CORRELATION OF HAEMATOLOGICAL PROFILE AND SICKLE CELL DISEASE: A STUDY IN RAIPUR AND DURG DISTRICTS OF CHHATTISGARH

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Abstract:

Sickle cell disease is an inherited structural disorder of hemoglobin and a major public health issue in the state of Chhattisgarh, India. Government of Chhattisgarh passed 'Sickle cell bill' in the Assembly looking to its previous impact on society. The present study was executed on sickle cell disease patients and controls. A cross-sectional study was done for screening of sickle cell hemoglobin with individual informed written consent, 90 subjects of Raipur & Durg districts, belonging to different caste category, ranging in age between 1.5year to 45 year were screened using 2% sodium meta bisulphate solution. Genotype of individuals with sickle cell hemoglobin as done by Allele specific polymerase chain reaction (ASPCR) analysis and normal individuals were treated as control. ERMA total blood cell counter machine (PCE-210) was used for the analysis of various hematological parameters.

The study focused on two aspects. Firstly mutant beta chain of HbSS was distinguished form normal beta chain of HbAA by using technique of PCR followed by gel electrophoresis. Secondly HbSS, HbAS & HbAA individuals correlated with various hematological indices. We found significant statistical difference for hematological indices – DLC, Leucocytes, bleeding time & clotting time between Sickle cell patients (HbSS & HbAS) & controls.

Keywords: Chhattisgarh, Haematological profile, Sickle cell disease, Cross-sectional, ASPCR,

INTRODUCTION

Every milliter of human blood contain approximately 5 billion erythrocytes and each erythrocyte is packed with 280 million hemoglobin (Hb) molecules, which is the oxygen carrier of blood4,21,34.Among the inherited disorders of blood, haemoglobinopathy constitute a major bulk of non-communicable by the in India. The haemoglobinopathies are characterized by the molecule. When the biological function is distorted due to a mutation in the Hb, the state of abnormal Hb is sickle cell haemoglobin, in which 6th amino acid, Glutamic acid, is replaced by Valine in Beta (β) globinchain[,2,16,17,19,25]

Sickle cell disease is one of the most widespread heredity diseases occurring global, which may affect any organ or organism of the human body. Sickle cell was first reported in 1910 by Dr. James B. Herrick in Chicago. He found them during a blood examination of a West Indian student who

complained of tiredness and pains in the body. He observed that they have an unsual shape instead of the normal round one i.e. the 'Sickle' shape which later gave the disease it name. [20]

It is an inherited disease and therefore it is not infections inherited by birth i.e. if you have one form sickle cell disease there is a chance that one of your offspring's world inherit the disease. In the Western world this believes has been eradicated but it is still a common believe in other parts of the world. In India, the S gene is prevalent especially in the tribal populations and the prevalence rate varies from 0-40% in different population groups [22, 24, 33, 38]

The disease was named "Sickle cell anaemia" by Vernon Mason in 1922.

Reason that sickle cell disease was allied with an alternation of haemoglobin was published in India by Linus Pauling and Croorkers. This was the first time a genetic disease was linked to a mutation of specific protein, a landmark in the past of molecular biology. The origin of the mutation that led to the Sickle cell gene was initially thought to be in the Arabian Peninsula, spreading to Asia and Africa. It is now know, from assessment of chromosome structures, that there have been at least four independent mutational events, three in Africa and fourth in either Saudi Arabia or Central India. These independent events occurred between 3,000 and 6,000 generations ago, approximately 70-150,000 years B.P. [7, 16, 24, 25, 26, 30, 35, 37]

India caters to almost 20 million people with sickle cell disease. The sickle cell gene in India was first described among tribal groups in South India, which spread the wrong memorandum that the disease is confined to tribal community. However the topical data unfolds that the disease is not classified only to tribal belt but is widely prevalent and has penetrated unlike caste and communities in our country. It is extensively identified mainly in the central parts of India. The highest incidence of sickle cell gene in India is reported in Orissa followed by Assam, MP, UP, Tamilnadu and Gujrat. The typical frequency of Sickle cell disease in India is 4.3% and that of Orissa is as high as 9.1% children comprised 52% of sickle cell patients and the three predominant forms of the disease (SS, SB and SD) are clinically and haematologically indistinguishable [5,22,33]

Sickle cell anaemia is an autosomal recessive disease caused by a point mutation in the hemoblobin beta gene (HBB) found on chromosome 11p15.5. In Sickle cell anaemia, inheritance of the sickle cell trait involves two co-dominant beta-globin alleles, the normal allele and the sickle-cell S allele. Individuals which are AS heterozygous produce both normal and abnormal and are frequently heating but may have some symptoms of sickle cell anaemia at high attitudes when blood oxygen is low and may pass the S allele to their offspring, carriers, heterozygous for sickle cell anaemia possessed only one recessive allele and were not horizontal to suffering throught " anaemia, joint pain, swelling of the spleen and frequent, severe infection" like their homozygous counterparts [11,16,25] Additionally, these individuals tended to live extended lives, as they were resistant to a prominent strain of malaria, responsible for the majority of deaths in Africa during the 1940s. When infected by a malaria causing parasite (transferred from the salivary glands of an affected mosquito) red blood cells that restricted some abnormal hemoglobin would sickle and flow through to the irritation. The irregular shaped cells were then eradicated from the blood stream, preventing the parasite within them from affecting their host, homozygous (SS) offspring manifesting SCA (Nagel and Steinberg, 2001).

If a person inborn only one copy of the sickle cell gene (from one parent), then he will not have sickle cell anaemia. Instead, he will have sickle cell trait. People who have sickle cell trait usually have no symptoms and lead normal lives. However, they can pass the sickle cell gene to their children.

This study conducted correlation of hematological profile and sickle cell disease among the different categories of Durg and Raipur district people using sample through ASPCR and gel electrophoresis techniques.

SAMPLES

The Blood sample used for this analysis consists of 90 subject were screened for Hbs out of which in

Category	HbSS	HbAS	HbAA
General Category	0	1	3
OBC Category	7	21	30
SC Category	1	8	9
ST Category	0	7	3
Total	HbSS-8	HbAS-37	HbAA-45

The biological Blood Sample collected in the EDTAfrom each subject and used for DNA extraction. Blood samples were transported in ice pack to Human Genomic Lab, School of Studies in Anthropology Department, Pt.Ravishankar Shukla University, Raipur for further analysis.

The biological Blood sample collected from Raipur & Durg district of Chhattisgarh and their diagnosis hospital & clinic of Raipur & Durg district of Chhattisgarh. The entire subject ranged was age 1.5-45 yrs.

The present study includes only data of unrelated individuals belongs to different ethnic groups. Data on age, gender, general food habit & medical history were collected from each subject.

METHODS

Total number of Biological samples 90 which in 45 samples were taken from patients and 45 samples were taken from normal person.

Method of collection of Biological samples were collected in the EDTA used for DNA extraction samples were transported in ice pack to Human Genomic Lab, School of Studies in Anthropology Department, Pt.Ravishankar Shukla University, Raipur for further analysis. Total blood cell machine ERMA INC.PCE.210 at Human genomic lab was used for heamatological analyisis blood samples which in blood counts, hemoglobin, total Leukocytes count (TLC), Neutrocytes, Lymphocytes, Monocytes, Eosinocytes, Basocytes, Erythrocytes sedimentation rate (ESR), Bleeding time, Clotting time.

Isolation of Genomic DNA from human blood was isolated from following procedure Miller et.al. Genomic DNA from blood samples were extracted & used for further analysis. Genotype of individuals for sickle cell disease was done by Allele-specific polymerase chain reaction (ASPCR) analysis (Ugozzoli and Wallace, 1991). The PCR reaction mix, PCR cycling protocols & electrophoresis protocols used were as under.

PCR (Polymerase chain reaction), A, PCR is a technique to amplify a single or few copies of a piece of a DNA across several orders of magnitude, generating millions or more copies of a particular DNA sequence. PCR were used to amplify specific regions of a DNA strand (the DNA traged) from the blood samples by DNA isolation method. Generally contains 4 stages used in PCR initialization, denaturation, annealing, and elongation/extension.

Gel Electrophoresis was also used in the early days of DNA manipulation DNA fragments were laboriously separated by gravity. 10µl of PCR product + 2ml of loading dye were electrophoresed in 2% agarose gel stained with Ethidium Bromide solution (1mg/ml) and visualized under UV-transilluminator in Gel Electrophoresis.

The data were analyzed using the statistical package for MS-Exel. The simple statistical techniques such as Mean, SD and SE had been used to substantiate the discussion on the basis of the finding of the present study.

Mean - $0 = A + 3fd'/N * i$

SD - $\sqrt{3fd'/N^2 - (3fd'/N)^2} * i$

SE of mean χ - $\sigma/\sqrt{N}$

SE of SD (σ) - $\sigma/\sqrt{2N}$

Descriptive statistics were calculated and student t-test for equal variances was applied to assess the difference between the means of the hamatological parameters HbSS x HbAS, HbAS x HbAA, HbSS x HbAA, Hb g% , TLC, DLC, ESR, BT , CT

Formula of 't' test = $\frac{M_1 - M_2}{\sqrt{(SE_1)^2 + (SE_2)^2}}$

The PCR in protocol for Sickle Cell Anaemia where reaction mixture-

Total volume 10µl, 100ng, 200nm dntps, 1.5mm $MgCl_2$, 25ng, each primer, 1.25 U Taq polymerase, 50mM KCl, 10Mm Tris-HCl (Ph 8-4) of the extracted DNA.

30 cycle of amplification were carried out, with each cycle consisting of (denaturation) at 94°C for 4 min after than next cycle X 30: denaturation at 94°C for 30 sec., anneling at 56°C for 30 sec., and extension at 72°C for 2min after than extension at 72°C for 3min.

Electrophoresis condition were 10µl of PCR product + 2ml of loading with Ethidium Bromide solution (1mg/ml) and contain HB-14A, HB-14S and BGP2 Markers for 7 samples with mixture of 10µl volume where H_2O – 5-9µl x 8 = 47.2, 10 x Buffer (with Mg_2+) – 1.0µl x 8 = 8.0. dNTPS – 0.8µl x 8 = 6.4, BGP2 – 0.5 µl x 7 = 3.5 (0.5µl NatF), A/S – 0.5µl x 7 = 3.5 (0.5µl NatR), Taq – 0.25µl x 8 = 2.0, DNA – 1µl.

RESULTS

This method for DNA based Sickle Cell anaemia utilizes fixed differences between the AS, SS and AA.

Table. 1: The distribution of Sex in HdSS, HbAS (patients) & HbAA (controls)

SEX	**PATIENTS**				**CONTROLS**	
	HbSS		HbAS		HbAA	
	No.	%	No.	%	No.	%
MALES	3	35.5	17	45.94	20	44.44
FEMALES	5	62.5	20	54.06	25	55.56
TOTAL	8	100	37	100	45	100

In table 1 result of the HbSS & HbAA in male in female. A total 90 samples were collected including 45 patients & 45 controls. Out of the 45 patients 8 samples were HbSS & 37 samples were HbAS genotype. Among the 45 patients, 25 samples were of women and the rest 20 samples were collected from males. The same no. of samples belonging to each gender were collected as controls samples.

Table. 2: The distribution of Caste in HdSS, HbAS (patients) & HbAA (controls)

CATEGORY	PATIENTS				CONTROLS	
	HbSS		HbAS		HbAA	
	No.	%	No.	%	No.	%
GENERAL	0	0	1	2.7	3	6.67
OBC	7	87.5	21	56.75	30	66.66
SC	1	12.5	8	21.62	9	20
ST	0	0	7	18.91	3	6.67
TOTAL	8	100	37	100	45	100

In table 2 results of HbSS & HbAA in different caste category group. Among the 45 patients samples 87.5% belong to OBC category, 12.5% belong to SC category while in HbAS patients 56.75% belong to OBC category, 21.62% belong to SC category, 18.91% belong to ST category and 2.7% belong to General category.

Table.3: The distribution of Hb value in **females** of HbSS, HbAS (Patients) & HbAA (Controls) genotypes

RANGE	PATIENTS				CONTROLS	
	HbSS		HbAS		HbAA	
	No.	%	No.	%	No.	%
Mild 10.0-11.9	4	80	19	95	3	12
Moderate 7.0-10.9	1	20	0	0	0	0
Severe 4.0-6.9	0	0	0	0	0	0
Very Severe <5.0	0	0	0	0	0	0
Normal >12	0	0	0	5	22	88
Total	5	100	20	100	25	100

When we compared the Hb level of HbSS, HbAS & HbAA Females it was observed that 88% Controls had normal Hb. Only 12% Controls had mild anaemia. 100% HbSS Patients had mild (80%) to moderate (20%) type of anaemia. 5% HbAS Patients had normal Hb where 95% had mild.

Table.4: The distribution of Hb value in **males** of HbSS, HbAS (Patients) & HbAA (Controls) genotypes

Range	**PATIENTS**				**CONTROLS**	
	HbSS		HbAS		HbAA	
	No.	%	No.	%	No.	%
Mild 11.0-13.0	2	66.66	13	76.48	1	5
Moderate 8.0-10.9	1	33.34	2	11.76	0	0
Severe 5.0-8.0	0	0	0	0	0	0
Very Severe <5.0	0	0	0	0	0	0
Normal >13	0	0	2	11.76	19	95
Total	3	100	17	100	20	100

When we compare the Hb level of HbSS, HbAS & HbAA Males it was observed that 95% Controls had normal Hb. Only 5% Controls had mild anaemia.100% HbSS Patients had mild (66.66%) to moderate (33.34%) type of anaemia. 2% HbAS Patients had normal Hb where 95% had mild and 11.76% had moderate

Table. 5: Hematological parameter among Sickle Cell Disease (SS) individuals

S. NO.	**Hematological Parameter**	**N**	**Mean±SE**	**S.D.**
1.	Hb g%	8	10.83±0.288	0.8158
2.	TLC	8	6137.5±265.207	750.11
3.	DLC	8		
	a. N	8	66.125±2.715	7.67
	b. L	8	26.25±0.958	2.712
	c. M	8	5.875±1.757	4.969
	d. E	8	2.375±0.2630	0.744
	e. B	8	0.375±0.182	0.517
4.	ESR	8	11.125±1.076	3.044
5.	BT	8	2.025±0.156	0.443
6.	CT	8	3.262±0.046	0.130

Table: 6: Hematological parameter among Sickle Cell Trait (AS) individuals

S. NO.	Hematological Parameter	N	Mean±SE	S.D.
1.	Hb g%	45	13.162±0.1257	0.8437
2.	TLC	45	5231.111±91.387	613.048
3.	DLC	45		
	a. N	45	64.066±1.8912	5.978
	b. L	45	25.822±0.3307	2.218
	c. M	45	5.222±0.235	1.579
	d. E	45	2.888±0.1083	0.726
	e. B	45	0.666±0.1054	0.707
4.	ESR	45	9.5111±0.386	2.590
5.	BT	45	1.908±0.047	0.316
6.	CT	45	20911±0.067	0.454

Table. 7: Hematological parameter among Control Samples (AA) individuals

SNO.	Hematological Parameter	N	Mean±SE	S.D.
1.	Hb g%	37	13.1622±0.1257	0.8437
2.	TLC	37	5231.111±91.387	613.048
3.	DLC	37		
	a. N	37	64.066±0.8912	5.976
	b. L	37	25.822±0.3307	2.218
	c. M	37	5.222±0.235	1.579
	d. E	37	2.288±0.1083	0.726
	e. B	37	0.666±0.1054	0.707
4.	ESR	37	9.5111±0.386	2.590
5.	BT	37	1.908±0.047	0.316
6.	CT	37	2.917±0.067	0.454

Table: 8't' test in different Hematological Parameters **Significant at 5% level (t - * significant)**

S. NO.	Hematological Parameter	t-value HbSS x HbAS	t-value HbAS x HbAA	t-value HbSS x HbAA
1.	Hb g%	1.5939	8.1645*	7.2416*
2.	TLC	1.9837	9.2070*	3.728*
3.	DLC			
	a. N	0.4274	2.4578*	0.8601
	b. L	2.1771*	4.9629*	0.4867
	c. M	0.6716	0.5421	0.7325
	d. E	1.6008	3.7264*	0.3111
	e. B	0.3248	0.9948	1.1090
4.	ESR	0.7325	0.4353	1.5863
5.	BT	2.3297*	5.9581*	0.9092
6.	CT	2.4665*	7.439*	2.1768*

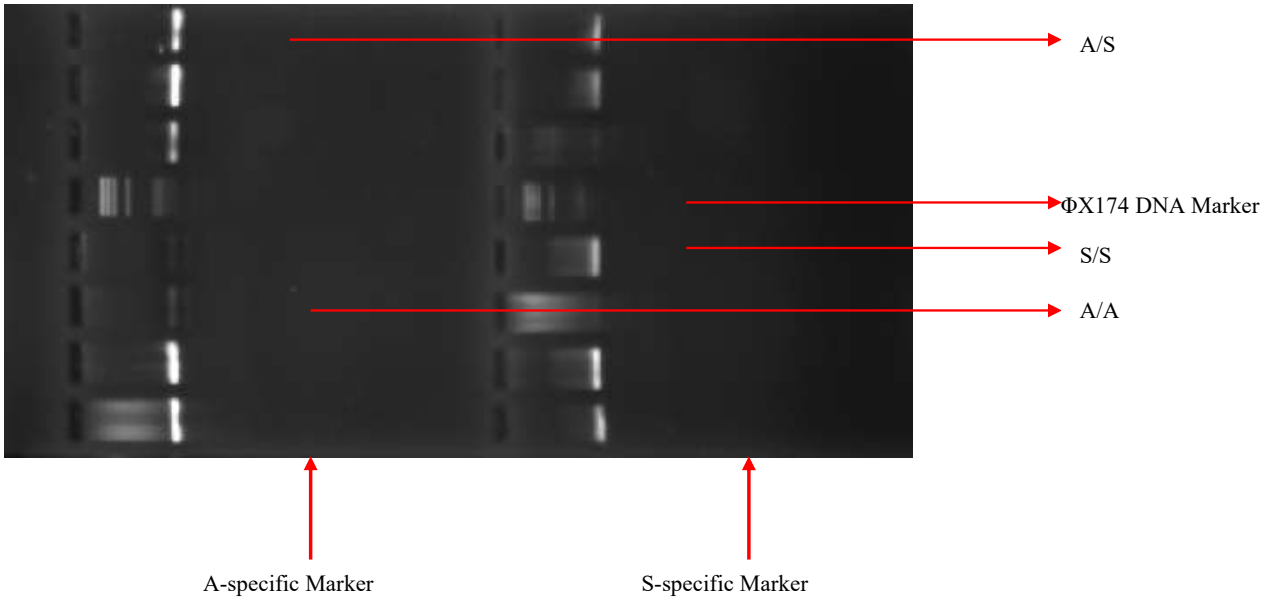
Mean Hb g% show that HbSS patients were severe anamic as compare to HbAS & HbAA subjects. However, Control subjects had mild to moderately anaemic statistical analysis reveals significant difference between Control & HbSS & Control & HbAS patients. HbSS X HbAS patients did not show any significant difference in Hb level. Other haematological profile under study show significant difference between Controls & Sickle cell trail, Control & Sickle cell anemic patients are Total leukocyte count (TLC) & Clotting time (CT). Control Sickle Cell trait (HbAS) subject also show significant difference for DLC, Neutocytes (N), Lymphocypte (L) & Eosinocytes (E) and Bleeding time (BT).

Among the patients (HbAS & HbAS) there is statistical significant difference found in DLC Lymphocytes (L), bleeding time (BT) & Clotting time (CT) hematological profile.

Table.9: Genotype & Phenotype constitution of the individuals screened for HbS

Genotype	Phenotype	No.	Percentage
AA	Normal	45	50%
AS	Trait/Carrier	37	41.11%
SS	Disease/End	8	8.89%
Total		90	100%

The present study, prevalence of sickle cell heamoglobin sample and hematological profile of samples under study on sickle cell anaemia sickle cell trait/carrier and non-sickle individuals were undertaken with a view to asses the genetic predisposition of sickle cell anaemia in relation to band pattern of HB-14A, HB-14S and BGP2 markers.



Photograph showing the band pattern of HB-14A, HB-14S and BGP2 markers on Sickle Cell Anemia samples

DISCUSSION & CONCLUSION

The present study conducted prevalence of sickle cell heamoglobin and hematological profile of samples on sickle cell anaemia, sickle cell trait/carrier and non-sickle individuals were undertaken with a view to asses the genetic predisposition of sickle cell anaemia in relation to band pattern of HB-14A, HB-14S and BGP2 markers.

Blood sample were collected from all the patients with their individual informed consent and DNA were isolated using the method of Miller et.al (1988). PCR was performed on these DNA samples that were sickle cell anaemia to detect the presence or absence of HB-14A, HB-14S and BGP2 markers.

For this method, 30 cycles of amplification were carried out, with each cycle consisting of denaturation at 94°C after than next cycle X 30: Initial denaturation at 94°C for 30 sec., annealing at 56°C for 30 sec., and extension at 72°C for 2min. after them extension at 72°C for 3min.

Blood samples were profiled for the selected haematological parameters using an automated particle counter; standard method was used for statistical analysis.

The data were analyzed using the statistical package for MS-Exel. The simple statistical technique such as mean, SD and SE had been used to substantiate the discussion on the basis of the finding of the present study.

Descriptive statistical were calculated and a student t-test for equal variances was applied to assess the difference between the mean of the haematological parameters. HbSS X HbAS, HbAS X HbAA, HbSS X HbAA

The sickle test was performed by gel method using 2% freshly prepared of Doland & Castle (1948). Further the genotypes were determined by molecular genetic analysis. Genomic DNA was extracted

from whole blood by statistical method (Miller et.al, 1988). Genotypes of individuals (who were Hbs positive) were determined by the allele-specific polymerase reaction (ASPCR) analysis (Ugozzoli & Wallace, 1991).

Gel electrophoresis was also used in the early days of DNA manipulation. DNA fragments were laboriously separated by gravity.

Haemoglobinathies in India are public health problem. Among haemoglobinpathies, sickle cell disease constitutes major genetic problems in India particularly in Chhattisgarh. These cause a high degree of morbidity, moderate of servers haemolytic anaemia among vulnerable segments of society like infants & children, adolescent girls, pregnant women etc. mortality (Balgir, 2000). Chhattisgarh state Government passed sickle cell bill in the assembly looking to its important & impact on society.

Haematological profile with sickle cell anaemia can be valuable for genetic counseling.

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