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Prevalence of Sickle Cell Anemia in Association of *Plasmodium falciparum* from Rajnandgaon, India

Tripathi S[1], Mishra N[2] and Kumar A[2]*

[1]Department of Science and Technology, Women Scientist-A, India

[2]Department of Biotechnology, Government VYT PG Autonomous College, India

***Corresponding author:** Anil Kumar, Department of Biotechnology, Government VYT PG Autonomous College, Durg-491001, Chhattisgarh, India

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Abstract

Sickle cell anemia is a blood disorder resulting from the inheritance of abnormal genes from parents. It is caused due to mutation in the β-globulin gene. Sickle cell anemia is widespread across the world and in Central India. The present study was undertaken to study the prevalence of the disorder in the Rajnandgaon district of Central India. A random sampling of 6088 people was done to test the sickle cell anemia problem by slide test method and a total of 249 (4.09%) people were found sickled positive. Further electrophoresis test was performed for all 249 of which 67 were found homozygous (HbSS) and 182 were found heterozygous (HbAS) positive.

Besides the above analysis, chloroquine prophylaxis associated with a high prevalence of *Plasmodium falciparum Pfcrt* K76T mutation in people (n=26) with sickle cell anemia was also analyzed. The genotype of the subject was screened using the hemoglobin electrophoresis system and the *P. falciparum Pfcrt* genotyping was carried out using PCR-Restriction Fragment Length Polymorphism (RFLP). The prevalence rate of *Pfcrt* K76T mutant gene was proportionately found higher in the hemoglobin SS (n=40, m=32, r=0.67) genotype individuals than the hemoglobin AS (n=52, m= 27, r=0.519) and AA (n=182, m= 68, r= 0.37).

Keywords: Sickle cell anemia; *P. falciparum*; Chloroquine; *Pfcrt* K76T, PCR-RFLP

Introduction

Sickle Cell Hemoglobin (HbS) is the first molecular disease known to man [1]. It is a structural variant of hemoglobin in which glutamic acid (an amino acid), at position no.6 of a β-globin chain of hemoglobin is replaced by valine. This happens due to the change of nucleotide, adenine to thymine (GAG/GTG) of codon 6 of a β-globin gene, located on the short arm of chromosome 11. This substitution of amino acid changes the net charge of hemoglobin, oxygen affinity, and three-dimensional structure of hemoglobin, thus rendering it unstable hemoglobin. Sickle hemoglobin gets polymerized at low oxygen tension and deforms the red cell from discoid shape to sickle-like form causing a lot of pathogenicities [2]. Due to its genetic nature of origin, the disease is inheritable following the Mendelian Principle. In malaria-infested areas, the high frequency of hemoglobinopathies, such as Sickle-Cell Disease (SCD), exhibits their protective role against *P. falciparum* malaria [3]. However, in homozygous sickle hemoglobin (SS) cases, the tenacity of *P. falciparum* could set off acute hemolytic events [4] and/or recurrence of Vaso-Occlusive Crises (VOCs).

The exact mechanism by which sickle-cell trait (AS) condition imparts resistance to malaria is not known. Various factors are likely to be involved that contribute to varying degrees of defense against malaria [5]. Erythrocytes of sickle cell trait, infected with the *P. falciparum* parasite are deformed since the parasite drastically decreases the oxygen tension within the red cells as it carries out its metabolism also. This deformation of sickle trait (AS) erythrocytes makes these cells abnormal and obvious targets for phagocytosis [6].

Plasmodium genus is approximated to have evolved around 150 million years ago, even before the evolution of *Homo sapiens* [7]. So, humans have evolved in the presence of malaria, and this co-evolution has shaped the human genome [8]. Various polymorphisms are protective against severe forms of malaria. The worldwide distributions of Hemoglobinopathies like sickle cell anemia, thalassemias, Glucose-6-phosphate dehydrogenase (G6PD) deficiency, and blood group polymorphisms suggests the prevalence of malaria, indicating that malaria has been a selective force for such mutations [9][7]. Also, genetic variations in human P450 genes (CYP2C8, CYP3A4, and CYP2A6) results in differential metabolism of anti-malarial drugs in humans and have important imputations in both anti-malarial drug efficacy and bearability [10,11].

Chloroquine is a 4-amino-quinoline anti-malarial drug that interferes with the sequestration of toxic heme, which is produced when hemoglobin is digested by an intra-blood parasite to obtain essential amino acids. The parasite crystallizes heme into hemozoin in its acidic digestive vacuoles.

Chloroquine (and other similar drugs) binds to heme and prevents the detoxification process [12]. Parasite resistance is believed to be achieved by the decreased accumulation of chloroquine in the digestive vacuole of the parasite.

In 2000, the *P. falciparum chloroquine resistance transporter* (*pfcrt*), the key gene involved in this resistance, was discovered [13]. CQ resistance is associated with a T76 mutation of the *P. falciparum* chloroquine resistance transporter gene (*Pfcrt*) [13] while a multidrug resistance analog (*Pfmdr1*) Y86 variation may modulate its degree

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[14]. The gene is present on chromosome 7 and encodes a drug and metabolite transporter protein (*PfCRT*) located on the membrane of the digestive vacuole. A unique mutation *pfcrt* K76T, was found in connection with other compensatory amino acid residue changes at loci *PfCRT* 72-76, where *PfCRT* 72-76 CVMNK is the sensitive haplotype and CVIET and SVMNT are considered the most common resistant haplotypes [15].

Chloroquine resistance depends upon the genetic background of the parasite line i.e., *pfcrt* K76T enhances the tolerance to chloroquine so that resurgence is likely to occur, but it does not always result in clinical dereliction [12].

According to a hypothesis regarding the resistance is the protonation of chloroquine in the acidic environment of the digestive vacuole. Efflux of the chloroquine (CQ^{2+}) is reduced by the charged lysine (*PfCRT* K76) amino acid residue in the Chloroquine sensitive strain. When lysine is replaced by neutral amino acid residue threonine (*PfCRT* 76T) then CQ^{2+} can egresses down its concentration gradient through *PfCRT*, eliminating the drug from its target site. Another drug, Verapamil, which is a calcium (Ca^{2+}) channel blocker, is can reverse this resistance by competing with chloroquine for binding at *PfCRT*, thereby blocking the efflux of the drug from the digestive vacuole.

For a very long time, it is supposed that the malarial parasite has tempted RBC for various mutations for protection. Probably sickling is one of them. A lot of Epidemiological and biochemical evidence is available related to the concept. Chhattisgarh state (India) is one of the major centers of malaria epidemics, especially for *Plasmodium falciparum*. We have noticed the development of malaria among the sickled population against the previous concept.

So banking on the prevalence of both malaria epidemics and sickling in the society of Chhattisgarh state (India), we have undertaken the present study to detect mutation in *Plasmodium falciparum* in an association of Heterozygous Sickling (HbAS) and Homozygous Sickling (HbSS) with a correlation of normal hemoglobin.

Material and Method

For the study, seven villages of Rajnandgaon District of Chhattisgarh state (India) within a radius of 60-70kms from district headquarters were selected. Two milliliters of intravenous blood was collected from each person by paramedical staff following ICMR and Institutional Ethics Committee norms. From the first village total, 6088 samples were collected from all seven villages (Table 1). During sample collection, it was taken into note that some of the donors were suffering from malaria and had taken chloroquine as medicine.

After sample collection primary screening was done by the slide test method, followed by electrophoresis in Starch-Agarose Gel electrophoresis for confirmation and hemoglobin type was determined by comparing with the standard. Blood from those persons who were suffering from sickling with malaria and malaria without sickling was blotted on a piece of blotting paper. DNA of *Plasmodium falciparum* was extracted from dried blood spots following the modified Saponin Chelex method. Genotyping of the resistance marker gene *Pfcrt* K76T was carried out by PCR - RFLP. Two primers viz. 5'AATTAAAgTTgAgTTTCggA3' and 5'TgTgCTCATgTgTTTAAACTT3' (Bgenei) for amplification of *Pfcrt* sequences were used, following amplification of the *Pfcrt* sequence restriction digestion of the product was carried out by restriction endonuclease Apo I (Bgenei). The product was observed in 2% agarose gel on electrophoresis, stained with EtBr, and examined in Gel Documentation System.

Result

The study showed that out of a total of 6088 samples collected, 249 were found sickle positive (4.09%). From the first village out of 982, 52 were found sickle positive (18-SS and 34-AS); from the second village out of 767, 19 were found sickle positive (6-SS and 13-AS); from the third village out of 1012, 58 were found sickle positive (11-SS and 47-AS); from the fourth village out of 894, 43 were found sickle positive (9-SS and 34-AS); from the fifth village, out of 759, 24 were found sickle positive (6-SS and 18-AS); from the sixth village, out of 852, 33 were found sickle positive (12-SS and 21-AS) and

Table 1: Prevalence of Sickle cell Anemia with a homozygous and heterozygous variation.

S.No.	Village	Total Sample	Sickle Positive after Slide Test	Percentage (%)	Electrophoresis Test	
					Homozygous (SS)	Heterozygous (AS)
1.	Village First	982	52	5.29%	18	34
2.	Village Second	767	19	2.47%	6	13
3.	Village Third	1012	58	5.73%	11	47
4.	Village Fourth	894	43	4.80%	9	34
5.	Village Fifth	759	24	3.16%	6	18
6	Village Sixth	852	33	3.87%	12	21
7.	Village Seventh	822	20	2.43%	5	15
Total		6088	249	**4.09%**	67 (26.90%)	182 (73.09%)

Table 2: Prevalence of *Pfcrt* K76T mutation in *P. falciparum* among Hb AA, HbSS and HbAS.

S. No.	Total Malaria patients & (%)	Patients with normal genotype (AA)		Patients with homozygous genotype (SS)		Patients with heterozygous genotype (AS)	
		No. of Patients	Patients with Mutant (76T)	No. of Patients	Patients with Mutant (76T)	No. of Patients	Patients Mutant (76T)
1	274 (4.38%)	182	68	40	27	52	21

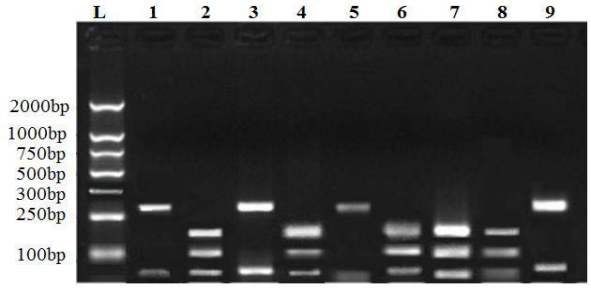

Figure 1: Agarose gel depicting the K76 and 76T after RFLP digestion with Apo I: Lane 1 is DNA Molecular Weight Ladder (100bp), Lane 2,4,6,7 & 8 showing the K76 (Wild type) bands (with 137,124 and 10bp bands); Lanes 1, 3, 5 & 9 showing the 76T (mutant) bands (with 260bp and 10bp bands).

from the seventh village, out of 822 samples collected, 20 were found sickle positive (5-SS and 15-AS). Out of a total of 6088 samples, 274 (4.38%) persons were found infected with malaria parasites (Table 2). Following amplification of the *Pfcrt* fragments of codon 76 and restriction digestion of the amplified products with *Apo I*, we found the prevalence rate of mutant genes and the prevalence rate of *Pfcrt* K76T mutant gene was reported higher in hemoglobin SS (n=40, m=32, r=0.67) genotype individuals than the hemoglobin AS (n=52, m=27, r=0.519) and normal hemoglobin AA (n=182, m=68, r=0.37) subjects (Table 2, Figure1). This indicates a kind of resistance in term of the proliferation of parasites occurred in the hemoglobin AS and SS individuals. It was found that there is a significant difference ($p<0.05$) in the prevalence rate of mutant *Pfcrt* genes between hemoglobin SS, AA, and AS.

Discussion

Our finding indicates that maximum resistance in terms of proliferation of parasites occurred in hemoglobin SS, but moderate resistance was also reported from hemoglobin AS. However according to an author, genes that select against "severe" falciparum malaria include hemoglobin S gene, Thalassemia gene, Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency gene, Ovulocytosis, and Duffy gene, and protection is afforded only to heterozygous individuals (hemoglobin AS) in severe malaria [16]. Several factors have been earmarked to contribute to the emergence of these resistant genes notably environmental factors, host factors, and drug pressure. This high prevalence in the hemoglobin SS genotyped individuals could be as a result of uncontrolled usage of chloroquine as prophylaxis. There is also a report that chloroquine prophylaxis is responsible for increased drug consumption and increased drug pressure that may lead to the selection of drug-resistant parasites [17].

Shrivastava et al., in 2014 analyzed the correlation of *Pfcrt*-K76T and Pfmdr1-N86Y mutations with Chloroquine (CQ) resistance in Northeast Indian *P. falciparum* isolates. They reported that out of 115 *P. falciparum* isolates, 100 isolates were found to resistant to CQ by the *in vitro* susceptibility test whereas 15 were found CQ sensitive. They further reported that all the CQ resistant isolates exhibited the presence of Pfmdr1 and *Pfcrt* mutations. On the other hand, CQ sensitive isolates didn't exhibit such mutations. They also observed strong linkage disequilibrium between the alleles at *Pfmdr1*-N86Y and *Pfcrt*-K76T loci. Their results indicated that *Pfmdr1*-N86Y and *Pfcrt*-K76T mutations can be used as molecular markers for the identification of CQ resistance in *P. falciparum* and recommended the importance of evaluation of CQ in vivo therapeutic efficacy in endemic areas for more effective malaria control strategies [18].

Another study also reported hemoglobin AS resistance against *Plasmodium falciparum* in comparison to hemoglobin SS [19]. But our findings differ from previous workers we found maximum resistance in hemoglobin SS than AS. Although the mechanism of resistance either in hemoglobin SS and in AS is yet to be worked out, the relevance of the present investigation is that there is an urgent need to control the indiscriminate use of chloroquine and development of substitute therapeutic mechanism to prevent the evolution of resistance gene in *Plasmodium falciparum*.

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