International Journal of Medical and Pharmaceutical Research
Website: https://ijmpr.in/| Print ISSN: 2958-3675 | Online ISSN: 2958-3683
NLM ID: 9918523075206676
Volume: 4 Issue:3 (May-June 2023); Page No: 20-24

Original Article

Efficacy of Paper Based Screening Test for Sickle Cell Disease

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ABSTRACT

Introduction: Sickle-cell disease (SCD) refers to a group of inherited disorders affecting hemoglobin resulting sickle hemoglobin variant (HbS). A simple, low-cost, paper-based test for SCD offers advantages over existing technologies for screening programs in resource-limited settings. The primary aim is to test the efficacy of paper-based test as a simple low cost and electricity free test. The main objectives are to determine the sensitivity and specificity of paper-based screening test for SCD in compare to HPLC test and to validate the paper based test.

Materials and Method: A comparative study was conducted in the Dept. of Physiology from April to June 2021 as ICMR approved STS project, after obtaining the ethical clearance (SVIEC/ON/MEDI/SRP/20027). A total of 60 samples (n=60) were collected from individuals ranged between 18-40 years for the study. Blood samples collected in EDTA anticoagulant vacutainers were used for SCD diagnosis by using paper-based test and then confirmed by HPLC test, using HPLC test as the gold standard test.

Result and Conclusion: True positive(TP), false positive(FP), true negative(TN), and false negative(FN) were determined on the basis of result obtained from HPLC test. The result obtained includes TP=36, FP=4, T N=10 and FP=10. The sensitivity and specificity of paper-based screening test was evaluated to be 78.2% and 71.4% respectively for HbS (HbAS and HbSS). ROC curve was obtained and the area under curve is 0.88 for discriminating between HbAA from HbAS and HbSS by paper-based test.

In conclusion, the paper-based test can be used as a substitute for screening HbS. The study validates the paper-based test as a simple low-cost and electricity free test capable of detecting sickle cell trait and disease and demonstrate its practicality in resource-limited clinical settings.

Key Words: *Hemoglobin, paper based screening, sickle cell disease, sickle cell screening*

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INTRODUCTION

"Sickle-cell disease (SCD) refers to a group of inherited disorders affecting hemoglobin caused by a single nucleotide substitution at position 6 of the β-globin, its pathophysiology stems from the polymerization of the resulting sickle hemoglobin variant (HbS), triggering a cascade of alterations in erythrocytes" [1-3].

Some of the highest βS allele frequencies have been reported in Indian populations [4,5] and India has been ranked the second worst affected country in terms of predicted sickle cell anemic (SCA) births [6]. SCD is predominantly found amongst the socioeconomically disadvantaged population subgroups majority of these patients remain undiagnosed. Thus, leading to high morbidity and mortality reported in some of the states including Gujarat. Gujarat with 89.12 lakh tribal populations is expected to have at least 9,00,000 SCT and 70,000 SCD patients [2]. The Dhodia, Dubla, Kukna, Gamit, Chaudhary, Halpati, Varli, Kokni, Kathodi, Kolcha, Kotwadia, etc. are major tribes with documented issues of Sickle cell disease (SCD) in Gujarat [7].

According to an estimate around 37,500 sickle cell disease patients are born annually and which inflict around 360 million Rupees ($ 5.4 million) economic burden on India annually [8]. The nationwide screening conducted by Ministry of Tribal Affairs (MoTA) and ICMR in 2016-2018 screened 1,13,83,664 individuals out of which 8.75% tested positive for sickle cell [9].

The high cost, complexity and reliance on other sources, specific equipment and their operation, diagnostic methods limits the scope of the screening of SCD, moreover the approach of rural population towards the diagnostic center is also

limited. Thus, in India, the disease is largely undocumented and there is an urgent need to document the disease with low cost community based screening approach, so that locally appropriate models of care may be evolved.

A simple, low-cost, and inherently portable test for sickle cell disease has exciting implications for screening programs in resource-limited settings. Paper-based test offers important advantages over existing technologies designed to enable low-cost SCD diagnostics in resource-limited settings i.e. act as a useful low-cost tool for screening adults and children for sickle trait and disease and demonstrate its practicality in remote settings. The objective of the study was to determine the sensitivity and specificity of the paper-based screening test and to validate paper-based test as a simple low cost and electricity free test capable of detecting sickle cell hemoglobin.

MATERIALS AND METHODS

It was an interventional comparative study, conducted in the Department of Physiology, Smt B K Shah Medical Institute and Research Center, Sumandeep Vidyapeeth, Gujarat as a part of ICMR approved (2020-04429) research project. Ethical clearance was obtained from Institutional Ethics Committee (SVIEC/ON/MEDI/SRP/20027) and written informed consent was obtained from the participants and those who volunteered were recruited for the study. A total of 60 samples of patients were approved and referred to central laboratory during the study duration. The patients with history of blood transfusion and blood disorder were excluded from the study. The samples were collected in vacutainer containing EDTA as an anti-coagulant and stored in laboratory for 15 days at 4°Cfordiagnosis by HPLC test. The same sample was assessed for The "Paper Based Diagnosis Test". The findings for both tests were recorded and compared.

TEST PROCEDURE

Blood sample is diluted with the "hemoglobin solubility buffer" at a volume ratio of 1:10. A 20 µL drop of this mixture is deposited onto chromatography paper (Whatman no. 3) using a pipette. The Paper with sample drop was allowed to dry for 5 minutes. After 5 minutes the blood satin pattern was visually interpreted. The insoluble polymerized deoxy-HbS and cellular debris are entangled by the paper fibers, remaining within the original outline of the droplet deposited on paper while soluble forms of hemoglobin are transported laterally outwards by capillary action. This process produces a blood stain with an easily recognizable pattern representative of the insoluble HbS content of the blood as seen in Figure 1. [10]

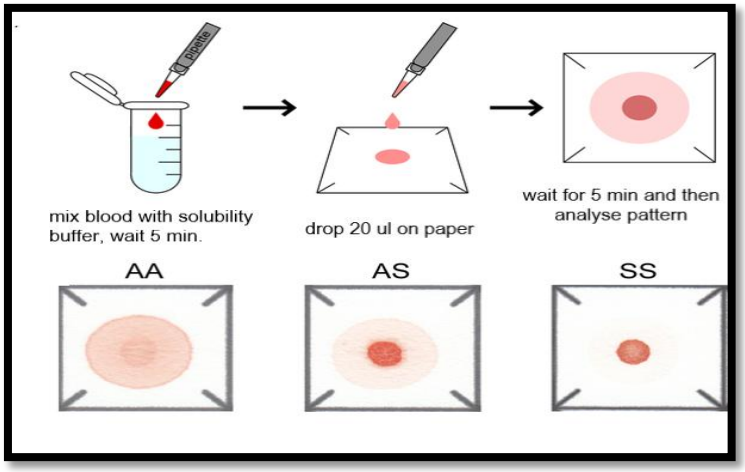

Figure 1: The Paper Based Test Procedure to identify the sickle cell hemoglobin.

Hemoglobin solubility buffer:

The "hemoglobin solubility buffer" consists of three components: Saponin, Sodium hydrosulfite and a concentrated phosphate buffer. [11,12] Saponin (4g/L) irreversibly lyses red blood cells (RBCs) by creating holes in the lipid bilayer, thereby releasing hemoglobin into the buffer. Sodium hydrosulfite (30 g/L) then converts the released hemoglobin into deoxy-Hb that is either soluble (HbA, HbE, HbF or HbC) or insoluble (HbS) in the phosphate buffer. Potassium phosphate buffer at 2.49M was made by dissolving solid 1.24M (169 g/L) monobasic and 1.25M (217 g/L) dibasic potassium phosphate in deionized water (final concentration of 2.49M). All three components were stored as dry reagents and the solubility buffer was made on site at the time of testing. The stability and longevity of buffer at different temperature and storing condition is not known thus the buffer prepared for the study was discarded after the test.

STATISTICAL ANALYSIS

The study prepared the data sheet in MS Office-Excel latest version with sample number, their HPLC findings and Paper based test findings. We analyzed the data to find the sensitivity and specificity of the paper based screening test. Evaluation of test performance includes:

Sensitivity = TP / (TP + FN);

Specificity = TN / (FP + TN);

Positive predictive value (PPV) = TP / (TP + FP);

Negative predictive value (NPV) = TN / (TN + FN); and
Accuracy = (TP + TN) / (TP + FP + TN + FN) were calculated;
Where TP = true positive, FP = false positive, TN = true negative and FN = false negative was taken in account with reference to HPLC test. ROC Curve was obtained to analyze to discriminate between HbAA from HbAS and HbSS by paper-based test

RESULTS

The paper-based test was performed for all the samples included in the study. The sample received was of patients between 18 to 40 years of age group and informed consent was taken from them. To perform the test a sample of blood is diluted with the hemoglobin solubility buffer and then a 20 μL droplet of this mixture is deposited onto chromatography paper. This process produces a blood stain with an easily visually recognizable pattern.All samples were analysed and compared with the standard HPLC data. The hypothesis was that the characteristic differences between the patterns of blood stains produced by samples from individuals with normal hemoglobin expression (HbAA), sickle cell trait carriers (HbAS) and sickle cell anemia patients (HbSS) would permit conclusive identification of eachpatient's type with visual evaluation. The tested hypothesis produced the following result seen in Figure 2.

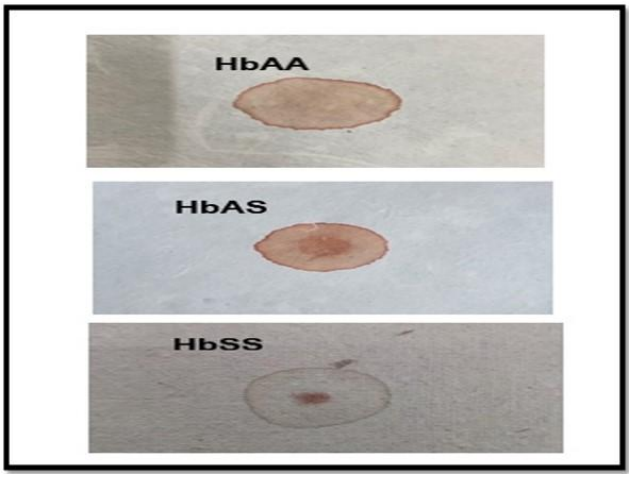

Figure 2: The patterns of blood spotting on paper with normal hemoglobin expression (HbAA), sickle cell trait carriers (HbAS) and sickle cell anemia patients (HbSS)

Table-1 shows that Paper-based study identifies 20/60 (33.3%)HbAA, 32/60 (53.4%) HbAS, and 8/60 (13.3%) HbSS. The HPLC test identifies 12/60 (20%) HbAA, 38/60 (63.3%) HbAS, and 10/60 (16.7%) HbSS. The results obtained includes TP=36, FP=4, TN=10 and FP=10. The test has 78.26% Sensitivity and 71.43% Specificityfor sickle hemoglobin (HbAS and HbSS). The calculated Positive predictive value (PPV), Negative Predictive value (NPV) and Accuracy are 0.9, 0.5, and 76.67% respectively.

Table 1: Comparison of samples for HPLC and Paper Based Test

Hb Content (N=60)	HPLC Result	Paper-Based Test
HbAA	12	20
HbAS	38	32
HbSS	10	8
Total	60	60

The Receiver Operative characteristic (ROC) curve was calculated and shown in Figure 3. ROC curve obtainedto discriminate between HbAA from HbAS and HbSS by paper-based test. ROC curve provides the details of AUC (area under curve) is 0.88 for discriminating HbAA from HbAS and HbSS by paper-based test which suggests paper-based screening test has excellent discriminating power. The positivity of the test can be matched with the curve range of the descriptive roc curve. The length of the curve determines the strength of spot and the width signifies the percentage of the sickling variation. Figure 3

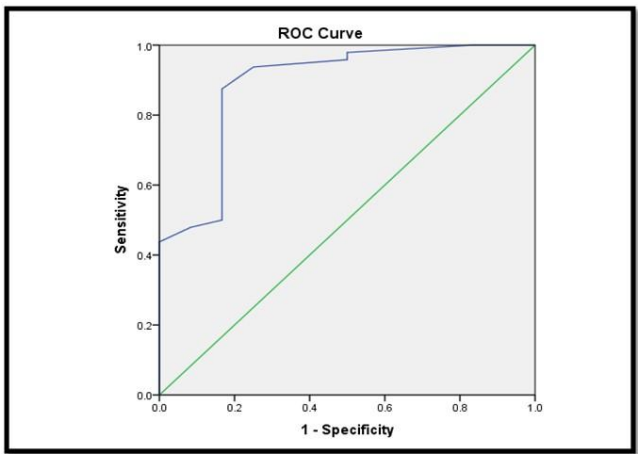

Figure 3: ROC curve to discriminate between HbAA from HbAS and HbSS by paper-based test

DISCUSSION

The paper based screening test was able to differentiate patients with normal hemoglobin with those who had the sickle cell hemoglobin in their blood. The present study conducted by an undergraduate student as an ICMR-STS project (2020-04429) in laboratory, signifies the simplicity and portability of paper-based screening test. The result obtained validates the test for screening programs in resource-limited settings.

In the present study, the differential absorption through capillary action of insoluble sickle hemoglobin and soluble non-sickle hemoglobin in paper forms the basis of the test with 76.67% accuracy. The test result indicates that 90% proportion of patients who tested positive actually have the disease, in turn establishes the credibility of the Paper-based test to be used as a screening tool in community and resource limited settings. Community-wise screening for sickle cell disease using conventional diagnostic methods such as electrophoresis, HPLC is quiet difficult and impractical because of the unaffordable high cost and lack of access to the technical infrastructure required for such testing.A more feasible and practical approach, given the limited resources available for healthcare in developing countries like India could be to use a low-cost screening assay with a low false-negative and low false-positive rate for general population screening, and then perform high-cost laboratory testing to confirm the diagnosis only for equivocal cases.

Yang et al. [11] in 2013 described a simple, quick and inexpensive paper-based sickle cell screening method that could differentiate between the sickle cell disease (SS), sickle cell trait (AS) and normal individuals (AA). They mixed the whole blood with the Hb solubility assay (SickleDex) solution and applied the mixture onto a paper. Blood stain patterns were visually interpretable. Further, Piety et al. [10] improved this assay using Hb solubility buffer (phosphate buffer saline, saponin and sodium hydrosulfite) and digitized the images of the blood stain pattern that could be quantified for HbS using the mean red color intensity in the center of the spot. They reported 93% sensitivity and 94% specificity for visual evaluation and 100% sensitivity and 97% specificity for automated analysis.

Torbian et al. [13] in 2017, by replacing the sodium hydrosulfite with sodium metabisulfite. Sodium metabisulfite is stable up to a year as a solid at room temperature and at least 6 months in aqueous solution in presence of oxygen.

India is a tropical country with a large variation in temperature throughout the year and at different geographical locations. Any variation in temperature may result in erroneous findings rendering the assay ineffective for community screening. Kumar et al. [14] in study evaluated the stability and longevity of the paper-based screening test for the sickle cell disease in relation to different temperatures and storage time. They reported 100% sensitivity and 100% specificity in identification of HbS and 97.7% sensitivity and 100% specificity for differentiating the sickle cell trait (AS) with disease (SS) with visual analysis. Such diagnostic accuracy approaches the accuracy of a lot more technologically complex methods.

The paper-based test also offers a number of advantages that are highly applicable in remote settings. It is simple and rapid to use, requiring only three steps for completion and permitting reliable visual scoring of results within 20 minutes of sample collection. The simplicity makes the test portable for use in remote facilities where standard laboratory equipment may not be available and distance, turn-around time, and poor communication infrastructure can limit the usefulness of screening outreach.

The estimated per-test cost for all components necessary to perform the paper-based test is Rs. 50 per test, which can be further reduced if bulk of samples is tested together. In contrast, the estimated per-test cost for conventional HPLC at clinical centers in India is approximately Rs. 800. With the advances in medical sciences and development of health resources and government health insurance policies (Ayushman Bharat), it is now possible to provide adequate treatment, prevent and control these disorders in India [15]. In this connection, Government of India has also issued in the union

budget 2023-24, a welcome initiative to test-track-educate-treat-council program impacting 7 crore Indians in tribal area to eliminate the sickle cell anemia by 2047, but these conventional methods require specialized equipment and trained technicians available at only tertiary health center thus further limiting the desired population to reach centers.

Currently sickling and solubility test are being used for community screening programs in India. Sickling slide test is based on the principle that in the presence of reducing agent RBC's become sickle shaped and can be viewed under microscope. This requires longer incubation period and intensive labor work. Furthermore, this test cannot differentiate between AS and SS. Similarly, solubility test based on the principle of decreased solubility of HbS in hypotonic buffers results in turbidity. Both these tests often produce false negative results in patients with severe anemia or low RBC count. Other point of care testing methods for the screening of SCD are either under development and/or at testing stages. The current screening test focuses on adapting available diagnostic tools for feasible operation at the field, especially in resource poor settings. The evolving technologiessuch as Sickle SCANTM and Hemo Type SC have varied towards overcoming concerns of cost, fabrication complexity, portability, as well as the need for highly-trained operators associated with conventional techniques [14].

Observation drawn from this study and from Kumar at al. [14] will be helpful in screening the mass population of tribal groups especially in a country like India where prevalence of sickle cell disease stretches up to 35% of their population. Early detection with this simple low cost and electricity free test can decrease the burden of disease complications and rapid treatment facility can be provided to them.

The main limitation of the present study was the availability of limited sample size. The limit detection of HbS was not evaluated in this study. The reliability of hemoglobin solubility buffer solution at different temperature in not studied in this study.

In conclusion, the paper-based test was able to detect sickled hemoglobin, thus can be used as a substitute for screening sickle cell hemoglobin in community and remote settings in India. The study validates the paper-based test as a simple low-cost and electricity free test capable of detecting sickle cell trait and disease, thus demonstrate its practicality in resource-limited clinical settings.

Acknowledgments: I would like to thank ICMR-STS for giving the opportunity to do this research. I extend my thanks to central lab staff for the help and cooperation. At last I am thankful to all the participants for the consent.

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