Sickle cell solubility test: evaluation of an in-house method

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ABSTRACT

This study aims to assess the performance of an in-house sickle cell solubility test (SCT) and compare it against data published on other in-house and commercial methods. Also assessed are the effects of possible interference due to haemoglobin (Hb) level, Hb F and Hb S levels, as well as lipaemia, icterus and haemolysis index. A total of 1030 patient samples were tested by the SCT and subsequently confirmed by high-performance liquid chromatography (HPLC). Seventy-five (7.3%) samples revealed a positive SCT whereas 955 (92.7%) were negative. The sensitivity and specificity of the method was 100% and it was shown to detect low levels of Hb S (12.1%). These data show that the authors' in-house SCT method has an excellent performance against other documented methods (including some commercial kits), which is probably explained by its robustness against low levels of Hb S and interfering substances such as lipaemia. The authors believe this shows that in-house tests are able to produce highly reliable results comparable to, if not better than, certain commercially available CE-marked kits.

KEY WORDS: Sickle cell.
Sensitivity.
Specificity.

Introduction

Sickle haemoglobin is a variant that results from a mutation in the sixth amino acid on the β-globin gene (β6; glutamic acid replaced by valine), commonly resulting in Hb S, although there are 13 other rare Hb variants incorporating this mutation (e.g., Hb C-Harlem or Hb S-Antilles, which contain the β6 mutation of Hb S and another substitution on the same β-gene).[1,2]

This condition may be inherited in its carrier form, where the individual inherits one copy of the Hb S variant and one copy of the normal β-globin gene (sickle cell carrier, Hb AS), or as a major form of the condition, sickle cell disease (SCD). The latter may present as one of two distinct groups: inheritance of two copies of the Hb S variant (sickle cell anaemia, Hb SS) or compound heterozygosity for Hb S and an interacting gene such as Hb C, β-thalassaemia, Hb E, Hb D-Punjab, Hb O-Arab or hereditary persistence of fetal haemoglobin (HPFH).[1]

The high frequency of sickle cell conditions observed among individuals of African and Mediterranean ancestries has been linked to the resistance it confers against malaria infection, with lower rates of severe complications of malaria infection (e.g., cerebral malaria) being reported in Hb S carriers compared with non-carriers.[3] As a result of migration, this condition is now seen in many other parts of the world, including the UK. The most recent research estimates that there are about 240,000 sickle cell carriers and 15,000 SCD patients in the UK. The highest prevalence is among Black Caribbean, Black African and Black British groups.[4]

Several sickle cell solubility tests have been developed over the years,[5–8] and these may be used as a rapid screening test for sickle cell status (e.g., urgent screening prior to surgery) or for confirmation of Hb S, when high-performance liquid chromatography (HPLC) is used as a screening test.

The aim of this study is to assess the performance of an in-house sickle cell solubility test (SCT) and compare it against data published on other in-house and commercial methods.

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In-house diagnostic tests have recently been put in the spotlight, as the European Parliament debated new legislation to replace the current directive on *in vitro* diagnostic medical devices, with concerns over quality, performance and safety of such tests in comparison to CE-marked tests being raised during the consultation period.[9]

Materials and methods

Samples referred for haemoglobinopathy screening were tested over a one-month period. A total of 1030 venous blood samples collected into 4-mL anticoagulated BD Vacutainer tubes containing dipotassium EDTA (0.184 mol/L; BD, Oxford, UK) were tested by the authors' in-house SCT and subsequently confirmed by HPLC (other techniques such as Hb electrophoresis and DNA analysis were also performed for confirmation of certain Hb variants).

Both tests were performed on fresh blood samples within 24 hours of collection. Newborns and infants less than six months old, and patients transfused within the previous four months were excluded from the study.

In order to assess the effect of possible interference on the SCT, the following results were collected: haemoglobin (Hb, g/L; DxH 800, Beckman Coulter, Brea, CA, USA); Hb F % and Hb S % when present (Variant II, Bio-Rad

Laboratories, Hercules, CA, USA); lipaemia, icterus and haemolysis (LIH) indices were also recorded when available (AU5800, Beckman Coulter).

Furthermore, a group of 100 transfused SCD patients was also tested by SCT and HPLC techniques in order to determine if low levels of Hb S affect the accuracy of the in-house method, which was based on a modification of the Itano solubility test,[5] adapted by Huntsman *et al*.[6] for whole blood samples, and has been in constant use for over 40 years.

Sickle cell solubility test protocol

The SCT entailed diluting 50-µL whole blood in 1 mL working Itano buffer solution. This was mixed well and centrifuged at 4000 x*g* for 2 min. A double volume of whole blood or packed cells was used in very anaemic samples (i.e., typically less than 80 g/L) if unclear results were obtained after centrifugation. The basic principle of the test is that saponin lyses the red cells, releasing haemoglobin into solution, and the sodium dithionite de-oxygenates the haemoglobin. Upon de-oxygenation, Hb S becomes insoluble and precipitates, producing a dark band that can be seen at the top of the solution after centrifugation. The phosphate buffer is essential to maintain the pH and ionic strength of the test. A known positive control was included in every batch of samples in order to validate the results, and the test was subjected to regular external quality assessment exercises (UK NEQAS).

Itano buffer

Potassium dihydrogen orthophosphate (KH_2PO_4): 675 g
Dipotassium hydrogen orthophosphate (K_2HPO_4): 1190 g
Saponin: 50 g
Dissolved in deionised water up to a final volume of 5 L.
This buffer was validated and stored routinely at 4°C for up to three months.

Working Itano buffer solution

Sodium dithionite: 0.5 g
Itano buffer: 50 mL
The working buffer solution was validated and stored at room temperature for up to 6 h (the integrity of the working solution was ensured by testing a known positive control with every batch of samples).

Haemoglobinopathy screen

All samples were tested by HPLC (Bio-Rad Variant II, β-thal short programme). Other techniques such as haemoglobin electrophoresis and/or molecular techniques were also performed, where indicated. α^0-thalassaemia was screened using an Hb H preparation, therefore it was not possible to exclude α-thalassaemia in certain cases (not relevant to the present study).

Lipaemia, icterus and haemolysis screen

The LIH screen was performed on the corresponding serum sample (when available) collected into 5-mL BD Vacutainer SST II gel tubes (BD, Oxford, UK), using the Beckman Coulter LIH reagent kit on the Beckman Coulter AU5800. Serum gel tubes were centrifuged at 1500 x*g* for 10 min within 2 h of sample collection, and the separated serum analysed within 4 h. The following interference cut-off values were established, as per the manufacturer's recommendations: lipaemia ≥0.4 g/L intralipid; icterus ≥0.025 g/L bilirubin; haemolysis: ≥0.5 g/L haemoglobin.

Statistical analysis

Descriptive statistics were calculated using SPSS version 14.0 (SPSS, Chicago, Illinois, USA). Sensitivity and specificity were calculated after determining the percentage of false-positive and false-negative results compared to the haemoglobinopathy screen.

Results

A total of 1030 patient samples were tested by the SCT and subsequently confirmed by HPLC. Patient age range was 1–95 years old (mean: 36.6±0.5), and 810 (78.6%) were female (most of the haemoglobinopathy screens processed in the authors' laboratory are part of the NHS sickle cell and thalassaemia antenatal screening programme).

Of the 1030 samples tested, 955 (92.7%) showed a negative SCT, whereas 75 (7.3%) resulted in a positive SCT (Table 1). All positive SCT results were consistent with the HPLC screen: 68 samples were Hb AS, five samples were Hb SS, one was Hb SC, and one was Hb S/HPFH. Of the 955 negative SCT, 883 revealed a normal HPLC screen and 72 showed haemoglobinopathy other than Hb S. The sensitivity and specificity of the method was 100%, based on the fact that no false-negative or false-positive results were observed.

As anaemia, low levels of Hb S or raised Hb F levels are usually regarded as factors that may cause erroneous sickle cell solubility test results, the study compared these parameters in both groups (Table 2).

The levels of Hb S detected in the positive SCT group

Table 1. Comparison of results obtained by in-house sickle cell solubility test versus haemoglobinopathy screen.

	Haemoglobinopathy screen (*n*=1030)					
	Normal	AS*	SS	SC	S/HPFH	Other haemoglobinopathy†
Positive SCT	0 (0%)	68 (6.6%)	5 (0.5%)	1 (0.1%)	1 (0.1%)	0 (0%)
Negative SCT	883 (85.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	72 (7.0%)
Total	883 (85.7%)	68 (6.6%)	5 (0.5%)	1 (0.1%)	1 (0.1%)	72 (7.0%)

SCT: in-house sickle cell solubility test.

*including eight cases of possible co-existent α-thalassaemia and/or iron deficiency (not confirmed).

†including 31 β-thalassaemia carrier, 15 Hb AC, 11 δ-chain variant, four HPFH, three Hb AE, three α-thalassaemia carrier, two Hb A/D-Punjab, one Hb A/O-Arab, one Hb A/Q-Iran, one Hb Constant Spring + Hb AE/Barts disease.

Table 2. Hb level, Hb F and Hb S levels in the positive and negative in-house sickle cell solubility test groups.

	Negative SCT (n=955)		Positive SCT (n=75)		
	Hb (g/L)	Hb F (%)	Hb (g/L)	Hb F (%)	Hb S (%)
Range	55–180*	0.1–28.2	39–179*	0.1–31.9	25.5–84.4
Mean	124.6±0.5	0.6±0.06	120.1±2.5	1.8±0.5	38.6±1.5

SCT: in-house sickle cell solubility test.

*Anaemic samples corrected using SCT protocol described.

varied (25.5–84.4%) and raised Hb F levels did not appear to affect the performance of the method (highest Hb F level tested was 31.9%). Anaemic samples did not impact the results of this study due to the fact that these were corrected to a normal haemoglobin level prior to performing the SCT.

The LIH screen was performed on 405 samples and 24 (5.9%) were positive for at least one interferent (Table 3). This suggests that approximately 6% of the samples contained a potentially interfering concentration of substances that could have affected the performance of the test, yet no false-positive or false-negative results were noted.

Low levels of Hb S may occur in neonates (<6 months old), following transfusion, in iron deficiency, or co-existent α-thalassaemia. Hb AS patients with co-existent α-thalassaemia may present with Hb S levels of 22–35%, depending on the number of α genes affected,[10] and this has been reported as a cause of false-negative results in sickle cell solubility tests.[11,12]

In order to determine if low levels of Hb S affect the accuracy of the SCT method, and to establish the lowest level of Hb S detectable by the method, the authors tested a group of 100 transfused SCD patients by SCT and HPLC. All revealed a positive SCT, and the Hb level, Hb F and Hb S levels are shown in Table 4. The levels of Hb S detected in the transfused SCD group varied (12.1–90.4%) and no false-negative results were obtained.

Discussion

The in-house SCT was shown to detect low levels of Hb S (12.1%). Theoretically, this would make it possible to pick up all cases of Hb AS with co-existent α-thalassaemia, a well reported cause of false-negative results in sickle cell solubility tests.[11,12] In fact, 11% of this group of samples showed <22% of Hb S (range: 12.1–21.5%), lower levels than reported in Hb AS with co-existent α-thalassaemia,[10] yet they all resulted in a positive SCT.

The sensitivity and specificity of the authors' method was 100%. Some studies reported similar findings but these involved a relatively small number of samples (range: 110–200),[7,8,13] whereas larger studies were associated with lower sensitivity (93.8–98.9%) and comparable specificity (99.9–100%).[12,14] The variation in sensitivity, specificity, accuracy and precision of different commercial solubility test kits was reported by Schmidt and Wilson.[15]

Confirmation of positive SCT results is required to differentiate between sickle cell carriers and major forms of the condition, or any of the other 13 known rare sickle variants.[1,2] None of these rare mutations were found in the samples tested in the present study.

The authors conclude that their in-house SCT method and the protocol used shows excellent performance against other documented methods (including some commercial kits), perhaps explained by its robustness against low levels of Hb S and interfering substances such as lipaemia, which are well-documented causes of erroneous results in commercial kits.[11,12,15] Another important characteristic of the in-house SCT (which most commercial methods lack) is centrifugation of the test sample prior to reading the results, thus concentrating the precipitated sickle haemoglobin. The authors believe their data show that in-house tests are able to produce highly reliable results comparable to, if not better than, certain commercially available kits that usually carry significantly higher costs.

As a result of the debate around the proposed new legislation to replace the current directive on *in vitro* diagnostic medical devices and in-house testing, and follow up by the British In Vitro Diagnostics Association (BIVDA) report,[9] the authors believe it is worth considering the role that in-house testing plays in diagnostic laboratories and their current performance. The assumption that in-house test performance is inferior to CE-marked methods is not justified or rational. In-house tests play an important role in specialist areas and may form the basis for the development of new commercial CE-marked tests. It is important that this avenue is not stifled to the extent that the use of an in-house test becomes untenable or is regarded as offering second-rate performance. □

Table 3. Lipaemia, icterus and haemolysis (LIH) screen.

	LIH screen (n=405)		
	Lipaemia	Icterus	Haemolysis
Present	4	4	16
Absent	401	401	389

Table 4. Hb level, Hb F and Hb S levels in the transfused sickle cell disease group.

	Transfused SCD (n=100)		
	Hb (g/L)	Hb F (%)	Hb S (%)
Range	60–122*	0.1–24.7	12.1–90.4
Mean	88.7±1.5	4.0±0.4	45.4±2.3

SCD: sickle cell disease.

*Anaemic samples corrected using SCT protocol described.

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