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Optimization of DTT Buffer Volume in Dithionite Tube Test (DTT) With Respect to Hemoglobin Concentration Used for Field Screening of Sickle Hemoglobin (HbS)

KEYWORDS: Sickle hemoglobin, Solubility test, DTT test: Dithionite Tube Test, DTT Buffer

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ABSTRACT *For field screening of sickle hemoglobin, the first method of choice is Dithionite Tube Test (DTT). This is because DTT is a simple, rapid and user friendly test. Though DTT test is not 100% sensitive and specific, its user friendly features makes it the first choice for mass screening as well as for routine laboratory testing. There are various DTT buffer compositions and test procedures for performing this test are prevailing in literature. This study was performed to find out the most appropriate DTT buffer volume to achieve highest sensitivity and specificity which is suitable for the field conditions. Here we have also focused on hemoglobin concentration of test samples. In order to perform this experiment, total 50 well characterized clinical samples were analyzed. We found that 1.5 mL and 2.0 mL of DTT buffer 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 of DTT buffer along with 20 µL of blood sample has given the easiest and accurate result interpretation which adds in userfriendlyness of the test.*

By adopting this test parameter, one can easily decrease the rate of false positive and false negative test results during sickle cell screening.

INTRODUCTION:

Haemoglobinopathies such as Sickle Cell Disease (SCD) is the most common autosomal, recessive, genetically inherited monogenic disorder of hemoglobin. A point mutation[GAG (Glutamic acid) to GTG (Valine)] in 6th codon of β-globin gene present on chromosome 11 leads to formation of sickle hemoglobin (HbS) (Ghosh *et al.*, 2014; Patki *et al.*, 2013; Doshi *et al.*, 2011; Wajcman and Moradkhani, 2011). WHO (2006) estimated around 5-10 million SCD people in India and 20-25 million SCD people in all around the globe (Urade, 2013). SCD causes a major health burden in India as every year 5,200 live SCD babies born in India (Ghosh *et al.*, 2014; Urade, 2012). This data shows that SCD is a significant 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. This indicates the importance of highly precise and accurate screening method for diagnosis of individual and large population screening for HbS.

Dithionite Tube Test (DTT) is a solubility test used as a primary screening method for detecting sickle haemoglobin (HbS). It is a simple and rapid screening test (Rupani *et al.*, 2012; Dhumne and Jawade, 2011). 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 (Okwi *et al.*, 2010; Robert *et al.*, 1973; Loh, 1971). So the present study was carried out to establish the most suitable volume of DTT buffer 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 1.

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

Sr. No.	Type / characteristics of 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	--	--

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

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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 (Government of Gujarat, Sickle cell anaemia control program manual [GOG-SCACP]). The above mentioned DTT buffer composition and blood sample volume (20 µL) were already optimized in our previous study and were directly adopted to perform the present study (Rajput & Naik, 2015; Rajput, Naik, & Italia, 2015).

Principle of DTT test is, if sickle hemoglobin is present in the test sample, it is reduced by sodium dithionite- a reducing agent. This reduced HbS is insoluble in nature and forms turbidity in DTT buffer solution. Whereas normal hemoglobin (HbA) gives clear solution, as not able to form turbidity (Dacie and Lewis, 1995; Chanwin, 1989).

Methodology:

Complete hemogram: Calibrated sysmex Poch-100*i* particle counter was used for getting complete hemogram of EDTA whole blood samples, and hemoglobin 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.

***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.

High Performance Liquid Chromatography (HPLC): For finalizing the sickle status of test sample, HPLC test (VARIANT β thalassaemia short program from Bio Rad laboratories) was used as a gold standard method. It is based on the principles of cation-exchange high –performance liquid chromatography (HPLC).

Test procedure for optimization of DTT buffer volume:

Before starting the test procedure, all the reagents and test samples were allowed to attain room temperature. 20 µL of whole blood specimen was added to 12 x 75 mm test tube containing different volumes of working DTT buffer. Total six different volumes (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mL) of DTT buffer were tested with 20 µL of same whole blood specimen. After mixing properly, tubes were left at room temperature for 10-15 minutes. Results were interpreted visually after proper mixing the contents of each test tube. Results were noted in adequate illumination by keeping the tubes in front of white paper having dark black lines.

RESULTS & DISCUSSION:

This experiment was performed to establish the optimum volume of DTT buffer with respect to different hemoglobin concentration of test sample for getting high sensitivity and specificity of DTT test without compromising the ease of use of the test in field screening of large populations for HbS. In this study total 50 different types of well characterized EDTA anti-coagulated whole blood samples (as mentioned in table 1) were analyzed.

When 0.5 mL and 1.0 mL of DTT buffer tested with 20 µL of normal (AA) blood samples containing high Hb concentration (*e.g.*, 17.1 g/dL), moderate Hb concentration (*e.g.*, 9.2 g/dL) and very low Hb concentration (*e.g.*, 6.1 g/dL), small red clumps were formed along with very dark coloration of the solution. This might cause hindrance in reading results and may leads to false positive results as shown in figure 1-a, b, c. Whereas the normal (AA) blood samples having different level of Hb concentrations gave accurate results with 1.5 mL and 2.0 mL of DTT buffer volumes with very easy and user friendly visual result interpretation as shown in figure 1- a, b, c. More than 2.0 mL (*e.g.*, 2.5 mL and 3.0 mL) of DTT buffer with 20 µL of blood samples gave very light coloration with normal AA samples (as shown in figure 1-b, c), and hence not suitable for interpretation of the results.

20 µL blood sample volume of sickle positive samples like AS (Hb:12 g/dL and Hb: 7.1 g/dL), and SS (Hb:11.1 g/dL and Hb:9.7 g/dL) with 0.5 mL and 1.5 mL of DTT buffer formed small red clumps and a very dark colored solution, which might interfere with result interpretation as shown in figure 2-a, b & figure 3-a, b. In case of AS and SS type of samples, if more than 2 mL of buffer was used with 20 µL of blood sample volume, then the amount of turbidity formed was very less as shown in figure 2 & 3.

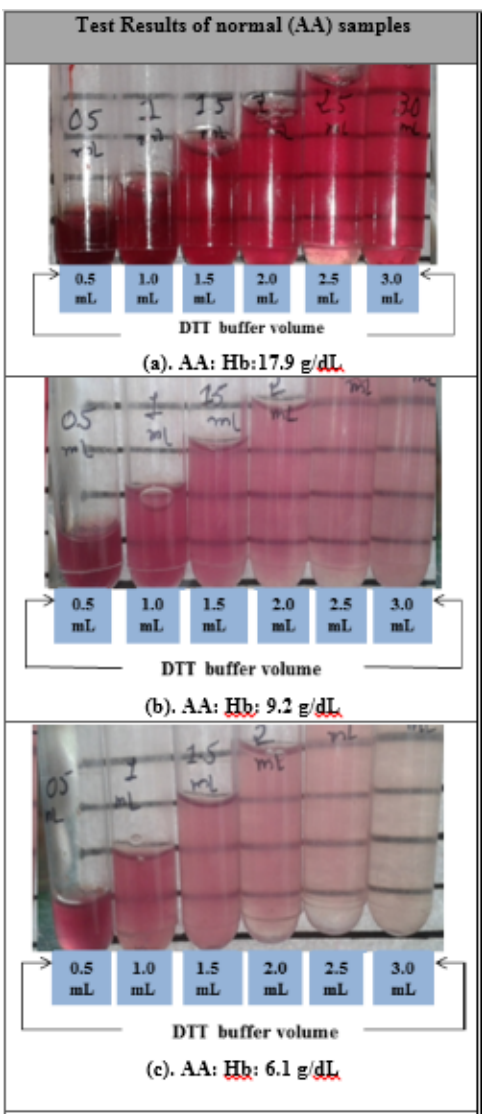

Figure 1: Results of DTT test (different volumes of DTT buffer + 20 µL of blood sample) with normal (AA) blood samples having different hemoglobin concentrations, a. Hb: 17.9 g/dL, b. Hb: 9.2 g/dL and c. Hb: 6.1 g/dL.

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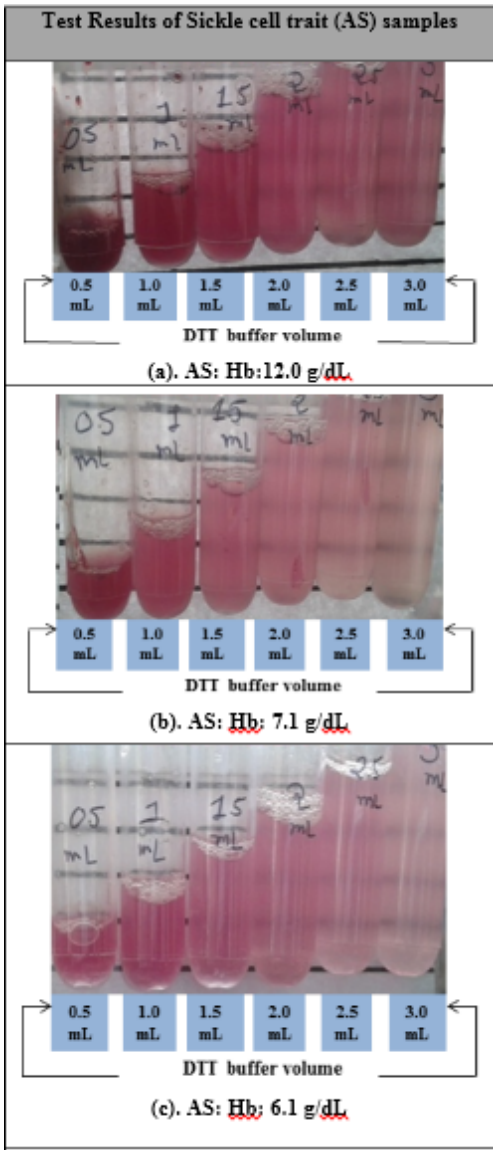

Figure 2: Results of DTT test (different volumes of DTT buffer + 20 µL of blood sample) with sickle cell trait (AS) blood samples having different hemoglobin concentrations, a. Hb: 12 g/dL, b. Hb: 7.1 g/dL and c. Hb: 6.1 g/dL.

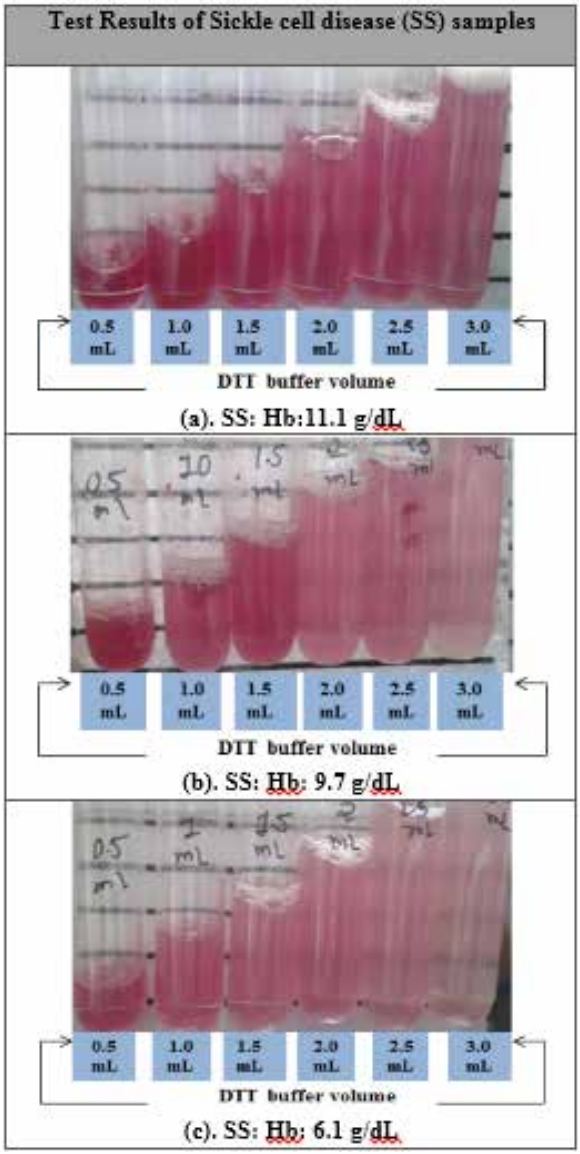

Figure 3: Results of DTT test (different volumes of DTT buffer + 20 µL of blood sample) with sickle cell anaemia (SS) blood samples having different hemoglobin concentrations, a. Hb: 11.1 g/dL, b. Hb: 9.7 g/dL and c. Hb: 6.1 g/dL.

In this experiment, along with AA, AS and SS type of samples, 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 also analyzed. Both of them shown accurate results with 1.5 mL and 2 mL of DTT buffer along with 20 µL of blood sample volume.

1.5 mL and 2.0 mL of DTT buffer with 20 µL of sickle positive and sickle negative samples having different Hb concentrations (like high, moderate and low Hb concentrations) did not give false (positive and negative) results as shown in figure 1, 2, and 3. In addition, this blood to buffer volume ratio also enhanced the ease of visual result interpretation.

Thus 1.5 mL and 2.0 mL of DTT buffer volumes with 20 µL of blood sample volume gave satisfactory results in terms of ease of visual result interpretation. But there are few conditions, like test sample which is normal for HbS, but having leucocytosis, erythrocytosis, multiple myeloma, cryoglobulinemia and other dysglobulinemia or highly lipemic sample normally cause coarse flocculation (Chasen *et al.*, 1999; Loh, 1971; Robert *et al.*, 1973). This coarse flocculation may be incorrectly interpreted as positive for sickle hemoglobin if less than 2.0 mL of DTT buffer was used with 20 µL of blood sample without washing the red blood cells. Thus even though the results of this experiment indicated that 1.5 mL and 2.0 mL both were giving satisfactory results, we recommend the use of 2.0 mL of DTT buffer in combination with 20 µL of blood sample volume to minimize false positive and negative results.

1.5 mL and 2.0 mL of DTT buffer volumes gave the most reliable results with respect to highest sensitivity and specificity with 20 and 25 µL of blood sample volumes. With less than 1.5 mL of DTT buffer, visual result interpretation was difficult and gave false positive results with samples

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containing high hemoglobin concentration. Whereas use of more than 2.0 mL of DTT buffer shown false negative results with samples containing low hemoglobin, in addition to this, it was difficult to mix sample and buffer properly with more than 2.0 mL of DTT buffer.

CONCLUSION:

Conclusively, based on this experimental result, we recommend the use of 2 mL of DTT buffer volume with 20 µL of blood sample volume 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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