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Clinical Evaluation and Enhancement of Dithionite tube turbidity (DTT) test reagents used for field screening of Sickle Haemoglobin (HbS)

Jignisha H. Rajput [1*], Jigna P. Naik[1], and Yazdi M. Italia[2]
1, Arts, Science & Commerce College, Department of Microbiology (PGDMLT), Kholwad, Surat, (Gujarat) - India
2, Valsad Raktdan Kendra (A Voluntary Blood Bank & Research Centre), R.N.C Free Eye Hospital compound, Valsad, (Gujarat) - India

Abstract

Dithionite Tube Turbidity (DTT) Test is used as a primary screening method for sickle hemoglobin in field screening as well as in routine laboratories. Even though it is not very reliable in terms of sensitivity and specificity, due to its simplicity and cost effectiveness, it is the only method feasible for mass field screening. Various DTT test buffer compositions and test procedures for performing test are prevailing in literature. This study was performed to find out the most appropriate DTT test buffer to enhance sensitivity and specificity which is suitable for the field conditions. We have found total of thirteen different chemical compositions for DTT test buffer preparation from literature review and visited existing users of the test. In order to know the best composition of DTT test buffer, total 128 well characterized clinical samples were analyzed with all different types of DTT buffers. Out of these thirteen DTT test buffer compositions, seven compositions have shown higher sensitivity and specificity. By considering the importance of visual result interpretation of the DTT test, out of these seven DTT test buffer compositions, four compositions have shown the easiest visual result interpretation, which would be user friendly and can be very easily adopted for field screening. By adopting this test procedure and DTT test buffer composition, one can easily decrease the rate of false positive and false negative test results during sickle cell screening.

Key-Words: HbS: Haemoglobin S / Sickle haemoglobin, Solubility test, DTT test: Dithionite Tube Turbidity Test

Introduction

Sickle cell disease is an inherited, lifelong disease comprises a group of genetic disorders of haemoglobinopathies characterized by the presence of sickle hemoglobin (HbS) from both parents or HbS gene from one parent and a gene for an abnormal hemoglobin or β-thalassaemia from the other parent[1-3]. Due to the structural abnormality of sickle haemoglobin, in the process of deoxygenation and reoxygenation red blood cell shrinkages to sickle shape and resumes back to its normal biconcave disc shape, respectively, repetition of this phenomenon resultant into the destruction of red blood cell, a condition called anemia[1, 3-4].

Sickle hemoglobin can be inherited in the homozygous state (SS), which results in sickle cell anaemia, or in the heterozygous state (AS), which is usually the benign, asymptomatic sickle cell trait.

*** Corresponding Author**
E.Mail: jignisha.r@gmail.com
Mob.:+91-9974215333

Hemoglobin S can also occur in the presence of other abnormal hemoglobin, *i.e.,* HbC (SC), thalassaemia (S-Thal), or HbD (SD). These double heterozygous are referred to as the sickle cell variants and can produce symptoms of varying severity[5-6].

Sickle cell anaemia is widely sprayed around the globe affecting millions of people. It is highly manifested in people whose ancestors belong to Sub-Saharan Africa, South America, Cuba, Central America, Saudi Arabia, India, and Mediterranean countries such as Turkey, Greece, and Italy[7]. The occurrence of sickle cell disease in India was unknown over the half 20th century as the first case was identified by Lehman & Catbush in Nilgiri hills amongst laborers in the tea garden of Assam in 1952[1, 3, 8]. Over the time the existence of sickle gene was also known in different parts of India among different communities especially scheduled cast and scheduled tribes[1]. It is important to note that WHO in 2006 gave an estimation of about 20-25 million sickle cell disease individuals in the world and out of which 5-10 million are living in India [9]. In India, an approximately 5,200 live babies are born with inherited

SCD every year[10]. According to Urade (2013) the frequency of sickle cell gene mostly occurred in south and central Indian tribal population which ranges from 5 to 35 percent[9]. In India sickle cell census of 2011 exhibits around 1.80 crore sickle cell trait and 14 lakhs SCD out of a large number of 18 crore tribal community. Particularly for Gujarat, census record of 2011 shows 89.12 lakh tribal population and out of which 9 lakh and 70 thousands are sickle cell trait and SCD patients, respectively. As per one of the ICMR survey in three indigenous Guajarati tribes, mortality rate in SCD individuals is 30% before the age of 14 years whereas 70% by the age of 50 years [10]. This census of sickle cell indicates a major public health concern in India and also a financial burden on Indian government treasure.

Taking into account the non curability of SCD at present, the early and accurate diagnosis could play a pivotal role in the prevention, alleviation and management of SCD. Thus it is important to note that for individual and large population screening, highly precise and accurate diagnostic screening test is essential. There are different clinical methods available for the detection of sickle haemoglobin (HbS) like hemoglobin electrophoresis, Iso Electric Focusing (IEF), Cation-Exchange High Performance Liquid Chromatography (CE-HPLC) and more recently Capillary Electrophoresis (CE) [2, 11-12]. In addition to these methods, various moleculre diagnostic methods are also available which includes: Allele-Specific Oligonucleotide (ASO) hybridization or dot-blot analysis, reverse dot blot analysis, allele specific priming or Amplification Refractory Mutation System (ARMS), restriction enzyme analysis, amplification created restriction analysis, mutagenically separated PCR, gap-PCR etc are also available for the detection of sickle gene[13]. All these advance techniques are highly sensitive and 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) [14-15]. DTT is a simple and rapid screening test for detecting HbS[15]. In order to perform this solubility test, there are different test procedures available along with various DTT buffer compositions [10, 16-27]. Present study was carried out to evaluate best composition and enhancement of the test procedure.

Material 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 128 EDTA anti-coagulated whole blood samples were used. Different types and numbers of samples used for this study are mentioned in table no.1.

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

Sr. No.	Type / characteristics of samples	Number of samples used
1	Normal (AA)	38
2	Sickle cell trait (AS)	52
3	Sickle cell anaemia (SS)	32
4	Sickle cell β thalassaemia (S.Thal)	05
5	Sickle Punjab D (SD)	01
	Total	128

Glassware and Chemicals

12 x 75 mm test tubes and glassware of good quality were used for performing the test. All the chemicals used are 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

Thirteen different chemical compositions were used for preparing thirteen different types of solubility test buffers. These compositions are mentioned in table no.2. All thirteen compositions have Saponin, KH_2PO_4, K_2HPO_4, and Sodium dithionite powder as common ingredients with varying concentration. Some of these compositions have some special chemicals which others do not have. All the above mentioned chemicals were dissolved in distilled water except saponin. Saponin was dissolved separately and then mixed with the buffer to avoid foaming. This buffer solution was stored at 2-8 °C. This test is based on the principle that if sickle hemoglobin is present in the test sample, get reduced in the presence of reducing agent-sodium dithionite and form turbidity in hypertonic organic buffer solution. This is because the solubility of sickle hemoglobin is less as compared to other hemoglobins in presence of reducing agent. Thus the normal hemoglobin - HbA produces no turbidity and the buffer solution remains clear [10, 16-27].

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Table 2: Composition of different DTT buffer solutions

Composition / Buffer No.	KH_2PO_4 (gm)	K_2HPO_4 (gm)	Saponin (gm)	Benzoic Acid (gm)	Final Volume (mL)	Na-Dithionite (gm)	Ammonium Sulphate $(NH_4)_2So_4$ (gm)
Composition-1	33.78	59.33	2.5	---	250	2.5	---
Composition-2	14.35	25	0.25	---	100	**	---
Composition-3	160.48	281.88	10	---	1000	20	---
Composition-4	143.5	250	2	2.5	1000	30	---
Composition-5	540	952	40	---	4000	40	---
Composition-6	169	215	1	---	1000	10	----
Composition-7	38.8	59.3	2.5	---	250	2.5	---
Composition-8	169	215	1	---	1000	5	---
Composition-9	125	217	2.5	---	1000	10	---
Composition-10	---	***	10	---	1000	10	280
Composition-11	169	216	10	---	1000	5	---
Composition-12	33.8	43.4	****	---	200	2	---
Composition-13	10.7	26	0.2	---	100	5	---

; **: take a pinch of Na-Dithionite powder in a test tube containing 2 mL of buffer at the time of testing, *** : adjust the pH of 280 gm/Lit $(NH_4)_2SO_4$ solution to 7.1 $\pm$ 0.1 with K_2HPO_4 (1mol/Lit), **** : 1 mL of 10 gm/100 mL aqueous Saponin solution.

Methodology

Complete hemogram and Blood grouping

On arrival of the EDTA whole blood test samples, blood group and complete hemogram tests were performed using calibrated sysmex pocH-100*i* particle counter to get important clinical information of haematocrit and hemoglobin concentration of the test samples. All the blood samples were stored at 2-8°C when not in use.

Haemoglobin electrophoresis

All the samples were subjected to hemoglobin electrophoresis using cellulose acetate membrane in alkaline TEB buffer at pH 8.4 for knowing the hemoglobin pattern of test sample. The order of hemoglobin fractions separated from cathode is HbA, HbF, HbS, & HbD and HbA2 [11].

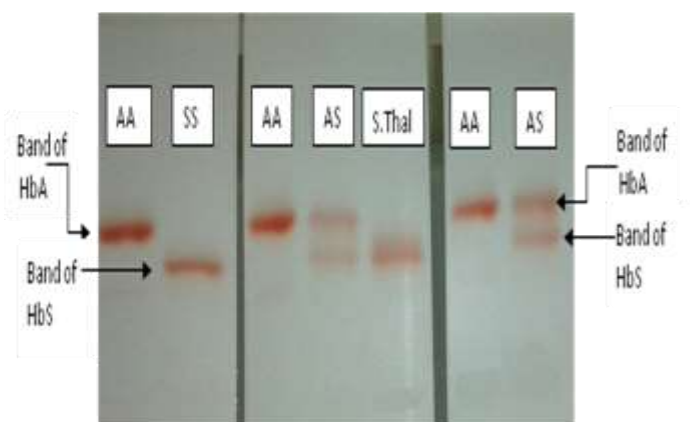
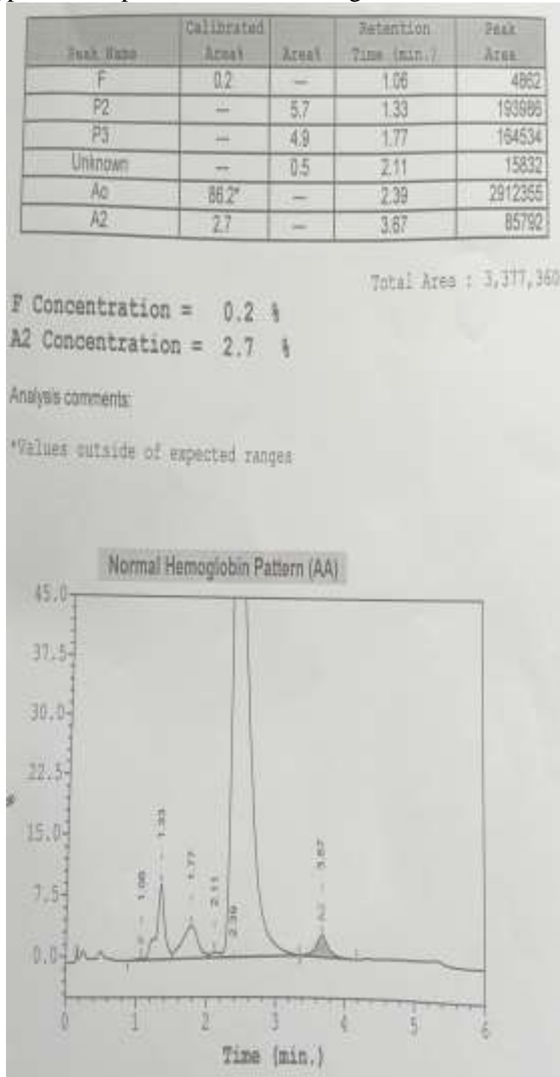


Fig. 1: Cellulose acetate electrophoresis test results

High Performance Liquid Chromatography (HPLC)

VARIANT β thalassaemia short program from Bio Rad laboratories was used as a gold standard method to finalise the sickle status of blood samples. It utilizes 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. Retention time is the elapsed time from the injection of the sample to the apex of a hemoglobin peak. Eachhemoglobin has a characteristic retention time. Results of the different types of samples were shown in figure.2.



Peak Name	Calibrated Area%	Area%	Retention Time (min.)	Peak Area
F	0.2	—	1.06	4862
P2	—	5.7	1.33	193986
P3	—	4.9	1.77	164534
Unknown	—	0.5	2.11	15832
Ao	86.2*	—	2.39	2912355
A2	2.7	—	3.67	85792

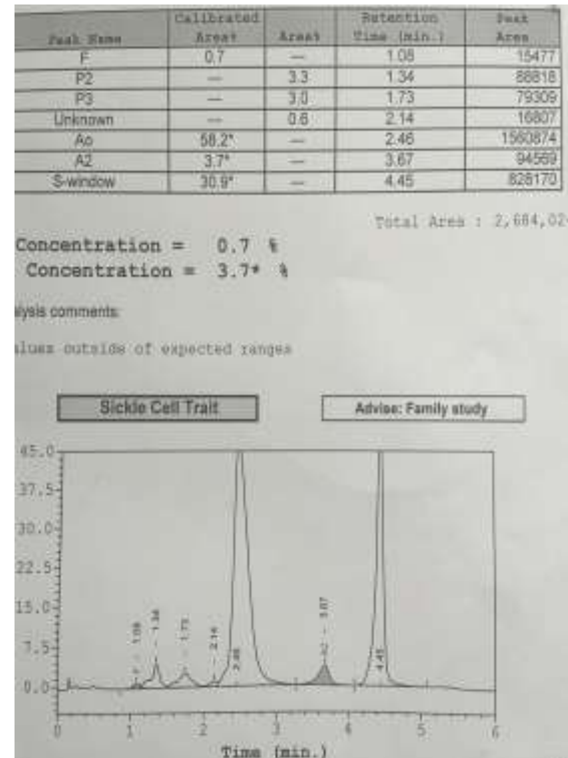
Total Area : 3,377,360

F Concentration = 0.2 %
A2 Concentration = 2.7 %

Analysis comments:

*Values outside of expected ranges

Fig. 2.a: Normal Sample (AA)



Peak Name	Calibrated Area%	Area%	Retention Time (min.)	Peak Area
F	0.7	—	1.08	15477
P2	—	3.3	1.34	88818
P3	—	3.0	1.73	79309
Unknown	—	0.6	2.14	16807
Ao	58.2*	—	2.46	1560874
A2	3.7*	—	3.67	94569
S-window	30.9*	—	4.45	828170

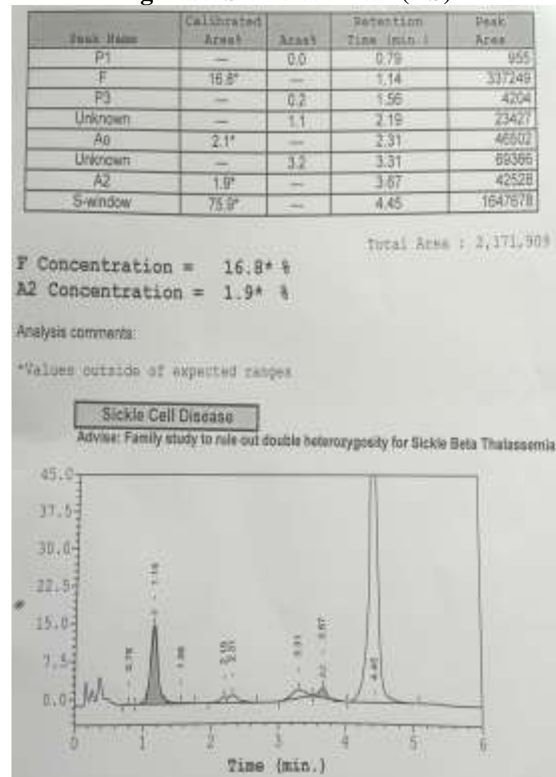
Total Area : 2,684,02

Concentration = 0.7 %
Concentration = 3.7* %

lysis comments:

lues outside of expected ranges

Fig. 2.b: Sickle cell trait (AS)



Peak Name	Calibrated Area%	Area%	Retention Time (min.)	Peak Area
P1	—	0.0	0.79	955
F	16.8*	—	1.14	337249
P3	—	0.2	1.56	4204
Unknown	—	1.1	2.19	23427
Ao	2.1*	—	2.31	46602
Unknown	—	3.2	3.31	69366
A2	1.9*	—	3.67	42528
S-window	75.9*	—	4.45	1647678

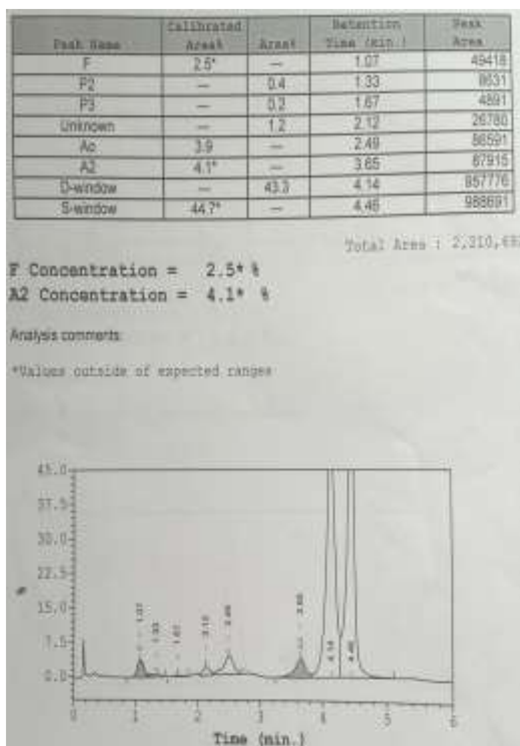
Total Area : 2,171,909

F Concentration = 16.8* %
A2 Concentration = 1.9* %

Analysis comments:

*Values outside of expected ranges

Fig. 2.c: Sickle cell anaemia (SS)



Peak Name	Calibrated Area%	Area%	Retention Time (min.)	Peak Area
F	2.5*	—	1.07	49418
P2	—	0.4	1.33	8631
P3	—	0.2	1.67	4891
Unknown	—	1.2	2.12	26780
Ao	3.9	—	2.49	86591
A2	4.1*	—	3.65	87915
D-window	—	43.3	4.14	857776
S-window	44.7*	—	4.46	988691

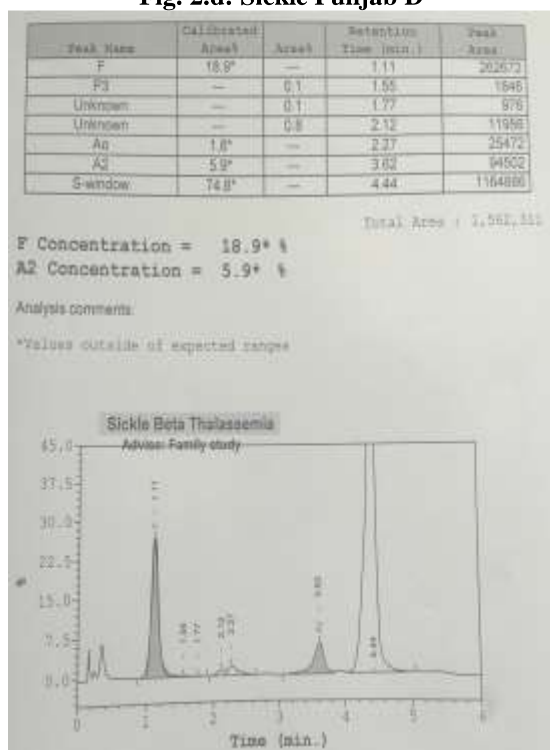
Total Area : 2,310,6[illegible]

F Concentration = 2.5* %
A2 Concentration = 4.1* %

Analysis comments:

*Values outside of expected ranges

Fig. 2.d: Sickle Punjab D



Peak Name	Calibrated Area%	Area%	Retention Time (min.)	Peak Area
F	18.9*	—	1.11	262673
P3	—	0.1	1.55	1646
Unknown	—	0.1	1.77	976
Unknown	—	0.8	2.12	11956
Ao	1.8*	—	2.37	25472
A2	5.9*	—	3.62	94502
S-window	74.8*	—	4.44	1164886

Total Area : [illegible]

F Concentration = 18.9* %
A2 Concentration = 5.9* %

Analysis comments:

*Values outside of expected ranges

Fig. 2.e: Sickle Thalassaemia

Fig. 2: Variant HPLC results of different types of blood samples

Common test procedure for solubility test

The common test procedure was adopted and finalized from different references [10, 16-27]. The assay protocol adopted for evaluating the thirteen different DTT test buffer compositions was mentioned below:

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 20 µL of whole blood specimen were added to 12 x 75 mm test tube. After mixing properly, it was left at room temperature for 10 minutes. Finally, the visual result interpretations of the test results were made by well mixing the contents of the test tube. Results were noted 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 3.

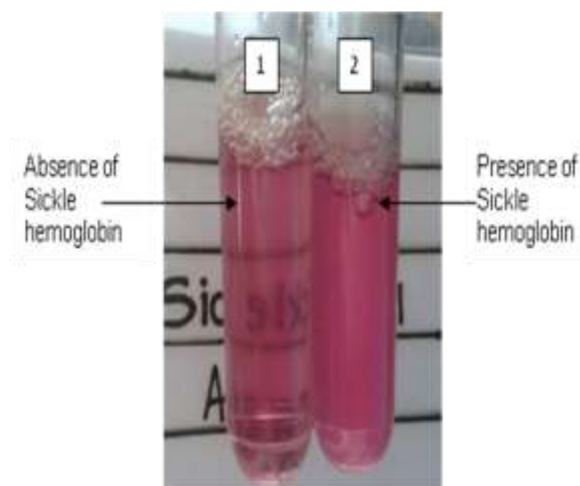


Fig. 3: Results of DTT test

In the next step, by keeping the same test procedure as mentioned above, we have performed the DTT test by using thirteen different buffer compositions.

In this test, we have used total 128 different types of blood samples which include Normal (AA), Sickle Cell Trait (AS), Sickle Cell Anaemia (SS), Sickle Thalassaemia, & Sickle Punjab D type of blood samples as mentioned in table no 1.

Results and Discussion

To the best of our knowledge, this was the first study to compare the thirteen different chemical compositions of DTT buffer with the test procedure performed in this study. This experiment was performed to provide the best composition for performing DTT test in the field, with the achievement of high sensitivity and

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specificity, without compromising the ease of use of the test in the field screening of large populations.

In this study total 128 blood samples were analyzed. All these compositions could reliably detect samples with homozygous state (sickle cell anaemia -SS). But some of them showed variability in their ability to detect heterozygous state (sickle cell trait-AS) and presence of double heterozygous state (sickle gene with some other abnormal hemoglobin gene) such as in this study sickle-thalassaemia and sickle-Punjab D. We found that seven compositions (C-1, C-2, C-4, C-6, C-8, C-11, & C-12) were giving the best results in terms of high sensitivity (100%) and specificity (100%). For two DTT buffer compositions (C-9 & C-10), our finding is in co-relation with the findings of Okwi *et al*, Chasen *et al* and Hicks *et al* that the solubility test is not 100% sensitive for the sickle cell trait (AS) stage, whereas for remaining eleven compositions, AS stage is correctly diagnosed [28-30]. In addition to this, we found that double heterozygous conditions (sickle-thalassaemia and sickle-Punjab D) are also not correctly detected by two DTT buffer compositions (C-9 & C-10), whereas no false negative results found with eleven chemical compositions. Out of thirteen compositions, four compositions (C-3, C-5, C-7, & C-13) gave false positive results with true negative samples.

Table 3: Results of primary screening with thirteen different DTT Buffer compositions:

Sample type	Normal (AA) N=38		Sickle cell trait (AS) N=52		Sickle cell anaemia (SS) N=32		Sickle-thalassaemia N=5		Sickle-Punjab D N=1		Sensitivity %	Specificity %
Composition (C) No.	P	N	P	N	P	N	P	N	P	N		
C-1	00	38	52	00	32	00	05	00	01	00	**100%**	**100%**
C-2	00	38	52	00	32	00	05	00	01	00	**100%**	**100%**
C-3	38	00	52	00	32	00	05	00	01	00	100%	50%
C-4	00	38	52	00	32	00	05	00	01	00	**100%**	**100%**
C-5	38	00	52	00	32	00	05	00	01	00	100%	50%
C-6	00	38	52	00	32	00	05	00	01	00	**100%**	**100%**
C-7	33	05	52	00	32	00	05	00	01	00	100%	53.52%
C-8	00	38	52	00	32	00	05	00	01	00	**100%**	**100%**
C-9	00	38	48	04	32	00	05	00	00	01	94.74%	100%
C-10	00	38	06	46	07	25	00	05	00	01	53.89%	100%
C-11	00	38	52	00	32	00	05	00	01	00	**100%**	**100%**
C-12	00	38	52	00	32	00	05	00	01	00	**100%**	**100%**
C-13	38	00	52	00	32	00	05	00	01	00	100%	50%

; P=Positive result, N= Negative result, C=Composition

We have also considered the importance of visual result interpretation of DTT test result. In co-relation to this, out of the seven compositions having 100% sensitivity and specificity, four compositions (C-2, C-4, C-8 & C-12) were giving the clear visual result interpretation in terms of clarity of solution with all different types of clinical samples. Though the remaining three compositions (C-1, C-6, & C-11) shown the 100% sensitivity and specificity, during test result interpretation we noted the presence of small red clumps and also less turbidity with few true positive samples and hazy/turbid solution which may interact during result interpretation with few of the true negative samples. For the user friendliness of the test procedure for the field screening, our results are matching with findings of Nalbandian *et al* and Clark K. G. A. that the procedure finalized for this study from different sources is simple, rapid, and non instrumental based, so suitable for mass screening in the field [18, 26].

Conclusion

By considering the above mentioned advantages, use of any one out of these four finalized chemical compositions (C-2, C-4, C-8 & C-12) for the field screening purpose is most accurate & precise. In addition to this, the procedure finalized for this study from different sources is simple, rapid, and non instrument based, so suitable and appropriate for mass

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screening in the field. The main limitation of this study was the use of less number of compound heterozygous samples (e.g. in this case Sickle-Thalassaemia & Sickle-Punjab D). So further study with more number of common double heterozygous samples (Sickle-Thalassaemia & Sickle Punjab D) along with other rare forms like sickle cell anaemia with high HbF, HbS /Hb O-Arab, HbS /HbC, HbS /HbE, HbS /Hb Lepore etc would be of great importance.

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