revealed a strong association of severe manifestation of *P. falciparum* malaria with the presence of both MGI and novel types of *P. falciparum* clones. This is at par with the results of the study conducted in Gabon and Sudan, where clinical attacks of malaria were found to be associated with increased complexity of infection (Ntoumi *et al.*, 2000; Roper *et al.*, 1998). But, contrast to our findings studies in Senegal, Tanzania and Papua New Guinea have reported lower genetic diversity of *P. falciparum* in individuals with clinical disease than in asymptomatic infections despite higher parasite density (Contamin *et al.*, 1996; Beck *et al.*, 1997; Al-Yaman *et al.*, 1997). Sometimes high parasite density may not only lead to unspecific amplification and generation of artefact fragments, potentially interpreted as large number of genotypes, but also to underestimation of minor clones, which may be missed by PCR. However, in our study the proportion of infections containing different concurrent family specific alleles is a conservative estimate and not affected by artefact amplification due to high parasite density. Nevertheless, the possible cause of variations between the above studies might be the level of transmission, socio-economic conditions and at large the ethnicity (host genetics) of the population. *An infection can have several genotypes due to super infections and/or inoculation of multiple genotypes during a single mosquito bite.* The circulation of multi genotype infections in severe malaria cases in our study area

might have been due to lack of strain specific immunity to limit the number of parasite clones (Eisen *et al.*, 2002). Presumably, our observation is simply a record of an outcome of a process, but we do not have information about the process itself.

To explore the genetic makeup of our parasite population further, we had analysed three MS markers. This showed the significant association of the C4 allele of C4M69 MS with severe malaria. In severe malaria *P. falciparum* parasitized red blood cells adhere to host endothelial cells, leading to mechanical blockade of microvessels, as well as cytokine secretion and modification of the T cell repertoire (Mazier *et al.*, 2000). pRBC sequestration obstructs capillaries and venules through various mechanisms, including cytoadherence, agglutination, rosetting and reduced red blood cell deformability (Pongponratn *et al.*, 2003, Dondrop *et al.*, 2004). *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP1), a surface antigen (encoded by 60 different *var* genes) which is expressed on pRBCs acts as a ligand during adhesion of the pRBCs to host endothelial receptor. However, the pRBCs continuously switch expression of PfEMP1 proteins and thereby evade immune destruction. Hence, a single pRBC only express a single variant surface antigen type at a given time and can switch to another variant surface antigen at other point of time, regardless of the variant surface antigen expressed by other pRBC. In human cytoadhesion involves more than 11 receptors present in endothelial cell surfaces, which have different affinity for different PfEMP1 subtypes (Chakravorty & Craig, 2005). The over representation of C4 allele of C4M69 MS in our parasite population indicates that the multiadhesion of the pRBCs found in the patients with severe malaria is possibly due to co expression of multiple binding adhesins in a single clonal population of parasites. Moreover, since the MS are neutral markers, the difference in proportion of a particular allele between mild and severe malaria supports the hypothesis of stratification of *P. falciparum* parasite population into strains of differing virulence (Gupta *et al.*, 1994).

Evolution of virulence in *P. falciparum* and the acquisition of natural immunity to malaria by the host are not properly known. Since human malaria infections are frequently composed of genetically diverse populations of parasites, the relationship between the number of different parasite clones in infection samples (multiplicity) and immunity and pathology has come under scrutiny. Recently four different models have been conceived to explain the evolution of virulence in *P. falciparum*. In a situation like ours where mixed genotype parasitism occurs in severe malaria cases, it is possible that the natural selection may favour levels of virulence that are higher than those optimal for single genotype infections as predicted by the kin and group selection model. Thus, if a parasite has the capacity to adopt a facultative strategy of increasing its growth (and thus increasing the rate of host exploitation) when in the presence of competing unrelated clones, then more virulence will result from multiple than single clone infections. Such models imply that *P. falciparum* infections, irrespective of clone multiplicity, may have evolved relatively high levels of intrinsic virulence because immune selection for antigenic diversity in the parasite population necessarily leads to inter-clone competition. Therefore, increase in clone multiplicity might be associated with increase virulence in human infections (Read *et al.*, 2002).

Parasite virulence and its relationship with transmissibility is another important theme in analysis of host-parasite interactions in natural populations (Anderson & May, 1991). In a situation where there is strong competition between different parasite types (because of shared immune responses, for instance), coexistence is only possible if the strains have very similar basic reproductive rates (Gupta *et al.*, 1994). Thus, a more virulent strain must compensate for higher host mortality (which reduces the duration of infectiousness, and transmissibility) through higher infectiousness. Prevalence of high gametocyte density ($P = 0.0147$, Fisher's exact test) and multi genotype infections of *P. falciparum* among severe malaria

cases in the present study tempting to predict that the mixed strains are possibly in competition and have positive influence on transmissibility. Interventions aimed at reducing the frequency of mixed-strain infections could indeed lead to reduced levels of virulence by taking away the evolutionary pressure on the maintenance of competitiveness (Adler & Losada, 2002). Therefore; use of bed nets can reduce the incidence of severe disease in this type of epidemiological set up without inducing any significant change in prevalence of infection.

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REFERENCES

- Adler, F.R. & Mosquera Losada, J. (2002). Adaptive Dynamics of Infectious Diseases In: *Pursuit of Virulence Management* (Editors, U. Dieckmann, J.A.J. Metz, M.A. Sabelis & K. Sigmund) pp. 139–149. Cambridge Univ. Press, Cambridge, U.K.
- Alano, P., Premawansa, S., Bruce, M.C. & Carter, R. (1991). A stage specific gene expressed at the onset of gametocytogenesis in *Plasmodium falciparum*. *Molecular Biochemical Parasitology* **46(1)**: 81-88.
- Al-Yaman, F., Genton, B., Reeder, J.C., Anders, R.F., Smith, T. & Alpers, M.P. (1997). Reduced risk of clinical malaria in children infected with multiple clones of *Plasmodium falciparum* in a highly endemic area: a prospective community study. *Transaction of Royal Society of Tropical Medicine and Hygiene* **91**: 602-605.

- Anderson, R.M. & May & R.M. (1991). *Infectious Diseases of humans: Dynamica and Control*. Oxford Univ. Press, Oxford.
- Beck, H.P., Felger, I., Huber, W., Steiger, S., Smit, T., Weiss, N., Alonso, P. & Tanner, M. (1997). Analysis of multiple *Plasmodium falciparum* infections in Tanzanian children during the phase III trial of malaria vaccine SPf66. *Journal of Infectious Diseases* **175**: 921-926.
- Branch, O.H., Takalo, S., Kariuki, S., Nahlen, B.L., Kolczak, M., Hawley, W. & Lal, A.A. (2001). *P. falciparum* genotypes, low complexity of infection, and resistance to subsequent malaria in participants in the Asembo Bay cohort project. *Infection and Immunity* **69**: 7783-7792.
- Breman, J.G. (2001). The ears of hippopotamus: manifestations, determinants, and estimates of the malaria burden. *American Journal of Tropical Medicine and Hygiene* **64**: S1-S11.
- Census of India. (2001). Provisional population totals, series-22. Orissa.
- Chakravorty, S.J. & Craig, A. (2005). The role of ICAM-1 in *Plasmodium falciparum* cytoadherence. *European Journal of Cell Biology* **84**: 15-27.
- Chotivanich, K., Udomsangpetch, R., Simpson, J.A., Newton, P., Pukrittayakamee, S., Looareesuwan, S. & White, N.J. (2000). Parasite multiplication potential and the severity of falciparum malaria. *Journal of Infectious Diseases* **181**:1206-1209.
- Contamin, H., Fandeur, T., Rogier, C., Bonnefoy, S., Konate, L., Trap, J.F. & Mercereau-Puijalon, O. (1996). Different genetic characteristics of *Plasmodium falciparum* isolates collected during successive clinical malaria episodes in Senegalese children. *American Journal of Tropical Medicine and Hygiene* **54**: 632-643.
- Dondorp, A.M., Pongponratn, E. & White, N.J. (2004). Reduced microcirculatory flow in severe falciparum malaria: pathophysiology and electron-microscopic pathology. *Acta Tropica* **89**: 309-17.
- Eisen, D.P., Saul, A., Fryauff, D.J., Reeder, J.C. & Coppen, R.L. (2002). Alternations in *P. falciparum* genotypes during sequential infections suggest the presence of strain specific immunity. *American Journal of Tropical Medicine and Hygiene* **67**: 8-16.
- Gupta, S., Hill, A.V.S., Kwiatkowskitt, D., Greenwoodt, A.M., Greenwoodf, B.M. & Day, K.P. (1994). Parasite virulence and disease patterns in *Plasmodium falciparum* malaria. *Proceedings of National. Academy of Sciences USA* **91**: 3715-3719.
- Kumar, A., Valecha, N., Jain, T. & Dash, A.P. (2007). Burden of malaria in India: Retrospective and prospective view. *American Journal of Tropical Medicine and Hygiene* **77**: 69-78.
- Mazier, D., Nitcheu, J. & Idrissa-Boubou, M. (2000). Cerebral malaria and immunogenetics. *Parasite Immunology* **22**: 613-623.
- Menegon, M., Severini, C., Sannella, A., Paglia, M.G., Sangare, D., Abdel-Wahab, A., Abdel-Muhsin, A., Babiker, A.H., Walliker, D. & Alano, P. (2000). Genotyping of *Plasmodium falciparum* gametocytes by RT-PCR. *Molecular Biochemical Parasitology* **111**: 413-415.
- Ntoumi, F., Ngoundou-Landji, J., Lekoulou, F., Luty, A., Deloron, P. & Ringwald, P. (2000). Site-based study on polymorphism of *Plasmodium falciparum* MSP-1 and MSP-2 genes in isolates from two villages in Central Africa. *Parasitologia* **42**: 97-203.
- Pongponratn, E., Turner, G.D., Day, N.P., Phu, N.H., Simpson, J.A., Stepniewska, K., Mai, N.T., Viriyavejakul, P., Looareesuwan, S., Hien, T.T., Ferguson, D.J. & White, N.J. (2003). An ultrastructural study of the brain in fatal *Plasmodium* malaria. *American Journal of Tropical Medicine and Hygiene* **69**: 345-359.

- Read, A.F., Mackinnon, M.J., Anwar, M.A. & Taylor, L.H. (2002). Kin-selection Models as Evolutionary Explanations of Malaria. In: *Adaptive Dynamics of Infectious Diseases: In Pursuit of Virulence Management* (Editors, U. Dieckmann, J.A.J. Metz, M.A. Sabelis & K. Sigmund) pp. 165–178. Cambridge Univ. Press, Cambridge, U.K.
- Roper, C., Richardson, W., Elhassan, I.M., Giha, H., Hviid, L., Satti, G.M.H., Theander, T.G. & Arnot, D.E. (1998). Seasonal changes in the *Plasmodium falciparum* population in individuals and their relationship to clinical malaria: A longitudinal study in a Sudanese village. *Parasitology* **116**: 501–510.
- Sambrook, J. & Russell, D.W. (2001). Molecular cloning – A laboratory manual (Vol-1), third ed. Col Spring Harbor Laboratory Press, New York. pp. 6.4-6.11.
- Snounou, G., Zhu, X., Siripoon, N., Jarra, W., Thaithong, S., Brown, K.N. & Viriyakosol, S. (1999). Biased distribution of msp1 and msp2 allelic variants in *Plasmodium falciparum* populations in Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **93**: 369–374.
- Su, X., Ferdig, M.T., Huang, Y., Huynh, C.Q., Liu, A., You, J., Wootton, J.C. & Wellems, T.E. (1999). A genetic map and recombination parameters of the human malaria parasite *Plasmodium falciparum*. *Science* **286**: 1351–1353.
- WHO (2000). Severe falciparum malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **94**: S1-90.