E-ISSN:2456-1487
P-ISSN:2456-9887
RNI:MPENG/2017/70771

Research Article

HPLC

Tropical Journal of Pathology and Microbiology

2020 Volume 6 Number 2 February

Publisher

MEDRESEARCH

www.medresearch.in

The efficacy and reliability of Solubility test followed by High-Performance Liquid Chromatography (HPLC) for sickle cell disorders in Gujarat- An original research article

Jain R.[1], Saxena S.[2*]

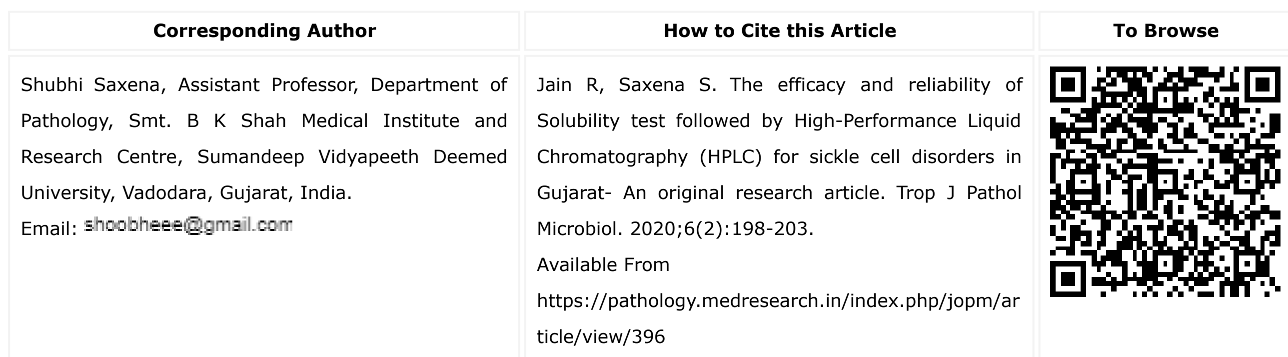
DOI: https://doi.org/10.17511/jopm.2020.i02.13

[1] Richa Jain, Assistant Professor, Department of Pathology, Smt. B K Shah Medical Institute and Research Centre, Sumandeep Vidyapeeth Deemed University, Vadodara, Gujarat, India. https://orcid.org/0000-0001-8141-2602

[2*] Shubhi Saxena, Assistant Professor, Department of Pathology, Smt. B K Shah Medical Institute and Research Centre, Sumandeep Vidyapeeth Deemed University, Vadodara, Gujarat, India. https://orcid.org/0000-0002-0616-5741

Introduction: Sickle cell anemia (SCA) is a genetic disorder characterized by severe hemolytic anemia. Diagnosis is an important aspect of the management of the disease. In the present study, the efficacy of the solubility test was assessed and High-performance liquid chromatography (HPLC) in the diagnosis of Sickle cell disorders. **Material and Methods:** A total of 1890 patients were screened. Among them, 1022 samples were tested positive for Sickle cell disorders. All the samples were observed for peripheral smear, subjected to solubility test and were further analyzed on HPLC 'BIO-RAD' analyzer for the confirmation along with the distinction of Sickle cell trait (SCT), sickle cell disease (homozygous) and Heterozygous sickle cell and beta-thalassemia. **Results:** Out of all 1890 patients screened, 1022 samples were found to be positive with the solubility test. A total of 686 samples were diagnosed as Sickle cell Trait, 247 for sickle cell disease (homozygous) and 89 samples were diagnosed as Sickle cell beta-thalassemia (heterozygous) with HPLC. **Conclusion:** Solubility test is very effective with 96.8% sensitivity. Specificity calibrated was on average 29.6%, with the predictive value of positive test 87.9% and a predictive value of negative test 64.0%. The most commonly used method now in Gujarat is HPLC although the solubility test is also sensitive and cheap.

Keywords: Sickle Cell Anaemia, Peripheral blood film, Solubility test, HPLC, Efficacy



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Shubhi Saxena, Assistant Professor, Department of Pathology, Smt. B K Shah Medical Institute and Research Centre, Sumandeep Vidyapeeth Deemed University, Vadodara, Gujarat, India. Email: shoobheee@gmail.com	Jain R, Saxena S. The efficacy and reliability of Solubility test followed by High-Performance Liquid Chromatography (HPLC) for sickle cell disorders in Gujarat- An original research article. Trop J Pathol Microbiol. 2020;6(2):198-203. Available From https://pathology.medresearch.in/index.php/jopm/article/view/396	

Manuscript Received	Review Round 1	Review Round 2	Review Round 3	Accepted
10-01-2020	20-01-2020	27-01-2020		31-01-2020
Conflict of Interest	**Funding**	**Ethical Approval**	**Plagiarism X-checker**	**Note**
No	Nil	Yes	18%	

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Introduction

Sickle cell disease is a hereditary blood disorder due to defective hemoglobin structure [1]. It is one of the most common monogenic disorders found globally with an autosomal recessive inheritance [2]. There is a replacement of valine with glutamic acid at the 6th position of the beta chain of hemoglobin [1].

In 1910, Sir James Herrick reported peculiar elongated sickle-shaped red cells in a severely anemic medical student from Grenada [3]. Linus Pauling and his colleagues showed that sickle hemoglobin (HbS) had altered electrophoretic mobility and they were the first to define it as a molecular disease in 1949.

A few years later in 1957, Vernon Ingram discovered that sickle hemoglobin resulted from a single amino acid substitution in the hemoglobin molecule [4,5]. The disease results from a single base Adenine>Thiamine mutation in the triplet encoding the sixth residue of the β-globin chain, leading to a substitution of valine for glutamic acid and the abnormal hemoglobin S (HbS).

Its clinical severity varies from the milder sickle cell trait (heterozygous) to severe sickle cell anemia (homozygous). The clinical manifestation of sickle cell disease (SCD) arises as sickle hemoglobin tends to polymerize at reduced oxygen tension resulting in the deformation of red cells into the characteristic rigid sickle shape.

Such inflexible red cells cannot pass through microcirculation efficiently, resulting in the destruction of the red cell and intermittent vaso-occlusion. It causes anemia, tissue damage, and pain [6].

The primary pathophysiology is based on the polymerization of deoxyHbS with the formation of long fibers within the RBCs causing a distorted sickle shape which eventually leads to increased hemolysis and vaso-occlusion of sickle red cells.

The sickle gene is an example of balanced polymorphism. Heterozygotes have a selective advantage and are protected against *Plasmodium falciparum* malaria while there is an increased premature death rate of homozygotes [4].

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 abnormalities of nitric oxide metabolism in the pathophysiology of this multi-organ disease [7].

Co-inheritance of the sickle gene with other Hb defects: Silvestroni and Bianco [8] were the first to describe the compound heterozygosity ($\beta s/\beta thal$) for the sickle gene and a β-thalassaemia gene in 1944.

Since then, HbS-β thalassemia cases have been reported from different ethnic groups.

Materials and Methods

The study is performed in the Tertiary health care center,1360 bedded hospital. Its an Observational Study. A total of 1890 patients of the In-Patient Department (IPD) and Out Patient Department (OPD) were examined. Among them, 1022 samples were tested positive for Sickle cell disorders. The study is conducted in patients with one year period data.

Inclusion Criteria

01. Detection of Sickle-shaped cells on peripheral smear.
02. Microcytic Hypochromic anemia.
03. Positive sickling solubility test.
04. Positive family history.

Exclusion Criteria

There were no exclusion criteria.

Sample type: Whole blood

Sample Additives, Preservatives: Vacuum collection tube containing EDTA.

Sample storage: Can be stored up to 4 days at 2-8 degrees Celsius or 1 day at room temperature at 15-30 degrees Celsius.

01. **Peripheral blood film method:** Thin blood films, stained with Field stain were examined under light microscopy.
02. **Solubility test:** The principle of solubility method was based on turbidity created when Haemoglobin S is incubated with sodium dithionate. Twenty microliters of each sample were mixed with 2mL of 0.02% sodium dithionate in a test tube and left to stand at room temperature for 5 minutes. The samples were examined using light against the background of black lines. The results were interpreted as positive when the black lines

Were not visible.

01. **High-Performance Liquid Chromatography (HPLC):** The machine used for estimating HPLC was Bio-Rad D-10 Dual Program analyzer with

Lot number 70468 (Bio-Rad Laboratories) which is an automated cation exchange HPLC instrument. The Bio-Rad D-10 operates on the principle of HPLC and the column comprises a small cation exchange cartridge, with a requirement of only 2ml of the blood sample, and each sample taking only 6.5 minutes for analysis.

The samples are injected into the analysis stream and separated by the cation exchange cartridge using a phosphate ion gradient generated by mixing 2 buffers of different ionic strengths to elute the different hemoglobins. A dual-wavelength filter photometer monitors the eluent from the cartridge as it passes through the photometer cell.

Changes in optical density at 415nm are measured. A secondary filter at 690nm corrects the effects caused by mixing buffers of different ionic strengths.

The data is processed and the report giving the chromatogram where the different peaks are identified in defined windows with relevant information like retention time, relative percentage and area.[9]

Table 1: Proportion of Different Haemoglobins in Normal Individuals and in Haemoglobin Disorders according to Surface Area percentage [10]

Condition	HbA	HbF	HbA2	HbS
Normal Adults	97%	<1%	1-3%	0
Sickle cell trait	56-60%	0	1-3%	40%
Sickle cell anemia	0	5-10%	1-3%	90-95%
Sickle cell Beta Thalassemia	56-60%	<1%	4-7%	40%

Table 1 shows established ranges in which common variants have been observed to elute using the extended program.

The printed chromatogram of HPLC shows all the hemoglobin fractions eluted, the retention times, the areas of the peaks and the values (%) of different hemoglobin components.

If a peak elutes at a retention time that is not pre-defined, it is labeled as an unknown.

Table-2: Proportion of Different Haemoglobins in Normal Individuals according to Retention time in minutes:[11]

Peak Name	Retention Time (minutes)
HbA	1.55-1.85
HbF	0.38-0.58
HbA2	2.80-3.50
S window	4.02-4.30

Observation and Results

Out of all 1890 patients screened, 1022 samples were found to be positive with the solubility test.

A total of 686 samples were diagnosed as Sickle cell Trait, 247 for sickle cell disease (homozygous) and 89 samples were diagnosed as Sickle cell beta-thalassemia (heterozygous) with HPLC (Table 5).

Table-3: The summary of the Haemoglobin AS, HbS-beta Thalassemia, SS detected by the sickling solubility test and by High-Performance Liquid Chromatography (HPLC)

S. No.	Variable	Solubility Test	HPLC (Gold Standard)
1.	True negative for sickle cells	48	0
2.	False-positive for sickle cells	114	0
3.	True positive for sickle cells	833	833
4.	False-negative for sickle cells	27	189
5.	Total	1022	1022

Table-4: Solubility Test as a screening method

Parameter	Estimate
Sensitivity	96.8%
Specificity	29.6%
Positive Predictive Value	87.9%
Negative Predictive Value	64.0%

Table-5: HPLC Interpretation with the distribution of the total number of cases percentage-wise.

HPLC Interpretation	No of Patients	%
Sickle cell trait	686	67.1
Sickle cell disease	247	24.2
S-beta Double heterozygous	89	8.7
Grand Total	1022	100

Discussion

Sickle cell disease is a common hereditary hemoglobinopathy caused by a point mutation in beta-globin that promotes the polymerization of deoxygenated hemoglobin, leading to red cell

Distortion. The thalassemia syndromes are a heterogeneous group of disorders caused by inherited mutations that decrease the synthesis of either the alpha-globin or beta-globin chains that compose adult hemoglobin, HbA (alpha2 beta2), leading to anemia, tissue hypoxia, and red cell hemolysis related to the imbalance in globin chain synthesis [12]. Herrick first described a case of sickle cell disease in 1910 [13].

The hemoglobin abnormalities are mainly confined to certain areas, religions, casts, and tribes particularly with endogamous norms of marriages, and by knowing the prevalence awareness can be spread regarding the diseases and their outcomes to the general population and take measurements to treat them and help in prevention [14].

Ost of the early studies on the epidemiology of sickle hemoglobin in different parts of the country used the sickling or the solubility test and in many reports, this was followed by HPLC analysis to determine the phenotypes.

However, in recent years, HPLC analysis has been used in many large programs to identify carriers of both sickle hemoglobin as well as β-thalassaemia. Nonetheless, even the simple and cost-effective solubility test has been shown to have a sensitivity and specificity of 97.4 and 100 percent, respectively in comparison to HPLC and could still serve as a good first-line screen for sickle hemoglobin in remote areas where other facilities are not available [15].

Sickle solubility tests identify hemoglobin S with high sensitivity and specificity. False-negatives are seen in patients with severe anemia, in those with a hemoglobin S fraction<10%, or in patients with high levels of hemoglobin F [16].

Coinheritance of alpha-thalassemia trait or hereditary persistence of fetal hemoglobin may lead to false-negative results. False-positives may be observed in conditions associated with increased serum viscosity (eg, polycythemia, hyperlipidemia, elevated serum proteins) and with some hemoglobin variants (eg, hemoglobin I, hemoglobin Bart's, hemoglobin Jamaica-Plain).

Unfortunately, sickle solubility tests do not distinguish SCT from SCD, nor do they identify Non-S hemoglobin variants [17]. For many healthy adults and even some health care providers, a positive result on a solubility test may cause confusion about the diagnosis of SCT or SCD.

More than 50% of the total global sickle cell anemia (SCA) cases are in India [18].

In the current study, the sensitivity and specificity of the solubility test were 96.8% and 29.6% respectively for the samples. Solubility test cannot differentiate between Sickle cell trait and Sickle cell disease [19] hence, confirmation is needed by HPLC. The causes of the false-positive test are Leucocytosis, Paraproteinaemia, Hyperlipidaemia, and Polycythaemia [20].

Automated cation exchange HPLC is being increasingly used as the initial diagnostic method in the diagnosis of Haemoglobinopathies [21].

High-Performance Liquid Chromatography (HPLC) is used for detection, identification, and quantification of hemoglobin variants. In this automated technique, a blood sample (hemolysate) is introduced into a column packed with silica gel.

Different hemoglobins get adsorbed onto the resin. Hemoglobin fractions are detected as they pass through a detector and recorded by a computer [22]. It is emerging as one of the best methods for screening and detection of various hemoglobinopathies with rapid, reproducible and precise results.

Limitation of the Present Study: Solubility test is only a screening method. This method is not a substitute for High-Performance Liquid Chromatography (HPLC).

Recent blood transfusions may alter the results. Samples with hypergammaglobulinemia may show the false-positive result which can be avoided by using saline washed cells instead of whole blood.

Samples with hemoglobin more than 19gm/dl and higher fetal hemoglobin lead to a false-positive result. Very anemic patient or sample with less RBC may lead to false-negative observation.

Proper visibility needs appropriate illumination. Each analysis of the solubility test should be tested against known positive and negative blood samples.

Conclusion

The solubility test is very effective with 96.8% sensitivity. Specificity calibrated was on average 29.6%, with a predictive value of positive test 87.9% and a predictive value of negative test 64.0%. The most commonly used method now in Gujarat is HPLC although the solubility test is also

Sensitive and cheap. This test cannot differentiate between Sickle cell trait and Sickle cell disease hence, confirmation is needed by HPLC.

What does the study add to the existing knowledge?

Sickling Solubility test is better for mass screening, and is still used in tertiary care centers in Gujarat because it is rapid (takes just about 5 min), reliable with minimal observer variation, does not need any microscope and requires a very small blood sample. It is also a cost-effective test. The sensitivity is 96.8% while specificity is on an average 29.6%.

Author's contribution

Dr. Richa Jain: Concepts, design, the definition of intellectual content, literature search, data acquisition, manuscript preparation, manuscript editing

Dr. Shubhi Saxena: Concepts, design, the definition of intellectual content, literature search, data acquisition, manuscript preparation, manuscript editing

Abbreviation

SCA- Sickle Cell Anaemia

HPLC- High-Performance Liquid Chromatography

SCT- Sickle Cell Trait

SCD- Sickle Cell Disease

Hb S- Sickle Haemoglobin

RBC's- Red Blood Cells

Hb- Haemoglobin

HbS- Beta Thalassemia- Heterozygous for Sickle Haemoglobin and Beta Thalassemia

EDTA- Ethylene Diamine Tetra Acetic Acid

Hb A- Adult Haemoglobin

Hb F- Foetal Haemoglobin

S Window- Sickle Window

Hb AS- Haemoglobin Adult and Sickle (Sickle Cell Trait)

Hb SS- Haemoglobin Sickle (Homozygous for Sickle Cell Disease)

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