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Optimization of Blood Sample Volume in Dithionite Tube Turbidity (DTT) Test With Respect to Hemoglobin Concentration used for Field Screening of Sickle Hemoglobin (HbS)

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ABSTRACT

Dithionite Tube Turbidity (DTT) test is a first method of choice for easy and rapid detection of sickle hemoglobin. Even though DTT is not 100% sensitive and specific, due to its user friendly features, it is a primary screening method for mass field screening as well as for routine laboratory testing. Various DTT test buffer compositions and test procedures for performing this test are prevailing in literature. This study was performed to find out the most appropriate sample volume to achieve highest sensitivity and specificity which is suitable for the field conditions. Here we have focused on hemoglobin concentration. In order to perform this experiment, total fifty well characterized clinical samples were analyzed. We found that 20 & 25 µL of sample volumes have shown higher sensitivity and specificity with different types of blood samples. But by considering the importance of visual result interpretation of DTT test, 2 mL buffer along with 20 µL of blood sample has given the easiest and accurate result interpretation which adds in userfriendlyness of test.

By adopting this test parameter, one can easily decrease the rate of false positive and false negative test results during sickle cell screening. This test procedure is easy to perform, user friendly and does not require any instrument like centrifuge which makes it most suitable for field screening.

Keywords*: HbS: Hemoglobin S/Sickle hemoglobin, Solubility test, DTT test: Dithionite Tube Turbidity Test, Sample volume.*

INTRODUCTION

Sickle cell disease (SCD) is a genetically inherited haemoglobinopathy. It is an autosomal, recessive genetic disorder caused by a point mutation in 6th codon of β-globin gene present on chromosome 11. Due to this mutation (GAG to GTG) Glutamic acid is changed by Valine and leads to formation of Sickle hemoglobin (HbS) [1-3]. In deoxygenated state red blood cell containing HbS converts its shape from biconcave disc shape to sickle or crescent shape, which is the most important characteristic of sickle cell disease, leads to the lose in flexibility, and thus becomes rigid to move through small capillaries. Finally, this leads to breakdown of red blood cell and sudden drop in hemoglobin concentration, a condition known as anaemia[2, 4]. Sickle cell disease (SCD) comprises a group of disorders in which sickle hemoglobin is accompanied by other abnormal hemoglobin variants such as HbS/S, S/C, S/D Punjab, S/O Arab, S/Lepore, S/β Thalassaemia[5-6]. Many countries in the world are affected with SCD[7]. WHO (2006) estimated around 5-10 million SCD people in India and 20-25 million SCD people in all around the globe[8]. Every year 5,200 live SCD babies born in India[4]. This data indicates that SCD is a major public health concern in India and requires early and accurate diagnosis which could play a pivotal role in the prevention, alleviation and management of SCD[9]. This proves the importance of highly precise and accurate screening method for diagnosis of individual and large population screening for HbS.

Different diagnostic methods like hemoglobin electrophoresis, Iso Electric Focusing (IEF), Cation-Exchange High Performance Liquid Chromatography (CE-HPLC) and more recently Capillary Electrophoresis (CE) are available to screen HbS[3, 10]. Now- a-days different molecular methods based on PCR are also playing important role in diagnosis of HbS[11]. All these advance techniques are highly sensitive & specific; moreover they also make a definitive diagnosis. But all these methods are not rapid, in addition, they are resources demanding, costly and as practically not feasible for field screening as well as for the routine laboratory diagnosis. Skilled technician is required for performing them. In contrast to this, DTT test full fill all the requirements for the test which makes it suitable for field screening.

Dithionite Tube Test (DTT) is a solubility test used as a primary screening method for detecting Sickle haemoglobin (HbS). DTT is a simple and rapid screening test for detecting HbS[12-13]. In order to perform this solubility test, there are different test procedures available along with various DTT buffer compositions[14]. Contrary, DTT test may give false negative results with blood samples containing low hemoglobin concentration and also false positive results with samples having high protein as well as lipid concentration[15-17]. It is not useful for screening of infants younger than 6 months of age because they have low level of sickle hemoglobin[18]. So the present study was carried out to know the most suitable volume of test blood sample to reduce the false positive and false negative results, and as to achieve highest sensitivity and specificity in addition to feasibility of test procedure for field screening.

MATERIALS AND METHODS

This study was carried out at Valsad Raktdan Kendra (VRK), a Regional Blood Bank and Haematological Research Centre, Sickle Cell Department, Valsad, Gujarat, India from January, 2014 to January, 2015. Due approval of the project has been obtained from Scientific Advisory Committee and Institutional Ethical Committee of VRK.

Test sample:

In this study total fifty EDTA anti-coagulated whole blood samples were used. Different types and numbers of samples used for this study are mentioned in table no.1.

Glassware and Chemicals:

12 x 75 mm test tubes and glassware of good quality were used for performing the test. All the chemicals used were of analytical grade procured from Qualigens Fine Chemicals, Thermo Fisher Scientific India Pvt. Ltd., SD Fine Chem Limited (SDFCL), and Laboratory reagents & fine chemicals (Loba Chemie).

Preparation of DTT buffer solution:

For preparation of DTT buffer (stock solution), Potassium dihydrogen orthophosphate (KH_2PO_4): 1.05 M, Di-potassium hydrogen orthophosphate (K_2HPO_4): 1.44 M, Saponin: 2 gm and Benzoic Acid: 0.02 M were used. All these chemicals were dissolved in distilled water except saponin. Saponin was dissolved separately and then mixed with the buffer to avoid foaming. pH of the solution was 7. This stock solution was stored at 2-8 ºC when not in use. Working DTT buffer solution was prepared freshly before starting the experiment by preparing 3% w/v solution of Sodium dithionite powder with stock buffer solution[14].

Principle of this test is, if the test sample contains HbS, it gets reduced by Na-dithionite- a reducing agent. Reduced HbS is insoluble in nature which makes the DTT buffer solution turbid. On the other hand normal hemoglobin (HbA) gives clear solution, as not able to form turbidity[19].

Methodology:

Complete hemogram:

Calibrated sysmex Poch-100*i* particle counter was used for getting complete hemogram of EDTA whole blood test samples, and Hb concentration was noted. This test was performed as soon as the samples arrived to the laboratory. When not in use, all the samples were stored at 2-8°C.

Table 1: Different types of whole blood samples used for testing

Sr. No.	Type / characteristics of whole blood samples	Number of samples used	Hemoglobin Concentration (g/dL)	
			Lowest Hb	Highest Hb
1	Normal (AA)	16	6.1	17.9
2	Sickle cell trait (AS)	12	6.1	12
3	Sickle cell anaemia (SS)	18	6.1	11.1
4	Sickle cell β thalassaemia (S.Thal)	02	8.1	9.5
5	Heterozygote for δ β Thalassaemia	01	7.1	-
6	Double heterozygote for HbS + δ β Thalassaemia	01	12.5	-
	Total	50	-	-

***Hemoglobin electrophoresis*:**

For determining the hemoglobin pattern (AA, AS, SS) of test sample, Hb electrophoresis was performed by using the cellulose acetate membrane with TEB buffer (pH 8.4). By this method the sequence of different hemoglobins separated from cathode is HbA, HbF, HbS, & HbD and HbA2[10].

High Performance Liquid Chromatography (HPLC):

For finalizing the sickle status of test sample, HPLC (VARIANT β thalassaemia short program from Bio Rad laboratories) test was used as a gold standard method. It is based on the principles of cation-exchange high –performance liquid chromatography (HPLC). For interpretation of results, windows (*e.g.,* ranges) have been established for the most frequently occurring hemoglobins based on their characteristic retention times.

Test procedure for solubility test:

Before starting the test procedure, all the reagents and test samples were allowed to attain room temperature. Then, 2.0 mL of working DTT buffer and different volumes of whole blood specimen were added to 12 x 75 mm test tube. Here from one EDTA whole blood sample, total eight different volumes (5, 10, 15, 20, 25, 30, 35, & 40 µL) were tested with 2 mL of DTT buffer solution. After mixing properly, tubes were left at room temperature for 10-15 minutes. Finally, the visual result interpretations of the test results were made by well mixing the contents of the test tube. Results were noted in adequate illumination by keeping the tubes in front of white paper having dark black lines. The clear visibility and invisibility of lines through the test tube indicated the absence and presence of sickle hemoglobin, respectively as shown in figure 1.

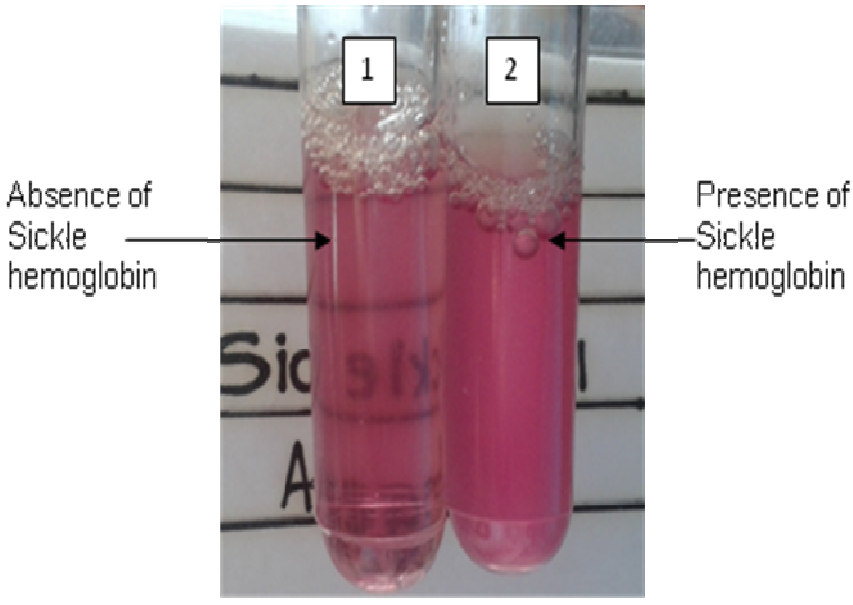

Fig. 1: Results of DTT test: 1. Normal blood sample (AA), 2. Sickle cell positive blood sample (AS/SS)

RESULTS AND DISCUSSION

This experiment was performed to know the best volume of test sample having different hemoglobin concentrations for getting high sensitivity and specificity of DTT test without compromising the ease of use of the test in the field screening of large populations. In this study total 50 different types of blood samples were analyzed as mentioned in table no.1.

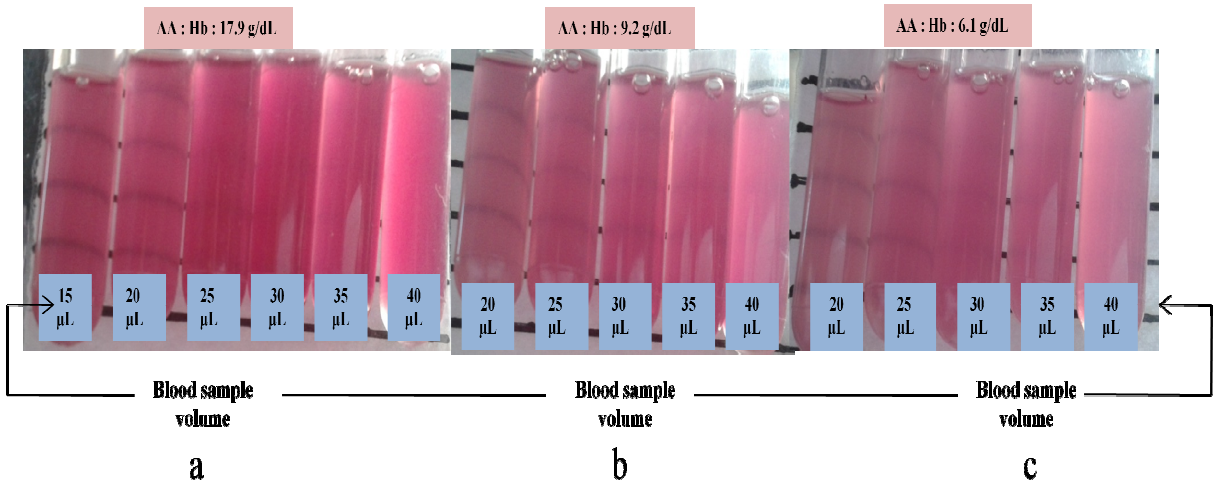

Fig. 2: Results of DTT test with normal (AA) blood samples having different hemoglobin concentrations
a. Hb: 17.9 g/dL, **b.** Hb: 9.2 g/dL and **c.** Hb: 6.1 g/dL

We found that normal (AA) blood samples with very high Hb concentration (*e.g.*, 17.1 g/dL), 25 µL, 30 µL, 35 µL & 40 µL gave small red clumps along with very dark red coloration of solution, which may interfere with result interpretation and leads to false positive results as shown in figure 2-a. Whereas with normal (AA) samples having very low Hb concentration (< 7 g/dL - *e.g.*, 6.1 g/dL), if we use less than 20 µL of blood sample then very less coloration of solution occurs thus difficult to interpret the test result as shown in figure 2-c.

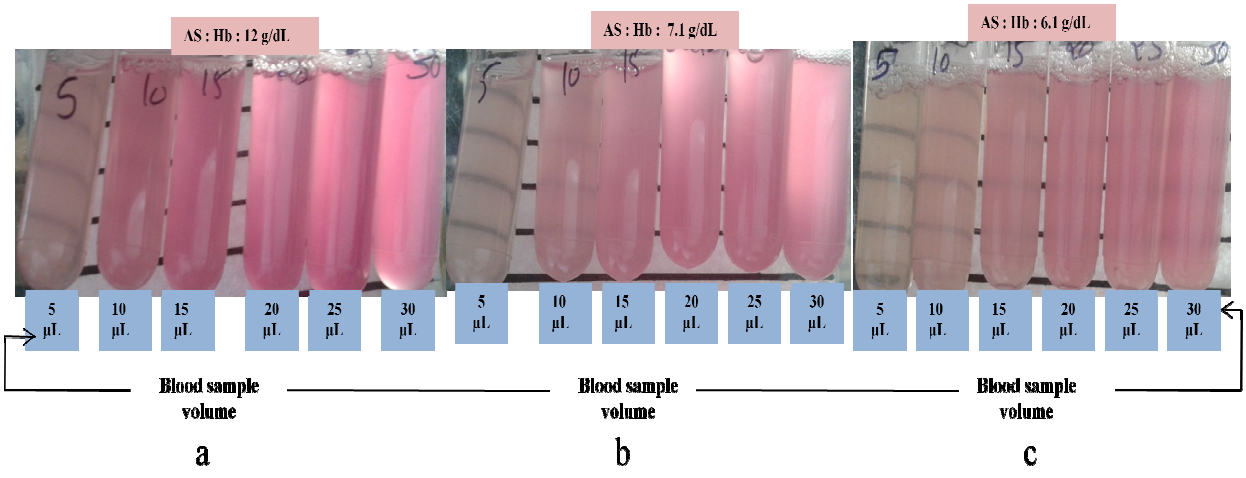

Fig. 3: Results of DTT test with sickle cell trait (AS) blood samples having different hemoglobin concentrations
a. Hb: 12 g/dL, **b.** Hb: 7.1 g/dL and **c.** Hb: 6.1 g/dL

But with sickle positive samples like sickle cell anaemia (SS), sickle cell trait (AS), and double heterozygote such as HbS + β-thalassaemia, and HbS + δ β-thalassaemia, with low Hb concentration (< 7 g/dL - *e.g.*, 6.1 g/dL), may lead to false negative results due to very low turbidity formation as shown in figure 3-c & 4-c.

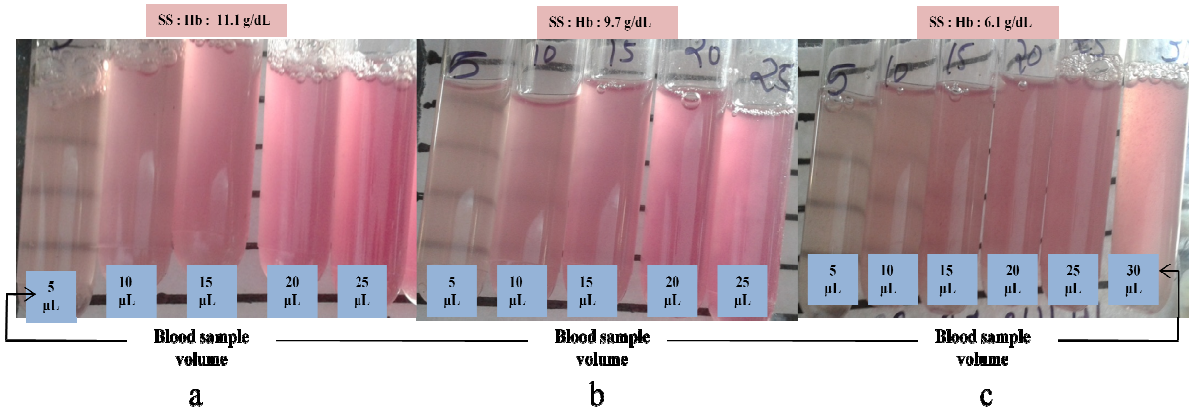

Fig. 4: Results of DTT test with sickle cell anaemia (SS) blood samples having different hemoglobin concentrations
a. Hb: 11.1 g/dL, **b.** Hb: 9.7 g/dL and **c.** Hb: 6.1 g/dL

In this study, one sample of double heterozygous state - HbS + δ β-thalassaemia (Hb: 12.5 g/dL) and one sample of heterozygote for δ β-thalassaemia (Hb: 7.1 g/dL) were used. Both of them shown accurate result when 20 µL of blood sample was used with 2 mL of DTT buffer. Sickle positive and sickle negative samples with normal Hb concentration (from 7 to 14 g/dL) did not give false (positive and negative) results with blood sample volumes 15 to 35 µL / 2 mL of DTT buffer as shown in figure 2-b, 3-b, and 4-b. But in accordance to Chasen *et al.*, (1999), Robert *et al.*, (1973) and Loh (1971), if any sickle negative sample with extreme leucocytosis, erythrocytosis, multiple myeloma, cryoglobulinemia and other dysglobulinemia or highly lipemic sample, normally cause coarse flocculation. This coarse flocculation may be incorrectly interpreted as positive for sickle hemoglobin if more than 20 µL of blood is used with 2 ml of buffer without washing the red blood cells. Many guidelines including Dacie and Lewis (1995), recommend the use of three times saline washed packed red blood cells instead of whole blood to decrease the rate of false positive and false negative results. But washing of red blood cells is not suitable for mass screening in the field as it is time consuming and also requires use of centrifuge. Thus for field screening of sickle hemoglobin, volume of test sample as well as DTT buffer should be precisely and accurately selected to decrease the rate of false positive and false negative results.

CONCLUSION

Conclusively, based on this experimental result, we recommend the use of 20 µL to 25 µL of blood sample volume with 2 mL of DTT buffer to perform this test in field. So that false positive results in case of high Hb or protein or lipid concentration and false negative results in case of low Hb concentration can be decreased significantly during the field screening of sickle hemoglobin.

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