VOLUME - 11, ISSUE - 08, AUGUST - 2022 • PRINT ISSN No. 2277 - 8160 • DOI : 10.36106/gjra

Original Research Paper

Pathology

SCREENING OF HAEMOGLOBINOPATHIES BY HPLC (HIGH PERFORMANCE LIQUID CHROMATOGRAPHY) METHOD IN NEWBORNS.

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ABSTRACT

Background: Haemoglobinopathies place a large burden on the patients, their families, and even on their communities. They are generally not curable but can be prevented by population screening and genetic counselling. Early initiation of supportive care for infants with hemoglobinopathies such as sickle cell disease and thalassemia have been shown to decrease mortality and morbidity. **Aims:** To assess the pattern of Haemoglobinopathies by HPLC (High Performance Liquid Chromatography) method in New-borns in a tertiary care centre and to classify them into Sickle cell disorder and other hemoglobinothies. Settings and design: Observational cross-sectional study. **Material And Methods:** Total 581 samples of every full term newborn's samples for routine hematological investigation were taken into account for the screening for complete blood count, peripheral smear and HPLC. Pattern of haemoglobins on Newborn screening by HPLC was interpreted according to NBS criteria. Statistical analysis was done using MedCalc software. **Results:** A total of 581 cases were studied. Out of these, 577 cases displayed normal "FA" pattern of chromatogram on HPLC. Four cases displayed abnormal hemoglobin fractions on HPLC. The major abnormality observed was S-window in all four cases. Parents of Positive cases were also screened by HPLC. Three out of four mothers showed sickle cell trait; and one patient's mother and father; both showed sickle cell trait. **Conclusion:** We concluded that neonates with or without any positive history of haemoglobinopathies should be screened at birth as we found 0.69% frequency of SCA in screening done in normal population.

KEYWORDS : Newborn screening (NBS), Hemoglobinopathies, Sickle cell disease, Thalassaemia

INTRODUCTION:

Among the inherited disorders of blood, hemoglobinopathies and thalassaemias constitute a major bulk of non-communicable genetic diseases in India.[1] Newborn screening is mainly recommended for sickle cell disorders among those tribal and nontribal populations where the prevalence of HbS is high. Ideally, universal screening should be done where all newborn babies in these high risk groups are screened as this would allow identification of other clinically significant disorders such as homozygous β-thalassemia and all cases of Hb S–β thalassemia. Babies with sickle cell disease (SCD) or sickle β-thalassemia should be followed-up every 3 months clinically and timely penicillin prophylaxis and pneumococcal vaccination should be given.[2]

Sickle cell anemia is predominantly seen in tribal population of India. Gujarat has 89.12 lakh tribal population and expected to have 9 lakh trait and 70,000 diseased patients. Major tribes presenting with sickle cell anemia in Gujarat are Dhodhia, Dubla, Gavit, Chaudhary, Tadvi, Rathod, Kolcha, Bariya.[3]

There has been recent interest from the Indian government to further expand sickle cell disease control measures in endemic areas. The state of Gujarat was the first state in India to implement a Sickle Cell Anemia Control Programme in 2006.[4]

Prevention of complications is important step in reduction of morbidity and mortality, so for this early diagnosis of sickling status from newborn period is required to implement various preventive strategies like – special vaccination (Pneumococcal, H.Influenza, Typhoid), penicillin prophylaxis, early detection and treatment of anemia, folic acid supplementation, hydroxyurea therapy initiation, growth and development assessment.[5]

The prevalence of β-thalassemia carriers in the Indian population is 3-4%. Some ethnic groups like Sindhis, Kutchis, Lohanas, Punjabis, few Muslim groups as well as few tribal populations have a higher prevalence (5-17%).[6]

The overall prevalence of α-thalassemia carriers (single α gene deletion) is around 13% but varies from 3% to 18% in the caste populations, however, it is very high in some tribal groups reaching over 90% in some groups. Hb H disease is uncommon.[6]

Patient and family history, clinical evaluation, and the origin of the family should be recorded. Full blood count (FBC) on an automated/semi automated hematology analyzer as well as a well stained peripheral blood smear should first be assessed. In cases with microcytosis, iron deficiency anemia and anemia of chronic disease should be ruled out and specific investigations for thalassemia and other hemoglobinopathies considered. The method of choice for Hb analysis is

automated cation exchange HPLC. This gives an accurate estimate of HbA_2, Hb F, and identifies and quantifies the common variant Hbs like Hb E, Hb S, and Hb D^{Punjab}. Hb H is not identified but may give a sharp spike at the start of the chromatogram before 1 min.[6]

The Aim of the present study was to assess the pattern of Haemoglobinopathies by HPLC (High Performance Liquid Chromatography) method in New-borns recorded in a tertiary care centre and to classify them into Sickle cell disorder and other hemoglobinothies.

MATERIAL AND METHODS:

This study was carried out as an observational cross sectional study.

It was a "Time bound study" of Screening of Newborns by HPLC done over a period of 10 months; December 2018 to September 2019 after approval of Institutional Ethics Committee for Human Research(IECHR) in a tertiary care hospital. Total 581 samples of every full term newborn's samples coming to the Central Clinical laboratory of the Department of Pathology for routine hematological investigation were taken into account for the screening.

Detailed history of the subjects including personal data and family history was taken from Department of Pediatrics.

Inclusion Criteria:

All full term Newborns' whole blood samples collected within seven days after birth; received for the routine haematological investigation in Central Clinical Laboratory, Department of Pathology were analyzed.

Exclusion Criteria:

Samples from Preterm newborns, Inadequate sample, Clotted sample, Hemolysed sample, Post-blood transfusion sample, Improper labelling and samples of newborn with congenital disorder were excluded.

Received samples were subjected for screening work up which involves complete blood count, along with HPLC for Haemoglobin variants analysis.

Complete blood count was done by 5 part automated cell counter (HORIBA Pentra XLR) and peripheral smear examination.

Then the blood samples were subjected further for, High Performance Liquid Chromatography (HPLC) on Bio-rad VARIANT II HPLC device.

Principle:

HPLC depends on the interchange of charged groups on the ion exchange material with charged groups on the hemoglobin molecules. A typical column packing is 5□m spherical silica gel. The surface of the support is modified by carboxyl groups to have a weakly cationic charge, which allows the separation of the hemoglobin molecules with different charges by ion exchange. When a hemolysate containing a mixture of hemoglobins is adsorbed onto the resin, the rate of elution of different hemoglobins is determined by the pH and ionic strength of any buffer applied to the column.

Elution of the charged molecules is achieved by continually changing salt gradient; fractions are detected as they pass through an ultraviolet/visible light detector and are recorded on an integrating computer system. Analysis of the area under these absorption peaks gives the percentage of the fraction detected. The time of elution (retention time) of any normal or variant hemoglobin present is compared with that of known hemoglobin, providing quantification of both normal hemoglobins (A, F and A_2) and many variants.

Pattern of haemoglobins on Newborn screening by HPLC (High Performance Liquid Chromatography) and their Interpretations are as given below.[7] The results were interpreted according to this criteria.

Table 1: Patterns Of Hemoglobins On Newborn Screening And Their Interpretations

New born screening Pattern	Interpretation	Recommendation
FA	Normal	None
F only	Premature infant â thalassemia major	Repeat testing, if persistent only F, needs education, genetic counseling, confirmatory testing and hematology referral
AF	Likely post-blood transfusion	Repeat testing 3-4 months after last transfusion
FS	SCD (HbSS) Sickle â thalassemia Sickle HPFH	Education, genetic counseling, confirmatory testing, and hematology referral
FSA	Sickle â thalassemia SCD with transfusion	Education, genetic counseling, confirmatory testing, and hematology referral. Repeat testing in 3-4 months if transfused.
FAS	Sickle cell trait Sickle â thalassemia	Education and genetic counseling. Repeat testing at 3 months of age – rule out sickle â thalassemia
FSC	Hemoglobin SC disease	Education, genetic counseling, confirmatory testing, and hematology referral
FC	Hemoglobin C disease Hemoglobin C â thalassemia	Education, genetic counseling, confirmatory testing, and hematology referral
FE	Hemoglobin E disease Hemoglobin E â thalassemia	Education, genetic counseling, confirmatory testing, and hematology referral
FA + variant (FAE, FAC, FAD)	Hemoglobin variant trait	Education and genetic counseling
FA Bart's	Silent á thalassemia carrier á thalassemia trait HbH disease HbH Constant Spring disease	If Bart's < 10%, patient will need education and genetic counseling If Bart's > 10%, patient will need further testing for evaluation of HbH disease and hematology referral

Statistical analyses were done by using unpaired Student's t-test to find out significance of difference between two groups. Interpretation was done according to p-values as follows:

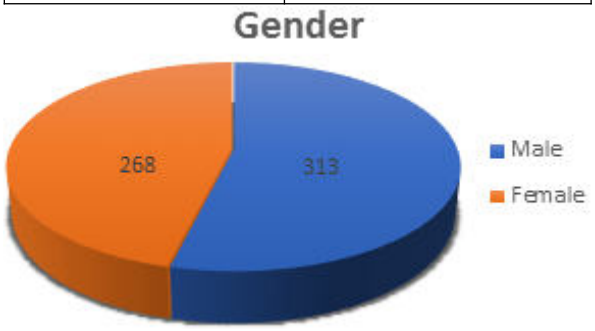
$P < 0.05$ was considered significant
$P < 0.001$ was considered highly significant
$P \geq 0.05$ was considered not significant
Statistical analysis was done using MedCalc software.

RESULTS:

The observations made with respect to various aspects of the study are as follows:

Table-2: Gender Distribution (n=581)

Gender	Number of Subjects(Total 581)
Males	313
Females	268

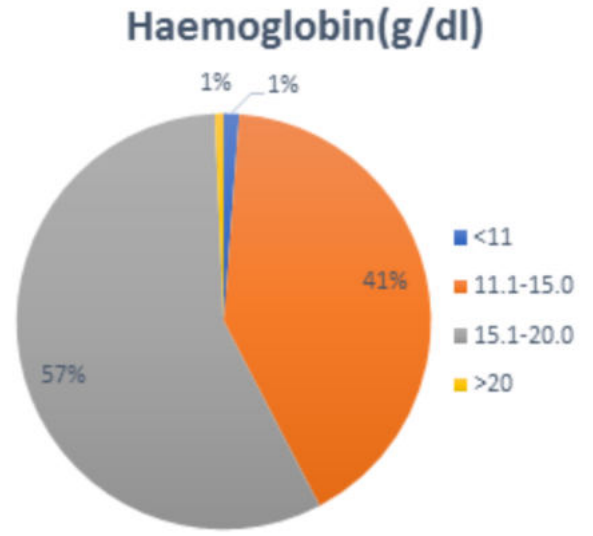


Graph-2: Gender Distribution (n=581)

Table-2 and Graph-2 show that there were 313 males and 268 females participants involved in this study.

Table-3: Haemoglobin Analysis

Hemoglobin values(g/dl)	Individuals(n=581)
<11	7
11.1-15.0	239
15.1-20.0	331
>20	4



Graph-3: Haemoglobin Analysis

Table-2 and Graph-2 show that there were 7 individuals having haemoglobin values less <11g/dl, 239 individuals were having hemoglobin values between 11.1-15.0 g/dl, 331 had haemoglobin values between 15.1-20.0g/dl and 4 were having values > 20.1 g/dl.

Table-4: Complete Blood Count Analysis

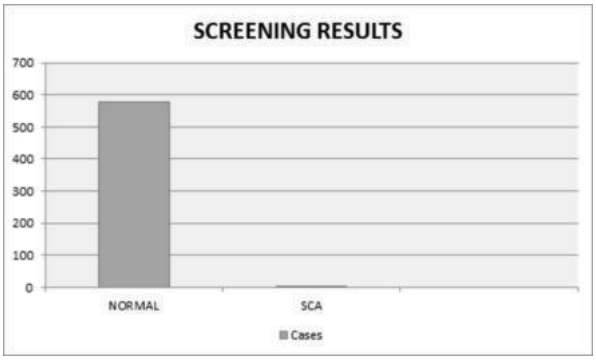
Group		Normal		SCA		P-value
No. of patients		577		4		
Parameter	Reference Range	Mean	SD	Mean	SD	
Hb g/dl	14-22	15.49	2.20	13.28	0.64	0.0444
RBC Mili/ cmm	3.5-6.6	4.07	0.76	4.06	0.95	0.9598
PCV %	35-50	44.61	4.60	49.15	3.14	0.0489
MCV fL	92-118	98.76	9.33	94	5.72	0.3082
MCH Pg	31-37	34.97	3.79	36.3	1.66	0.4827
MCHC %	30-38	36.30	2.44	33.30	1.16	0.0144
RDW %	15.5-20	17.98	1.67	17.20	1.06	0.3506

In the present study, no statistically significant difference was found in RBC, MCV, MCH and RDW values between Normal and SCA Group ($p>0.05$).

Statistically significant difference was found in Hb, PCV and MCHC values between Normal and SCA Group ($p<0.05$) having mean ± SD 15.49 ± 2.20, 44.61 ± 4.6 and 36.30 ± 2.44 respectively for Normal Group and 13.28 ± 0.64, 49.15 ± 3.14 and 33.30 ± 1.16 for SCA Group respectively.

Mean of hemoglobin F and hemoglobin A was also calculated. The mean value for hemoglobin F in normal group was 81.11% and for SCA group it was 70.37%. Mean value for hemoglobin A for normal people was 16.71% and SCA group it was19.85%.

A total of 581 cases were studied. Out of these, 577 cases displayed normal "FA" pattern of chromatogram on HPLC. 4 cases displayed abnormal hemoglobin fractions on HPLC. The major abnormality observed was S-window in all four cases.



Graph-4 Screening Result of 581 Cases

The positive cases were identified and as the HPLC findings of this cases showed F>A>S, which give us presumptive diagnosis of either sickle cell trait or Sickle β-thalassemia.

Parents of Positive cases were also screened by HPLC. Three out of four mothers showed sickle cell trait; and one patient's mother and father; both showed sickle cell trait.

Parents of Positive cases were educated about the disease, and instructed to go for Confirmatory Test after three months to rule out Sickle β- thalassemia. They were also made aware about other management protocols.

DISCUSSION:

The initial HPLC- based newborn screening program is highly sensitive in detecting clinically significant hemoglobin disorders. Unlike other hemoglobinopathies screening programs, HPLC is exceptional in that it applies both quantitative capabilities and extreme sensitivity and specificity[8,9,10,11]

Cation exchange HPLC offers a reliable tool for early, accurate detection thereby aiding in prevention and management of various hemoglobinopathies.[2]

In our study, we screened the newborn blood samples by Bio-Rad Variant II HPLC method. Generally, hemoglobin identified by neonatal screening is reported as a percentage. At birth, HbF is the more predominant than HbA. Hence, most normal infants show HbFA pattern. Even in most of the patients with hemoglobinopathies, HbF is still the predominant hemoglobin at birth.

On NBS, hemoglobin concentration is reported in decreasing order. For example, a report of "FAS" on NBS indicates that in the given sample the quantity of HbF is greater than that of HbA, and the quantity of HbA is greater than that of HbS.[7]

In present study out of 581 cases, 4 cases showed abnormal hemoglobin fractions on HPLC and 577 cases showed normal "FA" pattern of chromatogram on HPLC. The major abnormality observed was S-window in all four cases. The HPLC findings of these cases showed F>A>S pattern, which gave us provisional diagnosis of either sickle cell trait or Sickle β-thalassemia.

Parents of Positive cases were also screened by HPLC. Three out of four mothers showed sickle cell trait; and one patient's mother and father; both showed sickle cell trait. These parents were counselled about the disease, and Confirmatory Test after three months to rule out Sickle β- thalassemia. They were advised about further management of the child.

Panigrahi et al. did similar type of study using Bio-Rad Hemoglobin Variant II, where 1158 babies were screened for sickle cell anaemia in a hospital in Chhattisgarh; found out that 0.4% had SCD, 5.26 % has sickle cell trait and 0.08% were double heterozygous for Sickle and beta-thalassemia.[12]

In a similar study by Jain DL et al.; newborns of 1178 sickle positive mothers were screened by HPLC; 536 babies were reported as Sickle heterozygous and 88 were reported as Sickle homozygous.[13]Which indicates that doing screening of newborns of sickle positive mothers than doing the normal screening on all the newborns is more effective. Even in our study we also found out that all the positive babies' mothers tested as positive for of sickle cell trait.

In a similar study, Shafer FE et al; California's NBS (New Born screening) program for hemoglobinopathies includes a complex follow-up strategy that employs regional nurses to track positive results and ensure timely enrollment of infants into treatment systems. In a 4-year review, 97.6% of infants with positive initial results were successfully recalled for confirmatory testing.[10]

Study of Yazdi Italia et al screened total of 5467 newborn babies using High-performance liquid chromatography, with diagnosis by molecular analysis. Thirty-three babies (0.60%) were sickle homozygous, 13 (0.23%) were-sickle-b-thalassaemia, 687 (12.5%) were sickle heterozygous, and 4736 were unaffected.[14]

Such established newborn screening programs have not been initiated in India although the load of sickle cell disease is very high in Central India. First open initial pilot airport was made in the Raipur district in Chhattisgarh in India, on screening few babies (1158) however the affected babies where not followed up.[12] Screening of sickle cell disease in newborn was undertaken in Kalahandi district in Orissa in Eastern India which has a large tribal population. 13 out of 761 newborn screening homozygous for sickle cell disease study show the feasibility of undertaking newborn screening in district hospital which help to identify hotspot area of sickle cell disease in this region.[15]

In our study, we also found that HbS is more prevalent in people coming from the tribal areas near Vadodara, where tribes like Bariya, Parmar, Vasava, Tadvi and others are coming to Vadodara hospital for treatment.

In India, Hb S has been detected in more than 50 distinct subgroups predominantly tribals. In one similar study by Brahme et al in Gujarat, showed prevalence of HbS in tribes like Bariya, Parmar, Solanki, ,Vasava, Tadvi and others.[16]

Study by Colah et al showed that sickle gene is prevalent in the tribal populations of India who are considered the original inhabitants living mainly in rural area and in some non-tribal populations groups like the scheduled castes and other backward classes belonging to a low socio-economic status.[6] Earlier report on newborn screening for sickle cell disease by Jain D et al, in Central India showed a very high birth rate of sickle cell anemia babies (1.1%) with the highest incidence in the Mahar community (2%)[13]

Earlier studies by Mukherjee MB et al from Western India had shown that disease is more severe in the non-tribal populations in Maharashtra then in tribal groups of Gujrat[17]

More recent retrospective analysis of sickle cell disease in children from Central India has shown that in some cases the disease can be as severe as in the African cohorts.[18]

Neonatal screening helps to recognize the early signs of the Sickle cell disease and minimize morbidity and mortality by early comprehensive care profile active treatment.[19]

Studies on the natural history of sickle cell diseases show that the greatest morbidity and mortality occurs between 6 and 12 months and that early identification of affected infants by neonatal screening, careful follow up coupled with relatively simple measures decrease the mortality rate.[19]

In infants, increased susceptibility to pneumococcal infection, dactilitis and splenic sequestration are common in Sickle cell anaemia.[20.21]

Certain clinical abnormalities have been identified in patients with sickle cell traits. Urine concentration defect due to microscopic infarction of the renal medulla is one the most common abnormalities found in Sickle cell trait.[21]

Starting in 2000, universal childhood vaccination with a 7-valent pneumococcal conjugate vaccine beginning at 2 months of age has resulted in a sharp decline in invasive pneumococcal disease in all children, including those with Sickle cell anemia.[22]

Furthermore, nearly 90% of infants diagnosed with SCD began prophylactic penicillin therapy by age 24 weeks [10]

Studies have found that many primary care physicians are not prepared to manage follow up care of children with positive newborn screen.[23]

In the Jamaican sickle-cell cohort study 40% of the children died in the first two years of life when early age interventions when not implemented compared to less than 1% when the preventive strategies where available. Neonatal hemoglobinopathy screening of 1917 83 newborns in Belgium identified 123 babies with sickle cell disease of someone died of septicemia when profile active treatment was interrupted.[24] In another study on newborn screening among tribal population in South Gujarat in Western India, showed 21.8 percent of the sickle cell disease babies having CVR clinical complications before the 5 years of age.[14]

In our study we educated the parents of positive cases for future confirmatory testing and further management protocols.

There is a limited data on the comprehensive care delivered on complications or ,long term outcomes of newborns diagnosed with haemoglobinopathies in India. Future projects must include the establishment of centers which provide comprehensive care and capture data on long term outcome of newborns diagnosed with screenable conditions.

Conclusions drawn from the present study are as under:
We concluded that neonates with or without any positive history of haemoglobinopathies should be screened at birth as we found 0.69% frequency of SCA in screening done in normal population.

Early initiation of supportive care for neonates and infants with haemoglobinopathies have been shown to decrease mortality and morbidity. Hence accurate interpretation of newborn screening test is very much important. Once a hemoglobinopathy is suspected, patient should be referred to haematologist at the early stages of the disease which can be very much beneficial for their lives.

The following points can be taken care of in positive cases after screening of NBS.
1. Morbidity prevention for the affected newborns;
2. Retrospective primary prevention for the parents who are at risk and had an affected child;
3. Prospective primary prevention for those couples who are at risk and had a carrier child;
4. Health gain in the treatment of an anaemic carrier and accident prevention particularly during anaesthesia for HbS carriers
5. Long term information for healthy carriers for future partner testing and reproductive choices.

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