Reliability of Screening Tests against HPLC for the Detection of Sickle Cell Disease in Betul District – A Hospital Based Study from Central India

Smita Rathi*, Nutan Rathi**

*consultant Pathologist, Nidan Diagnostics, **consultant Physician, Shree Goverdhan Rathi Hospital, Betul, Madhya Pradesh - 460001

Abstract: Introduction: The role of laboratory in the diagnosis of haemoglobinopathies is crucial. Sickle cell disease (SCD) is the most common life threatening disease worldwide. Peripheral blood film method, Sickling and Solubility tests are used as screening methods of sickle cell disease. Aim of the study: This study was conducted to determine the reliability of Peripheral blood film; sickling and solubility tests against High pressure Liquid Chromatography as gold standard. Methods: This was a cross sectional laboratory based study carried out at Nidan Diagnostics, Shree Goverdhan Rathi Hospital, Betul. A total of 132 samples of children aged up to 1 year and adults up 30 years were studied over a period of six months. High Performance Liquid Chromatography was used as the gold standard. Results: Sickling, solubility and peripheral blood film tests had sensitivities of 87.5%, 75%% and 81% respectively. Sickling, solubility and peripheral blood film tests had specificities of 95%, 84% and 89.3% respectively. The positive predictive values for Sickling, solubility and peripheral blood film tests were 84.85%, 60% and 70.27% respectively. The negative predictive values for Sickling, solubility and peripheral blood film tests were 95.96%, 91% and 93.68% respectively. Conclusion: The Sickling test was the most reliable, cheapest and easiest to perform; it had high specificity, sensitivity. The solubility test was found expensive, cumbersome and unreliable for sickle cell screening, it had low sensitivity. Sickling would therefore be the most recommended test for screening children for sickle cell disease using HPLC as confirmatory method. [Smita R NJIRM 2017; 8(6):83-86]

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

Author for correspondence: Nutan Rathi, Shri Govardhan Rathi Hospital, Link Road, Betul, Madhya Pradesh - 460001 E-Mail: drnutanvallabh@gmail.com M: 9993703468

Introduction: Sickle cell disease (SCD) is one of the most common monogenic disorders globally with an autosomal recessive inheritance[1]. James Herrick, a physician first described the characteristic sickle shaped red cells in a medical student from Grenada in 1910. Linus Pauling and his colleagues showed that sickle haemoglobin (HbS) had an 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 haemoglobin resulted from a single amino acid substitution in the haemoglobin molecule[2,3]. The disease results from a single base A>T 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 haemoglobin S (HbS)[4].

Methods including hemoglobin (Hb) electrophoresis, iso-electric focusing (IEF) and High Performance Liquid Chromatography (HPLC) are used to screen for hemoglobinopathies in some developed countries[5]. However, there are other affordable methods 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.

Most of the early studies on 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 Hb electrophoresis to determine the phenotypes. However, in recent years, high performance liquid chromatography (HPLC) analysis has been used in many large programmes to identify carriers of both sickle hemoglobin as well as β-thalassaemia. Capillary electrophoresis has also now been introduced at some centers. Nonetheless, even the simple and cost-effective solubility test has been shown to have a sensitivity and specificity of 97.4 and 100 per cent, 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[5].

Methods:

Study design: It was laboratory based cross sectional study

Study Place: The study was conducted in NIDAN diagnostics, further samples were forwarded to higher centers for HPLC (high performance liquid chromatography).

Sample size: A total of 132 samples which were come during the last six months were included in the study.

Procedure: Study purpose was explained before performing the tests and informed consent was taken from all the subjects.

Solubility test: The principle of solubility method was based on solubility difference between HbS and HbA in concentrated phosphate buffer solution. Red blood cells under test are lysed by a powerful hemolytic agent and the released hemoglobin is then reduced by sodium dithionite in a concentrated phosphate buffer. In the presence of sodium dithionite HBS precipitates causing turbidity of the reaction mixture. Under the same conditions, HbA, as well as most other hemoglobins, are soluble. When subjected to a centrifugal force the precipitated hemoglobin (HbS) forms a red precipitate on top layer leaving the lower solution clear and colorless. The soluble hemoglobin (HbA) gives a clear red lower solution with a grey precipitate on the top layer and most HbAs which contains both precipitated and soluble hemoglobin gives a red precipitate ring on top layer with a light to pink color lower solution.

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

Peripheral blood film method: Wright-Giemsa–stained peripheral blood smears were evaluated under microscope (100X). Results were considered positive when the sickle cell count was greater than 25% of the total red cell count.

High Performance Liquid Chromatography (HPLC): It is highly reproducible, offers simplicity with automation, superior resolution and rapid results. HPLC based method used at higher centers were used to determine the presence of AA, AS, and SS in the samples and to confirm the results generated by the above methods. The underlying principle of HPLC is based on hemoglobin separation by an analytical cartridge in cation exchange HPLC using a preprogrammed buffer gradient with increasing ionic strength to the cartridge .The hemoglobin fractions separate based on their ionic interaction with the cartridge .The separated fractions pass through a flow cell, where absorbance is measured at 415 nm and again at 690 nm to reduce background noise. Changes in absorbance are monitored over time producing a chromatogram (absorbance v/s time). Each hemoglobin has its own characteristic retention time and is measured from the time of sample injection into the HPLC to the maximum point of each peak. Identification of unknown hemoglobin is achieved through comparison with known hemoglobin retention time. HPLC achieves good separation and quantitation of Hb F and HbA2.

Quality control: Each batch of assays included a known negative control (AA) and a known positive control (Hbss). All procedures were undertaken by three independent experienced senior technologists and the results were included in the analysis only when at least two observers were in agreement.

Statistical Analysis: Online statistical software was used to analyze the data.

Results:

Table no 1: The summary of the haemoglobin AA, AS/SS detected by the sickling, solubility and peripheral blood film methods and by Hb electrophoresis (Gold standard)

Variable	Sickling	Solubility	Peripheral blood film	HPLC
True positive for sickle cells	28	24	14	48
False negative for sickle cells	04	09	12	0
True negative for sickle cells	95	91	102	84
False positive for sickle cells	05	08	04	0
Total	132	132	132	132

Table no 2: Comparison of various screening methods against HPLC (gold standard)

Parameter	Sickling	95 % CI	Solubility	95 % CI	Peripheral blood film	95 % CI
Sensitivity	87.5 %	71.01 to 96.49	75.00%	56.60% to 88.54%	81.25 %	63.56% to 92.79%
Specificity	95 %	88.72 to 98.36	84.00 %	75.32% to 90.57%	89.00 %	81.17% to 94.38%
Positive Predictive Value	84.85 %	70.23 to 93.00	60.00% (*)	47.85% to 71.04%	70.27 % (*)	56.91% to 80.88%
Negative Predictive Value	95.96 %	90.46 to 98.35	91.30 % (*)	85.13% to 95.06%	93.68 % (*)	87.79% to 96.84%
Diagnostic Accuracy	93.18 %	-	81.8%	-	87.1 %	-

Discussion: Basically, all these methods could reliably demonstrate patients with SS; they showed variability in their ability to detect the carrier state of hemoglobin (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.[6]

The solubility test was not sensitive for the detection of carriers and was unsuitable for screening purposes. Besides the solubility test could lead to stigmatization and unnecessary referrals because of its high false positive rate which is characteristic of a test with low diagnostic accuracy. The probable reason for this high false positive rate was that some of the samples might shown erythrocytosis, highly marked leucocytosis and /or hiyperlipidemia, unfortunately. It was not possible to link these hypotheses to false positivity of solubility test because none of these parameters were measured in this study. However, these observations were in conformity with Robert et al.[7]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 anemia false positive results were obtained.

In a large scale study conducted by Sumanta Panigrahi, P. K. Patra, and P. K. Khodiar in Chhattisgarh in 2014. 15,701 (7,959 were males and 7,742 were females) were screened for sickle cell anemia. 1,769 tested positive for solubility. Their venous blood were collected in EDTA and subjected to alkali Hb electrophoresis. 1,672 (10.6 %) cases were having sickle cell trait (AS) and 97 (0.6 %) cases were having sickle cell disease and other associated bands[8].

In a study conducted by Schneider et al. they observed that the false positivity rate of sickling test increases as the hemoglobin decreases. In contrast we observed that rate of false positivity increases in solubility test as hemoglobin decreases[9].

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[10].

When using the sickling test false negative result in sickling tests were obtained, either due to inappropriate reagent preparation or due to subjective errors like inadequate packing of slides with wax.

Conclusion and recommendation: The sickling test was the most reliable, sickling test was found most reliable for the detection of hemoglobin as although there were some false positive cases, it had high specificity, sensitivity. The solubility test was found expensive, cumbersome and unreliable for sickle cell screening, it had low sensitivity. Sickling would therefore be the most recommended test for screening children for sickle cell .The peripheral blood smear was found unreliable in detecting carriers as they had very few circulating sickle cells. Sickling would therefore be the most recommended screening test, using HPLC as confirmatory method.

References:

1. Serjeant GR. Sickle-cell disease. Lancet 1997; 350:725-30.
2. Stuart MJ, Nagel RL. Sickle cell disease. Lancet. 2004; 364:1343–60.
3. Rees DC, Williams TM, Gladwin MT. Sickle cell disease. Lancet. 2010; 376:2018–31.
4. https://www.cdc.gov/ncbddd/sicklecell/documents/nbs_hemoglobinopathy-testing_122015.pdf

(Available online, As assessed on 28th September 2017)
5. Surve RR, Mukherjee MB, Kate SL, Nagtilak SB, Wadia M, Tamankar AA, et al. Detection of the beta S gene: an evaluation of the solubility test against automated chromatography and haemoglobin electrophoresis. Br J Biomed Sci. 2000; 57:292–4.
6. L. Okwi, W. Byarugaba, A. Parkes, M. Ocaido. The Reliability of Sickling and Solubility Tests and Peripheral Blood Film Method for Sickle Cell Disease Screening at District Health Centers in Uganda. Clinics in Mother and Child Health. 2010: 7:1-5.
7. Panigrahi S, Patra PK, Khodiar PK. The screening and morbidity pattern of sickle cell anemia in Chattisgarh. Ind J Hematol Blood transfusion. 2015;31(1):104–09.
8. R.G. Schneider, J.B. Alperin, H. Lehmann, Sickling tests pitfalls in performance and interpretation, JAMA. (1967): 202: 419-421.
9. S. Chasen, Z.S. Loeb, and E. Landsberger. Haemoglobinopathy screening in pregnancy: Comparison of two protocols, Am J Perinat. (1999): 16: 175-180
10. M. Robert, M.D. Schmidt, M. W. Sylvia. Standardization in detection of abnormal hemoglobins. Solubility tests for hemoglobins.S.JAMA,225(1973),1225-1230

Conflict of interest: None
Funding: None
Cite this Article as: Smita R, Nutan R. Reliability of Screening Tests against HPLC for the Detection of Sickle Cell Disease in Betul District. Natl J Integr Res Med 2017; 8(6):83-86