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ORIGINAL ARTICLE **OPEN ACCESS**

Baseline Survey and Hbb Gene Amplification For Screening Of Sickel Cell Disease among Tribal and Non Tribal Population Of Kumaun Region of Uttarakhand

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ABSTRACT
The aim of the present investigation was to provide an in-depth analysis of the current status of sickle cell disease and strategies to address prevention of this disease as well as to provide some new information on frequencies of alleles among various tribal/ non-tribal population of Kumaun region. Questionnaire based investigation was performed o collect information from doctors and population in general. Total Fifty four (54) Doctors and One thousand three hundred sixty nine (1,369) individuals were contacted to understand their awareness status about Sickle cell disease (SCD). Most of the tribal population where sickle cell disease is common usually rely on the primary health care services of rural and often isolated areas. In the present investigation total 30 individuals from five tribal families (with SCD history) were examined by collecting their blood samples. Genomic DNA was isolated from each sample followed by PCR amplification of β-globin gene using HBB-1 and HBB-2 primers. Gel analysis indicates homozygous patients revealed amplified product sizes (APS) of 231bp and heterozygous revealed 231 and 400bp, while healthy individuals' revealed at 231 bp. The findings of the present study are unique kind of report from Kumaun region of Uttarakhand and it would be very helpful for early identification of SCD. The present study concluded that this procedures used during the investigation would be best for rapid identification and screening of large populations suffering with SCD at a low cost.
Key words: Sickle cell disease; Haematological analysis; Tribal; Non-tribal; Kumaun region

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INTRODUCTION

Prior to the year 1960s, Sickle cell disease (SCD) diagnosis was based on routine hematological studies and clinical manifestations [1]. Sickle cell disease (SCD) is one such blood disorder caused by the abnormal hemoglobin that damages and deforms red blood cells. The breakdown of abnormal red cells causes anemia and obstruct blood vessels leading to recurrent episodes of severe pain and multi-organ ischemic damage. In SCD, clinical severity varies, ranging from mild and sometimes asymptomatic states to severe symptoms [2]. Symptomatic treatments exist and newborn screening (NBS) for SCD can reduce the burden of this disease in children. The increasing gene occurrence of haemoglobinopathies in India is approximately 4.2%. With a population of over one billion and a birth rate of 28 per 1000 individuals, there are over 42 million carriers and over 12,000 infants take birth every year with clinical haemoglobinopathy [3-6]. Hemoglobin is a tetramer composed of two α-globin and two non- α -globin chains working in conjunction with heme to transport oxygen in the blood.

Normal adult hemoglobin (HbA) is designated $\alpha^A2\beta^A2$ [7] Variant hemoglobin is derived from gene abnormalities affecting the α-globin genes (HBA1 or HBA2) or β-globin (HBB) structural genes (exons). The Homo sapiens truncated hemoglobin beta chain (HBB) gene provides instructions for making a protein called beta-globin [8]. β-globin is a component (subunit) of a larger protein called hemoglobin, which is present inside red blood corpuscles. In adults, hemoglobin normally consists of four protein subunits: two subunits of beta-globin and two sub-units of another protein called alpha-globin, which is produced by gene called *HBA* these protein subunits are attached (bound) to an iron-containing molecule called heme; which contains an iron molecule in its center and bind to one oxygen molecule. Sickle cell anemia, a common form of sickle cell disease, is caused by a particular mutation in the *HBB* gene. This

mutation results in to the production of an abnormal version of beta-globin called hemoglobin S or HbS, in this condition, hemoglobin S replaces both beta-globin subunits in hemoglobin.

MATERIAL AND METHOD

Location of the study:

This imminent, cross sectional study was conducted at Department of Zoology, D.S.B. Campus, Kumaun University, Nainital, Uttarakhand. Study was conducted over a period of three years. The questionnaire was designed specifically to extract the information. The importance of piloting the questionnaire was recognized and questionnaire was piloted in a small group of people.

Participants and Sample size:

Total fifty four (54) Doctor's observations were included in the investigation based on questionnaire having ten basic questions on treatment and facilities. One thousand three hundred sixty nine (1,369) individuals were screened for their general awareness about SCD and their knowledge about risk factors. Total thirty one (31) persons screened for molecular studies.

Laboratory based investigation:

Collection Blood Samples: Blood samples were collected using EDTA as anticoagulant by disposable syringes and needles from infected persons and people free from disease and blood transfusion history. The blood groups of respondent were examined with blood group testing kit.

Cellulose acetate electrophoresis (CAE):

Hb electrophoresis performed on cellulose acetate paper as per [9-10] with minor modifications. Identification of sickle cell trait (SCT) and sickle cell disease (SCD) by cellulose acetate paper electrophoresis with TBE buffer at pH-8.9 [11].

DNA isolation and HBB gene amplification:

Homo sapiens truncated hemoglobin beta chain (HBB) gene primers were designed with the help of online tool Primer 3 and NCBI. The DNA sequence of the HBB gene was derived from NCBI's GenBank: DQ115318.1 (Table 1). The genomic DNA of blood from selected respondents was isolated by standard method [12] quantified and analyzed on agarose gel electrophoresis [13]. *Homo sapiens* truncated hemoglobin beta chain (HBB) gene primers were used for the survey too (Table 1). PCR amplification was performed as per the standard protocol using 50-100 ng of template DNA, 30 ng of primer (IDT), 0.1 mM dNTPS , 1.5 U Taq DNA polymerase (Bangalore Genei, India), 1X PCR buffer (10mM Tris pH-8.0, 50 mM KCl and 1.8 mM $MgCl_2$) in a volume of 25 µl. Amplification was performed with thermal cycler (Eppendorf, Germany). The standardized amplification was Initial denaturation 95°C for 2 minutes followed by 35 cycles of denaturation at 94°C for 1 min; Primer annealing for 1 min; primer extension at 72°C for 30 sec; and final primer extension at 72°C for 7 min respectively. Amplified PCR product were resolved on 1.5% agarose gel ethidium bromide was used as dye.

Table 1: *Homo sapiens* truncated hemoglobin beta chain (HBB) gene primers**.**

S. No.	Name of Primer	Length (bp)	Tm*	GC Content	Sequence	Product Size (bp)	NCBI Reference Sequence
1	DCM-HBBL-1	20	60.08	50.00	5'-TTGGACCCAGAGGTTCTTTG-3'	161	DQ115318.1
2	DCM-HBBR-1	20	60.35	55.00	3'-CACTCAGTGTGGCAAAGGTG-5'		
3	DCM-HBBL-2	20	59.52	50.00	5'-TGGATGAAGTTGTGGTGAGG-3'	231	
4	DCM-HBBR-2	20	59.98	55.00	3'-CAGCATCAGGAGTGGACAGA-5'		

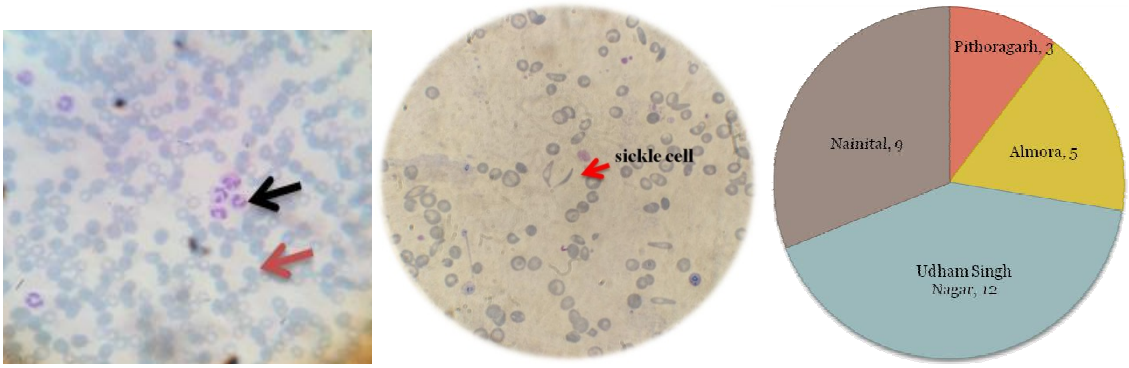
RESULTS AND DISCUSSION

Several general and tribal communities are found in Kumaun region in which popular general communities are Pandey , Pant, Joshi, Bisht , Negi , Parihaar , Dhaik , Fartiyal , Samant , Bhatt , etc. and four communities belong to tribal population which are Bhotiya in Dharchula and Munsyari region of Pithoragarh district , Vanrawat in Chapawat district , Tharu in Khatima and Sitarganj region of U.S. Nagar and Buksaa in Bazpur , U.S. Nagar . In comparison to general communities Sickle cell diseases is very common in tribal communities especially it was prevalent in Tharu communities in Sitarganj area.

District wise numbers of reported patients of Sickle cell disease are as follows Pithoragarh: 03; Almora: 05; Udham Singh Nagar: 12; Nainital: 09 and eight new cases were investigated in Nainital and

Almora. The positive cases of sickle cell disease during investigation of Kumaun are total twenty nine cases.

Sickle cell identification by microscope and blood group frequency in Kumaun:



(A) **(B)** **(C)**

Figure 1: Images showing the (A) blood cells and black arrow showing white blood cells (WBC) and red arrow showing the red blood cells (RBC) (Images are 20X magnification) and (B) Sickle cell in blood smear (arrow) and the magnification is 40X. (C): Graphical representation for district wise number of patients of Sickle cell disease.

The blood grouping was performed to understand the allele variation ,it was found that 24.11 % respondents with $A^{+,}$ 03.00% A^{-}, 25.57 % B^{+}, and B^{-} 02.62%, AB^{+} 12.80%, AB^{-}02.77% O^{+} 26.13% and O^{-} 03.00% respectively (**Figure 2**).

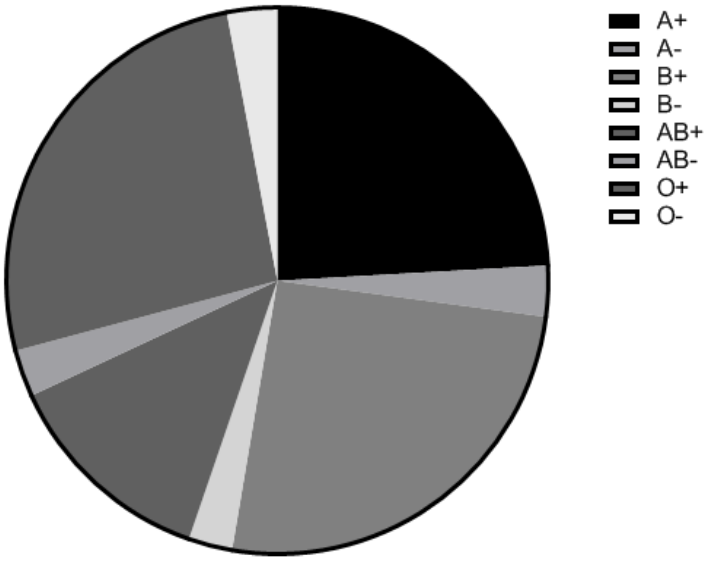


Figure 2: The blood group of respondents A^{+} (24.11%), A^{-} (03.00%), B^{+} (25.57%), B^{-} (02.62%), AB^{+} (12.80%), AB^{-} (02.77%), O^{+} (26.13%) and O^{-} (03.00%) **.**

Doctor's observations:

Based on baseline investigation it was observed that facilities are not of desired level in the hospitals of Kumaun region for the SCD patients specially the diagnosis facilities, Patient family record and registration units are not operational in the study areas (**Figure 3**). SCA and SCT patients were observed in very low strength and Sickle cell Beta Thalassemia (SC-BT), Hemoglobin C (H-C) were not detected.

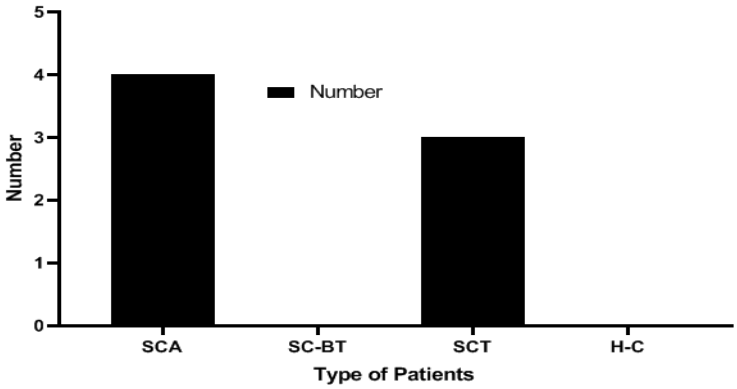
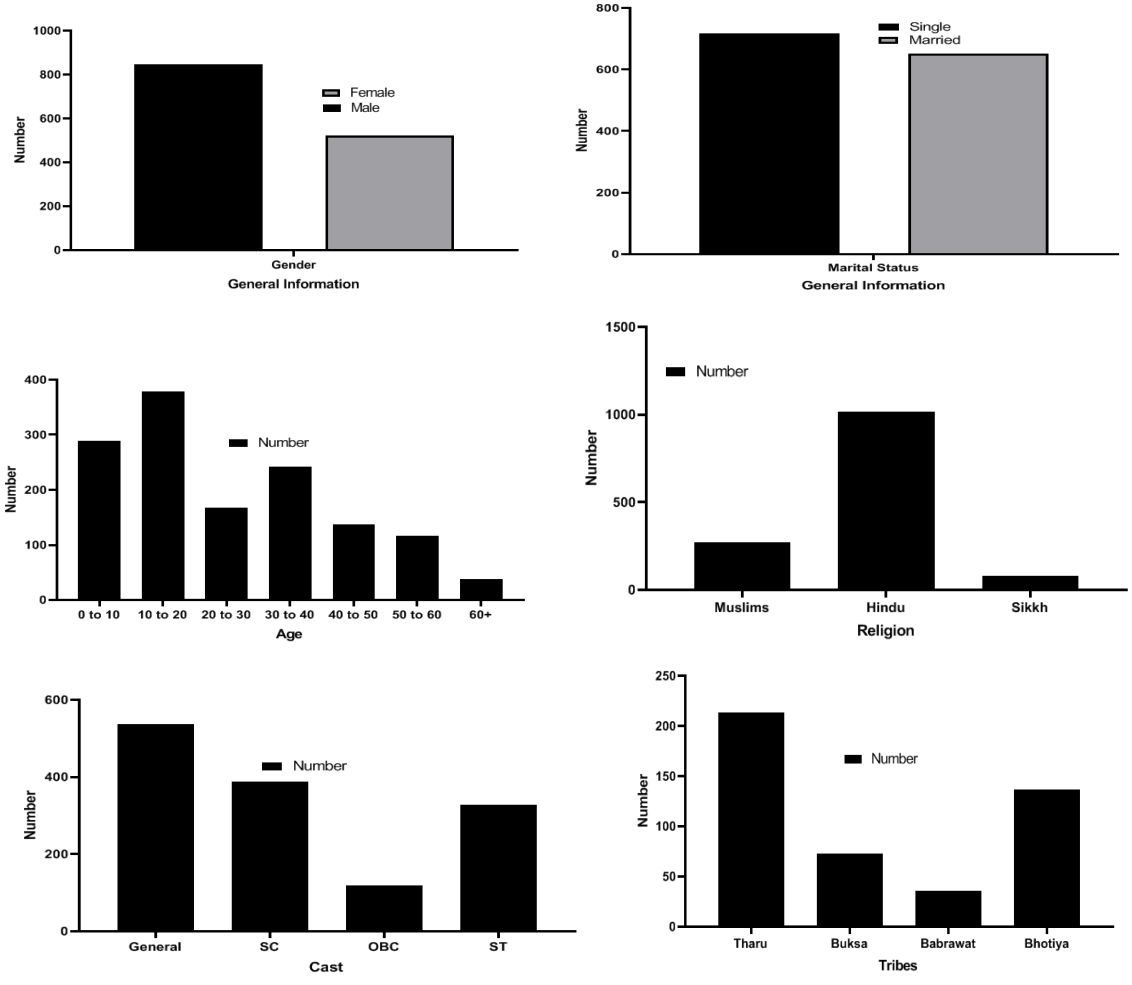


Figure 3: Base line survey of SCD patients with doctor's observation (SCA; Sickle cell Anemia, SC-BT; Sickle cell Beta Thalassemia, SCT; Sickle Cell Trait, H-C; Hemoglobin C).

POPULATION OBSERVATIONS: Total 1, 369 individuals were screened for their knowledge about SCA and SCD of Kumaun region of Uttarakhand based on questioners.

General Information: In the present study non-significant difference was observed between male and female (Gender) and Marital status. People from general category are more than the SC, ST and lowest was OBC. Tharu tribes were higher than Bhotiya, Buksa and Banrawat were lowest in number. Education label is a very important factor to create awareness of SCD or SCT on population, High school and intermediate educated were maximum and then uneducated but graduate and post graduate were very low in the number. The main occupation of respondents is Agriculture **(**figure **4**).



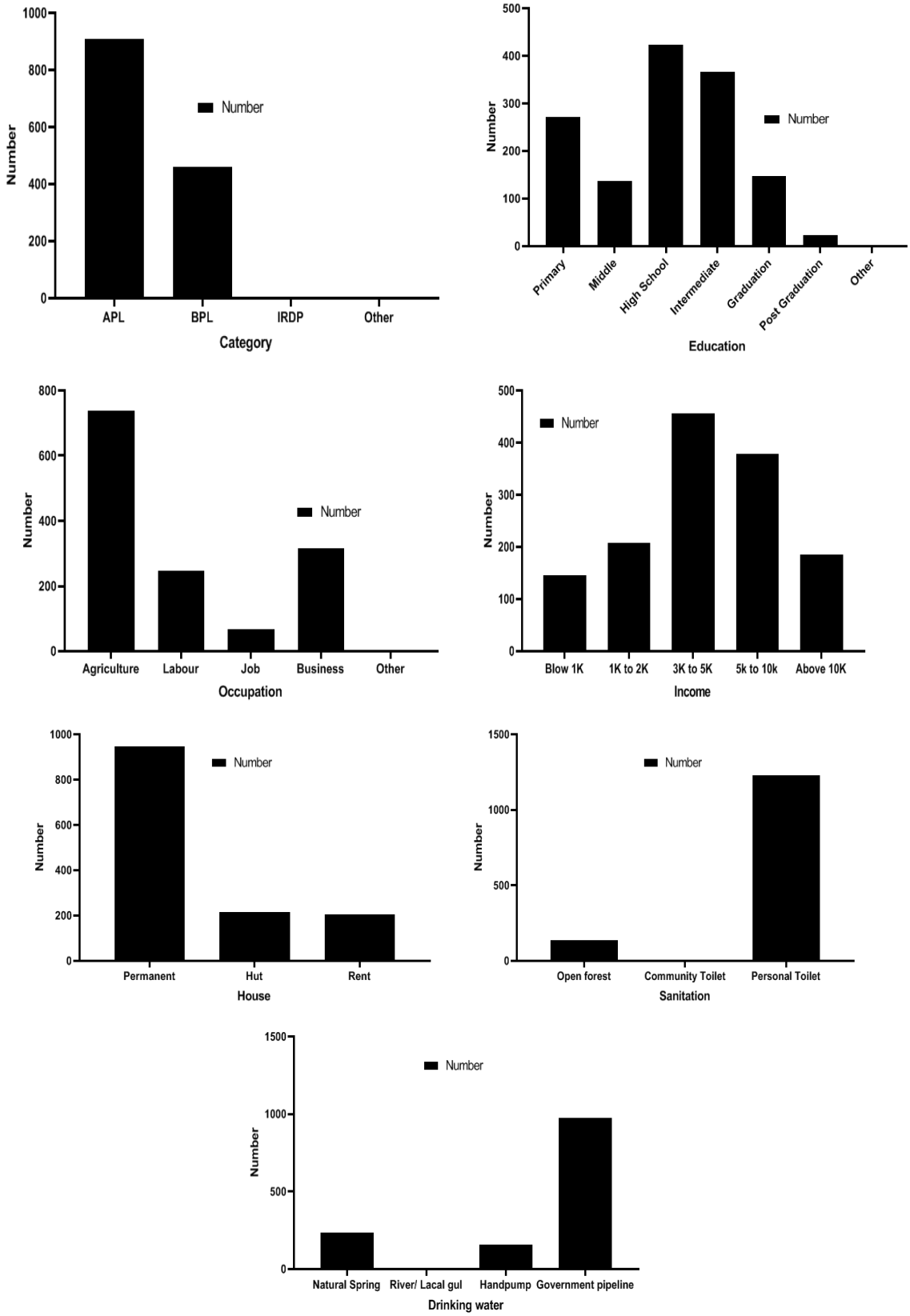


Figure 4: General information of screened population based on Gender, Marital status, Age, Religion, Cast, Tribes, Category, Education, Occupation, Income, House, Sanitation and Drinking water.

General Information (Medical): Significant number people found aware of blood grouping and medical facility in hospitals but they have no idea about blood transfusion (**Figure 5**).

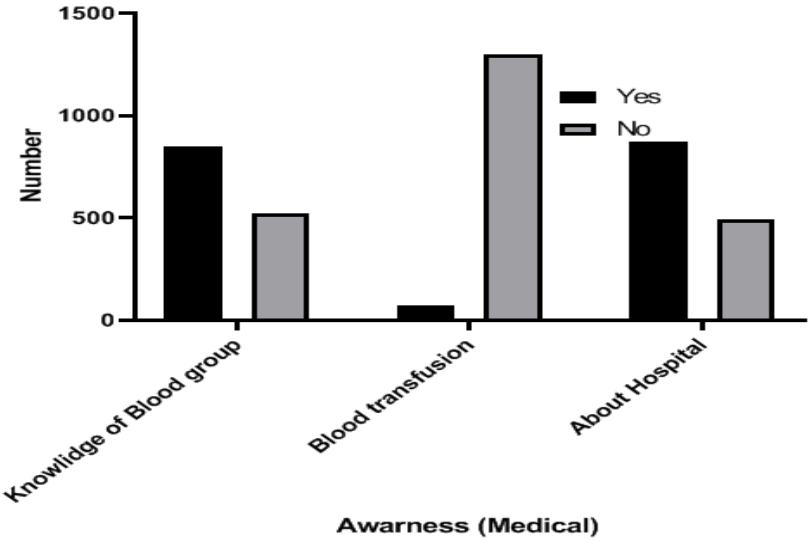


Figure 5: Awareness level about medical facility among study group.

Awareness (Disease): Very limited number of individuals found aware about SCA, Causes of disease, symptoms, suffering family, local person and known treatment etc (**Figure 5**). Certain tribes are now very less in number therefore it is needed to address the issue at medical and social level to conserve unique human genome.

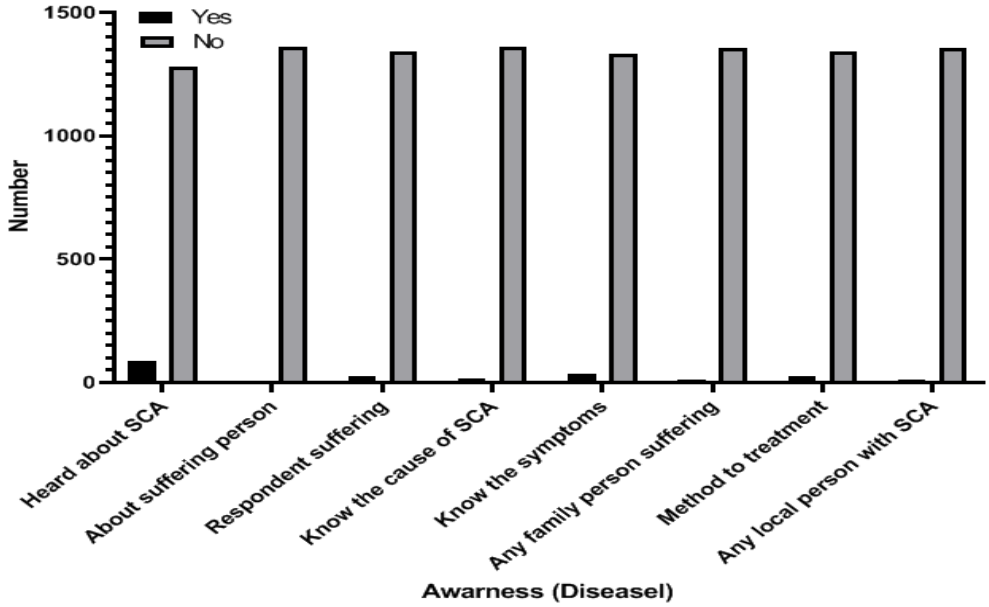


Figure 5: Awareness status of SCD among study population.

Societal awareness in the Community.

During the study Societal awareness and belief was analysed and it was found that status of marriage in close relations is less. Very low or negligible SCA occur in respondent and they admitted it due to close marriage and maximum respondent were not interested to know about SCA (**Figure 7**).

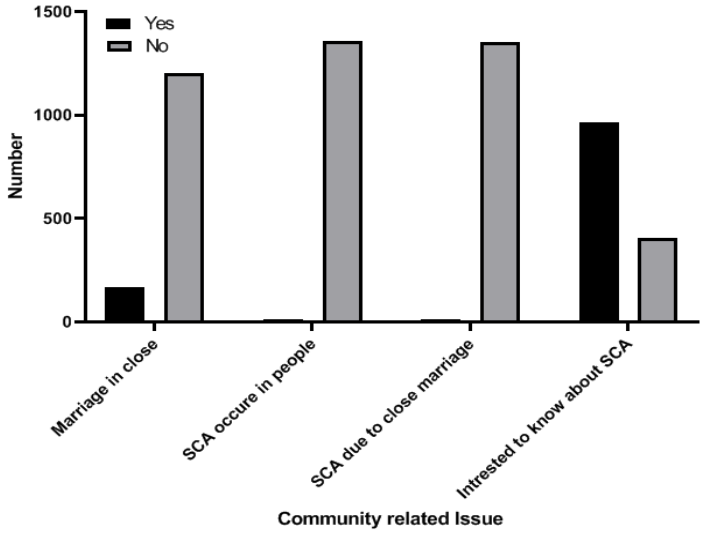


Figure 7: Community awareness related to premarital carrier screening.

Cellulose acetate electrophoresis (CAE):
Total thirty one (31) cases were selected for further investigation and it was found that eight were suffering from SCA (**Figure 8**) and after quantification of the bands for HbF, HbA2, HbA0 and HbS percent was studded (**Table 2**). The CAE (also called zone electrophoresis) is easy, quick and cost effective technique to process large number of samples at the same time **[14]**.

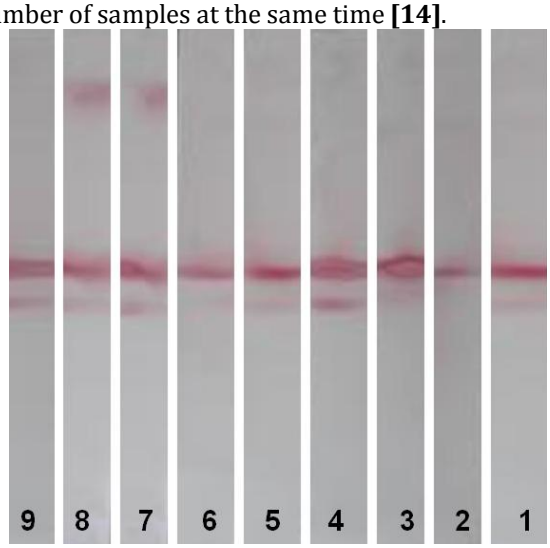


Figure 8: Cellulose acetate membrane electrophoresis identification of sickle cell trait (SCT) and sickle cell disease (SCD).

Table 2: Hb fractionation results of nine positive samples from five families.

Subject	HbF (%)	HbA2 (%)	HbA0 (%)	HbS (%)
1	47.1	3.5	1.8	49.1
2	1.0	3.5	58.2	29.9
3	15.6	2.9	56.25	23.4
4	43.8	3.8	2.1	50.85
5	4.00	6.20	89.80	58.49
6	5.5	2.6	90.8	14.0
7	8.1	5.5	78.8	4.5
8	2.8	3,6	3.4	82.4
9	05.00	5.20	87.80	56.49

The community related study resulted that, all the strategies for the deterrence of SCD or SCA will be effective only if they are utilized to its maximal extent by creating more awareness to the population **[15]**

reports say that ideally a person suffering from SCD or SCA should test the hemoglobin levels checked in every two to three months **[16]**.

PCR amplification of HBB gene:

50-100ng purified DNA was used for Polymerase Chain Reaction (PCR) amplification and two micro liters purified DNA was loaded in 1% agarose gel for purity confirmation (**Figure 9**). After analysis of gel it was clearly observed that some templates were not amplified on their product sizes 231 and 161bp (**Figure 10**). This may be due to some non-available of complimentary sequences for used primers. The PCR products need more exploration as nucleotide sequencing to exploration point mutation. In the **figure (10A)** there was not significant variation observed, in **figure (10B)** there was additional band approx 400bp was observed in some sample may be due to allelic variations. Amplification results of all homozygous patients revealed amplified product sizes (APS) at 231bp only and heterozygous revealed (APS) at both 231 and 400bp, while healthy individuals' revealed (APS) at 231bp only (**Figure 10B**).

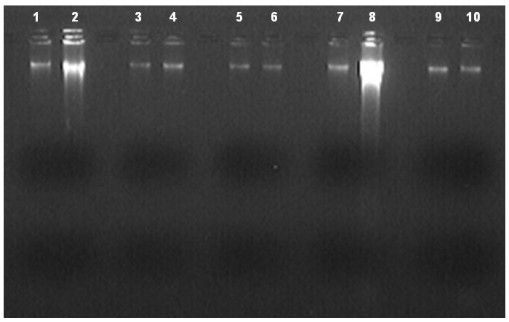


Figure 9: Purified DNA isolated from blood of *homo-sapiens.*

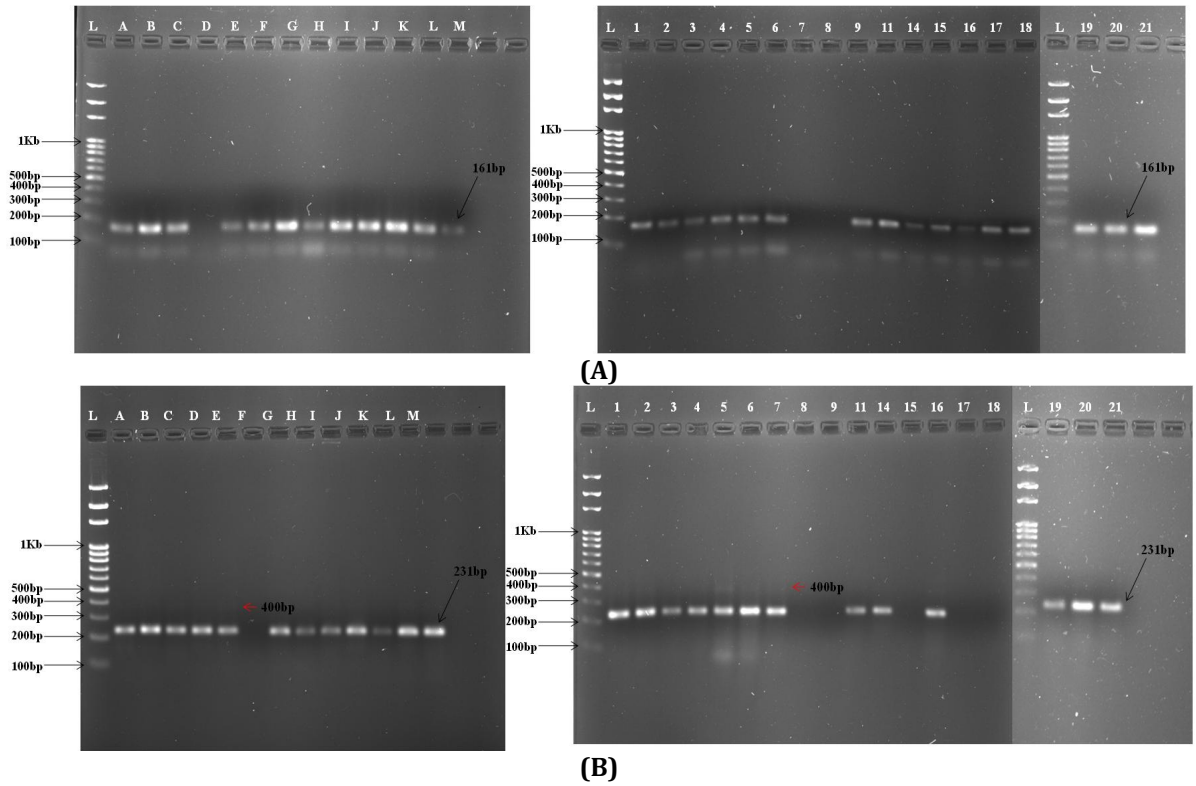


Figure 10: *Homo-sapiens* truncated hemoglobin beta chain (*HBB)-1*gene has been amplified to identification of allelic variations for SCD and amplification product size is 161bp (A) and 231bp with additional 400bp (B). Loading of samples 1 to 9 family (one), 10 to 15 family (Two), 16 to 21 family (Three), A to F family (Four) and G to M family (Five).

Sickle cell disease (SCD) is an autosomal recessive disease caused by a transversion type of point mutation in the beta globin (HBB) gene **[17]**. The alpha (HBA) and beta (HBB) loci determine the structure of the 2 types of polypeptide chains in adult hemoglobin, Hb A. The normal adult hemoglobin tetramer consists of two alpha chains and two beta chains. Mutant beta globin causes sickle cell anemia.

Absence of beta chain causes beta-zero-thalassemia. Allele specific PCR correctly and unmistakably detected the presence or absence of haemoglobins A and S from all samples for serene, representing its accuracy and accuracy for the screening of SCA [18]. It is evident from the study that those PCR products are visible at regions 517bp for HbA and 267bp for HbS.

CONCLUSION

The findings and techniques used in the present investigation could be employed in identification of groups at high risk through mass screening at the community level. The urgent attention of the hour is to start community based screening programmes at large, to target the high risk inhabitants. Implementation and monitoring can be done to reduce the prevalence of haemoglobinopathies in the Uttarakhand. This study is a unique kind of report from Kumaun region of Uttarakhand and investigation would be very helpful for early identification of SCD using the tools and techniques used during the present investigation.

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Conflict of interest: Authors have no conflict of interest.

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