ORIGINAL ARTICLE

Screening of haemoglobinopathies by high performance liquid chromatography and its molecular characterization using amplification refractory mutation system and direct DNA sequencing techniques among college students in central Gujarat

Minal T[1], Hitesh S[2], Prakash P[3], Navneet S[4]*

[1]Shree P M Patel College of Paramedical Science and Technology, Anand People's Medicare Society, Anand-388001 (Gujarat), India, [2]Department of Biochemistry, Shree Krishna Hospital and Pramukh Swami Medical College, Bhaikaka University, Karamsad-388325 (Gujarat), India, [3]Indian Red Cross Society, Gujarat State Branch, Ahmadabad- 380013 (Gujarat), India, [4]Department of Paramedical Sciences, Sumandeep Vidyapeeth (Deemed to be University), Vadodara-391760 (Gujarat) India

Abstract

Background: Haemoglobinopathies are inherited blood disorders that can have significant health implications and are common in certain populations. *Aim and Objectives*: To investigate the prevalence of haemoglobinopathies among college students in central Gujarat, India. *Material and Methods:* A combination of techniques were employed to identify haemoglobinopathies. Chromatograms were examined to identify variant haemoglobin based on characteristics like proportion, retention time, and peak features. Prior to DNA analysis, prevalent Indian thalassemia mutations, as well as HbS and HbE, were identified using the Amplification Refractory Mutation System (ARMS) Polymerase Chain Reaction (PCR) technique. DNA sequencing was performed at SN Gene Lab, Surat. *Results:* The findings revealed that HbS codon 6 (A→T) heterozygosity, Hb S Codon 6 (A→T) double heterozygosity, and HbE: codon 26 (G→ A) were relatively common in the population. Among carrier students screened for haemoglobinopathies, IVS-1, nt5 (G→C), and Codon41/42 (-CTTT) were the most prevalent beta-thalassemia mutations in India. Codon 88 (C-T) and other less common mutations were also detected. *Conclusion:* The study underscores the importance of raising awareness and providing counselling for individuals affected by haemoglobinopathies. By utilizing High Performance Liquid Chromatography (HPLC) screening and molecular techniques for characterization, the study contributes to the understanding of mutation patterns in central Gujarat. This knowledge is crucial for the effective detection of mutations in screening programs and prenatal diagnostics, ultimately enhancing the quality of life for affected individuals and preventing further transmission of these disorders.

Keywords: Amplification Refractory Mutation System Polymerase Chain Reaction, DNA sequencing, Haemoglobinopathies, High Performance Liquid Chromatography

Introduction

High Performance Liquid Chromatography (HPLC) is a technique for confirming the diagnosis of hemoglobinopathies. Inherited disorder of haemoglobin includes multiple abnormalities ranging from structurally abnormal Hb variant to reduced production of haemoglobin [1]. Hemoglobinopathies is a group of disorders inherited from parents in which there is abnormal structure and abnormal/reduced production of haemoglobin. In thalassemia, there is decreased production of

one of the globin chain while in sickle cell abnormal globin chain is produced [2].

Thalassemia and hemoglobinopathies are most prevalent single gene disorder in the world including India. Every year about 32,400 babies are born with serious haemoglobin disorder in India [3]. According to estimates from the WHO, 5% of the world-wide population may be carriers transmitting this abnormal haemoglobin gene. Thalassemia trait and sickle cell hemoglobinopathy incidence range from 3–17% and 4–44%, respectively, in India [4]. Recognition of these disorders is crucial for epidemiologic purposes in order to prevent significant thalassemia and clinically severe hemoglobinopathies [4].

HPLC is now the technique utilised to quantify Hb-A2, Hb-S, Hb-F, and detect other haemoglobin subtypes for the diagnosis of hemoglobinopathies [5]. HPLC is a very sensitive, focused, and high-resolution automated technique [4]. For estimation of Hb-A2, Hb-F and other Hb subtype, several methods are available like electrophoretic and chromatographic procedures but HPLC and Cation Exchange (CE) HPLC using automated technology have become the methods of choice [5]. Advantage of the HPLC system is the required small volume sample (5 µl), excellent resolution, reliability, and quantification of normal and other variant haemoglobin resulting in accurate diagnosis of hemoglobinopathies [1]. HPLC usually detects haemoglobin A, A2, C, D (Punjab), F, G (Philadelphia) and S. Hb-lepore as well as Hb-E are often removed by Hb-A2, although they can also be detected using other methods [2]. Hb-D Punjab cannot be distinguished from Hb-S in traditional CAE techniques, while Hb O-Arab may be confused with Hb-C or Hb-E. However, using HPLC, these two variants may be separated from one another [6]. Because preventive measures like population screening, genetic counselling, and prenatal diagnosis are more economical than curative treatments like bone marrow transplants and also because of its quick diagnosis and the ability to safely end a pregnancy, if necessary, DNA analysis is the most acceptable test among the several techniques used to identify mutations [7]. There are 785 mutations on the cluster of the beta-globin genes; 232 of these variants are responsible for clinical symptoms of beta-thalassemia, albeit only a small number of these specific mutations have been recorded for each community. Various regions of India have different sets of mutations and rates of hemoglobinopathy [7]. In India, around 22 β-thalassemia variants have been identified with IVS I-5 (G→C), IVS I-I (G→ T), codon 41/42 (-TCTT), codon 8/9 (+G), and 619bp deletion. Eighty percent of common mutations are caused by this type of deletion [8]. Overall, this research paper seeks to investigate the prevalence of haemoglobinopathies among college students in central Gujarat using HPLC screening and subsequently characterize the identified variants through molecular techniques. The study also emphasizes the significance of raising awareness and providing counselling to individuals affected by haemoglobinopathies in order to improve their quality of life and prevent further transmission of these disorders.

Material and Methods

The current study was a voluntary cross-sectional screening program. This study was carried out at Anand People's Medicare Society, Anand, Gujarat in collaboration with the Indian Red Cross Society, Gujarat state branch, Ahmedabad, Gujarat. Total number of students participated in this study were 395 during October 2021 to November 2021 with age group starting from 18 years up to 30 years. For this study, 2 ml venous blood samples from cubital vein were withdrawn in EDTA bulb for CBC and HPLC tests. Screening for anemia with haemoglobin concentration less than 12 g% and CBC estimation were carried out by automated method using cell counter by (Sysmex K4500). According to the instructions in the user's manual for the Variant Haemoglobin Testing System (Biorad Variant II),the VARIANT II Beta-Thalassemia Short Program uses a sodium phosphate buffer as elution buffers 1 and 2, with 0.05% sodium azide added as a preservative. The wash/diluent solution is deionized water with 0.05% sodium azide. No sample preparation is necessary. Whole blood samples collected in EDTA-filled vacuum collection tubes are loaded onto the VARIANT II sampling station. If the sample is in an unusually shaped or sized tube or if the sample's height is less than 25 mm, it is pre-diluted 1:200 with the wash or diluent solution. The VARIANT II system utilizes a cation exchange cartridge for the separation and analysis of haemoglobins. The two pumps of the VARIANT II chromatographic station supply an established buffer gradient of increasing ionic strength to the cartridge, separating the HbA2/F based on their electrostatic attraction. Retention time windows, such as D-window, S-window, and C-window, are used to presumptively identify haemoglobin variations. The retention time for haemoglobin A2 is 3.65 ± 0.10 minutes. The methodology involves automatic dilution and blending of the samples, separation of HbA2/F on a cation exchange cartridge using an established buffer gradient, measurement of absorbance at 415 nm, and calculation of Hb A2/F values using calibration factors. The VARIANT II system provides automated sample collection, dilution, and analysis.The various haemoglobinopathies were identified according to the percentage, residence time, and peak characteristics of mutant haemoglobin in a chromatogram of HPLC. A cut-off HbA2 level of >4.0% was used to identify carriers of β-thalassemia.Total 21 haemoglobinopathy carrier samples of students were processed for DNA analysis by ARMS PCR at Indian Red Cross Society, Ahmedabad. Samples were characterized for common mutations like IVS I-5 (G→C), IVS I-I (G→T), Codon 41/42 (-TCTT), Codon 8/9 (+G) and 619bp deletion by ARMS-PCR technique. A stepwise identification approach had been applied to characterize the mutation. This involved the following [9-10]: A. DNA isolation from blood with commercially available Qiamp DNA Mini Kit and Maxwell® RSC Whole Blood DNA kit from the peripheral venous blood of the subjects having BTT, SCT and other haemoglobin variants. The QIAxpert high-speed microfluidic UV/VIS Spectrophotometer was used to detect exact quantities of DNA and profile sample content to discriminate between DNA, RNA, and sample impurities. B. Detection of Hb S and Hb E as well as the five prevalent Indian β-thalassemia variants using ARMS PCR.C. Uncharacterized

materials were transferred to the SN gene lab in Surat for DNA sequencing.

Ethical considerations

This study was authorised by the H M Patel Centre for Medical Care and Education's ethical committee, Karamsad, District-Anand, under Faculty of Medicine. All participating students included in the study signed their written, informed consent.

Results

A total of 395 students from the Anand People's Medicare Society were examined for thalassemia and hemoglobinopathies; 374 of them were found to be normal. However, 21 of the 395 students had hemoglobinopathies as determined by the HPLC method, with 6 males and 15 females among them. Students who had been diagnosed with compound heterozygous for sickle cell disease had HbS value greater than 30%, HbF value greater than 2%, and HbA2 value greater than 3%. Eleven students were detected as having sickle cell trait (with co-existence / co- inheritance of one or more alpha gene) with HbS range among 8 students was 21-30 % and > 30% among the remaining 3 students. HbF value was found within the normal range (0.8-2%) except 1 who had HbF value more than 2% and HbA2 value between 3–7%. HPLC method detected 7 students as having typical β- thalassemia minor with HbA2between 3-7% and normal HbF value of < 2%. Whereas in Hb -E trait, HbF was > 2 with peak in A2 region as shown in Table 1. By applying Cross-Tabulation, we can gain a better understanding of the distribution and relationships between different types of hemoglobinopathies and their associated haemoglobin levels in Anand district. The samples from all 21 carriers were examined for mutations related to various hemoglobinopathies. Five frequent mutations, including the Hb-S and Hb-E and the β-thalassemia mutation were reported using the ARMS-PCR approach for mutation characterization. A total of 11 (52.38%) students had the Hb-S Codon 6 (A→ T) heterozygous mutation, 2 (9.52%) had the Hb-S Codon 6 (A→ T) double heterozygous mutation, 1 (4.76%) had the Hb- E Codon 26 (G→A) mutation, 6 (28.5%) had the five common mutations, and 1 (4.7%) had the unusual mutation. IVS-1, nt5 (G→C) was the most frequent mutation of the five identified mutations, occurring in 4 (19.04%) and Codon41/42 (-CTTT) occurring in 2 (9.52%). Any carrier students who were examined for hemoglobinopathies did not have the IVS-1, nt1 (G→ T), Codon8/9 (+G), or 619bp del mutation. In addition to these five prevalent mutations, codon 88 (C→ T) (4.76%) was also found. Table 2 displays the frequency of these mutations. According to the prevalence of hemoglobinopathies in various communities, SC/ST/OBC people are the most at risk, with 57.1%. There is also a prevalence in communities like Rajput (28.5%), Patel (9.5%), and Lohana (4.7%). Table 3 displays the distribution of various mutations in relation to communities. According to this data, the most prevalent mutation across all populations, with the exception of SC/ST/OBC caste, was IVS-1, nt5 (G→ C). Second common mutation was Codon 41/42 (-CTTT) found in Rajput and SC/ST/OBC caste. Hb S Codon6 (A→ T) Heterozygous and doble heterozygous mutation was more prevalent in SC/ST/OBC caste. Where Hb E Codon 26 (G→ A) was found in Muslim as well SC/ST/OBC caste followed by Rajput. Rare mutation Codon -88 (C→ T) was identified in Rajput community.

Table 1: Cross-tabulation of HPLC results correlating the hemoglobinopathies type

Hemoglobinopathies (21)	HBA2		HBF		HBS	
	< 3%	3–7%	<2%	> 2%	21-30%	> 30%
Sickle cell trait (with co-existence /co-inheritance of one or more alpha gene) (11)	0	11	10	1	8	3
Double heterozygous for sickle cell anaemia (02)	0	2	0	2	0	2
Typical β thalassemia trait (07)	1	6	5	2	-	-
Hb-E trait (01)	0	1	0	1	-	-

Table 2: Distribution of various mutations in different hemoglobinopathies

Types of mutation	Number of case positive	Mutation frequency (%)
Common (n= 20) by ARMS-PCR		
Hb-S Codon 6 (A→T) Heterozygous	11	52.38
Hb-S Codon 6 (A→T) Double Heterozygous	2	9.52
IVS-1, nt5 (G→C)	4	19.04
Codon 41/42 (-CTTT)	2	9.52
IVS-1, nt1 (G→T)	0	0
Codon8/9 (+G)	0	0
619bp del	0	0
Hb-E: Codon 26 (G→A)	1	4.76
Rare (n= 01) by DNA sequencing		
Codon -88 (C→T)	1	4.76
Total	**21**	**100**

Table 3: Frequency distribution of mutations with respect to community

Community	IVS-1 and nt5 (G→C)	Codon 41/42 and (-CTTT)	Hb-S Codon 6 (A→T) Heterozygous	Hb-E: Codon 26 (G→A)	Codon -88 (C→T)	Total (%)
SC/ST/OBC	00	01	10	01	00	**12 (57.1)**
Rajput	01	01	03	00	01	**06 (28.5)**
Patel	02	00	00	00	00	**02 (9.5)**
Lohana	01	00	00	00	00	**01 (4.7)**
Total	**04**	**02**	**13**	**01**	**01**	**21 (100)**

Table 4: Comparative account for levels of Hb-A2, Hb-S, Hb-F, in various hemoglobinopathies

Hemoglobinopathies		Panda *et al.* [15]	Balgir *et al.* [16]	Sawaimul *et al.* [17]	Present study
β–thal trait	Hb-F	0.60	1-5	0.30	0.3-12
	Hb -A2	4.50	3.50-7.00	5.40	4.8-6.2
Sickle cell trait	Hb -F	0.80	N	0.9	0.3-4.3
	Hb -A2	1.75	2-4	3.30	2.80-3.80
	Hb -S	25.22	38-45	34.60	28-37
Sickle Cell disease	Hb -F	-	1-20	12.90	-
	Hb -A2	-	2-4	2.80	-
	Hb -S	-	75-95	80.50	-
Double heterozygous	Hb -F	-	5-30	18.70	7.90-15.60
	Hb -A2	-	4-8	3.90	3.40-3.80
	Hb -S	-	60-85	48.20	76.50-84.00
HbE- Punjab	Hb -F	-	-	-	0.50
	Hb -A2	-	-	-	27.30

Figure 1: HPLC chromatogram for HbE trait, typical beta thalassemia trait, sickle cell trait and double heterozygous for sickle cell anaemia

Bio-Rad CDM System **PATIENT REPORT**
Bio-Rad Variant V-II Instrument #3 **V2_00064F**

Patient Data		Analysis Data	
Sample ID:	O22161874	Analysis Performed:	29/07/2022 17:43:41
Patient ID:		Injection Number:	699
Name:		Run Number:	27
Physician:		Rack ID:	
Sex:		Tube Number:	5
DOB:		Report Generated:	30/07/2022 16:05:14
Comments:		Operator ID:	

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	1.01	2579
F	1.4	---	1.11	32235
Unknown	---	0.7	1.23	15375
P2	2.5	---	1.34	59427
Unknown	---	2.0	1.74	46523
P3	2.2	---	1.83	52976
Ao	62.5*	---	2.39	1474811
A2	28.9*	---	3.69	677163

Total Area: 2,361,089

F Concentration = 1.4 %
A2 Concentration = 28.9* %

*Values outside of expected ranges

Analysis comments:

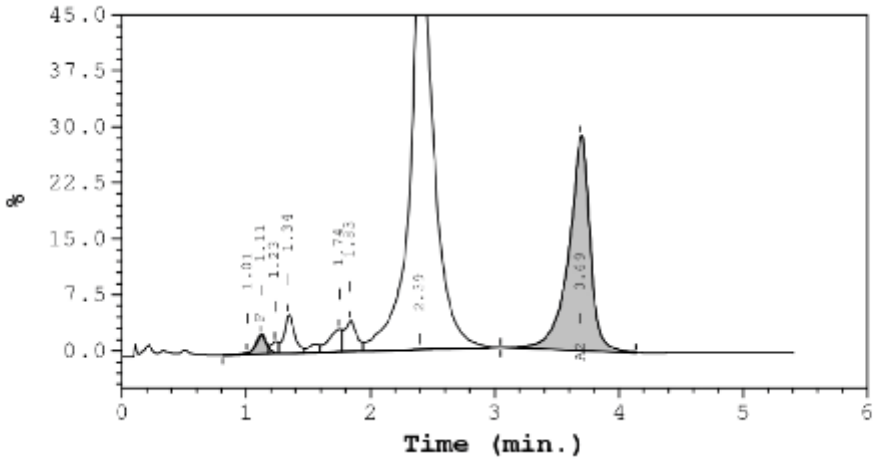

Figure 1a: Chromatogram of HbE trait

Bio-Rad CDM System **PATIENT REPORT**
Bio-Rad Variant V-II Instrument #2 **V2-80106AE**

Patient Data		Analysis Data	
Sample ID:	C22109618-R	Analysis Performed:	29/07/2022 14:32:27
Patient ID:		Injection Number:	485U
Name:		Run Number:	16
Physician:		Rack ID:	
Sex:		Tube Number:	5
DOB:		Report Generated:	30/07/2022 16:05:59
Comments:		Operator ID:	

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	1.02	2811
F	1.0	---	1.12	26132
Unknown	---	1.0	1.24	27793
P2	3.8	---	1.34	101981
P3	4.9	---	1.75	132087
Ao	85.6	---	2.39	2287489
A2	3.8*	---	3.66	92462

Total Area: 2,670,755

F Concentration = 1.0 %
A2 Concentration = 3.8* %

*Values outside of expected ranges

Analysis comments:

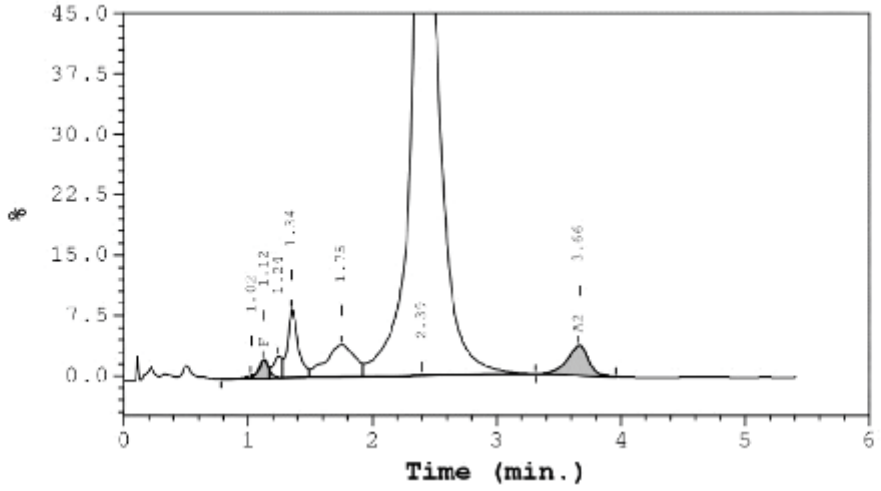

Figure 1b: Chromatogram of typical beta thalassemia trait

Bio-Rad CDM System **PATIENT REPORT**
Bio-Rad Variant V-II Instrument #2 **V2-80106AE**

Patient Data		Analysis Data	
Sample ID:	C22110449	Analysis Performed:	29/07/2022 18:57:54
Patient ID:		Injection Number:	525
Name:		Run Number:	16
Physician:		Rack ID:	0007
Sex:		Tube Number:	5
DOB:		Report Generated:	30/07/2022 16:10:16
Comments:		Operator ID:	

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	1.04	1277
F	0.5	---	1.13	6222
Unknown	---	1.1	1.27	14078
P2	3.1	---	1.36	39668
P3	4.5	---	1.74	58376
Ao	62.1*	---	2.46	799925
A2	3.0	---	3.66	35418
S-window	25.9*	---	4.36	333241

Total Area: 1,288,205

F Concentration = 0.5 %
A2 Concentration = 3.0 %

*Values outside of expected ranges

Analysis comments:

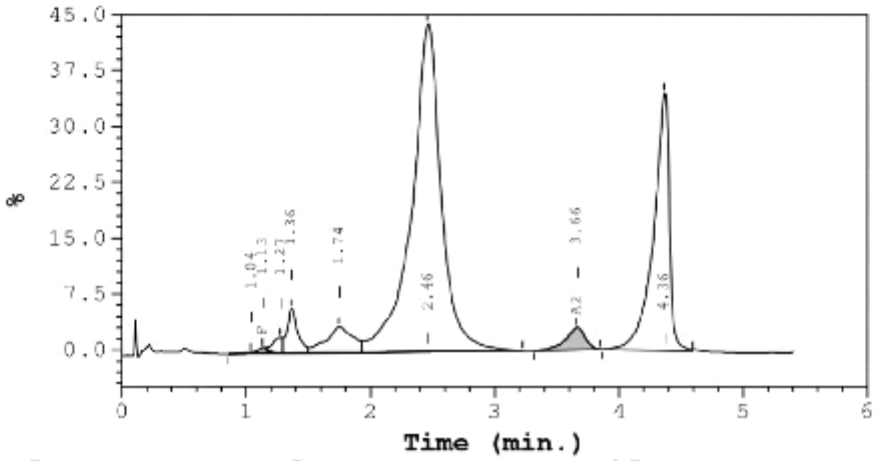

Figure 1c: Chromatogram of sickle cell trait

Bio-Rad CDM System **PATIENT REPORT**
Bio-Rad Variant V-II Instrument #2 **V2-80106AE**

Patient Data		Analysis Data	
Sample ID:	O22165162-R	Analysis Performed:	29/07/2022 13:39:22
Patient ID:		Injection Number:	477U
Name:		Run Number:	16
Physician:		Rack ID:	
Sex:		Tube Number:	7
DOB:		Report Generated:	30/07/2022 16:01:20
Comments:		Operator ID:	

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	0.1*	---	0.87	1237
F	17.7*	---	1.16	236898
Ac	2.6*	---	2.26	34721
A2	4.3*	---	3.66	53978
S-window	75.8*	---	4.39	1026119

Total Area: 1,352,953

F Concentration = 17.7* %
A2 Concentration = 4.3* %

*Values outside of expected ranges

Analysis comments:

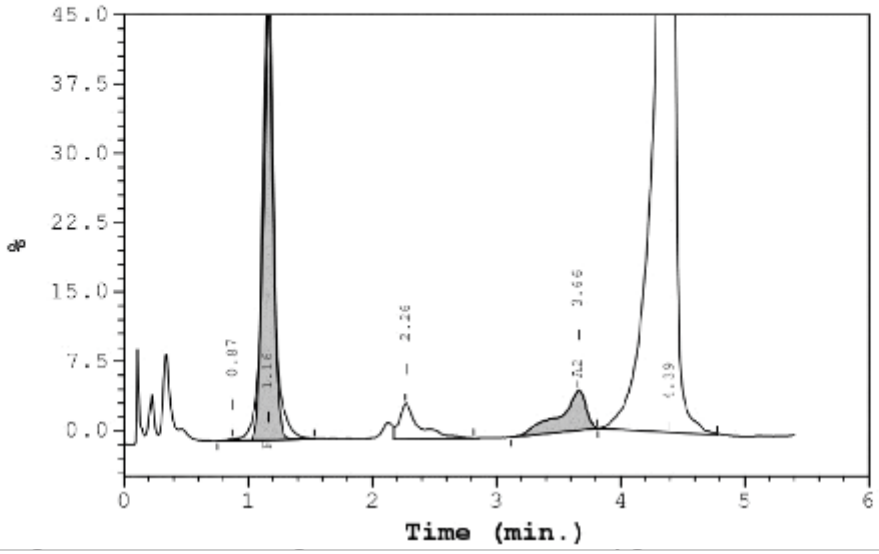

Figure 1d: Chromatogram of double heterozygous for sickle cell anaemia

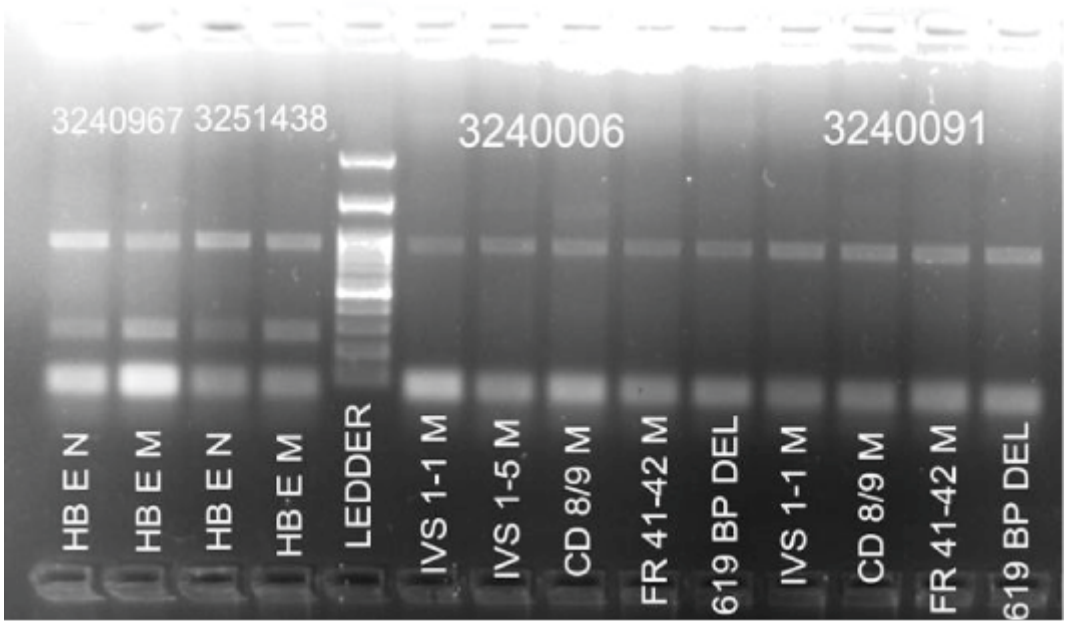

Figure 2: Agarose gel of ARMS-PCR showing IVS-1, nt5 (G→C), Codon 41/42 (-CTTT), IVS-1, nt1 (G→T), Codon 8/9 (+G), 619bp del and HbE: Codon 26 (G→A) mutation

Discussion

The most prevalent types of Hb diseases world-wide are hemoglobinopathies. These haemoglobin disorders, which were formerly mostly diagnosed among people of a certain religion, caste, tribe, and region, are now seen almost everywhere [11]. The prevalence of hemoglobinopathies globally was significantly impacted by increased global human migration, inter-caste marriages, and the endogamous norm. Several ethnic groups in India are more frequently impacted, including the Gujarati, Punjabi, Sindhi, Lohana, and Bengali. Hemoglobinopathies were discovered in the Patel, Lohana, Rajput, and SC/ST/OBC groups in the current study (Table 3) [11-12]. The prevalence of hemoglobinopathies was determined to be 5.3% in the current study. In the current study, beta thalassemia carriers were found to have 1.7% and 3.2% sickle cell disease, as well as 0.2% HbE. The World Health Organization claims that 2.3% of persons have sickle cell disease, while 3.9% have beta thalassemia [11, 13]. In our study, 16 students were found to have borderline HbA2 levels (3.4–3.8) in sickle cell trait, sickle cell trait with co-existence or co-inheritance of one or more alpha thalassemia genes, complex heterozygous for sickle cell and beta thalassemia, and also high borderline HbA2 levels found in negative cases for all hemoglobinopathies. These findings require careful evaluation and interpretation. Low HbA2 levels are associated with conditions such as beta-thalassemia trait and iron deficiency anemia, on the other hand, elevated HbA2 levels are not typically associated with vitamin B12/folate deficiency. In these situations, careful RBC index examination in conjunction with clinical correlation of haemato-logical characteristics is typically helpful.

Low HbA2 (1.7–1.9%) in a prior study indicated iron deficiency anaemia [11]. High HbA2 has been recorded in the β-thalassemia minor as well as other Hb variants: Hb S, Hb D Punjab, δ β-thalassemia [14]. Our study recorded 7 students as having β- thalassemia minor. It is caused by mutation of chromosome no.11 in β- globin gene. In students with beta-thalassemia minor, slightly decreased or normal Hb with microcytic hypochromic red cell were seen [3, 11]. In India, the prevalence of sickle cell disease varies widely. Stroke symptoms that result from erythrocyte (RBC) sickling include seizure, numbness or paralysis in legs and arms, haemolytic anaemia, unexpected speech issues, and organ damage [18]. Hb-S was detected in the current study at 3.29%; however, in other studies, a much higher prevalence of SCD/SCT is found than the current study. Among the Hb-S identified by HPLC results in the current investigation, were sickle cell trait, sickle cell trait with co-existence or co-inheritance of one or more alpha thalassemia genes, and compound heterozygous for sickle cell and beta thalassemia [11, 18]. However, in the various investigations conducted, delta beta thalassemia has consistently shown normal Hb-A2 levels. Warghade *et al.* state that the co-inherence of delta thalassemia with other thalassemia mutations may result in borderline HbA2 levels. In our study, one instance of HbE-Punjab phenotype was also noted [3]. The most cutting-edge method for diagnosing hemoglobinopathies in the current study is HPLC, which provides reliable results with respectable specificity and sensitivity. Six (28.5%) of the 21 carrier students had the five common mutations, and one (4.7%) had the uncommon mutation. Numerous additional regions of India, including Gujarat, South western Maharashtra, Punjab, and Southern India, have significant rates of mutation in IVS1-5 (G→C) [19-20]. The frequency of IVS1-5 (G→C) was estimated to be 72% in Eastern India [21]. According to a different study, the frequency of the IVS1-5 (G→C) mutation varies across India, from 44.8% in the north to 71.4% in the east, and from 54.7% to 44.8% in the west. The predominance of the IVS1-5 (G→ C) mutation is similar (57.1%) in the current study and is completely consistent with other research [22]. According to the study, the prevalence of the Codon 41/42 (-CTTT) mutation is relatively high at 28.5%. Twenty percent population of Bengal, 10% of Tamilnadu, 9.68% of Southwestern Maharashtra, 9% of Haryana, 7.2% of Maharashtra, 6% of eastern India, 5% of Punjab, 3% of Uttar Pradesh, and 2% of western India were discovered to have this mutation more frequently than elsewhere in the country [19, 23-24]. From carrier students who were evaluated for hemoglobinopathies, the current study has shown that none of them had IVS-1, nt1 (G→T), Codon8/9 (+G), or 619bp del mutations. Previous studies have shown high prevalence of these mutations from Gujarat [25-26]. According to the community distribution in Table 3, the Hb-S Codon 6 (A→T) heterozygous and doble heterozygous mutation in these communities puts them at high risk, followed by the Hb-E Codon 26 (G→ A) mutation and the Codon 41/42 (-CTTT) mutation. While IVS1-5 (G→ C) mutations had the paramount impact on the Rajput (28.5%), Patel (9.5%), and Lohana (4.7%) groups, who followed one another in that

order. The current study shows how native Indians have changed over time [25]. Codon 41/42 (-CTTT), the second common mutation, is more prevalent in the SC/ST/OBC and Rajput castes. The similarity to previously published data is evident [25]. The frameshift mutation Codon -88 (CT) is classified as a rare mutation in the Indian residents, while having a low incidence in the Rajput community group. Some cultures have a higher proportion of rare mutations, which has facilitated more targeted screening. Genetic testing is an essential method for accurately diagnosing hemoglobinopathies, offering a range of diagnostic tests such as polymerase chain reaction and DNA sequencing. However, these tests are advanced and not easily accessible in diagnostic setups with limited resources. Alternatively, hemoglobin electrophoresis using HPLC can provide valuable insights into the distribution of Hb fractions, serving as a diagnostic indicator and aiding in the development of suitable management strategies [26]. Characterizing the mutation pattern discovered through this study serves as the foundation for prenatal diagnosis and genetic counselling for those who are afflicted. To preserve rare resources, the most cost-effective techniques for their control and management should be created. It is vital to determine the cause of the unusual phenotypic heterogeneity and inherent history of these disorders [27]. In order to comply with the government's regulation that college students be screened for BTT, APMS, Anand college students were subjected to premarital screening using the HPLC method. To prevent marriage among Hb-disordered students, carriers will be strictly followed up in our college with adequate counselling and public awareness. In our study, automated HPLC for hemoglobinopathies screening provided 100% correct results. Rapidity, sensitivity, focus, reproducibility, and accuracy are all strengths of HPLC. When B-12 and folate insufficiency results in slightly elevated Hb-A2 and a misleading diagnosis of hemoglobinopathies, another method, such as the molecular technique ARMS-PCR, is employed.

Conclusion

This research combines HPLC screening and molecular techniques to identify and characterize haemoglobinopathies among college students in Central Gujarat. The study contributes to the existing knowledge by providing insights into the prevalence and specific mutations of these disorders in the region. It emphasizes the significance of awareness, counseling, and early detection to mitigate the impact of haemoglobinopathies on affected individuals and their communities. The findings serve as a valuable resource for screening programs and prenatal diagnostics in Central Gujarat and beyond.

Acknowledgments

We are deeply grateful to the President Shree Bipinchandra P Patel and management of Anand People's Medicare Society for making Thalassemia Screening compulsory to all students of affiliated colleges. We thank CEO and general secretory Dr. Prakash Parmar and all staff member of Indian Red Cross Society, Ahmedabad, Gujarat state Branch, for their active help in conducting screening camp for haemoglobinopathies. We are also thankful to all students who participated in biggest humanitarian Thalassemia prevention program which helps the human and society.

References

1. Das R, Datta S, Kaviraj A, Sanyal SN, Nielsen P, Nielsen I, Sharma P, *et al.* A decision support scheme for beta thalassemia and HbE carrier screening. *J Adv Res* 2020; 24:183-190.
2. Vitacolonna E, Succurro E, Lapolla A, Scavini M, Bonomo M, Di Cianni G, *et al.* Guidelines for the screening and diagnosis of gestational diabetes in Italy from 2010 to 2019: critical issues and the potential for improvement. *Acta Diabetol* 2019; 56(11):1159-1167.
3. Warghade S, Britto J, Haryan R, Dalvi T, Bendre R, Chheda P, *et al.* Prevalence of hemoglobin variants and hemoglobinopathies using cation-exchange high-performance liquid chromatography in central reference laboratory of India: A report of 65779 cases. *Lab Physicians* 2018; 10(01):073-079.
4. Raman S, Sahu N, Senapati U. A study of haemoglobinopathies and haemoglobin variants using high performance liquid chromatography (HPLC) in a teaching hospital of Odisha. *J Evol Med Dent Sci* 2017; 6(11):842-850.
5. Baig MA, Swamy KB, Baksh AD, Bahashwan A, Moshrif Y, Al Sawat A, *et al.* Evaluation of role of HPLC (Merits & Pitfalls), in the diagnosis of various hemoglobinopathies and thalassemic syndromes. *Indian J Pathol Microbiol* 2021; 64(3):518-523.
6. Livingstone FB, Marks J. Abnormal hemoglobins in human populations. Routledge, Taylor and Francis Group 2019. 1st edition.
7. Lama R, Yusof W, Shrestha TR, Hanafi S, Bhattarai M, Hassan R, *et al.* Prevalence and distribution of major β-thalassemia mutations and HbE/β-thalassemia variant in Nepalese ethnic groups. *Hematol/Oncol Stem Cell Ther* 2022; 15(1):279-284.
8. Saleh KK, Kakey ES. Some molecular characterization of β-thalassemia major in Koya City. *Int Conf Pure Appl Sci* 2018; 4: 64-68.
9. Westemeyer M, Saucier J, Wallace J, Prins SA, Shetty A, Malhotra M, *et al.* Clinical experience with carrier screening in a general population: support for a comprehensive pan-ethnic approach. *Genet Med* 2020; 22(8):1320-1328.
10. Old J, Harteveld CL, Traeger-Synodinos J, Petrou M, Angastiniotis M, Galanello R. Prevention of thalassaemias and other haemoglobin disorders: Volume 2: laboratory protocols. 2nd edition. Nicosia (Cyprus): Thalassaemia International Federation; 2012.
11. Colaco S, Colah R, Nadkarni A. Significance of borderline HbA2 levels in β thalassemia carrier screening. *Sci Rep* 2022; 12(1):5414.
12. Weatherall DJ, Clegg JB. The thalassaemia syndromes. 4th Edition. Oxford: Blackwell Science Ltd, 2001.
13. Ikawa Y, Miccio A, Magrin E, Kwiatkowski JL, Rivella S, Cavazzana M. Gene therapy of hemoglobinopathies: progress and future challenges. *Hum Mol Genet* 2019; 28(R1):R24-R30.
14. Trivedi DJ, Sagare A. Assessment of iron overload in homozygous and heterozygous beta thalassemic children below 5 years of age. *J Krishna Inst Med Sci* 2014; 3(2):17-22.
15. Panda A, Praveen B, Bisht SS. Clinical and pathological status of haemoglobinopathies among pregnant women in southern Orissa. *Indian J Biotechnol* 2009; 8(4):456-457.
16. Nigam N, Kushwaha R, Yadav G, Singh PK, Gupta N, Singh B, *et al.* A demographic prevalence of β thalassemia carrier and other hemoglobinopathies in adolescent of Tharu population. *J Family Med Prim Care* 2020; 9(8):4305–4310.
17. Sawaimul KD, Iqbal MB, Sawaimul VD, Kambale T, Hanmante R. Study to identify the role of high-performance liquid chromatography in detecting haemoglobinopathies in antenatal patients. *Indian J Pathol Oncol* 2018; 5(1):6-11.
18. Jandial R, Gupta I. Incidence of thalassaemia in Jammu and Kashmir, India. *J Lab Med* 2021; 10(1): PO11-PO13
19. Datkhile KD, Patil MN, Vavhal RD, Khamkar TS. The spectrum of β-globin gene mutations in thalassemia patients of south-western Maharashtra: A cross sectional study. *J Krishna Inst Med Sci* 2015; 4(3):49-58.
20. Mohanty SS, Parihar S, Huda RK, Toteja GS, Sharma AK. Prevalence of sickle cell anemia, β-thalassemia and glucose-6-phosphate dehydrogenase deficiency among the tribal population residing in the Aravali hills of Sirohi region of Rajasthan state. *Clin Epidemiology Glob Health* 2022; 13:1-4.
21. Patel CJ, Jadav PP, Marathe BJ. Molecular diagnosis of β-thalassemia in Indian population. *J Adv Sci Res* 2020; 11(4):38-45.
22. Yadav SS, Panchal P, Menon KC. Prevalence and management of β-thalassemia in India. *Hemoglobin* 2022; 46(1):27-32.

23. Sinha S, Dutta AK, Das MK, Bhattacharya P. The spectrum of rare and novel indel mutations responsible for β Thalassemia in eastern India. *Med Rxiv* 2022.
24. Vachhani NA, Vekariya DJ, Colah RB, Kashiyani HN, Nandani SL. Spectrum of β-thalassemia and other hemoglobinopathies in the Saurashtra region of Gujarat, India: Analysis of a large population screening program. *Hemoglobin* 2022; 46(5):285-289.
25. Gadhia PK, Vaniawala SN, Kachhadiya TB. Prevalence of beta thalassemia mutations in population of Gujarat using amplification refractory mutation system-polymerase chain reaction. *Int J Community Med Public Health* 2019; 6(8):3294-3297.
26. Singh VK, Prabhakar P, Belurkar S, Manohar C. Identification of delta-beta thalassemia in a family with elevated HbF: A case report. *J Krishna Inst Med Sci Univ* 2020; 9(2): 88-93.
27. Jaing TH, Chang TY, Chen SH, Lin CW, Wen YC, Chiu CC. Molecular genetics of β-thalassemia: A narrative review. *Medicine* 2021; 100(45):e27522.

Author for Correspondence:
Dr Navneet Kumar R Singh, Department of Paramedical Sciences, Sumandeep Vidyapeeth (Deemed to be University), Vadodara - 391760, Gujarat
Email: navneetspan@gmail.com Cell: 9898708184

How to cite this article:
Minal T, Hitesh S, Prakash P, Navneet S. Screening of haemoglobinopathies by high performance liquid chromatography and its molecular characterization using amplification refractory mutation system and direct DNA sequencing techniques among college students in central Gujarat. *J Krishna Inst Med Sci Univ* 2023; 12(2):20-34

Submitted: *02-Jan-2023* ***Accepted:*** *10-Mar-2023* ***Published:*** *01-Apr-2023*