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 (unarousable 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 55°C 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%Nusieve/ 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 $\chi^2$ 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/µl) compared to uncomplicated malaria patients (3827.6/µl).

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/µl), 21 (43.8%) had scizontaeemia, 19 (39.6%) had generalized convulsions, 7 (14.6%) had severe anaemia (Hb<5g/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 single150bp (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).
Table 1. The MSP1 and MSP2 allelic families found in P. falciparum isolates associated with mild and severe malaria cases

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<tr>
<th></th>
<th>MSP1 gene</th>
<th>MSP2 gene</th>
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<tr>
<td></td>
<td>K1</td>
<td>MAD20</td>
</tr>
<tr>
<td>Allelic Frequency (%)</td>
<td>M:53.4, S:46.9</td>
<td>M:25.6, S:31.8</td>
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<tr>
<td>Mean no. of clones</td>
<td>M:0.71, S:0.64 (P=0.501)</td>
<td>M:0.34, S:0.43 (P=0.408)</td>
</tr>
<tr>
<td>Length polymorphism (product size range, bp)</td>
<td>150-300</td>
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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 gametocyte 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 \textit{P. falciparum} malaria with the presence of both MGI and novel types of \textit{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 \textit{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 \textit{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). Plasmodium falciparum erythrocyte membrane protein-1 (PfEMP1), a surface antigen (encoded by 60 different \textit{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 \textit{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 interclone 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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