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STUDY ON RELIABILITY OF SCREENING TESTS AGAINST HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR DETECTION OF SICKLE CELL DISEASE ALONG WITH ANALYSIS OF HEMOGRAM PARAMETERS AT A TERTIARY CARE HOSPITAL IN WESTERN INDIA

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Abstract

Background: Sickle cell disease (SCD) and its variants are genetic disorders are due to presence of an abnormal form of haemoglobin, haemoglobin S (HBS). The role of Laboratory in the Diagnosis of Haemoglobinopathies is very important. Peripheral blood film method, sickling and sickle solubility tests are used as screening method of sickle cell disease. While High Performance Liquid Chromatography (HPLC) is confirmatory test.**Aim of the study:** This study was done to evaluate various types of haemoglobins and their relative percentage in sickle cell anaemia cases. Besides this, we analysed haemogram parameters such as haemoglobin (Hb), WBC, Platelets etc. Along with this we determined reliability of various screening test against HPLC technique.**Method:** We analysed blood samples from 95 patients suspected to have sickle cell haemoglobinopathies and all were tested for sickling screening test. All positive cases will be subjected to HPLC to separate constituent haemoglobins and complete blood count (CBC) analysis was done to check haemogram parameters.**Results:** In sickle cell trait (SCT) patients, there is a significantly higher level of HbA2 and sickle haemoglobin (HbS) and significantly lower level of adult haemoglobin (HbA) observed. In sickle cell disease patients, there were significantly higher levels of HbA2, HbF and HbS and significantly lower levels of HbA observed. Both sickle cell trait and sickle cell disease patients had significantly lower levels of haemoglobin and platelet, but sickle cell disease patient has high white blood cell (WBC) count, which is almost normal in sickle cell trait

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patients.**Conclusion:** While analysing HPLC patterns, presence of HbS, low levels of HbA and high levels of HbF and HbA2 should raise a suspicion for presence of sickle cell haemoglobinopathy. The sickling test is the most reliable, cheapest and easiest to perform; it has high specificity and sensitivity. The solubility test is found expensive, cumbersome and unreliable for sickle cell screening, it had low sensitivity. Therefore sickling test is most recommended test for screening children for sickle cell disease using HPLC as confirmatory method.

Key Words: HPLC, sickle cell disease, sickling test, sickle solubility test.

Introduction

Sickle cell disease (SCD) is one of the most common genetic disorders globally with an autosomal recessive inheritance. First described by James Herrick, a physician, characteristic sickle shaped red cells in a medical student from Grenada in 1910. Sickle haemoglobin (HbS) had an altered electrophoretic mobility and they were the first to define it as a molecular disease in 1949 by Linus Pauling and his colleagues. After few years in 1957, Vernon Ingram discovered that sickle haemoglobin (HbS) resulted from a single amino acid substitution in the haemoglobin molecule. The disease results from a single base A>T mutation in the triplet encoding the sixth postion of the β-globin chain, leading to a substitution of valine by glutamic acid and formation of abnormal haemoglobin S (HbS)

The primary pathophysiology is based on the polymerization of deoxy sickle haemoglobin with formation of long fibres within the Red Blood Cells (RBCs) causing an altered sickle shape rbc which eventually leads to increased haemolysis and vaso-occlusion by sickle shaped red cells. However, the clinical presentation of SCD patients is extremely variable and there are several events that may trigger vaso-occlusion crisis. Recent work has shown the importance of red cell dehydration, abnormal adhesion of RBCs to the vascular endothelium, inflammatory events, and activation of all the cells in the vessel and alteration of nitric oxide metabolism in the pathophysiology of this multi-organ involvement.

Methods including haemoglobin (HB) electrophoresis, iso-electric focusing (IEF) and High Performance Liquid Chromatography (HPLC) are used to screen for heamoglobinopathies in some developed countries. However, there are other affordable methods also available with of varying reliability, ease of applicability and cost effective for early screening of SCD. Slide method and solubility tests are the two methods used to screen sickling, these method are easily applicable cost effective. So, in this study we will analyse sensitivity and specificity various screening test against HPLC.

In this study we will profile various types of haemoglobins and their relative percentage in sickle cell trait & sickle cell disease cases. Also, we will analysis haemogram parameters like Hb, WBC, platelets in sickle cell anaemia cases.

Method

This study was done in a tertiary care hospital, P.D.U Medical College and Hospital, Rajkot, Gujarat, Western India. Our study population consists primarily of tribal population from Central Gujarat and southern Madhya Pradesh mostly.

We used 2 ml of venous blood collected in EDTA bulb under aseptic conditions from total 169 patients (April 2023 to March 2024) suspected to have sickle cell hemoglobinopathies

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and subjected them to sickling screening test. All positive cases were evaluated by HPLC to separate constituent haemoglobins and complete blood count analysis was done to check heamogram parameters.

Solubility test: The principle of solubility method was based on solubility difference between sickle haemoglobin (HbS) and adult haemoglobin (HbA) in concentrated phosphate buffer solution. Red blood cells in test solution are lysed by a powerful haemolytic agent and it release haemoglobin is then reduced by sodium dithionite in a concentrated phosphate buffer. In the presence of sodium dithionite reagent HBS precipitates causing turbidity of the solution. Under the same conditions, HbA, as well as most other haemoglobins, are soluble. Then we centrifuge the solution & precipitated haemoglobin (HbS) forms a red precipitate on top layer leaving the lower solution clear and colourless. The soluble haemoglobin (HbA) gives a clear red lower solution with a red precipitate ring on top layer with a light to pink colour lower solution.

Sickling test: In the sickling test we create the conditions at which oxygen tension decrees to induce the sickling process of HbS in RBCs. When a drop of blood is sealed between a cover slip and a slide, the decrease in oxygen tension is due to oxidative processes in the blood cells which leads to formation of sickling. In this method blood drop is added with freshly prepared sodium metabisulfite, a chemical reducing agents which rapidly reduces oxy-haemoglobin to reduced haemoglobin to accelerate sickling. The typical sickle-shaped red blood cells appear in positive samples.

Results

Criteria used for differentiating HPLC patterns

SERIAL NO	Haemoglobin pattern	Disease Types
1	A>S	Sickle cell trait, sickle alpha-thalassemia
2	S, F and no A	Sickle cell anaemia, Sickle-beta thalassemia
3	S> A and F	Sickle-beta thalassemia
4	A>C	HbC trait
5	C, F and no A	HbC disease, HbC-beta thalassemia
6	C>A	HbC-beta thalassemia

TABLE NO.1: SUMMARY OF HB-AA, AS /SS DETECTED BY SICKLING, SOLUBILITY AND HPLC

SERIAL NO.	VARIABLE	SICKLING	SOLUBILITY	HPLC
1	TRUE POSITIVE FOR SICKLE	54	54	117
2	FALSE NEGATIVE FOR SICKLE	6	3	0
3	TRUE NEGATIVE FOR	3	40	52

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	SICKLE			
4	FALSE POSITIVE FOR SICKLE	0	9	0

Table no 1 shows summary of true positive, true negative & false positive and false negative case of various screening test and HPLC. Total 117 patients HPLC graph shows S window, out of which total 95 patient's reporting was done successfully, as remaining have inconclusive results due to blood transfusion & sample contamination etc events.

TABLE NO.2: COMPARISION OF VARIOUS SCREENING METHOD AGAINST HPLC

SERIAL NO.	PARAMETER	SICKLING	SOLUBILITY	HPLC
1	SENSITIVITY	90%	85%	100%
2	SPECIFICITY	100%	81%	100%

Table no 2 shows summary of sensitivity & specificity of various screening test and HPLC, which is showing that sickling test is 100 % specific, while solubility was neither as much sensitive nor as much specific.

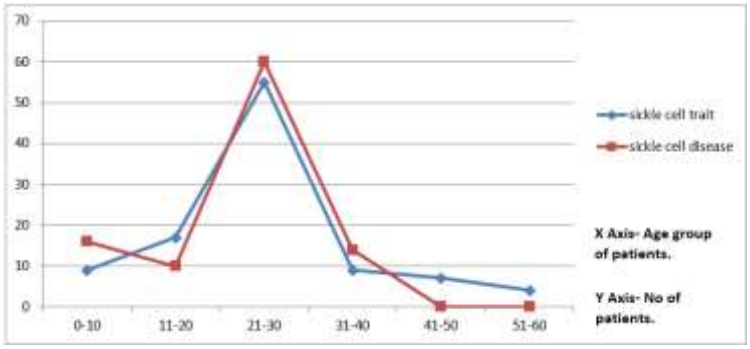

CHART 1: AGE WISE DISTRIBUTION OF SICKLE CELL TRAIT & SICKLE CELL DIASEASE CASES.

Line diagram shows age wise distribution of HPLC confirmed patients, X axis represent age groups & Y axis represents no. Of cases, in which total 88% patients are below the age of 40 years in sickle cell trait, in sickle cell disease patient all patients are below the age of 40 years respectively, Line diagram showing age wise distribution of sickle cell trait & sickle cell disease cases.

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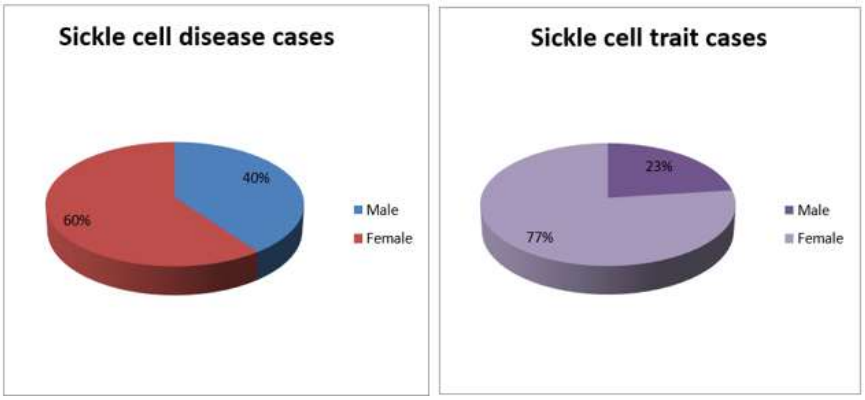
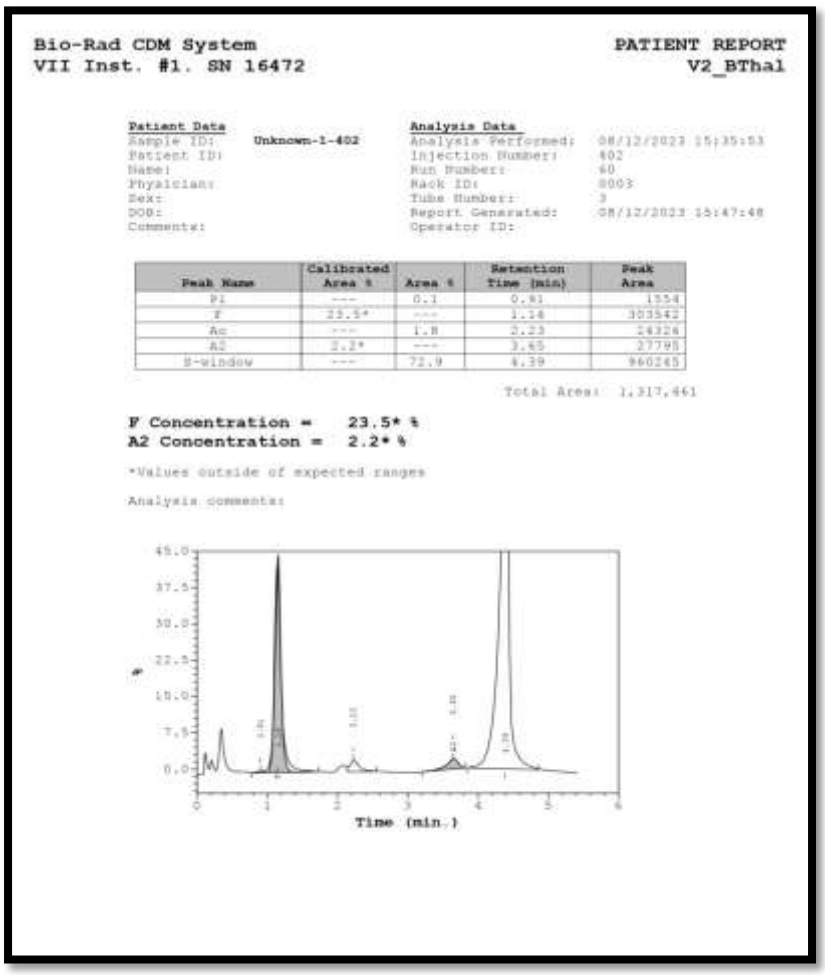


CHART 2: SEX WISE DISTRIBUTION OF SICKLE CELL TRAIT CASES & SICKLE CELL DISEASE CASES

Pie chart shows sex wise distribution of HPLC confirmed patient in sickle cell trait total 23 **%** patients are male and 77**%** patients are female, in sickle cell disease patient total 40 **%** patients are male and 60 **%** patients are female respectively. In our study both sickle cell trait & sickle cell disease more prevalent in females.



HPLC GRAPH SHOWS SICKLE WINDOW 71.9 % & HBF 9.0 % -SICKLE CELL DISEASE.

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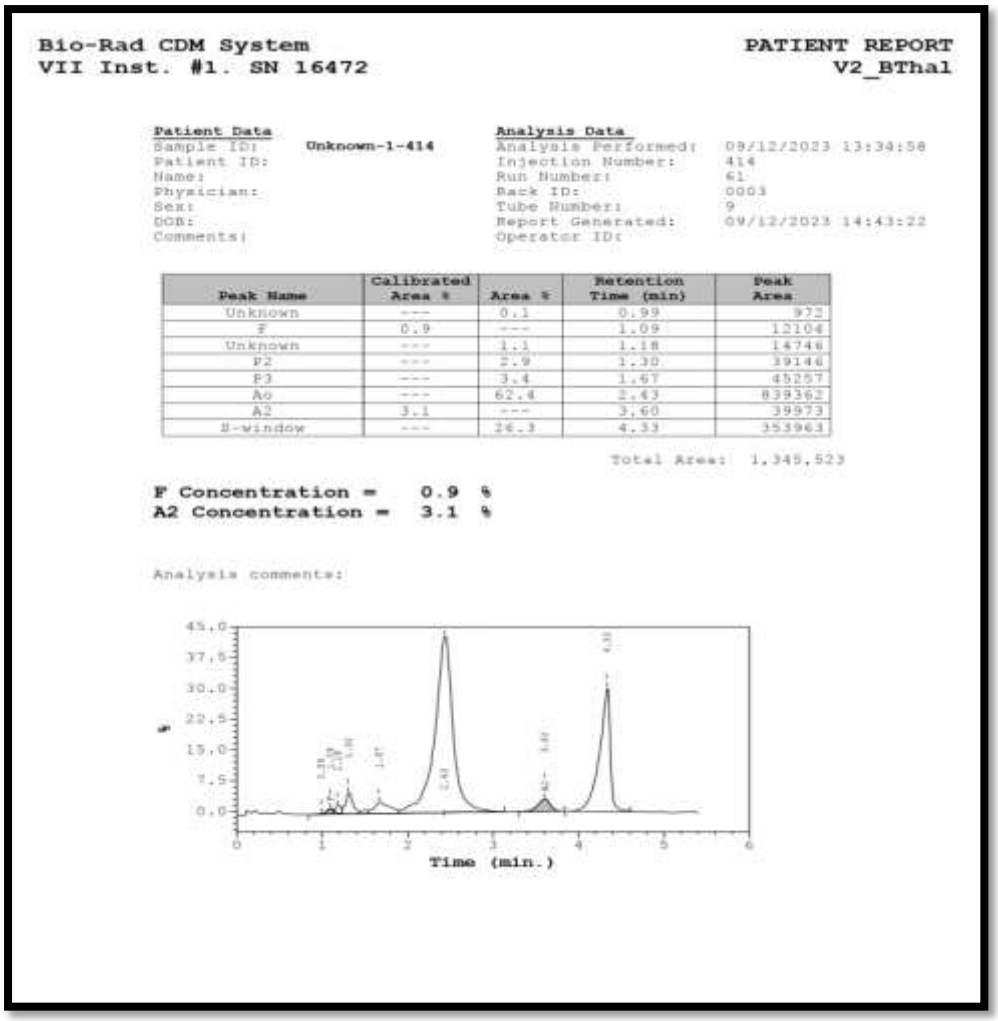

Bio-Rad CDM System
VII Inst. #1. SN 16472

PATIENT REPORT
V2_BThal

Patient Data
Sample ID: Unknown-1-414
Patient ID:
Name:
Physician:
Sex:
DOB:
Comments:

Analysis Data
Analysis Performed: 09/12/2023 13:34:58
Injection Number: 414
Run Number: 61
Rack ID: 0003
Tube Number: 9
Report Generated: 09/12/2023 14:43:22
Operator ID:

Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	0.1	0.99	972
F	0.9	---	1.09	12104
Unknown	---	1.1	1.18	14746
P2	---	2.9	1.30	39146
P3	---	3.4	1.67	45257
Ao	---	62.4	2.43	839362
A2	3.1	---	3.60	39973
S-window	---	26.3	4.33	353963

Total Area: 1,345,523

F Concentration = 0.9 %
A2 Concentration = 3.1 %

Analysis comments:

HPLC GRAPH SHOWS SICKLE WINDOW 26.3% & HBF 0.9% - SICKLE CELL TRAIT.

TABLE NO.5 THE MEAN LEVEL OF HBA, HBA2, HBF AND HBS IN SICKLE TRAIT & SICKLE CELL DISEASE

SERIAL NO.	TYPE OF HB	SICKLE CELL TRAIT CASES	SICKLE CELL DISEASE CASES
1	HBA	63.1	4.8
2	HBA2	3.1	3.4
3	HBF	1.1	16.2
4	HBS	27.4	76.2

Table no 5 shows HPLC patterns in sickle cell trait & sickle disease patient in which both group demonstrate significantly higher level of HBA2 & HBS, and lower level of HBA. Sickle cell disease patient has significantly higher level of HBF.

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TABLE NO.6: MEAN VALUE OF Hb, WBC, PLATELETS IN SICKLE CELL DISEASE & SICKLE CELL TRAIT PATIENTS.

SERIAL NO.	PARAMETER	SICKLE CELL DISEASE CASES	SICKLE CELL TRAIT CASES
1	Hb (gm/dl)	7.5	9.9
2	WBC (CELLS/CMM)	19000	10400
3	PLATELETS(LACS/CMM)	2.02	2.35

Table no.6 shows mean value of HB, WBC, platelets in sickle cell trait & sickle disease patient. In which sickle cell disease patient has low level of HB compared to sickle cell trait but high WBC count in sickle cell disease patient observed. Platelet values are almost normal in both groups.

Discussion

Basically, all these methods could reliably demonstrate patients with SS; they showed variability in their ability to detect the carrier state of Haemoglobin (AS). In a similar type of study conducted by A.L. Okwi and W. Byarugaba *et al.* in Uganda in 2010, they observed sickling to be the most sensitive and peripheral blood smear to be the most specific screening test for sickling. But they used Hb electrophoresis as a gold standard for their study.

Also we conclude that solubility test is not sensitive for the detection of carriers and unsuitable for screening purposes. Besides the solubility test could lead to stigmatization and unnecessary referrals due to its high false positive rate, which is suggestive of low diagnostic accuracy of test.

However, these observations were in conformity with Robert *et al.* who noted that factors such as Erythrocytosis, highly marked Leucocytosis and Hyperlipidemia were possibly be linked to false positivity by this method. Also in some cases of severe Anaemia false positive results were obtained. In a study conducted by Chasen *et al.* in 1999, they found that the solubility test was not sensitive for the detection of carriers. This was in affirmation with our study.

In case of false negative sickling test results, it can be due to inappropriate reagent preparation or due to subjective errors like inadequate packing of slides with wax.

Looking at the age distribution we could conclude that sickle cell trait patients had a higher life expectancy as compared to sickle cell disease patients (12 % of sickle cell trait patients were in above the age of 40 years whereas none of the single sickle cell disease patients were above the age of 40 years). This was in accordance with study done in Boston in 1994.

Our study reflected that in sickle cell trait patients, there is a significantly higher level of HbA2 and HbS and significantly lower level of HbA as compared to normal population. This is in accordance with studies done by S hirley L *et al.* and Eman A *et al.*

In sickle cell disease patients, there were significantly higher levels of HbA2, HbF and HbS and significantly lower levels of HbA as compared to normal population. This is in accordance with various studies done by Eman A *et al.* and Cotton F *et al.*

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Both sickle cell trait and sickle cell disease patients had significantly lower levels of Hb as compared to normal population. This correlates with various studies done by Walke *et al.* and Chikhlikar*et al.*

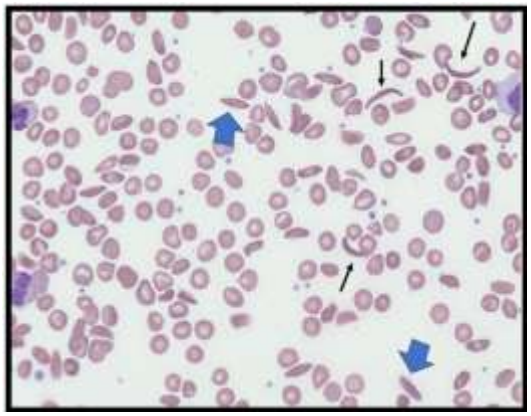

FIGURE1: SHOWS SICKLE SHAPED RBCS ON PERIPHERAL SMEAR.

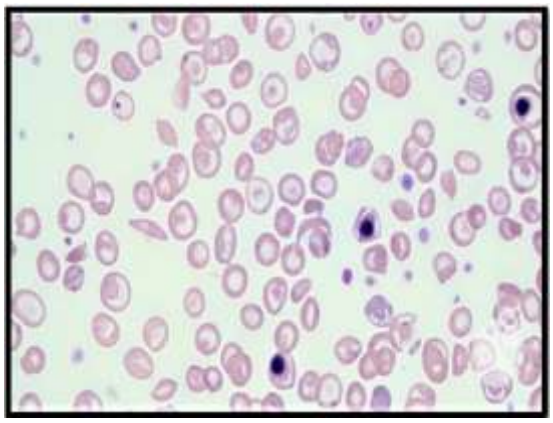

FIGURE2: SHOWS SICKLE SHAPED RBCS ALONG WITH NUCLEATED RBCS AND FRAGMENTED RBCS – HAEMOLYTIC PICTURE.

In WBC count of sickle cell disease patients have Leukocytosis& shift to left observed, which is not seen in sickle cell trait patients. Platelets are almost normal in both group, but some sickle cell disease patients reported with thrombocytopenia also, mostly during the period of sickle cell crisis.

Conclusion

The sickling test was the most reliable for the detection of haemoglobin it had high specificity and sensitivity, whereas the sickle solubility test was found expensive, cumbersome and unreliable for sickle cell screening, it had low sensitivity.

Sickling test would therefore be the most recommended screening test, using HPLC as confirmatory method. We conclude that sickle cell haemoglobinopathies are very common in our population which consists of a large proportion of tribal's due to consanguineous and same caste marriages and lack of prenatal counselling and detection.

Apart from appearance of HbS on HPLC, low levels of HbA and high levels of HbA2 should raise a suspicion for presence of sickle cell haemoglobinopathy.

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There was a statistical difference between level of Hb, WBC and platelets level compare to normal population.

We should be suspicious when these parameters are on lower side, especially in population who is prone to have sickle cell disorder such as tribal community.

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