Efficacy of a Modified Solubility Test in Detecting HB S and Differentiating HB SS & HBAS- An Institutional Study

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ABSTRACT

BACKGROUND

Sickle cell anaemia is the most common disease entity of all the monogenic disorders. In the absence of any definitive treatment, to reduce the disease burden and the associated morbidity and mortality, a simple, low-cost test to assess the presence of Hb S and also to differentiate the homozygous and heterozygous forms, is required.

METHODS

This study was carried out in the Department of Pathology of MKCG Medical College, Berhampur, where samples of 130 cases (50 cases of sickle cell trait and 50 cases of homozygous sickle cell disease along with 30 cases of normal haemoglobin pattern) diagnosed by CE-HPLC were subjected to modified haemoglobin solubility testing and cases were classified as Hb AA, Hb AS and Hb SS depending upon presence/absence of red precipitate and colour of lower layer.

RESULTS

Out of the 130 cases in the study, there was agreement with the findings of CE-HPLC in 123 cases (accuracy of 94.62%). All 50 cases of homozygous SCD formed a red precipitate, but in 4 of those cases a diagnosis of SCT was made by the solubility test. Similarly, 47 of 50 trait cases were correctly diagnosed but other 3 cases were diagnosed as having normal pattern. Sensitivity and specificity of the solubility test in differentiating cases of homozygous and heterozygous sickle cell disease came out to be 92% and 100% respectively.

CONCLUSIONS

The modified haemoglobin solubility test is a simple and cheap test with high degree of accuracy that can be carried out by health workers at the peripheral level, with minimum requirement of training and equipment. It can be used as a preliminary screening as well as differentiating test.

KEYWORDS

Sickle Cell Anaemia, CE-HPLC, Sickling Test, mHST

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BACKGROUND

Sickle cell anaemia is the most common disease entity of all the monogenic disorders. This is an autosomal recessive disorder. The prevalence is estimated to be increasing worldwide. Latest reports say that around 300,000 infants are born each year with Homozygous Sickle cell anaemia and the number is likely to increase by about one-third by 2050.[1-3] Because of evolutionary selection due to malaria protection, the highest frequencies of SCA are seen in tropical regions. The vast majority of newborns with SCA occur in low- and middle-income countries.[2,4] In India, in 1952, sickle cell disease was first described in the Nilgiri hills of northern Tamil Nadu. According to a statement released by the State health department in 2015 Odisha has 5.35 lac of the population affected by the disease.[1] In the long term, in the absence of a definitive treatment for SCA, the best intervention to reduce excess mortality caused by this disorder and to keep public health costs associated with the follow-up care of SCA patients through their lifetime manageable, especially in low income countries, is to avoid the births of affected newborns.[5,6] So, the need of the hour is a better diagnostic facility and a reduction of the disease load by providing pre-marital counseling and pre-pregnancy work-up.

Therefore, it is crucial to identify best screening methods for detecting sickle cell haemoglobin in individuals to assess the status of sickle cell mutations in high risk populations. Methods including haemoglobin (Hb) electrophoresis, iso-electric focusing (IEF) and high-performance liquid chromatography (HPLC) are used to screen for haemoglobinopathies in the developed countries. However, there are other affordable methods of varying reliability, ease of applicability and cost available for early screening for SCD in low income countries. These include solubility and sickling tests and peripheral blood film method.[7,8] However these tests are not able to differentiate between homozygous and heterozygous forms of sickle cell disease with certainty. It has long been desired to have a simple and low -cost and -equipment test to not only assess the presence of Hb S but also differentiate the homozygous and heterozygous forms of this condition, so that a preliminary diagnosis can be achieved at the peripheral level. The present study was carried out in the Department of Pathology, MKCG Medical College, Berhampur to assess the usefulness of a modified version of Haemoglobin solubility test (mHST), which is based on the principle of direct observation of turbidity resulting from the insoluble HBS in the presence of phosphate buffer solution, as a diagnostic, as well as differentiating test between heterozygous and homozygous forms of Hb S.

METHODS

The present study was carried out in the Department of Pathology of MKCG Medical College, Berhampur, where a total of 100 cases having Sickle cell disease (50 cases of trait and 50 cases of homozygous sickle cell disease) along with 30 cases of normal haemoglobin pattern, diagnosed by CE-HPLC at the MDRU (Multi-disciplinary Research Unit) of MKCG Medical College, were taken as the study group and the samples were subjected to a modified haemoglobin solubility testing. All persons were above 1 year of age and any person with history of recent blood transfusion was excluded from the study. This haemoglobin S screening test is based on the relative insolubility of haemoglobin S in concentrated phosphate buffer when combined with sodium dithionite, a reducing agent. When whole blood is mixed with the reagent, a powerful haemolytic (saponin) lyses the erythrocytes and haemoglobin is released. If haemoglobin S is present, in the presence of sodium dithionite, it will precipitate and give a turbid appearance to the solution. A transparent solution is seen with other haemoglobins that are more soluble in the reducing agent.[9] The Sicklevue Hb S Solubility test kit for Sickle cell anaemia manufactured by Tulip group was used for the study. Working solution of reagent was prepared by adding 5 mg sodium dithionite (2nd reagent) to 1 ml of first solution containing saponin and phosphate buffer. To 2 ml of this working solution, 20 µL of EDTA blood was added, mixed thoroughly and incubated at room temperature for 10 minutes. Then the test tube containing the mixture was observed for turbidity by holding the tube against a white paper having ruled black lines 0.5 cm apart.

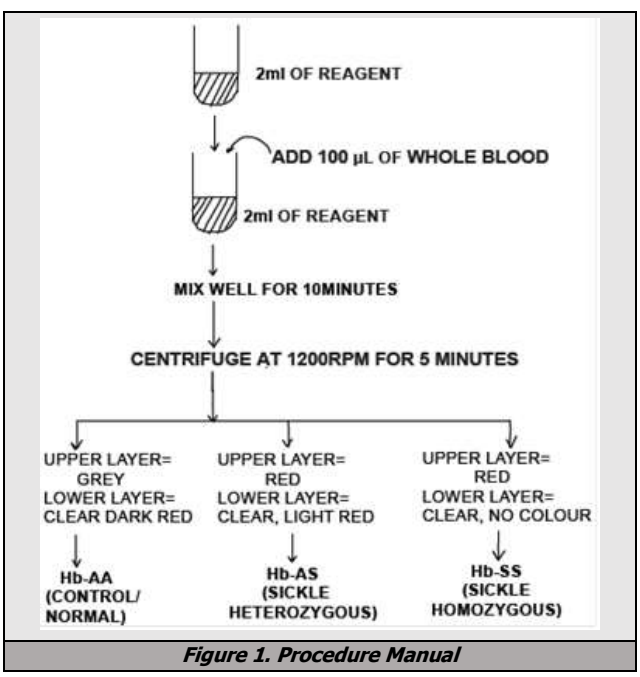

Figure 1. Procedure Manual

A positive result was indicated by a turbid suspension through which the ruled lines were not visible while a negative result was indicated by a transparent suspension through which the ruled lines were visible. However, the problem that is faced in this process was that a number of cases presented with what can be termed as inter-observer variability, i.e. in certain cases, while some reporting authority marked the lines as not visible, another marked it as visible. And heterozygous and homozygous forms could

not be differentiated. Therefore, a modified version of HST (mHST) was used where the mixed solution of reagent and blood sample (100 µL in this case) was subjected to centrifugation at 1200 rpm for 5 minutes, centrifuge was stopped without breaking and result was noted and cases were classified as per Table 1, Figure 1, Figure 2. Statistical analysis was done and sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated.

Type	Lower Layer	Upper Layer
Hb-AA (Normal)	Clear & dark red colour	Absent red precipitate
Hb-AS (Sickle cell trait)	Clear & light red to pink colour	Red precipitate
Hb-SS (Sickle cell anaemia)	Clear & colorless	Red precipitate

Table 1

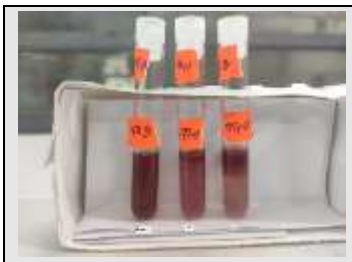

Figure 2

RESULTS

The present study was carried out with 100 cases of sickle cell disease patients and 30 persons with normal haemoglobin pattern diagnosed by CE-HPLC at the MRU of MKCG Medical College. Of the 100 sickle cell cases, 50 were homozygous and other 50 were heterozygous. The age of patients ranged from 1 year 2 months to 22 years with a mean of 8 years 06 months. The sex distribution was almost equal. The findings are shown in Table 2. Out of the 130 cases in the study, there was agreement with the findings of CE-HPLC in 123 cases (accuracy of 94.62%). In the present study, in 97 cases, there was formation of a red precipitate while in 33 cases, there was no red precipitate. Of these 33 cases, 30 were proven cases of Hb AA pattern, while 3 were sickle cell trait cases. Sensitivity and specificity of the solubility test in diagnosing cases with sickle cell disease came out to be 97% and 100% respectively while PPV and NPV were 100% and 90.1% respectively. All 50 cases of homozygous SCD formed a red precipitate, but in 4 of those cases the lower layer appeared to be pink in colour leading to a diagnosis of SCT by the solubility test. Similarly, 47 of 50 trait cases were correctly diagnosed but other 3 cases were diagnosed as having normal pattern. Sensitivity and specificity of the solubility test in differentiating cases of homozygous and heterozygous sickle cell disease came out to be 92% and 100% respectively while PPV and NPV were 100% and 92.6% respectively. Mean turnaround time for individual test was 49 minutes. But it was seen that when a batch of samples were tested (the largest batch in this study was of 12 cases), mean TAT came out to be 6 minutes (i.e. in 72 minutes, all 12 cases could be reported).

Cases Diagnosed By CE-HPLC	Total No.	Diagnosis BY mHST		
		Hb AA	Hb AS	Hb SS
Hb AA	30	30	-	-
Hb AS	50	03	47	-
Hb SS	50	-	04	46

Table 2

DISCUSSION

Sickle cell anaemia is a very common disease entity in this geographic region with a gradually increasing disease load. In the long term, in the absence of a definitive treatment for SCA, the best intervention to reduce excess mortality caused by this disorder and to keep public health costs associated with the follow-up care of SCA patients through their lifetime manageable, especially in developing countries, is to avoid the births of affected newborns,[5,6] for which a good diagnostic set up along with increasing awareness and pre-marital counseling are of paramount importance.

Although many tertiary care institutions in the state of Odisha have very good diagnostic facilities for detecting Hb S like Hb electrophoresis and CE- HPLC, it's not accessible to many people, especially those in the rural and tribal areas of the state where the prevalence of sickle cell disease is unfortunately disproportionately high and although everyone from those areas, particularly children and young adults, should be tested for presence of Hb S to know the carrier status so as to impart proper counseling, it would be impractical and highly cost intensive to collect samples from all the persons in these areas and run tests in the tertiary care centers. The need of the hour being to have a simple, low-cost and-equipment and rapid test that could detect both the presence of Hb S as well as differentiate the homozygous and heterozygous cases with accuracy and can be done at the peripheral health centers by the health care providers like pharmacists, laboratory technicians and ANMs, this study was carried out in the Department of Pathology, MKCG Medical College, Berhampur to assess the usefulness of a modified Haemoglobin solubility test (mHST). This test is based on the principle of direct observation of turbidity resulting from the insoluble HBS in the presence of phosphate buffer solution and a reducing agent.[9] Sickling test, which is being used as the screening test at the present moment, is also a simple and cost effective test but the disadvantages are that it is time consuming and instruments like microscope are required, it cannot differentiate between homozygous and heterozygous forms of SCD with any accuracy, as well as the need for a trained person, preferably a pathologist, to report the sickling slides. The mHST, on the other hand, is much more rapid, requires only a minor instrument like centrifuge machine which is at present available with all technicians at the PHC level, minimal training only is required and can differentiate between both forms of SCD. It however has some physiological limitations like- Erythrocytosis, hyperglobulinemia, extreme leukocytosis, or hyperlipidaemia may cause false positive results, severe anaemia may induce a false negative result, recent transfusion with normal erythrocytes may cause a

false negative result and in infants younger than 6 months, false negatives may occur.[7,10] In the present study, to compare the efficacy of solubility test in detecting and differentiating homozygous and heterozygous SCD, 100 cases of HPLC proven SCD blood samples (50 homozygous and 50 heterozygous) along with 30 samples with normal Hb pattern were tested by mHST. It was seen that 97 SCD cases in this study gave a positive result in the mHST with a specificity of 100% and sensitivity of 97%. Sensitivity and specificity of the test in differentiating homozygous and heterozygous forms were 92% and 100% respectively. Although we did not find any published articles on the accuracy of this modified HST method, the few published studies on HST found a higher level of inaccuracy in determining sickle cell trait. Okwi et al[7] found the solubility test to have low sensitivity for Hb AS. In the study by Chasen et al,[11] they also reported that the solubility test was not sensitive for the detection of carriers and was unsuitable for screening purposes. Robert et al[10] noted that factors such as erythrocytosis, highly marked leucocytosis and hyperlipidaemia were possibly linked to false positivity in HST. Mean TAT for individual test was 49 minutes; but when a batch of samples was tested, it came down to 6 minutes, indicating the ease and rapidity of the test when a large batch of tests is done. Nalbandian et al[12] also found solubility test easier to perform, but Okwi et al[7] found that solubility test had a high TAT because a lot of time was required for reagent preparation.

CONCLUSIONS

The modified haemoglobin solubility test is a simple and cheap test and can be carried out by health workers at the peripheral level, with minimum requirement of training and equipment, and a large number of samples can be processed in a short period of time. With sensitivity of 97% and 92% respectively in diagnosing and differentiating the two forms of SCD, and specificity of 100% in both instances, it can be used as a preliminary test for diagnosis of SCD at the primary or secondary health care centers. The recommendation of this study would be to use mHST as a preliminary diagnostic test at the primary and secondary health centers in areas with high prevalence of sickle cell disease and confirming positive samples at higher centers with facility for Hb electrophoresis and/ or CE-HPLC would be the most cost effective way. However further studies with larger number of unknown cases, followed by evaluation of accuracy of this method by comparing with the results of confirmatory tests like CE-HPLC, are required before mHST can be included in the state health policy.

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