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Genetic diversity in *Plasmodium falciparum* merozoite surface protein 1 and 2 coding genes and its implications in malaria epidemiology: a review of published studies from 1997–2007

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Abstract

A major characteristic of human malaria parasites is their genetic diversity and an increasing number of studies have been reported on the epidemiology of *Plasmodium falciparum*, mainly focusing on the polymorphism of merozoite surface protein (MSP) 1 and 2 genes. A myriad of information on the genetic diversity and multiplicity of *P. falciparum* infections has been generated from such studies, and a range of molecular tools for epidemiological studies were produced, creating both optimism and pessimism in regard to the global efforts to control malaria. The objective of this review is to provide current and comprehensive information on the diversity in the gene that encodes the merozoite surface protein (MSP) 1 and 2 of *P. falciparum* and its implications on the epidemiology of malaria, immunity and development of control measures, and point out some research themes that need to be explored further by utilizing molecular techniques currently at our disposal. Articles published in journals between 1997 and 2007 are herein reviewed.

Key words Genetic diversity – immune response – merozoite surface protein – multiplicity – *Plasmodium falciparum*

Introduction

Plasmodium falciparum is the most virulent of the four parasites which cause malaria in humans. These malaria parasites are genetically diverse at all levels of endemicity. The inherent variability of *P. falciparum* is particularly prevalent in merozoite surface antigens being targeted for malaria vaccines. This provides multiple effective evasion and drug resistance mechanisms for the parasite. It also represents a major challenge for development of an effective malaria vaccine. Thus, the study of genetic diversity in malaria parasites is expected to provide new insights for the deployment of control measures.

The genetic complexity of *P. falciparum* and in particular its ability to generate mutant variants, makes it a successful pathogen. Although information on the frequency of genes conferring, for example, resistance to a certain drug or a vaccine in a given area has obvious relevance to the implementation of control measures using such agents, a critical first step is to obtain information on the genetic polymorphism of the parasites in the human hosts in the community to be investigated. As such, many studies have used the polymorphic regions of merozoite surface proteins (MSP) 1 and 2 as genetic markers to determine the genetic diversity of *P. falciparum*, multiplicity of infection, assess the level of malaria transmission, as well as investigating the relationship of these factors with acquisition of natural immunity against malaria[1–9].

Additionally, MSP-1 and MSP-2 cause immune response in humans and have been identified as potential candidates for blood-stage malaria vaccine[10–13]

but recent reports have shown importance of another merozoite surface protein, MSP-3, and apical membrane protein antigen, AMA-1, in evoking the human immune system[14–16]. This discovery implies that we now have a number of potential malaria vaccine candidates, which if further investigated may provide more information on candidates which may provide better protection against malaria. It is assumed that the use of high-throughput assays like protein microarrays may offer opportunity to identify antigens that either alone or in combination, function as targets of natural acquired immunity against malaria infections.

Methods

Citation of relevant articles was searched in the following electronic databases: PubMed, Hinari, and Blackwell Synergy. Search terms included '*Plasmodium falciparum*', 'merozoite surface protein', 'genetic diversity', 'malaria', 'multiplicity', 'molecular epidemiology', 'sickle-cell trait', 'natural immunity', 'immune response' used in various combinations like 'merozoite surface protein + genetic diversity', 'sickle-cell trait + genetic diversity', 'merozoite surface protein + sickle-cell trait + *P. falciparum*', etc. Only papers originally published in English language between 1997 and 2007 were considered. Unpublished data were not considered.

Studies were selected if they satisfied the following criteria: (i) the study involved human subjects, (ii) the subjects were infected with only *P. falciparum* and no other *Plasmodium* species, and (iii) the study involved genotyping of *P. falciparum.* Selected studies were entered into an End Note library (End Note 7.0.0 Bld 98, Thomson ISI ResearchSoft). The following information was sought for from the selected papers: year of publication; level of malaria transmission or endemicity of malaria; genetic diversity determined using MSP-1 and/or MSP-2 as genetic markers; multiplicity of infection (MOI) which is the presence of multiple concurrent genetically diverse clones in an infection; if the study reported other factors such as red cell polymorphism, drug resistance and/or immune response in the context of genetic diversity; and conclusions from the studies.

Genetic diversity in *msp-1* and *msp-2* genes

It is well known that the *msp-1* gene is divided into 17 blocks, based on analysis of sequence diversity: seven highly variable blocks are interspersed with five conserved and five semi-conserved regions. Block 2 of the *msp-1* gene appears to be subjected to rapid intragenic recombination, and so, is highly polymorphic[3,4]. The *msp-2* gene, also known as merozoite surface antigen (*msa-2*) gene, codes for a merozoite surface polymorphic glycoprotein that has been widely studied as one of the major vaccine candidates. The sequencing of DNA has shown that a single copy of *msp-2* gene has conserved N- and C-terminal domains (blocks 1 and 5), two non-repetitive variable regions (blocks 2 and 4), and a polymorphic central region (block 3) containing variable numbers of tandem repeats, which also vary in sequence and length[6,7,17,18]. Genes in which polymorphism has arisen through intragenic recombination in repetitive segments are characterized by repeat motifs with length variability differing between strains.

A major mechanism for the generation of allelic diversity in the *P. falciparum msp-1* gene is meiotic recombination in the *Anopheles* mosquito, which is believed to be dependent on the intensity of transmission. It is suggested that frequent recombination events between MSP-1 alleles intermittently generate novel alleles in high transmission areas[19]. Single nucleotide polymorphisms (SNPs) contribute largely to the variability of *P. falciparum* and provide multiple effective evasion and drug resistance mechanisms for the parasite necessitating the use of molecular techniques to differentiate alleles responsible for recrudescences and re-infections after treatment.

Polymorphic genes within a parasite species are used as genetic markers, thus, providing a means to assess the composition of the parasite population. Several

strategies have been used for *Plasmodium* parasite genotyping. At present, there are various techniques based on DNA fingerprinting for microorganisms such as PCR-restriction fragment length polymorphism (PCR-RFLP)[18,20,21] and amplified fragment length polymorphism (AFLP)[22]. Other methods for typing *P. falciparum* are based on microsatellite (MS) analysis[23–25], reverse transcriptase (RT)[26], sequence analysis[6], fluorogenic PCR[27], minisatellite variant repeat (MVR) mapping[28], matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOFI)[29] and ligase detection reaction-fluorescent microsphere assay (LDR-FMA)[30]. The most widely used methods are PCR-based[4,7,8,18,31–36], since they require use of very little amount of blood. PCR techniques are also being used for the detection of drug resistance markers[37] and differentiation of recrudescences and re-infections after treatment[38,39] but a recent technique using microsatellites and capillary electrophoresis[40] showed higher sensitivity and specificity in differentiating recrudescences from new infections than conventional PCR methods.

It should be noted that the commonly used PCR methods have limitations: they underestimate allelic polymorphisms as there may be daily individual variations[41], presumably due to sequestration of different parasites every 24 h. Indeed, as a result, most studies are not able to show the daily individual variations of strains. In addition, alleles of identical size but with point mutations may not be differentiated[6,8,42] and alleles present at very low density in a complex blood infection are likely to remain undetected. Nevertheless, the analysis of parasite genotypes provides measures that can be used to characterize the malariological picture in human populations, and to extend our understanding of the epidemiological effects of natural immunity to *P. falciparum.*

Several studies have reported that MSP-1 allelic variants fall under three major types—MAD20, K1 and RO33[3,4,43] but their frequency varies in different geographical areas, even in neighbouring villages[44]. *msp-1* is associated with protection, especially the highly conserved *msp-1*$_{19}$-kDa fragment[45]; this is a major reason to consider *msp-1* block-2 (and loci in genetic disequilibrium with *msp-1* block-2) specifically rather than as only a marker for overall MOI. The extensive genetic disequilibrium in *msp-1*, results in block-2 genotypings not being indicative of the allelic types present in other regions of the gene[46]. This has implications for the interpretation of population genetic studies of selection on *P. falciparum* genes.

MSP-2 has been widely used to characterize *P. falciparum* field isolates[5,39,47], and some authors have reported that it is highly discriminatory and have used it alone to characterize *P. falciparum* populations[6,35,48]. However, the use of only one marker, no matter how polymorphic it is, would miss variation at other polymorphic loci, and thus, almost certainly underestimate the magnitude of multiple infections[34]. Nevertheless, the choice of a particular gene marker for typing natural *P. falciparum* clones depends on the question being addressed. MSP-2 alleles, which differ in number and sequence of intragenic repeats, can be grouped into two allelic families, FC27 and 3D7/IC, according to the central dimorphic domain as first observed over a decade ago.

Since *msp-1* and *msp-2* genes are under strong natural selection, interpretation of population structure using data derived from these loci is not easy, since it is not clear whether the patterns observed reflect population history or natural selection. MSP-1 and MSP-2 are non-neutral markers and may differ in their ability to discriminate between populations, even when they are equivalent in their ability to discriminate between clones and strains within the same population, especially when natural selection is a major source of variation in allele frequencies. To overcome these limitations, putatively neutral markers (i.e. polymorphisms located in non-coding DNA sequences, that are not under selection), such as most hypervariable microsatellite loci can be used[23].

Level of malaria transmission

Malaria transmission may be hyper- or holo-endemic, mesoendemic and hypoendemic. Areas with hyper- or holo-endemic malaria transmission are characterized by perennial malaria or stable malaria. Mesoendemic areas have low to medium malaria transmission, which is also seasonal, while hypo-endemic areas have unstable malaria transmission[49] and are prone to epidemics. The annual entomological inoculation rate (AEIR), which estimates the average number of infective mosquito bites per person per annum, is used to express the intensity of malaria parasite transmission. Malaria transmission intensity in Africa is highly variable with AEIR ranging from <1 to >1000 infective bites per person per year. Holoendemic transmission is characterized by >80 infective bites/person/year[50,51] while hypo-endemic transmission by <8 infective bites/person/year[49,52]; and mesoendemic transmission by values between those of holo- and hypo-endemic transmission[1].

Plasmodium falciparum diversity in Africa and Asia is thought to reflect endemicity. Classic studies in areas of holoendemic transmission showed that clinical immunity develops quite rapidly during childhood[53], although the processes through which increasing levels of resistance to infection are acquired are still not understood. However, holo-endemic transmission is one end of the spectrum of malaria epidemiology and the development of clinical immunity is also affected by factors such as the infection rate and the local parasite species composition[54]. It is suggested that in endemic areas, the number of clones of malaria parasites co-infecting a single host can be a useful indicator of the level of transmission and/or the immune status of the host[51,55–57]. Increase in transmission levels (as measured by entomological inoculation rates) is generally associated with progressive increase in the average number of malaria parasite clones per host[58] suggesting that in areas of low transmission intensity, significantly fewer parasite genotypes per infected person should be found. However, there is evidence of high genetic diversity of MSP-1 and MSP-2 in areas of low malaria transmission[31,59]. This may imply that the extent of allelic diversity is determined not only by the transmission intensity but also by the number of alleles prevalent in the local parasite population and the extent of multiplicity of infections.

Multiplicity of infection and morbidity

Parameters describing the infection dynamics of *P. falciparum* are important determinants of the potential impact of interventions and are potential outcome measurements for malaria intervention trials. Low parasite densities, periodic sequestration of parasites, and the presence of multiple concurrent infections make it essential to use molecular techniques to estimate the force of infection and duration of infections in endemic areas. To date, a high degree of polymorphism has been demonstrated at both the *msp-1* and *msp-2* loci in parasites from areas of stable malaria transmission. As a consequence, in such areas it is rare to find parasites of the same two-loci genotype in more than one subject. Some surveys have documented *P. falciparum* multiplicity of up to nine different parasite clones at a given time in a single asymptomatic host[35].

In the last decade, emphasis in several studies has been placed on investigating, if any relationship exists between multiplicity and variables like parasite density, age and infection outcomes. Studies in Tanzania[60] and in Papua New Guinea[61] suggested that in individuals with substantial previous exposure to malaria, co-infection with multiple clones of *P. falciparum* can protect against subsequent clinical malaria attacks. However, other studies, mainly of individuals with little previous exposure such as in infants, multiplicity was positively associated with parasite density and risk of clinical morbidity[21,47] contrary to the suggestion from many studies that high multiplicity is protective against clinical malaria. This finding probably reflects the immune status of these very young children; the absence of premunition in infants may be a major factor contributing to their great vulnerability to clinical malaria[62]. These observations imply that in highly endemic

areas MOI is not directly correlated with exposure to *P. falciparum*[35].

As with parasite density, the relationship between multiplicity and age is still unclear—some studies have reported decrease in multiplicity with age[51], others have observed a positive correlation in infants and children but not in older individuals[35,63]. Some reports have indicated decrease of MOI during adulthood to the levels found in infants[21] while others did not observe any relationship between the two parameters[47,50]. This suggests that the mechanisms controlling multiplicity of infection and parasite densities follow different profiles and so are different.

Several studies have investigated the association of specific *P. falciparum* genotypes with the clinical disease and virulence to find out why severe malaria is seen to occur in only a small percentage of patients but no definitive results are currently available. However, there are some indications that parasites differ in their virulence. Some studies have shown an overexpression[36,64] or complete absence[65] of one of the allelic types of either MSP-1 or MSP-2 in severe malaria. Others did not see any association between any MSP-1 or MSP-2 genotype and clinical status[9,66,67]. One study that investigated the effect of genetic diversity of *P. falciparum* on severe malaria by using four genetic markers indicated an MOI of 0.936, suggesting that virulence markers might be more diverse than expected[68]. Earlier on, a case-control study on adults with cerebral malaria showed high multiplicity in those patients than with asymptomatic malaria suggesting that the development of cerebral malaria in adults residing in endemic areas is more dependent on strain multiplicity rather than on a specific strain or strains of *P. falciparum*. In view of this, given the high polymorphism which has been detected in MSP-1 and MSP-2 in many malaria endemic areas, it is not likely that different geographical areas would have similar pairs or set of genotypes responsible for the clinical disease. Nevertheless, the discovery that *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which is encoded by *var* genes is an important virulence factor that may provide an explanation why certain MSP-1 genotypes have been associated with the clinical disease. Since MSP-1 has a key role in invasion of red blood cells just like PfEMP1, it is conceivable that specific MSP-1 allelic forms favour more efficient invasion than do others. Indeed, one study reported an association of a specific MSP-1 allele (B-K1) with a specific *var* gene (*var-D*), which was over represented among patients with severe malaria[69]. These findings provide us with a platform to further investigate not only the biological functions of PfEMP1 but also immune responses against it and their implications on severe malaria.

Genetic diversity of *P. falciparum* and acquisition of immunity

Genetic diversity of *P. falciparum* plays a major role in the natural acquisition of immunity to malaria infections and is also a concern to the development and deployment of control measures. In humans living in malaria endemic regions, immunity to *P. falciparum* is acquired as a result of natural exposure to multiple infections over many years. In holo- or hyper-endemic areas, immunity develops at a younger age than in areas where transmission is less intense[50]. It is usually considered that immunity to *P. falciparum* has two components: an anti-disease immunity, which develops rapidly; and an anti-parasite immunity, which is acquired slowly and leads to a marked decrease in parasite densities.

Acquired anti-*P. falciparum* immunity reduces parasite density, limits the number of parasite genotypes infecting an individual at any given time, and controls parasites against which a strong immune response has been mounted[50]. It has been suggested that asymptomatic infections protect against developing clinical malaria and that such protection is enhanced by the diversity of infecting strains[51]. This fact makes the hope of developing effective malaria vaccines a realistic goal but is being constrained by the antigenic diversity of *P. falciparum*. Nevertheless, a coherent theoretical framework of how protective immunity to *P. falciparum* malaria is acquired

following natural exposure to the parasites is beginning to emerge, as a result of combined clinical and epidemiological data with basic immunological research. This is based on the fact that IgG antibodies against the most frequent subtypes of block 2 of MSP-1 are important in acquired antimalaria immunity[70].

Influence of haemoglobin variants on genetic diversity of *P. falciparum*

The importance of human genetic background on malaria morbidity and infections has become increasingly recognized in the last decade. Haemoglobin (Hb) S, HbC, and a^+-thalassemia confer protection from malaria. Accordingly, these traits may influence MOI of *P. falciparum* and the presence of distinct genotypes. Of the three traits, HbS in the heterozygous form, sickle-cell trait (HbAS), has been studied most. In areas endemic for falciparum malaria, HbAS is widespread due to its advantage against fatal malaria[71,72], however, the mechanism of protection still remains controversial. In such areas, children with HbAS have lower parasite rates and densities than children with normal haemoglobin (HbAA). Sickle-cell trait protects against severe falciparum malaria and reduces susceptibility to mild malaria but does not prevent malaria infection[72,73], thus, the protection is not absolute. The protective effect of the trait is highest during the first 12 months, after the loss of passively acquired immunity and before specific protection mechanisms are developed. While this protection may involve innate factors such as the reduced ability of *P. falciparum* parasites to grow and multiply in HbAS erythrocytes, recent observations suggest that it might also involve the accelerated acquisition of malaria-specific immunity[74].

In a study conducted in Gabonese children with asymptomatic malaria[73], there was a significantly lower prevalence of infection in children with sickle-cell trait compared to their normal-haemoglobin counterparts. In addition, the proportion of children with multiple infections and MOI were higher in HbAS carriers than in children with HbAA. Although the effect of haemoglobin type on multiplicity is still unknown, these observations could mean that the effects of HbAS complement the immune system in such a way that acquired immunity in sickle-cell trait carriers is attained at a younger age than in those with normal haemoglobin. In a study conducted in Ghana[75], and in Senegal[44], haemoglobin type did not influence MOI; however, the former study involved symptomatic children. In symptomatic children, the effect of Hb variants on parasite multiplicity and diversity appears to be limited. It is suggested that this may reflect an actual lack of influence or indicate abrogation in symptomatic malaria[75]. Elsewhere, some authors reported influence of haemoglobin type on the distribution of MSP-1 alleles[32], few authors observed only limited influence[75], and few others did not observe this influence[44] implying that the influence of HbAS on *P. falciparum* genetic diversity is still largely unknown. Further studies, with standardized protocols are needed to validate the present contradicting findings.

Studies have been done to investigate the role of a^+-thalassemia on genetic diversity of *P. falciparum.* In Papua New Guinea, where a^+-thalassemia affects more than 90% of the population, this haemoglobinopathy protected children against severe malaria and other infections[76]; however, the relevant mechanism is unknown. Contrary to what was expected, a^+-thalassemia was seen to increase the incidence of contracting mild malaria in the first two years of life in Vanuatu. No clear explanation for this could be given and further studies are needed to re-evaluate these observations.

Elsewhere, in northern West Africa where both HbC and a^+-thalassemia are quite common[75], their influence on parasite density and MOI is largely unknown; it was observed that a^+-thalassemia influenced the distribution of MSP-2 IC-type alleles but not parasite density, in symptomatic children, while HbAS did not influence MOI but it reduced parasite density[75]. This suggests that the protective mechanisms of a^+-thalassemia and HbAS against malaria are different, at least partially. All this implies that

the genetic composition of humans may also play a role in the defence against the parasite, so the immune mechanisms responsible for the acquired immunity remain uncertain.

Implications of genetic diversity of *P. falciparum*

Genetic diversity provides *P. falciparum* with the potential capacity of avoiding the immune response, and possibly supporting the selection of drug or vaccine resistant parasites[77]. The acquisition of drug resistance by *P. falciparum* has severely curtailed global efforts to control malaria. Strategies to prevent the rapid spread of parasites resistant to novel drugs or vaccines require an understanding of the population structure of the parasites. It is suggested that multiplicity affects the prevalence of genes involved in resistance to antimalarial drugs[78]. Drug resistance seems to spread faster in higher transmission areas, regardless of drug pressure; in low transmission areas, drug pressure seems to be the critical factor[79,80]. However, it is reported that in some African countries, resistance to antimalarial drugs has resulted from gene flow rather than mutation[81]. Since there is evidence of multiplicity increasing with malaria transmission intensity, probably as a result of mutation in several genes caused by the frequency of parasites' sexual recombination, it is conceivable that reducing malaria transmission may help to slow the spread of drug resistance; it is important to note that severe malaria has been associated with resistance to antimalarial drugs[82,83]. With increasing resistance of *P. falciparum* against the cheap and commonly used drugs such as chloroquine and sulfadoxine-pyrimethamine, especially in malaria endemic areas, an effective malaria vaccine could be the ideal malaria control tool to bring down the number of cases that die from malaria annually.

There is a long-standing evidence that immunoglobulin IgG has a role in protection against clinical malaria[11–13,16]; however, the antigenic diversity of *P. falciparum* represents a significant challenge for the development of a malaria vaccine. Although it is recognized that individuals living in malaria endemic areas develop, with time, clinical and antiparasite immunity[17,70,84], currently, there is no assay or clinical parameter that could predict whether such persons are protected against malaria[14], which represents a major obstacle to vaccine development. There is, therefore, need to correlate this protection with serum profiles against multiple *P. falciparum* antigens in areas with different levels of malaria transmission. Most studies of vaccine development and immune response analysis have focused on analyzing either one or a few immunodominant antigens at a time[85]. It is assumed that the use of high-throughput assays like protein microarrays offers opportunity to identify microbial antigens that either alone or in combination, function as targets of natural acquired immunity against infectious diseases. This is because, recently, an unexpectedly complex antibody immune response against multiple leading malaria vaccine candidates using microarray immunoassays was reported[14]. This suggests that such profiles would provide means for simultaneous assessment of many distinct antigen-antibody reactions, and the ability to monitor temporal stability or lack thereoff of these patterns in every individual, which may dramatically facilitate the identification of the parasite antigens that, in combination, function as targets of the protective immune response, and hence, facilitate both the development, and evaluation of antimalarial vaccines.

Conclusion

A series of studies have confirmed the epidemiological significance of genetic diversity of *P. falciparum* infections. Antigenic diversity, especially in the antigens that are being developed as potential components of an asexual-stage vaccine, still poses a major setback to developing an effective vaccine. However, we now have several molecular techniques, which can be used not only to define the malariological picture in hosts in a given area, but also to isolate and evaluate particular proteins or antigens from the malaria parasite that could be used in development of an effective malaria vaccine and/or evaluation of those which are undergoing trials. Microarray immunoas-

says should be developed and used to detect antibody immune responses directed against multiple antigens in individuals living in endemic areas. More studies are still needed to define the mechanisms underlying the protective advantage of HbAS and a^+-thalassemia against malaria in endemic areas. A better understanding of these mechanisms may yield important insights of how acquired immunity is enhanced.

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