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Molecular characterization of *Plasmodium falciparum* in Arunachal Pradesh from Northeast India based on merozoite surface protein 1 & glutamate-rich protein

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***Background & objectives*: Northeast (NE) India is one of the high endemic regions for malaria with a preponderance of *Plasmodium falciparum*, resulting in high morbidity and mortality. The *P. falciparum* parasite of this region showed high polymorphism in drug-resistant molecular biomarkers. However, there is a paucity of information related to merozoite surface protein 1 (msp-1) and glutamate-rich protein (glurp) which have been extensively studied in various parts of the world. The present study was, therefore, aimed at investigating the genetic diversity of *P. falciparum* based on *msp-1* and *glurp* in Arunachal Pradesh, a State in NE India.**

***Methods*: Two hundred and forty nine patients with fever were screened for malaria, of whom 75 were positive for *P. falciparum*. Blood samples were collected from each microscopically confirmed patient. The DNA was extracted; nested polymerase chain reaction and sequencing were performed to study the genetic diversity of *msp-1* (block 2) and *glurp*.**

***Results*: The block 2 of *msp-1* gene was found to be highly polymorphic, and overall allelic distribution showed that RO33 was the dominant allele (63%), followed by MAD20 (29%) and K1 (8%) alleles. However, an extensive diversity (9 alleles and 4 genotypes) and 6-10 repeat regions exclusively of R2 type were observed in *glurp*.**

***Interpretation & conclusions*: The *P. falciparum* population of NE India was diverse which might be responsible for higher plasticity leading to the survival of the parasite and in turn to the higher endemicity of falciparum malaria of this region.**

Key words Genetic diversity - glutamate-rich protein - merozoite surface protein 1 - North East India - *Plasmodium falciparum*

Malaria, one of the major vector-borne public health problems, is endemic throughout tropics. Although, in recent years, significant progress has been made to reduce malaria morbidity and mortality[1], the

estimated global burden of malaria is still immense with 212 million cases, resulting 429,000 deaths annually[1]. There are several immunogenic antigens which are stage specific and have been targeted and characterized with respect to their use in vaccine development against *Plasmodium falciparum* with varying success[2]. However, several studies have shown high genetic polymorphisms as well as antigenic variation in *P. falciparum* vaccine candidates[3]. Hence, proper mapping of the genetic diversity of the malaria parasite is of utmost importance not only for the development of effective malaria vaccine but also for future vaccine trials.

The polymorphic regions of merozoite surface proteins-1 (*msp-1*) and glutamate-rich protein (*glurp*) have been the targeted markers for parasite genotyping in antimalarial drug trials to distinguish between recrudescence and reinfection[4]. The *msp-1* (block 2), the most abundant surface protein, is a leading vaccine candidate gene, as it is believed to play an important role in parasite invasion[5]. It is divided into 17 blocks that are further categorized as variables, conserved or semi-conserved blocks[6,7]. The block 2 region consists of three allele families (K1, MAD20 and RO33). Alleles in K1 and MAD20 have antigenically unique, tripeptide repeats[5]. Though the tripeptide repeats observed in the other two families were absent in RO33, but outside block 2, this allele was similar to the MAD20 type[8]. Fragment size in the three block allele families is commonly used as a molecular marker for malaria transmission dynamics as also for host immunity in *P. falciparum* malaria[9-12].

The *glurp* is an exoantigen of *P. falciparum* on which Phase I vaccine trials have been completed[13]. It is expressed in both the pre-erythrocytic and erythrocytic stages of the parasite life cycle and also on the surface of newly released merozoites[14]. Moreover, *glurp* has been found to inhibit the *in vitro* growth of *P. falciparum* with or without the cooperation of monocytes, indicating its important role in controlling parasitaemia[15]. It contains three repeat regions: the N-terminal non-repeat region (RO), a central repeat region (R1) and an immunodominant C-terminal repeat region (R2). The *glurp* is highly polymorphic, and this polymorphism mainly involves variations in the number of repeats of certain genomic sequences that affect the size of the gene and its protein product. The effect of malaria control interventions could not be assessed due to the lack of information regarding the genetic diversity of *P. falciparum* from NE India.

NE India comprises eight States, namely, Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Tripura. Although NE India represents only four per cent of the entire population of the country, it contributes to 8-12 per cent of the malaria cases in the country[16]. This indicates the high prevalence of malaria in the region. The region has an inimitable topography favourable for the spread of malaria, and its meagre socio-economic situation increases the transmission risk[17-19]. The earlier study conducted from NE India was on genetic polymorphism of only one antigenic marker *i.e*., *glurp* which was carried out in Darang district of Assam[20]. However, the present study was conducted in Arunachal Pradesh to investigate the genetic diversity of *P. falciparum* parasite in the region.

Material & Methods

During July-September 2013, a total of 249 fever cases were screened for malaria by microscopical examination using Giemsa staining at primary health centre of Miao area of Changlang district, Arunachal Pradesh, India. Of these, 75 were found to be positive for *P. falciparum*. Before treatment, 2 ml of blood was drawn from each microscopically confirmed patient in cryoprotectant vials and stored in liquid nitrogen and transported to the laboratory at Regional Medical Research Centre (RMRC), Dibrugarh, Assam, India.

Cases were selected based on the following criteria: greater than one year of age, devoid of severe malnutrition, not having any other illness and temperature >37.5°C. The study was approved by the Ethical Committee of the RMRC for NE Region (Indian Council of Medical Research), Dibrugarh, Assam, India. Written informed consents were obtained from all patients.

Preparation of DNA, PCR amplification and sequencing: Genomic DNA was extracted from blood samples using QIAamp DNA blood kit (Qiagen, CA, USA). In the final step of elution instead of 150 µl, 40 µl of elution buffer was used and stored at −80°C for further use. The allelic variation in polymorphic region (555 bp) of block 2 of *msp-1* was amplified using amplification conditions and primers as described by Bharti *et al*[21]. The R2 region (1063 bp) of *glurp* was determined by nested PCR using the primers GLPF: 5'-TGCAAGTGTTGATCCTGAAGT- 3' and GLPR: 5'-AATGTAGGTACCACGGGTTC- 3'(designed by WCMC-Q, Doha, Qatar). The primary PCR was

performed in 50 µl in platinum PCR SuperMix (Invitrogen, USA) with 0.4 µM each forward and reverse primers (GLPF and GLNF) and 3 µl extracted genomic DNA. The PCR reaction was initiated at 95°C for 5 min and followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 53.5°C for 2 min, extension at 72°C for 2 min and final extension of 10 min at 72°C. Primary PCR product was diluted (1:10) and further used for nested PCR using the primers GLNF: 5'-ATGTATCTGAAGTTGTTGAAGA-3' and GLNR: 5'- GTTTGTGATGGTACTTCTTCA-3'. The nested PCR was performed with annealing at 48.5°C for 35 cycles. Other nested PCR conditions were the same as those described for the primary PCR. The PCR products were resolved on 1.5 per cent agarose gel. A positive control (3D7-DNA) and a negative control (nuclease free water) were included in each amplification reaction. The nested PCR products were purified using High Pure PCR Product Purification Kit (Roche, Penzberg, Germany). Purified products were sequenced using the Sanger method (Genewiz INC, NJ, USA) with both respective forward and reverse primers.

Analysis of sequencing data: Obtained DNA sequences (*msp-1* 495 and *glurp* 972) were edited manually in BioEdit[22] and aligned using ClustalW[23]. A total of 75 sequences were obtained. Multiple sequence alignment (MSA) was performed to identify intraspecific variation, if any, amongst the sequences. The homology search was done using BLASTn programme with default parameters (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*) for *msp-1* and *glurp* genes. All nucleotide sequences were translated online using Expasy portal (*https://www.expasy.org/*) and obtained amino acid sequences were compared with available sequences in the NCBI database using ClustalW. The number of repeat regions of *glurp* in our isolates was determined based on the well-conserved amino acid repeat pattern DKNEKGQHEIVEVEEILPE of *glurp* R2 repeat region. The alleles of *glurp* were determined on the basis of the presence of GQ/VE/VQ on the 6th and 7th positions of the amino acid repeat region.

Results

Allelic polymorphisms of merozoite surface protein-1 (msp-1) (block 2): The polymorphic *msp-1* (block 2) was studied in the *P. falciparum*-infected patients for allelic diversity. In these patients, in the block 2 of *msp-1,* the nucleotide and the deduced amino acid sequence were found to be highly polymorphic. The nucleotide changes were non-synonymous; hence, the deduced amino acid variations corresponded to one or the other alleles. The samples comprised three alleles, namely, R033, MAD20 and K1. The allelic frequency in the studied population was 47 (63%), 22 (29%) and 6 (8%) for R033, MAD20 and K1, respectively. The samples were composed of two variants in R033 allele whereas MAD20 had only one variant and the remaining K1 allele was found to be composed of three variants. Further analysis showed that the R033 allelic sequences were identical and showed 80-100 per cent similarity with reported R033 isolates of Iran, Sudan, Brazil and Tanzania, whereas the MAD20 and K1 allelic sequences showed similarity with that from Tanzania and Thailand, respectively.

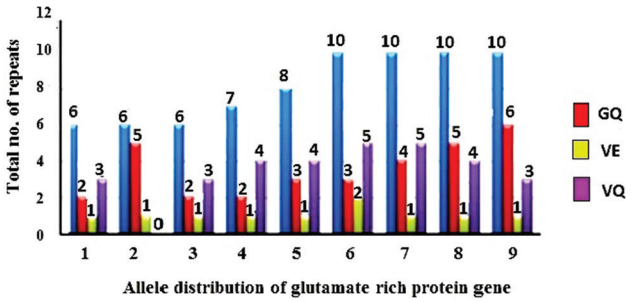

Figure. Distribution of repeat regions amongst the alleles. Alleles were identified based on the presence of GQ/VE/VQ on the 6th and 7th positions of the amino acid repeat region DKNEKGQHEIVEVEEILPE.

Allelic diversity of glutamate-rich protein (glurp) R2 repeat region: Of the 75 samples, four genotypes were observed based on the sequence length variation of the R2 repeat region of *glurp*. Genotype 1 consisted of six amino acid repeat sequence units (AAUs), whereas genotypes 2, 3 and 4 had 7, 8 and 10 AAUs, respectively. From the total of 75 samples, 43 (57.3%) samples were genotype 1 and 14 (18.7%), 11 (14.7%) and 7 (9.3%) were genotypes 2, 3 and 4, respectively. The changes at positions sixth and seventh (GQ/VE/VQ) yielded a total of nine alleles across the 75 samples of our study (Figure). The more conserved AAU of *glurp* R2 *i.e*. DKNEKGQHEIVEVEEILPE was present in 33 (44.0%) samples. However, at positions sixth and seventh, GQ was replaced by either VE or VQ in the remaining 42 (56.0%) samples. The repeat VQ was found in 31 (42.67%) samples and VE was present in 10 (10.33%) samples.

Discussion

The present study indicated considerable genetic polymorphism in *msp-1* (block 2) and *glurp* sequences

of *P. falciparum* from a malaria endemic region of NE India. Earlier reports suggested that genetic diversity and assortment of *Plasmodium* species were accountable for the natural success of the malaria parasite[24]. Genetic polymorphism in the malaria parasite helps it to evade the human immune response as well as to develop resistance to antimalarial drugs. Highly polymorphic vaccine candidate antigens such as *msp-1* elicit variant-specific immunity. Single-nucleotide replacement leads to amino acid diversity in malaria parasite antigens. Based on amino acid substitution, MSP-1 has been defined into 17 blocks. Most variations seen in malarial antigens have been due to genetic recombination.

Earlier studies carried on block 2 of *msp-1* from India have reported varying patterns of diversity[25,26]. Our study revealed that R033 alleles were the most dominant (63%), followed by MAD20 (29%) and K1 (8%), which was in agreement with the previous report[26]. In the present study, two variants of the dominant allele R033 were observed. Previous reports have suggested that the differences in the number of alleles for each gene associated with *P. falciparum* correlate with the degree of endemicity of a particular area[27,28]. The severity of malaria and the association between the distributions of allelic families have been investigated, and studies have yielded a variety of results. The RO33 allelic family has been frequently reported in asymptomatic malaria cases and K1 family in severe cases[29].

Low numbers of alleles have been reported from areas of low endemicity[30] whereas higher numbers of alleles have been reported from highly endemic areas of Africa and Asia[31,32]. Findings of the present study revealed diverse nature of *P. falciparum* from the malaria endemic region of Arunachal Pradesh with respect to the presence of all the three allelic families of *msp1*, which corroborated with previous findings from India[26,33]. These alleles are highly variable amongst the three groups but less variable within the group. The hypervariability in malaria antigens are mainly due to genetic recombination as it occurs in several orders of magnitude more frequently than mutation[34].

The *glurp* is considered to have an important role in the induction of protective immunity against *P. falciparum* malaria[35]. However, only a few studies[36] have been conducted on conserved amino acid sequences of R2 repeat region of *glurp* from field isolate of *P. falciparum,* and only one study has reported the polymorphism and amino acid repeat order of *glurp* in Assam, NE India[20]. Hence, the present study was an attempt to understand the overall population diversity of *P. falciparum,* and arrangement of amino acid sequence pair repeat in R2 region of *P. falciparum glurp* gene. Our data showed diversity in our populations, which was consistent with the findings reported earlier from high endemicity area of Africa and Asia[20,37] This finding, however, were in contrast with low endemic regions of Central South America, where frequencies of *glurp* allele varied from two to four[38]. In the present study, nine alleles and four genotypes were observed in the *glurp* gene and presence of a high number of repeats ranging from six to 10 signified a high level of diversity in the parasite population and malaria endemicity in this region.

In conclusion, the *P. falciparum* population of NE India was genetically diverse based on the two antigenic markers signifying this part of India as a malaria endemic zone. The information generated through this study will help in designing malaria vaccines in future.

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***Conflicts of Interest*:** None.

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