

THE RELIABILITY OF SICKLING AND SOLUBILITY TESTS AND PERIPHERAL BLOOD FILM METHOD FOR SICKLE CELL DISEASE SCREENING AT DISTRICT HEALTH CENTERS IN UGANDA.OKWI A.L.¹; BYARUGABA W.¹; PARKES A.²; OCAIDO M.³(Manuscript N°D194. Received 24/02/2010. Accepted in revised form 25/05/2010) **Clin Mother Child Health 2010; Vol 7, N° 1: 1205 - 1210****ABSTRACT**

Although sickling and solubility tests and peripheral blood film methods are today available for sickle cell disease screening in Uganda, their reliability and ease of applicability have not been determined. This study was therefore carried out to determine the reliability of sickling and solubility tests and peripheral blood film method for screening for SCD in Uganda. This was descriptive laboratory based study which was carried out at Makerere University College of Health Sciences. The 200 samples from children aged between 6 months and five years were independently analyzed using sickling and solubility tests and peripheral blood film method. Hemoglobin electrophoresis cellulose acetate was used as the gold standard. Sickling and solubility tests had sensitivities of 65.0 % and 45.0% respectively and peripheral film had 35.0%. Sickling, solubility and peripheral film had specificities of 95.6%, 90.0% and 96.7% respectively. Sickling had diagnostic accuracy of 92.5%, solubility (85.5%) and peripheral film (90.5%). Sickling had a Cohen's Kappa of 0.6, solubility 0.3 and peripheral film 0.4. Sickling test had turn around time (TAT) of 38 minutes, solubility 70 minutes and peripheral 44 minutes.

In conclusion, the sickling test was more reliable and easier to perform than solubility test and peripheral blood film method. It would therefore be a recommended test for preliminary screening of children for SCD at district health centers IV and confirming only positives using hemoglobin electrophoresis.

KEY WORDS: Screening - Sickling and solubility tests -Peripheral blood film - Sickle cell disease - Uganda.

LA FIABILITE DES TESTS DE FALCIFORMATION ET DE SOLUBILITE ET DE LA METHODE DE FROTTIS SANGUIN PERIPHERIQUE DANS LE DEPISTAGE DE LA DREPANOCYTOSE DANS LES DISTRICTS DE SANTE EN OUGANDA.**RESUME**

Bien que les analyses hématies falciformes, la solubilité et le film sanguin périphérique soient aujourd'hui disponibles pour le dépistage de la maladie drépanocytaire en Ouganda, leur fiabilité et aisance d'applicabilité n'ont pas été déterminées. Cette étude a été, par conséquent réalisée pour déterminer la fiabilité de la méthode de dépistage des analyses des hématies falciformes et de la solubilité ainsi que le film sanguin périphérique pour la SCD (sigle anglais pour Sickle cell disease) en Ouganda. Ceci a été une étude descriptive basée au laboratoire et réalisée par le Collège des Sciences de la Santé de l'Université de Makerere. Les 200 prélèvements des enfants ages de 6 mois à 5 ans ont été analysés de façon indépendante en utilisant la méthode des analyses d'hématies falciformes, la solubilité et le film sanguin périphérique. L'acétate de cellulose de l'électrophorèse de l'hémoglobine a été utilisé comme échantillon. Les analyses des hématies falciformes et de solubilité ont eu la sensibilité de 65,0% et 45,0%, respectivement, et le film sanguin périphérique a eu 35,0%/. Les hématies falciformes, la solubilité et le film sanguin périphérique ont eu les spécifications de 95,6%, 90,0% et 96,7% respectivement. Les hématies falciformes ont eu la précision diagnostique de 92,5%, la solubilité – 85,5% et le film sanguin périphérique – 90,5%. Les hématies falciformes ont eu le Kappa de Cohen de 0,6, la solubilité – 0,3 et le film sanguin périphérique – 0,4. L'analyse des hématies falciformes a eu une période de rotation de 38 minutes, la solubilité – 70 minutes et le film sanguin périphérique – 44 minutes.

En conclusion, l'analyse des hématies falciformes a été plus fiable et plus facile à exécuter que l'analyse de la solubilité et la méthode de film sanguin périphérique. Elle serait, par conséquent, une analyse recommandée pour le dépistage préliminaire des enfants pour la SCD aux centres de santé IV au niveau de districts et confirmer seulement les résultats positifs en utilisant l'électrophorèse de l'hémoglobine.

MOTS CLES: Dépistage – Tests de falciformation et de solubilité – Sang périphérique- Drépanocytose - Ouganda.

¹Department of Pathology, Makerere University College of Health Sciences, P.O. Box 7072, Kampala, Uganda

²Centre for Endocrine and Diabetes Sciences, Cardiff University, Cardiff CF14 4XN Wales, UK

³Department of Wildlife and Animal Resources, Faculty of Veterinary Medicine, Makerere University, P.O. Box

7062, Kampala, Uganda. Correspondences Dr. OKWI A.L. Department of Pathology, Makerere University College of Health Sciences, P.O. Box 7072, Kampala, Uganda;

E-mail: livexo@yahoo.co.uk, Tel: +256 772 481887

INTRODUCTION

Methods including hemoglobin (Hb) electrophoresis, iso-electric focusing (IEF) and high performance liquid chromatography (HPLC) are today used to screen for haemoglobinopathies in some developed countries [1]. In particular, polymerase chain reaction (PCR) is useful for both pre-natal and neonatal diagnosis of haemoglobinopathies, including sickle cell disease [2]. According to SCHULTZ [3], methods using monoclonal antibodies for sickle cell screening are also available, but they have not yet been clinically accepted because they are un-reliable. While Hb electrophoresis is extensively used in Jamaica as

a confirmatory procedure [4], its use in most of the resource constrained countries, including Uganda, is impractical due to high cost. In Uganda, the availability of Hb electrophoresis equipment is limited to some of the teaching hospitals and the national referral hospital at Mulago.

However, there are other affordable methods of varying reliability, ease of applicability and cost available for early screening for SCD in low income countries. These include solubility and sickling tests and peripheral blood film method. Although a developing country such as Malawi has carried out reliability and cost benefit analysis studies [5], and has recommended appropriate haemoglobin methods for use at district health centers, none of these studies have been undertaken in Uganda. This study was therefore conducted to evaluate the sensitivity, specificity and predictive values and ease of applicability of sickling and solubility tests and peripheral blood film method.

II- MATERIALS AND METHODS

- **Study design:** This was descriptive laboratory based study.

- **Study sites:** The study was conducted in the department of Pathology, Makerere University College of Health Sciences where approval of the study was made and Department of Clinical Chemistry, Mulago hospital Uganda where Hb electrophoresis analysis was performed on blood samples which were received from the study districts.

- **Sample size:** The 200 blood samples were estimated using standard graphs based on the assumption that at 95% confidence interval, the expected coefficient of reliability detection of sickle cell disease is 0.05 BROWNER et al [6].

- **Study procedure:** Two hundred samples (178 negative for SCD; 22 positive for SCD) were selected using

systematic sampling procedure from samples which had been collected for prevalence studies from children aged 6 months to 5 years. The samples were then independently analysed by three senior technologists using sickling and solubility tests and peripheral blood film method adapted after [7-8].

1. Sickling test

The principle of sickling test was based on microscopical observation of sickling of red blood cells when exposed to a low oxygen tension. Twenty micro litres of each blood sample, was mixed with 20 micro liters of 2% sodium metabisulphite on a cover slip. A slide was then gently pressed onto the cover slip and after inversion; the cover-slip was ringed with candle wax. The slide preparations were left in a humidified chamber for 15 minutes at room temperature and then examined under the microscope (x10). Further observations were taken after 30 minutes, 1 and 2 hours. In each test, an average of 5 films were randomly selected and wholly examined. The proportion of the number of red blood cells that were sickled was then expressed as percentage and results were considered positive when more than 25% of the red blood cells sickled.

2. Solubility test

The principle of solubility method was based on turbidity created when Hb S is incubated with sodium dithionate. Twenty micro-liters of each sample was mixed with 2 ml of 0.02% sodium dithionate in a test tube and left to stand at room temperature for 5 minutes. The samples were examined using light against the background of black lines. The results were interpreted as positive when the black lines were not visible.

3. Peripheral blood film method

Thin blood films, stained with Giemsa stain were examined by light microscopy (x100). The same procedure used in sickling test was also used to select and examine the films. The proportion of the number of sickled red blood cells was then expressed as percentage and results were considered positive when more than 25% of the red blood cells sickled. were also randomly selected results were considered positive when the sickle cell count was greater than 25% of the total red cell count.

4. Hb elecrophoresis

The cellulose acetate membrane Hb electrophoresis method at pH 9.2 adapted after JUNIUS et al [9], was used to determine the presence of AA, AS, and SS in the samples and to confirm the results generated by the above methods. The principle of this method was based on the fact that proteins normally have either positive or negative charge that is determined by the charged amino acid they

contain. When electric field is applied to a solution containing protein molecules, positively charged proteins will move to the cathode and negatively charged proteins will migrate to the anode. Depending on their charges, size and shape, different haemoglobins will separate and migrate at different rates. They are then stained and their bands compared with the known controls [9].

5. Measure of technical feasibility

The turn around time (TAT) of each method was also determined. This was done by timing each process starting from the preparation of working solutions, setting the test and final reading of the results. Sample collection was not timed.

5. Cost benefit analysis

Cost benefit analysis was separately done and the manuscript was accepted and published by South African Medical Journal [10].

- Quality control

Each batch of assays included a known negative control (AA) and a known positive control (HbSS). All procedures were undertaken by three independent experienced senior technologists and the results were included in the analysis only when at least two observers were in agreement.

- Analysis

The statistical analysis of the data was undertaken using the soft-ware package for social sciences 10.0 (SPSS 10.0). The performance difference of the methods was evaluated against Hb electrophoresis as a gold standard using Open Source Epidemiologic Statistics for Public Health Version 2.2.1 Openepi [11].

- Ethical clearance

Permission to carry out this study was sought from Faculty Research and Ethics Committee and Uganda National Council of Science and Technology (UNCST).

III- RESULTS

Of the 200 blood samples included in the study, Hb electrophoresis detected 178 samples with Hb AA, 20 AS and 2 SS. Figure 1 shows one of the results by Hb electrophoresis.

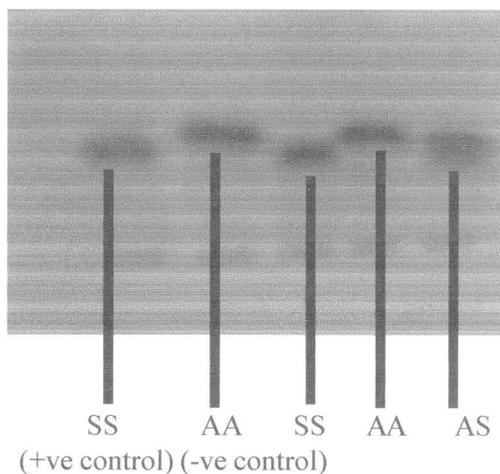


Figure 1- Hb Electrophoresis bands.

There was variability in the ability of the sickling and solubility tests and peripheral blood film methods to accurately demonstrate the presence of haemoglobin AA and sickle cells. The sickling method demonstrated the presence of 172 AA and 13 sickle cell cases, whilst the solubility method demonstrated 162 AA and 9 samples with sickle cells. The peripheral blood film method showed 174 AA and 7 cases with sickle cells. However, basing on Hb electrophoresis results each of these methods demonstrated the presence of 2 SS cases in these samples. Sickling and solubility tests demonstrated the presence of 11 AS and 7 AS respectively while peripheral blood film method demonstrated the presence of 5 AS. The summary of the results is shown in Table I.

Table I- The summary of the haemoglobin AA, AS /SS detected by the sickling, solubility and peripheral blood film methods and by Hb electrophoresis (Gold standard).

Variable	Sickling	Solubility	Peripheral blood film	Hb electrophoresis (gold standard)
True negative for sickle cells	172	162	174	178
False positive for sickle cells	8	18	6	0
True positive for sickle cells (11AS,2SS)	13	9 (7AS,2SS)	7 (5AS,2SS)	22 (18 AS, 2SS)
False negative for sickle cells	7	11	13	0
Total	200	200	200	200

Notably, the sickling method had sensitivity 65% (CI: 43.3, 81.9) and specificity of 95.6% (CI: 91.5, 97.7). It had positive and negative predictive values of 61.9% and 96.1% respectively. The level of agreement (diagnostic accuracy) between sickling method and the gold standard was 92.5% with a kappa score of 0.6 (CI: 0.5-0.7). The summary of the results is as shown in Table II.

Table II- Sickling screening method against Hb electrophoresis (gold standard)

Parameter	Estimate	95% CI
Sensitivity	65.0%	(43.3, 81.9)
Specificity	95.6%	(91.5, 97.7)
Positive Predictive Value	61.9%	(40.9, 79.3)
Negative Predictive Value	96.1%	(92.1, 98.1)
Diagnostic Accuracy	92.5%	(88.0, 95.4)
Likelihood ratio of a Positive Test	14.6	(10.6 - 20.3)
Likelihood ratio of a Negative Test	0.4	(0.3 - 0.5)
Diagnostic Odds	39.9	(12.5 - 127.4)
Cohen's kappa	0.6	(0.5 - 0.7)

The peripheral blood film method had sensitivity of 35.0% (CI: 18.1, 56.7) and specificity of 96.7% (CI: 92.9, 98.5). It had positive and negative predictive values of 53.9% and 93.1% respectively. The peripheral blood film had diagnostic accuracy of 90.5% and kappa score of 0.4 (CI:0.2-0.5) respectively. The summary of the results is as shown in Table IV.

Table IV- Peripheral blood film method against Hb electrophoresis (gold standard).

Parameter	Estimate	95% CI
Sensitivity	35.0%	(18.1, 56.7)
Specificity	96.7%	(92.9, 98.5)
Positive Predictive Value	53.9%	(29.1, 76.8)
Negative Predictive Value	93.1%	(88.4, 95.9)
Diagnostic Accuracy	90.5%	(85.6, 93.8)
Likelihood ratio of a Positive Test	10.5	(4.5 - 24.5)
Likelihood ratio of a Negative Test	0.7	(0.6 - 0.8)
Diagnostic Odds	15.6	(4.6 - 53.3)
Cohen's kappa	0.4	(0.2 - 0.5)

Measure of technical feasibility

The measure of technical feasibility showed that TAT for the sickling and solubility tests was 38 and 70 minutes respectively. While peripheral blood film method had TAT of 44 minutes.

Cost benefit analysis

The results on cost benefit analysis showed that screening the children at health centers and then confirming only the positive cases at regional hospital was both cheap and sensitive [10].

IV- DISCUSSION

Despite the availability of SCD screening methods such as the solubility, sickling and peripheral blood film methods, their reliability for SCD screening at district health centers in Uganda has, hitherto, not been ascertained. This was the first study to determine the reliability of these methods using 200 blood samples which were taken from children between 6 months and 5 years in the districts of Sironko and Mbale in eastern region of Uganda. The analysis of the samples was undertaken in the Departments of Pathology, Faculty of Medicine and Department of Clinical Chemistry, Mulago Hospital.

Whilst all these methods could reliably demonstrate patients with SS, they showed variability in their ability

The solubility method had sensitivity of 45% (CI: 25.8, 65.8) and specificity 90.0% (CI: 84.8, 93.6). It had positive predictive value of 33.3% and negative predictive value of 93.6%. Solubility method had diagnostic accuracy of 85.5% and Cohen kappa of 0.3 (CI: 0.2-0.4). The summary of the results is as shown in Table III.

Table III- Solubility screening method against Hb electrophoresis (gold standard).

Parameter	Estimate	95% CI
Sensitivity	45.0%	(25.8, 65.8)
Specificity	90.0%	(84.8, 93.6)
Positive Predictive Value	33.3%	(18.6, 52.2)
Negative Predictive Value	93.6%	(89.0, 96.4)
Diagnostic Accuracy	85.5%	(80.0, 89.7)
Likelihood ratio of a Positive Test	4.5	(3.1 - 6.5)
Likelihood ratio of a Negative Test	0.6	(0.5 - 0.7)
Diagnostic Odds	7.3	(2.7- 20.2)
Cohen's kappa	0.3	(0.2 - 0.4)

to detect the carrier state of haemoglobin (AS). The solubility test was in particular found to have low sensitivity for Hb AS. This finding is in agreement with a study by CHASEN et al; [12] which found that the solubility test was not sensitive for the detection of carriers and was unsuitable for screening purposes. Besides, the solubility test could lead to stigmatization and unnecessary referrals because of its high false positive rate which is characteristic of a test with low diagnostic accuracy and low positive likelihood ratio. The probable reason for this high false positive rate was that some of the samples might have shown erythrocytosis, highly marked leucocytosis and/or hyperlipidemia. Unfortunately, it was not possible to link these hypotheses to false positivity of solubility test because none of these parameters were measured in this study. However, these observations were in conformity with ROBERT et al; [13] who noted that factors such as erythrocytosis, highly marked leucocytosis and hyperlipidemia were possibly be linked to false positivity by this method.

Although NALBADIAN et al [14], found solubility test easier to perform, our study found that solubility test had a high turn around time because a lot of time was required for reagent preparation. Notably, sickling test was found most reliable for the detection of haemoglobin AS although there were some false positive cases. The false positivity of the sickling test was probably due to anemia. This observation was augmented by the fact that 5 out of 8 samples which tested false positive had haemoglobin concentration below 10 g/dl. This finding was in agreement with the study by SCHENEIDER et al [15] who found that the presence of anemia was associated with a false positive result when using the sickling test.

While the peripheral blood film method was found reliable in detecting normal haemoglobin AA, it was found un-reliable for the detection of haemoglobin AS. The probable reason for this was that most of the children who had been recruited into the study with hemoglobin AS, were normal looking (asymptomatic) and probably had very few circulating sickle cells in their blood at that time.

However a study on cost benefit analysis of these methods was separately carried out and its findings indicated that using sickling test to screen for SCD at district health centers IV and confirming only positive samples at either regional or district hospital was the most cost effective intervention [10].

V- CONCLUSION AND RECOMMENDATIONS

The sickling test was the most reliable, cheapest and easiest to perform. It had high specificity, sensitivity and kappa score. The solubility test was found expensive, cumbersome and unreliable for sickle cell screening. It had both low sensitivity and kappa score, and had high TAT. The peripheral film method had high specificity, low sensitivity and high TAT. Sickling would therefore be the most recommended test for screening children for SCD at district health centers in poor resource settings and using Hb electrophoresis as confirmatory method ■

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