Study of abnormal haemoglobin variants using cation exchange high performance liquid chromatography (HPLC) in paediatric population of Gujarat, India.

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Abstract

Introduction: Haemoglobinopathies particularly β thalassemia and sickle cell anemia are the most commonly encountered single gene disorders in the India; so as in Gujarat. The data pertaining to their occurrence and prevalence in the state of Gujarat are scarce and hence it was considered worthwhile to study the burden of haemoglobinopathies in Gujarat, India. The objective of current study is to find occurrence of haemoglobinopathies in paediatric population. **Materials and Methods:** Analysis of blood samples of 1560 cases referred to the pathology laboratory from paediatric department for the workup of anemia or other blood related disorders was done by Bio Rad D 10 Dual program HPLC instrument. **Results:** Of the 1560 paediatric cases, samples analyzed on Bio Rad D 10 Dual program HPLC for haemoglobinopathies, 1003 abnormal haemoglobin variants were detected in which maximum 652 (65%) as sickle cell trait, 174 (17.3%) as sickle cell disease, 139 (13.8%) were diagnosed as β-thalassemia trait, 34 (3.3%) were diagnosed as S-β double heterozygous, 02 (0.19%) as β-thalassemia major and 01 (0.09%) as HbE trait, 01 (0.09%) as HbD trait. **Conclusion:** The prevalence of haemoglobinopathies among children is more commonly seen in countries with limited resources, where priority tends to be given to tackling infant and child mortality from infections and malnutrition. This study indicates that almost all the common haemoglobinopathies are prevalent in Gujarat but sickle cell trait/anemia and β thalassemia are very common.

Keywords: Abnormal haemoglobin variants, Gujarat, High performance liquid chromatography (HPLC), Paediatric population.

Introduction

Abnormal haemoglobin variants or haemoglobinopathies are the most common genetically inherited disorders. Every year, there are over 42 million carriers and more than 12,000 infants born with a major and clinically significant haemoglobinopathy. In India, the cumulative gene frequency of haemoglobinopathies is around 4.2% [1].

The cost of optimal management for a child with thalassemia including regular blood transfusions, chelation therapy, investigations, hospitalization and specific vaccines which is not an affordable amount by most Indian families. The result is irregular transfusions without chelation therapy, leading to complications of iron overload and early death. Patients with sickle cell disease do not require transfusions but are disabled due to severe pain as a result of vaso occlusive episodes and susceptibility to infections. They require antibiotic prophylaxis, specific immunizations and frequent hospitalization [2].

Haemoglobinopathies form a significant proportion of hereditary disorders in paediatric population leading to a range of myriad complication, leading to mortality in large number of afflicted patients. Published literature includes various reports on screening patient using HPLC in adults as well as paediatric population. However, there is paucity of literature on studies on exclusive paediatric population. The main objective of the study was to know the prevalence of abnormal haemoglobin variants in paediatric population in the state of Gujarat, India and to review various strategies that could be implemented for the effective control and prevention of these disorder

Materials and Methods

Setting: 1560 anemic paediatric cases (6 months to 14 years of age).

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Duration of Study: One year (September 2018 to September 2019).
Type of Study: Hopsital based retrospective study.

Sampling method: After consent from parents, 2 ml of venous blood collected in EDTA (Ethylene diamine tetra acetic acid) coated vaccutainers from each patient were taken. The haematological profile of cases was done, which included peripheral smear examination, Complete Blood Count including RBC indices, reticulocyte count etc. With the help of Bio Rad D 10 Dual program HPLC exact percentage of HbS, HbF, HbA_2 and HbA was estimated to classify the cases.

Sample size calculation: 1560 random paediatric cases having clinical manisfestations like anemia, mild hepatosplenomegaly, weakness, repeated infections, aches and pain, fever along with family history were calculated.

Inclusion criteria

1. Patients with haemoglobin up to 11 gm% using machine Sysmex KX-21.
2. Paediatric age group.
3. Detection of sickle shaped cells on peripheral smear.
4. Positive sickling solubility test.
5. History of more than two blood transfusion in absence of trauma or any clinical morbidity.

Exclusion criteria: Adult age group

Data collection procedure: Paediatric patients taken from Outdoor Patients Department (OPD) and Indoor patient department (IPD).

Data analysis: done in tabular form and graphical presentation.

Ethical consideration & permission: Signed informed consent was obtained from the parents of study subjects. The study was done according to the rules of the Ethics Committee.

Scoring system: None
Surgical procedure: None

The D 10 Dual program is based on chromatographic separation of the analytes by ion-exchange high performance liquid chromatography (HPLC). The whole blood EDTA samples are automatically diluted on the D 10 and injected into analytical cartridge. It delivers a programmed buffer gradient of increasing ionic strength to the cartridge, where the haemoglobins are separated based on their ionic interactions with the cartridge material. The separated haemoglobins then pass through the flow cell of the filter photometer, where changes in the absorbance at 415 nm are measured [3].

Table 1: Proportion of different haemoglobins in normal individuals and in haemoglobin disorders according to area percent:[4].

CONDITION	HbA	HbF	HbA_2	HbS
Normal Newborn	25%	75%	<1%	0
Sickle cell trait	56-60%	0	1-3%	40%
Sickle cell anemia	0	5-10%	1-3%	90-95%
β thalassemia trait	90-95%	0-5%	4-7%	0
β thalassemia major	0	95-98%	2-5%	0

Table 2: Proportion of different haemoglobins in normal individuals according to retention time in minutes:[5].

Peak Name	Retention Time (minutes)
HbA	1.55-1.85
HbF	0.38-0.58
HbA_2	2.80-3.50
S window	4.02-4.30

Results

Of 1560 patients screened for haemoglobinopathies, 898 (58%) were males and 662 (42%) were females (Figure 1). Out of 1560 patients, 1003 (64.2%) variant haemoglobinopathies were diagnosed (Table 3).

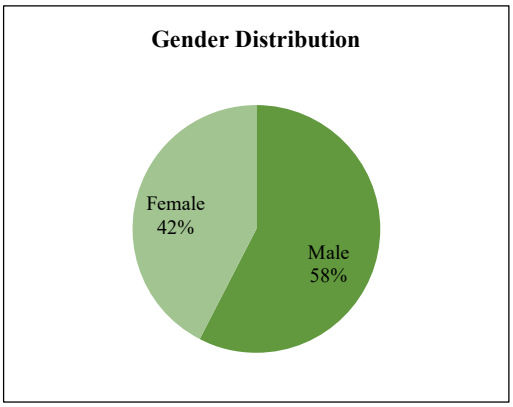

Fig-1: Gender distribution.

Presumptive identification of haemoglobin variants was made primarily by retention time (RT) windows and area percent. Of 1003 variant haemoglobinopathies detected, 543 (54.1%) belonged to the 0-5-year age group, followed by 104 (10.36%) to the 6-10-year age group and 356 (35.4%) to the 11-14-year age group (Table 4). Of the 1560 patients screened for haemoglobinopathies, pallor (anemia) was the most common clinical feature seen in 884 (56.6 %) patients, followed by weakness in 512 (32.8%) cases, other presenting complaints include fever, hepatomegaly, splenomegaly, jaundice, growth stunting, repeated blood transfusions, failure repeated blood transfusions, failure to thrive in infants, convulsion and a positive family history.

Table-3: Distribution of abnormal haemoglobin variants in Gujarat.

Abnormal Haemoglobin Variants	Number of patients	%
Sickle cell trait	652	65.0
Sickle cell disease	174	17.5
β thalassemia trait	139	13.8
S-β double heterozygous	34	3.38
Thalassemia major	2	0.19
HbD Trait	1	0.09
HbE Trait	1	0.09
Grand Total	**1003**	**100**

Table 4: HPLC interpretation according to age.

Age	HPLC INTERPRETATION								
	Beta Thal Trait	Thal Major	HbD Trait	HbE Trait	HbS	HbS Trait	S-Beta Thal	Normal	Grand Total
0-5 years	57	0	0	0	123	312	51	232	775
6-10 years	34	1	0	0	11	54	4	133	237
11-14 years	118	0	1	1	46	177	13	192	548
Grand Total	**209**	**1**	**1**	**1**	**180**	**543**	**68**	**557**	**1560**

Of the 1003 paediatric cases with variant haemoglobins, 759 (75.6%) parents have history of consanguineous marriage. Majority of the cases with abnormal haemoglobin variants have microcytic hypochromic picture 758 (75.5%) and 245 (24.4%) have normocytic normochromic picture (Table 5).

Of the 1560 paediatric cases, 557 shows normal HPLC pattern while 1003 cases shows abnormal haemoglobin variant 652 (65 %) as sickle cell trait, 174 (17.3 %) as sickle cell disease, 139 (13.8%) were diagnosed as β thalassemia trait, 34 (3.3 %) were diagnosed as S-β double heterozygous, 02 (0.19 %) as β-thalassemia major and 01 (0.09 %) as HbE trait, 01 (0.09 %) as HbD trait (Table 3).

Table-5: Peripheral smear findings.

Peripheral smear findings	No of patients	%
Microcytic Hypochromic (MCHC)	758	75.5
Normocytic Normochromic (NCNC)	245	24.5
Grand total	**1003**	**100**

Discussion

A study carried out by Christianson et al [6] estimate the prevalence of pathological haemoglobinopathies in India being 1.2/1000 live births. This suggests the annual birth of 32,400 babies with a serious Haemoglobin disorder.

These facts compel us in employing newer techniques for early detection, prevention, and treatment of hemoglobinopathies. Cation exchange HPLC is emerging as one of the best methods for screening and detection of various haemoglobinopathies with rapid, reproducible and precise results [7].

It has the advantage of quantifying HbF and HbA_2 along with haemoglobin variant screening in single and highly reproducible system. The simplicity of the automated system with internal sample preparation, superior resolution, rapid assay time and accurate quantification of haemoglobin fractions makes this an ideal methodology for routine clinical laboratory [8].

In this study, out of 1560 patients 1003 (52.67%) variant haemoglobinopathies cases diagnosed on Bio Rad D10 Dual program HPLC. In 2014 Mauchumisakia Pathak et al [9] studied 800 anaemic paediatric patients and found 522 (65.25%) variant haemoglobinopathy patients. In this study, majority cases of haemoglobinopathies were found in the age group of 0–5 year. Similar results were seen in the study by S.S Ambekar et al (2001) [10].

Out of 1560 cases of variant haemoglobinopathies detected, 898 (57.5%) were males and 662 (42.4%) were females and found comparable with Dr. Mauchumisaikia Pathak et al [9] in 2014 that is 522 cases, 268 (51.34%) were males and 254 (48.66%) were females. Endogamy and consanguinity are a common practice in the Indian subcontinent and it poses a major risk factor for the homozygous inheritance of haemoglobinopathies. Of the 1003 cases with variant haemoglobins, 759 (75.6 %) parents have history of consanguineous marriage. The study done by Shivashankara A.R et al [11] in 2008, J Sana et al [12] in 2008 and Colah et al [13] in 2010 reported 20%, 24.3% and 21% consanguinity rate in haemoglobinopathy cases. Majority of the cases in the present study belonged to tribal and low socioeconomic communities, where in the tradition of consanguineous marriages is common, thus the reason of higher percentage of consanguinity in the present study.

T. sahu et al [14] in 2003, Shah Sejal et al [15] in 2012, Dr. Mauchumisaikia Pathak et al [9] in 2014 found pallor (anemia) as most common presenting complaint in their studies which is comparable to the present study in which the most common presenting complaint was pallor in the afflicted (56.6%) cases. A patient with sickle cell disease or trait has normochromic normocytic anemia but majority of the patients in this study had hypochromic microcytic anemia (75.5 %) [16].

This could be due to associated iron deficiency or α-thalassaemia trait. High incidence of iron deficiency has been reported in patients with sickle cell disease from India.[17] On BIO-RAD D-10 HPLC, in thalassemia major group the average HbF levels were the high as compared to the other groups and it was (88.30±11.92). In 2011 C.Vani et al [18] reported HbF 88%. Elevated HbF levels were encountered in double heterozygous states of Sβ thal and Eβ thal patients. The cut off value of HbA_2 >4.0 %, was used to diagnose thalassemia trait, after exclusion of the other causes of increase HbA_2.

In the present study in thalassemia trait patients the average HbA_2 level was found to be (4.34±0.87**).** In 1993 G.B.Tan et al [19] reported 4.6% HbA_2 in thalassemia trait**.** The mean HbS levels in sickle cell disease patients was found

73.77±10.31, in sickle cell trait 33.81±6.32 and in S β thalassemia patients was 42.74±16.94.

The most common haemoglobinopathy detected was 652 (65%) as sickle cell trait, 174 (17.3 %) as sickle cell disease, 139 (13.8%) were diagnosed as β-thalassemia trait, 34 (3.3%) were diagnosed as S-β double heterozygous, 02 (0.19 %) as β-thalassemia major and 01 (0.09 %) as HbE trait, 01 (0.09%) as HbD trait. S.S. Ambekar et al in 2001 [10] also got very low percentage of thalassemia trait (0.5%). The reason may be because thalassemia trait patients are asymptomatic.

The frequency of sickle cell disease is 18.7 % in our data. The average frequency in India is 4.3% [20]. Timely detection of sickle cell trait can be helpful in warning patients of the possible complications and the preventive measures to be taken. Prenatal or early postnatal diagnosis of sickle cell disease helps in prompt therapy before the onset of serious complications of the disease [21].

HbE disease is most frequently found in Eastern and far Eastern parts of India [22,23]. HbE and HbD is not very common is Gujarat [24]. The incidence of HbE (0.09%) and HbD (0.09%) was very low in this study. These cases could have avoided only by doing premarital screening test of pre conceptional diagnostic tests.

The comprehensive data thus obtained can help us formulate, develop and shape infrastructure and policies for afflicted children care and provide impetus for research in the development of advanced techniques, newer drugs and diagnostic modalities. Likewise, newborn screening is primarily useful to identify infants with sickle cell disease so that early intervention with prophylactic penicillin can ameliorate complications and comprehensive care can reduce morbidity and mortality [25].

Effective prevention approaches to thalassaemia have now been demonstrated in many countries with diverse carrier screening programmes. For example, in Cyprus, Greece, the Islamic Republic of Iran and Italy, premarital screening for thalassemia is standard practice and national audit data are available; most at-risk couples are identified in time to be offered early diagnosis for the first pregnancy [26,27]. The majority of such couples use this service and produce healthy offspring.

Out of 1560 paediatric cases in the present study, 1003 (64.2%) cases showed some abnormalities in hemoglobin by HPLC. Detail investigation of anemia keeping in mind the possibilities of detecting abnormal haemoglobin is very much helpful in finding out more carriers of different hemoglobinopathies. Detection of other variants becomes important due to complex interactions in cases with double heterozygous and homozygous states, which may lead to severe hematological abnormalities. CE-HPLC findings must be supplemented by hemogram, family/sibling studies, and molecular studies. Combined approach of primary and secondary prevention needs to be followed. It will prove to be cost effective by preventing the birth of child with genetic homozygous inheritance disease.

Conclusion

Thus, haemoglobinopathies exert significant burden on India, especially in the western part of the country specially Gujarat. Screening is affordable and an accessible way to detect carriers and can be offered in a range of settings in different societies: in high school, before marriage, or in antenatal clinics.

What the study adds to the existing knowledge?

Haemoglobinopathies and thalassemia are public health problem in this region of India, emphasizing the need for neonatal screening and genetic counselling programs. The HPLC based Haemoglobin testing system forms a rapid and easy tool early detection and management of various haemoglobin disorders.

Nationwide Government sponsored programme can effectively reduce the occurrence of new cases of serious haemoglobin variants as well as thalassaemia major cases and thus making it possible to direct the available resources towards the optimization of treatment of the patients who are already present. Detection of these patients with abnormal haemoglobins will help in prevention of more serious haemoglobin variant cases

Author's contribution

Dr. Shubhi Saxena: Concept, Design, Definition of intellectual content, Literature search, Manuscript preparation
Dr. Richa Jain: Experimental studies, Data acquisition, Data analysis, Statistical analysis, Manuscript preparation

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Ethical Approval: This study was approved by the Institutional Ethics Committee

Abbreviations

HPLC: High performance liquid chromatography, **CE-HPLC:** Cation exchange high performance liquid chromatography, **HbF:** Foetal haemoglobin, **HbA:** Adult haemoglobin, **MCHC:** Microcytic hypochromic, **NCNC:** Normocytic normochromic

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