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Analysis of Complement Receptor Type 1 (CR1) Polymorphisms and Its Association With Malaria in Rural Population of Maharashtra

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Abstract— **The interaction between human host and the Plasmodium parasite is complex. The factors affecting the causality of infection and its severity are yet not completely understood. Single Nucleotide Polymorphisms (SNP) associated with CR1 may be associated with patho-physiology of malaria and its susceptibility to the disease. Methods: The objective of the present study was to calculate the incidence of various antigens of Knops blood group system and CR1 Exon22 polymorphisms in rural population from Chiplun Taluka of Ratnagiri district. The study included 112 malaria positive cases and 909 healthy controls, which were screened for CR1 Exon22 polymorphism. Knops (Kna/b), McCoy (McCa/b), Swain-Langley (Sl1/2) polymorphisms were screened in 93 cases and 321 healthy controls. The frequencies were determined using a PCR-RFLP technique. Results: Only wild types of the allele form were observed in Knops blood group system in malaria cases and healthy control. CR1 exon22 polymorphism was seen in the study population with all the 3 allele type distributed in the cases and control samples. No significant allelic or genotypic differences were found between the controls and the disease groups. Conclusion: The results of the present study demonstrate that common CR1 Exon22 and Knops blood group system are not associated with malaria in the endemic area.**

Index Terms— **Complement receptor 1, Knops, McCoy, Swain-Langley, Malaria, Single Nucleotide polymorphisms (SNP), PCR-RFLP..**

I. Introduction

Malaria is a widespread parasitic disease of humans. It can be caused by any of the four species of Plasmodium, of which *Plasmodium falciparum* is responsible for most of the cases of the disease and death across sub Sahara Africa, while *Plasmodium vivax* is the most prevalent parasite in other parts of the World [1]. According to WHO, there were about 198 million cases of malaria in 2013 and an estimated 5,84,000 deaths [2]. Even with significant decrease in malaria related mortality and morbidity, the disease still poses huge risk to humans.

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Several groups are engaged in determining the factors that lead to the development of malaria. The main factors that are being focused in research are parasite virulence phenotypes and host genetic factors. There is a complex interaction between host-parasite and the factors that influence severity of disease are still not fully understood. With little effect implicated by the environment, the primary factors that are involved, are that of the genomes of host and parasite. It is believed that the formation of rosette [3] is associated with the malaria pathology via obstruction in smaller capillaries [4-6]. Rosetting is mediated by the parasite ligand, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) on the surface of infected RBCs, which binds to various molecules like complement receptor 1 (CR1/ CD35) [7] and blood groups antigens [8]. The human C3b/C4b receptor (CR1) is present on the surface of erythrocytes, polymorphonuclear leukocytes, monocytes/macrophages, B lymphocytes, some T lymphocytes, follicular dendritic cells, and glomerular podocytes [9,10].

The major functions of CR1 as the receptor are: adherence to and removal of C3b- and C4b-containing immune complexes from the circulation, and regulation of the complement cascade to prevent autologous complement attack [11]. Several polymorphic traits of CR1 have been reported which result in variations in the number of C3b binding sites [12]. The gene encoding CR1 is located at long arm of chromosome 1 in humans. Studies showed that CR1 gene polymorphisms influence CR1 production or expression and are associated with malaria in several populations [13-15]. Clinical studies have showed a correlation between serum CR1 level and disease severity in patients with malaria [16-17]; few results were inconsistent [18-19]. National Center for Biotechnology Information SNP database showed that CR1 gene rs2274567 G/A, rs4844600 G/A, and rs2296160 C/T sites (all are located in coding region) have different frequencies of polymorphism distribution in different ethnic group. However, there have been very few studies to examine the association between SNPs of CR1 gene rs2274567 G/A with malaria in Indian population [20-22].

Several antigens have been described in the Knops blood group system which is a part of CR1 and this includes Knops antigens a/b (Kna/Knb), McCoy a/b (McCa/McCb), Swain-Langley 1/2 (Sl1/Sl2). The genetic polymorphisms that results in differential expression of these antigens are

related to the nucleotide change in the coding regions. The details of these polymorphisms and the associated changes are listed in the table [1].

Several groups have investigated the association between polymorphisms in the Knops blood group system and malaria. Moulds et al. in 2010 [23] described the molecular basis for McC and Sl allele. In the study, they determined the frequencies of these alleles in West Africa (pooled samples n= 182) and Malian Population (n=99). The allele frequencies were found to be 31% (McC^b) and 79% (Sl2) in West African samples and 30% (McC^b) and 76% (Sl2) from Mali. Later several groups have studied the association of Knops blood group and severe malaria, with inconsistent results [24-26].

The aim of the present study was to look for any possible association of various genetic markers with malaria infection. In the present publication we are presenting the data on antigens of Knops blood group system and CR1 Exon22 polymorphisms. The data obtained helped to calculate the incidence of various antigens of Knops blood group system and CR1 Exon22 polymorphisms in rural population from Chiplun Taluka of Ratnagiri district, Maharashtra.

II. MATERIALS AND METHOD

A. Ethics Statements:

The study and its protocols were approved by the Institutional Ethics Committee of National Institute of ImmunoHematology(NIIH-ICMR). Blood samples were collected after written consent of the healthy controls, and malaria patients.

B. Study Site and Sample Collection:

The study was conducted at the Primary Health Centre (PHC) at Dadar, Ratnagiri, Maharashtra. Ratnagiri is one of the district of Konkan region from south west Maharashtra. Chiplun is one of the taluka of this district. North east part of this taluka is a hilly malaria endemic area. The primary Health Centre (PHC) at Dadar covers this area. About 15-20 villages are under the PHC (Figure1). Katkaris, Dhangars tribal groups and Kunbis are the three main ethnic group seen in this region.

Katkaris are nomadic, forest dwelling tribals, scattered in small groups staying in an area known as “padas”. They are seen throughout the hill regions and forest of Ratnagiri, Raigad and Thane districts of Maharashtra. Dhangars, nomadic tribal groups, are traditionally being shepherds and buffalo keepers and live a socially isolated life, due to their occupation in forest or hills far away from the main village. Kunbis are cultivators and main rural population in the region.

During 2009-2011, more than 350 cases of malaria, of which 70-85% were caused due to *P vivax* were detected at Dadar PHC. Therefore in the present study, samples were collected from retrospective malaria positive cases and from normal healthy individuals, to screen fro various genetic markers in malaria endemic area.

C. DNA Isolation and Genotyping:

DNA was extracted from the lymphocyte by Phenol: $CHCl_3$ method. The lymphocytes were separated by preferential lysis of RBC and separation of lymphocytes by centrifugation. The extracted DNA was checked for its quality and quantity prior to PCR.

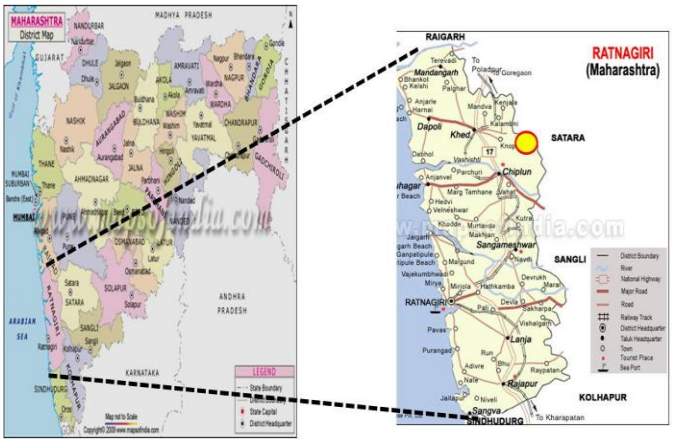

Figure1: Sample Collection Area in Chiplun

D. Polymerase chain reaction:

All amplification reactions were carried out in a final volume of 25µl, which included 0.3µM of each primer (forward and reverse), dNTP at 200µM, 2.5mM $MgCl_2$ and 1 Unit of DNA polymerase (Bangalore Genei) with 1X Taq DNA polymerase buffer. Approximately 100ng DNA was used in the PCR reaction. Amplification conditions included an initial denaturation at 95°C (5 min) and 34 cycles of denaturation at 95°C (45 sec), annealing of primers to the templates at specified temperature (45 sec) and extension at 72°C for 45 sec. A final extension step lasted for 5 min at 72°C followed by 4°C for 5 mins. 5µl of amplified PCR products from each amplification were run on a 1% agarose gel to check quality of amplification.

For CR1 Exon22 amplification, touch-down PCR (TD: 62-55 with -0.5°C for 13 cycles and 25 cycles as conventional PCR with annealing at 55°C) was employed to eliminate the non specific amplification and to increase the product yield.

Genetic variant of CR1 (A3650G RsaI) polymorphisms was determined by using PCR-RFLP technique (Table 1). DNA fragment containing the CR1 A3650G RsaI polymorphic site was amplified using primer sequences listed in the table. PCR

product of 682 bp also contains one non-polymorphic site for RsaI which cuts it into 520 bp and 162 bp fragments. In the presence of CR1 A3650G RsaI variant allele (G) 520 bp band was further cleaved into 458 and 62 bp, while the alternate allele (A) remained uncut.

Polymorphisms in Knops blood groups system were also assessed using PCR-RFLP. The polymorphism and the restriction enzyme utilized to detect the polymorphisms are listed in table2.

Table1: CR1 and Knops Blood Group Antigen Polymorphisms

Antigen	rs No.	Phenotype	Nucleotide Position	Amino Acid Change
Knops (27)	41274768	Kn^a	4708	V1561M
		Kn^b		
McCoy (28)	17047660	McC^a	4795	K1590E
		McC^b		
Swain-Langley (28,29)	17047661	Sl_1	4828	R1601G
		Sl_2		
CR1 Exon 22 (30,31)	2274567		3650	H1208R

Table2: PCR specifications for Amplification of Target region

Antigen	Primer Sequence (5'→3')	Ta	bp	Restriction Enzymes
Knops	FP:ACCAGTGCC ACACTGGACCA GATGGAGAACA GCTGTT TGAGCAT	57.3°C	305	NdeI
McCoy	RP: GGAGGAGTGTG GCAGCTTG			BsmI
Swain-Langley				MfeI
CR1 Exon 22	FP: TTCACATTGGAT AGCCAGAGC	TD (62-55)	682	RsaI
	RP: CCAGAGGTTAA TCTCCCTGGA			

III. RESULTS AND DISCUSSION

We in this study analysed the genetic diversity of complement regulatory gene by examining the SNPs associated with Knops blood group antigens viz., Knops (a/b); McCoy (a/b) and Swain-Langley (1/2) along with polymorphism in CR Exon22 in the above mentioned population group.

Examination of the of the Malaria cases and Normal Healthy control yielded only wild type haplotype involving Knops, McCoy and Swain-Langley polymorphisms. A study (n=200) conducted by Monika et al. 2009 [32] showed that only wild type and homozygous mutant type allele is predominant in Indian population. Here in our study (n=414) we found only the wild type form of the Kn^{a+}, $McCoy^{a+}$ and Sl1/1 alleles resulting in 100% of only one type allele of the antigen (Table-3).

CR1 Exon22 polymorphism was observed in the given subjects. However, the distribution of wild type (AA homozygous); heterozygous (A/G) and the mutant type (G/G) alleles of CR1 exon22 between malaria cases and their counter normals showed no difference in genotypic (Table4) or allelic (Table5) frequencies.

Table3: Genotypic Frequency for Knops Blood Group Antigen

Genotypic Frequency					
SNP	Genotype	Cases n=93(%)	Control n=321 (%)	Odds ratio (95% CI)	P-value
Kna/b	a/a	93 (100%)	321 (100%)	NS	NS
	a/b	0 (0%)	0 (0%)	NS	NS
	b/b	0 (0%)	0 (0%)	NS	NS
McCa/b	a/a	93 (100%)	321 (100%)	NS	NS
	a/b	0 (0%)	0 (0%)	NS	NS
	b/b	0 (0%)	0 (0%)	NS	NS
Sl1/Sl2	1/1	93 (100%)	321 (100%)	NS	NS
	½	0 (0%)	0 (0%)	NS	NS
	2/2	0 (0%)	0 (0%)	NS	NS

To assess the prevalence of CR1 polymorphism in exon 22 in studied population, 909 healthy controls were genotyped by PCR-RFLP and the results are shown in Table-4. For exon22 polymorphism, frequency of GG genotype was found to be 44% than AG which accounted for 41, while AA was seen only in 15% of the normal population. A study by Aditya el al [32] have demonstrated similar results (GG 44% AG 41% and AA15%). The distribution of exon22 genotypes in healthy controls was in agreement with HWE.

We used a case control study to examine whether polymorphism at CR1 gene is associated with malaria. The functional CR1 polymorphisms (exon22) in infected subjects were typed and results are shown in Table4. For the CR1 exon22 polymorphism in malaria cases, the frequency of the GG genotype was found to be 40%, AG 42%. Prevalence of homozygous wild type (AA) was lower (AA 18%).

Table4: Genotypic Frequency for CR1 Exon22

Genotypic Frequency					
SNP	Genotype	Cases n=112 (%)	Control n=909 (%)	Odds ratio (95% CI)	P-value
CR Exon 22	A/A	45 (40.17)	352 (38.72)	1.0628 (0.712-1.5865)	0.7657
	A/G	52 (46.42)	415 (45.65)	1.0316 (0.696-1.5292)	0.8767
	G/G	15 (13.39)	142 (15.62)	0.8353 (0.4712-1.4807)	0.5377

Table4: Allelic Frequency for CR1 Exon22

Allelic Frequency					
SNP	Allele	Cases n=224(%)	Control n=1818 (%)	Odds ratio (95% CI)	P-value
CR22	A	142 (63.39)	1119 (61.55)	1.0817 (0.8112-1.4425)	0.5926
	G	82 (36.6)	699 (38.44)	0.9244 (0.6932-1.2327)	0.5926

IV. CONCLUSIONS

The results from this study confirm earlier findings by Monika et al. 2009 [33]. Kna, McCa, and Sl1, being the only genotype found in the Indian population. Also Kn, McC and Sl do not seem to confer any protective advantage against malaria infection. Based on these findings, in a very well-characterized population, malaria does not seem to be the selective force on these alleles. Moulds et al [23] reported frequency of Knb in Bandiagara be 18.3%t. Other serologic studies have reported that the frequency of Knb ranged from 1.2% among African American persons living in Philadelphia [34] to 4.7% among 63 random Caucasian donors [35]. Helle et al 2013 [36] compared the allele frequencies of McC and Sl. In the comparison, it was found that the Ghanaian population differed significantly from the Gambian at the McC loci, having a lower frequency of the McCb allele ($p < 0.001$, 0.27 vs 0.39). The Sl loci in the Ghana differed from both Mali ($p = 0.02$, 0.83 vs 0.76) and Kenya ($p < 0.001$, 0.83 vs 0.68) by a higher frequency of the Sl2 allele and slightly differed from The Gambia ($p = 0.06$, 0.83 vs 0.80).

The present study did not revealed any association between CR1 gene rs2274567 G/A polymorphisms and malaria in the selected tribal population. Nevertheless, further studies are needed to explore the complex interaction between environmental factors and CR1 gene polymorphisms in the risk of malaria, especially in ethnically disparate populations.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Aarti P. and Shivam S. helped in sample collection and organization of the blood collection camps in Chiplun Taluka, Ratnagiri, Kanchan M and Namrata M, carried out the laboratory work, Roshan S. analysed the data, supervised DNA work and wrote the manuscript. Ajit C. G. contributed to study design and interpretation of data, and revised the manuscript for important intellectual content. Kanjaksha Ghosh contributed to concept development and study design. All authors read and approved the final manuscript.

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