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Different Methodologies to Characterize and Diagnose Sickle Cell Disease in Both Developed and Developing Nations

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Different methodologies to Characterize and Diagnose Sickle Cell Disease in Both
Developed and Developing Nations

By
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A culminating capstone project report submitted to the faculty of Dominican
University of California in partial fulfillment of the requirements for the degree of
Master of Science in Clinical Laboratory Sciences

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This Capstone Project, written under the direction of the candidate's First Reader/ Project Supervisor and approved by the Program Director, has been presented to and accepted by the Department of Natural Science and Mathematics in partial fulfillment of the requirements for the degree of Masters of Science in Clinical Laboratory Science. The content and research methodologies presented in this work represent the work of the candidate alone.

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Abbreviations

SCD- Sickle cell disease

SCT- Sickle cell trait

Hb- Hemoglobin

POC- Point of care

RBC- Red blood cells

WBC- White blood cells

IEF- Isoelectric focusing

HPLC- High performance liquid chromatography

SNP- Single-nucleotide polymorphism

GWAS- Genome-wide association studies

PND- Prenatal diagnosis

CZE- Capillary zone electrophoresis

pI- Isoelectric point

RFLP- Restriction fragment length polymorphism

WES- Whole exome sequencing

AMPS- Aqueous phase multiphase system

PMMA- Poly (methyl Methacrylate)

DI- Deionized

Abstract

Sickle cell disease (SCD) is a genetic blood disorder that causes the red blood cells (RBC) to become sickle shaped due to a mutation in the β -globin gene encoding the protein hemoglobin. This disease causes reduced oxygen carrying capacity of RBC resulting in painful crisis, vasculopathy, infarction, hemolytic anemia, and infection susceptibility. SCD affects around 100,000 individuals in USA alone and 14 million people globally. SCD affected individuals have high mortality rates. Early detection and constant monitoring of this disease is essential. The following review focuses on various methodologies that have emerged in the diagnosis of SCD. Also, low cost methods that can be easily adopted in developing nations are discussed. Various conventional screening techniques and emerging Point-of-care (POC) methodologies for detection of SCD are covered in this review. The advantages and limitations of these techniques are discussed. Some of the popular conventional techniques explained here in details include the following: hemoglobin electrophoresis, isoelectric focusing (IEF), high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) and deoxyribonucleic acid (DNA) analysis. Under DNA analysis there are again several methodologies, that include use of restriction enzyme along with polymerase chain reaction (PCR) to detect mutation in the SCD individuals; exome sequencing of DNA and direct genotyping for the single-nucleotide polymorphism (SNP) that encodes the sickle mutation and also genome-wide association studies (GWAS) used to detect SCD. The emerging POC technologies for SCD screening have also been covered here that include the following methodologies; paper-based hemoglobin solubility assays, Lateral Flow Immunoassays, Density-based separation methods and Micro engineered electrophoresis. The goal of this review is to give a comprehensive report of the current methodologies used for detection of SCD.

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1. Introduction

Sickle cell disease (SCD) is caused by a single nucleotide substitution (GAG to GTG) due to a point mutation in the β -globin gene, which results in a substitution change replacing amino acid glutamic acid with valine at the sixth position of the beta globin chain thus resulting in the production of the sickle hemoglobin (HbS) (Randolph *et al.* 2012). Individuals having both the β -globin gene allele mutated are called homozygous (HbSS) and show various symptoms of SCD whereas those with a single mutated β -globin gene are known as sickle cell trait (HbSA) or carriers and are asymptomatic. The oxyhemoglobin dissociation curve is right shifted in SCD patients indicating a lower affinity of the sickle hemoglobin for oxygen (Abdu *et al.* 2008). Reduced oxygen tension causes polymerization of this mutated form of hemoglobin, thus changing the shape of the red blood cells (RBC) to a sickle shape, which thereby results in development of chronic anemia, acute painful crisis that arises due to blockage of small vessels. Progressive damage to multiple organs sets in over time that includes damage to brain, kidneys, lungs, bones and cardiovascular system (Rees *et al.* 2010).

Around 14 million individuals are affected by SCD worldwide, where as in the United States alone 80,000-100,000 people primarily African Americans have this disease (Brousseau *et al.* 2010; Alapan *et al.* 2016). Annually, the overall healthcare cost of SCD in US alone is estimated to be over \$1 billion. (Ballas *et al.* 2009).

Like any hereditary disease, attempts to prevent its occurrence are the best approaches against SCD. The first level of prevention is known as prenatal diagnosis (PND), which makes parents who are homozygous for SCD be aware of the risk of having a child with SCD. People living in the remote areas and in developing nations are not aware of the risk of having a child born with SCD due to the lack of information and appropriate diagnostic tools.

Also, the second level of prevention is neonatal screening. Mortality due to infection and stroke is very common in newborns that are not diagnosed for SCD, which happens when newborns are not screened for the presence of sickle hemoglobin in the blood. Studies have shown that there is a greater risk of *Streptococcus pneumoniae* infection in children up to 3 years of age. The mortality rate in these children is as high as 30% (Yanni *et al.* 2009). Hence, it is essential to carry out neonatal screening for SCD so that proper management can be delivered to reduce newborn mortality rates. This problem is acute in developing countries where early diagnosis is not very prevalent and hence a relatively higher number of newborn mortality due to sickle cell disease is observed in these nations.

Developed nations rely on molecular and protein-based tests to diagnose SCD. Molecular tests are expensive and thus not a practical approach in underdeveloped countries. In African countries for instance, screening attempts have been unsuccessful mostly due to the high cost of screening kits available in the market (Hajer *et al.* 2012). In addition to the cost issue, other challenges faced by healthcare professional include the heterogeneities among SCD patients and the multiple organs effected in this disease. This review describes various SCD screening and monitoring methodologies. It also discusses the challenges involved with such technologies and future of SCD screening. The review also describes low cost techniques that can be implemented in developing nations to detect SCD cases in children at an early stage with significant lower costs.

2. Various methodologies for SCD Diagnosis

2.1 Conventional methodologies

RBCs of affected individuals have varying percentages and combinations of HbS, HbA, HbF, HbC, and HbA₂. Patients are diagnosed and categorized as belonging to homozygous HbSS (sickle cell anemia, SCA) and heterozygous HbSA (sickle cell trait, SCT), HbSC disease and HbS- β thalassemias based upon the percentages and combinations of the above-mentioned types of hemoglobin. The sickling test and the sickle solubility test are two of the basic tests that are popularly used to detect the sickle hemoglobin in the blood sample (Berg, A. O. 1994).

In the sickling test, polymerization of sickle hemoglobin is induced by sodium metabisulfite followed by sickling of the RBC by reducing oxygen tension. Microscopic analysis is conducted by taking a sample of this diluted blood and observed for the presence of sickled RBC. The test is very simple to carry but the major drawback of the test is it cannot differentiate between different forms of hemoglobin that include HbSS, HbSA, HbSC, or HbS- β thalassemias. The test requires an incubation time between 1-4 hours. after which the slide is examined under a microscope. Sickling test is performed for routine health screenings or if a disease or toxicity is suspected.

In the solubility test, a concentrated buffer solution made of phosphate is used to reduce HbS into an insoluble state. Precipitation of HbS thus occurs in reduced state to form tactoids, which makes the solution turbid. A positive and negative control blood sample is used to compare the light refraction (Diggs *et al.* 1975). The biggest drawback of this test is, newborns predominantly have HbF in their blood and do not have much of HbSS (SCA) or HbSA (SCT) to give rise to considerable amount of HbS, which this test detects. Hence lot of times although a newborn is homozygous for sickle hemoglobin may end up giving false-negative result in this test. This phenomenon is generally

observed when the HbS concentration in the newborn is less than 10% of the total hemoglobin.

This test is not considered as an optimal test to differentiate between SCD from SCT because it has the potential to cause confusion between them. Also, other tests, that can positively identify the homozygote from heterozygote are available (Tubman *et al.* 2015). Other clinically significant forms of sickle hemoglobinopathies that include HbC and β -thalassemia cannot be detected in this test (Fabry *et al.* 2003).

In order to determine the type of hemoglobin present, (SCA vs SCT), additional tests need to be carried out, which include the following: hemoglobin electrophoresis, high performance liquid chromatography (HPLC), isoelectric focusing (IEF), and DNA based analysis (Greene *et al.* 2015). The basic principle upon which electrophoresis-based techniques operate is that different types of hemoglobin molecules carry different charges on them and hence they migrate at different velocities in an electrophoresis gel when an electric current is applied. Each of the above mentioned are explained in detail below.

2.12 Hemoglobin electrophoresis:

Unaffected individual has the following proportions of hemoglobins - HbA = >95%; (95-98%); HbA₂ = 1.5-3.7%; HbF = <2%; (0.8-2%); HbS = 0%; HbC = 0% and Newborn HbF = 50-80%. Hemoglobin electrophoresis is a method used to discriminate between these different types of hemoglobin under alkaline conditions using variety of sieving materials that includes gel and paper. Under alkaline condition various forms of hemoglobin have a net negative charge and move towards the positive electrode. Different hemoglobin form migrates to different lengths on the gel based on the charge carried by these molecules, pore size of the medium and the ionic strength of the buffer solution. A condition can be determined based on the various bands observed on the gel. The advantage of this methodology is rapid screening of small number of samples

is possible. After electrophoretic separation, densitometry is used to quantify various forms of hemoglobin observed on the gel. However, at low sample concentration densitometry suffer from inaccuracy of result. The resolution between HbF and HbS is very low and hard to distinguish in neonates having high concentration of HbF.

For high-throughput screening a capillary zone electrophoresis (CZE) is carried out in a U-shaped narrow-bore fused-silica capillary tube. Like HPLC, CZE is an automated method and has been shown in various studies to be an effective methodology to detect monoclonal gammopathies and protein abnormalities present in the serum. Migration in CZE is similar to conventional electrophoretic methods in which the protein migrates based on its net charge when an electric field is applied. The buffers used in CZE differ from gel electrophoresis where barbital buffer is used. Barbital buffer has a disadvantage since it has a high absorbance at 200nm wavelength, which coincides with peptide bonds and thus interferes with the detection system. To avoid this CZE uses borate-based buffers that do not cause the above interference (Dolník *et al.* 1995).

The negative charge of the fused silica used to make the capillaries provides a negative surface charge to the migrating protein molecules. Also, the narrow lumen (about 50 μ m in diameter) inside the capillary tubes makes the surface area relatively large. This combined effect of large surface area and charge on the capillaries causes a more effective movement of proteins in the sample towards the cathode than could have been achieved by only voltage difference between cathode and anode. This high charge and surface area offered by the capillaries causes a higher resolution of the hemoglobin variants present in the samples. A detector is usually present at the cathodal end of the capillary, which records the optical density at a wavelength of 200nm (peptide bond absorption) as the proteins migrate past it. The Hb variants pass by the detector in the following order- γ , β , α_2 , and α_1 globulins (Keren *et al.* 1998).

High resolution, ease of automation, shorter run time at a high voltage and no additional requirement for densitometer are the advantages of the CZE methodology for diagnosis of SCD. However, CZE is a new technology and the limitations haven't been properly investigated so far. One major drawback is this methodology does not have any gel that can be examined. The bands represent those substances that have absorbency at around 200 nm, which are usually proteins. Non-protein substances having similar absorbency parameters can therefore also produce bands that can be confused with hemoglobin variants (Keren *et al.* 1998; Bain, B. J. 2008). CZE requires a detector, which records optical density at a wavelength of 200nm and this is connected to a computer for data acquisition and also needs high voltage power supply. All these arrangements are expensive and are not readily available in remote areas hence CZE-based SCD screening methodology is not easy to implement in developing or in underdeveloped countries.

2.13 Isoelectric focusing (IEF):

Every protein carries a net charge that varies according to the pH of the surrounding environment. An isoelectric point (pI) is defined as a point in pH gradient where a protein carries a net zero charge. Hemoglobin samples are run in a gel medium across which an electrical gradient is applied, and a fixed pH gradient is maintained throughout the gel (Fig. 1). Different types of hemoglobins present in the blood sample travel and stop at the location in the gel at their respective pI. Resolution of IEF is better than Hb electrophoresis and thus this technique is useful in distinguishing between higher populations of individuals with variant Hb (Bain, B. J. 2008). The disadvantage of this technique is that the interpretation of result sometimes becomes difficult due to the presence of a higher number of bands on the gel. Also, the technique is more expensive compared to Hb electrophoresis method. Like Hb electrophoresis, the quantitation depends on densitometric analysis, that sometimes gives inaccurate interpretation.

However, IEF is the method of choice for screening newborns in many clinical settings since this methodology can operate at a very small volume and even gives reliable results from dried blood spot samples (World Health Organization 2006; APLH & CDC - Hemoglobinopathies -Current Practices for Screening, Confirmation and Follow-up - DECEMBER 2015).

In a pilot study conducted in Tunisia, a low cost neonatal screening method using IEF assay for the detection of SCD and other Hb variants has been reported (Hajer *et al.* 2012). In this study samples from 9148 newborns were collected on blotting paper (Whatman grade BFC 180) at maternity centers and using a general office-use printer these blotting papers were printed. IEF methodology was then carried out by a lab-prepared agarose gel to test the dried blood samples from these newborns. This low cost IEF on lab prepared agarose gel along with office printer printed blotting paper for sample collection proved to be an effective method since this method successfully detected the newborns who had abnormal Hbs (HbS, HbC, HbO and HbG). The accuracy of the data collected in this screening method was also verified by comparing with previously established national data. The families of these newborns were made aware of the result and guidelines for treatment along with genetic counseling was also provided.

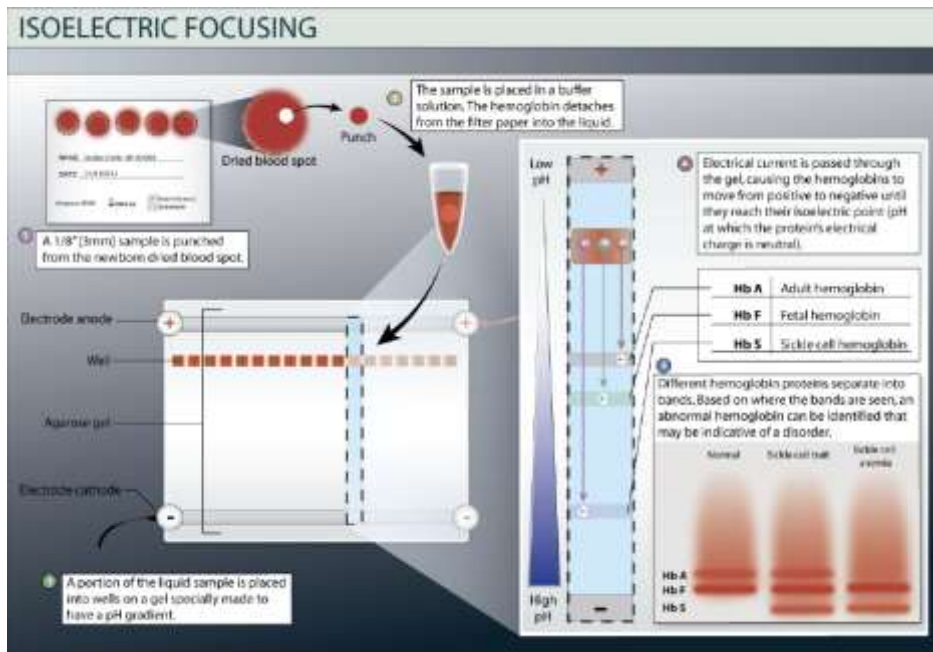


Figure 1 Isoelectric focusing (IEF): The illustration showing the various steps in IEF (APLH & CDC - Hemoglobinopathies -Current Practices for Screening, Confirmation and Follow-up -DECEMBER 2015).

2.14 High Performance Liquid Chromatography (HPLC):

Cation exchange HPLC uses charge to separate different types of hemoglobin in the blood. HPLC has a negatively charged stationary phase comprising of an absorbent material that can be either silica or other polymers. The mobile phase is a liquid with an increasing concentration of cations that passes through the chromatography column. A pressure pump is used to drive the fluid through the chromatography column. The HPLC is connected to the computer, which shows separation of the various hemoglobin types on a screen. HPLC offers the advantages such as full automation of the entire procedure and accurate quantification of the levels of hemoglobin present as long as a variant or known Hb in the patient sample does not interfere with the interpretation (Greene *et al.* 2015). The disadvantage of HPLC technique is the cost of the instrument that makes this technique not practical to operate in developing countries for screening of population for SCD. In developed countries like the US, HPLC is the methodology of choice for primary screening of SCD individuals over Hb electrophoresis and IEF

(Alapan *et al.* 2016). HPLC is much less labor intensive, accurate and less time-consuming method in comparison to the Hb electrophoresis. HPLC can also be used on SCD individuals on hydroxyurea (HU) or transfusion therapies (Makani *et al.* 2013). HU is the drug-of-choice to reduce the pain episode frequency in sickle cell patients. HU raises the level of HbF that significantly decreases the rate of painful episodes by about 50% (Agrawal *et al.* 2014). Blood transfusion lowers the amount of sickle hemoglobin in the body. Since the normal red blood cells increase in the blood stream thus increasing the supply of oxygen.

2.15 Liquid Chromatography-Mass spectrometry (LC-MS/MS):

It is important to also include liquid chromatography-mass spectrometry (LC-MS/MS) (also known as tandem mass spectrometry) under this category since it is a much faster and accurate and can be used for cost effective population of screening of sickle cell disease (Chace *et al.* 2003). For characterization of hemoglobinopathies Electrospray ionization in conjunction with tandem MS have been used. Masses of intact globin chains can be measured by scanning whole blood. This is followed by tryptic digestion of the blood specimen for detailed peptide analysis in order to specify the hemoglobin variant in the blood. This method allows unambiguous characterization of majority of globin mutations (Daniel *et al.* 2005).

2.16 DNA Analysis:

DNA based assays can be used to screen patients by looking for mutations in the β -globin gene that results in the production of the sickle hemoglobin (Clark *et al.* 2004). Compared to the methodologies discussed earlier, DNA analysis to screen SCD is usually more expensive. There are two ways DNA analysis can be performed. One popular method, known as Restriction Fragment Length Polymorphism (RFLP), looks for Ddel I restriction enzyme site in the β -globin gene. Mutation that causes SCD eliminates this restriction site. Thus, normal DNA without sickle mutation will be cut

with Ddel I whereas DNA having mutation for SCD will not be cut with this enzyme. To carry out this procedure first DNA sample from individuals are collected and a 125 bp region of the β -globin gene is PCR amplified using gene-specific primers and this amplified DNA is then subjected to digestion by Ddel I. After incubation with the restriction enzyme for a certain time, an aliquot of this reaction is loaded in a gel and electrophoresis is carried out. Individuals with mutation in the β -globin gene will have one big fragment (125 bp) whereas normal DNA will be cut into two fragments by the restriction enzyme (106 bp and 19 bp). It is hard to visualize 19 bp fragment since it's too small to visualize on a gel. If the individual is homozygous that is both the alleles have the S mutation (SS), then only the 125 bp fragment is seen in the gel. Whereas in case of sickle cell carriers (Trait) who have both the A and S allele (AS), both the 125 bp and 106 bp bands are seen.

RFLP is easy to perform and analyze and is an inexpensive method compared to other molecular techniques. However, the drawback of RFLP method is it's a low throughput method since most of the steps need to be performed manually. It's the method adopted in laboratories handling fewer samples.

Clinicians and researchers also use other DNA analysis methods that include whole-exome sequencing (WES) to identify pathogenic single-nucleotide variants (SNVs). This is achieved by sequencing only the protein-coding portions of the β -globin gene followed by copy number variation analysis of the beta globin locus to determine normal vs. sickle mutation. These sequencing methods provide the most accurate and comprehensive report of the beta-globin gene. However, the cost involved is much higher than other methodologies used to screen SCD.

2.2 SCD Diagnosis by using emerging Point-of-care (POC) methodologies:

The idea behind recent methodologies is to make SCD screening accessible by adapting to technologies that are available at the POC even in the most developing nations. The emerging technologies consider the cost of a particular methodology, complexity of use, accessibility and availability of trained personnel. The emerging technologies in the past few years have thus been categorized into four groups: (a) paper-based hemoglobin solubility assays, (b) lateral flow immunoassays, (c) density-based separation, and (d) micro engineered electrophoresis. Each of these methodologies has been explained in detail below.

2.21 Paper-based Hemoglobin Solubility Assay:

The principle behind this technology is the insoluble property of hemoglobin and the filtration ability of paper. This assay uses a microfluidic paper-based device known as μ PADs, to analyze the samples. In this way the HbS in the sample can be seen with our eyes. A drop of blood ($\sim 20\mu\text{L}$) is mixed with hemoglobin solubility buffer in a 1:10 ratio and then put onto a chromatography paper that is patterned (Fig. 2). Based on the different capillary action pattern of different hemoglobin types, various bloodstain patterns are observed for HbS that is polymerized and other hemoglobin types (Shevkoplyas *et al.* 2017). Screening can be achieved within 20 minutes and the method is 94.2% sensitive (see Table 1 below) (Yang *et al.* 2013). The advantage of this paper-based is that it is cheaper compared to other methods, easy to interpret and sample preparation is very easy. Individual tests can be performed without the need to batch samples.

The technique however suffers from few disadvantages. First, if the blood clots before adding to the μ PAD, then the capillary action of the blood is lost, and the blood would

not be absorbed in the paper. The interpretation depends on naked eye detection and hence the assay is completely operator based and thus prone to error. To minimize error an automated image-processing algorithm can be paired with this assay. The assay cannot distinguish between the hemoglobin types HbSC and HbAS. Newborns have high percentage of HbF which may hinder the polymerization of HbS and thus this test has limited application in terms of newborn screening (Mulumba *et al.* 2015).

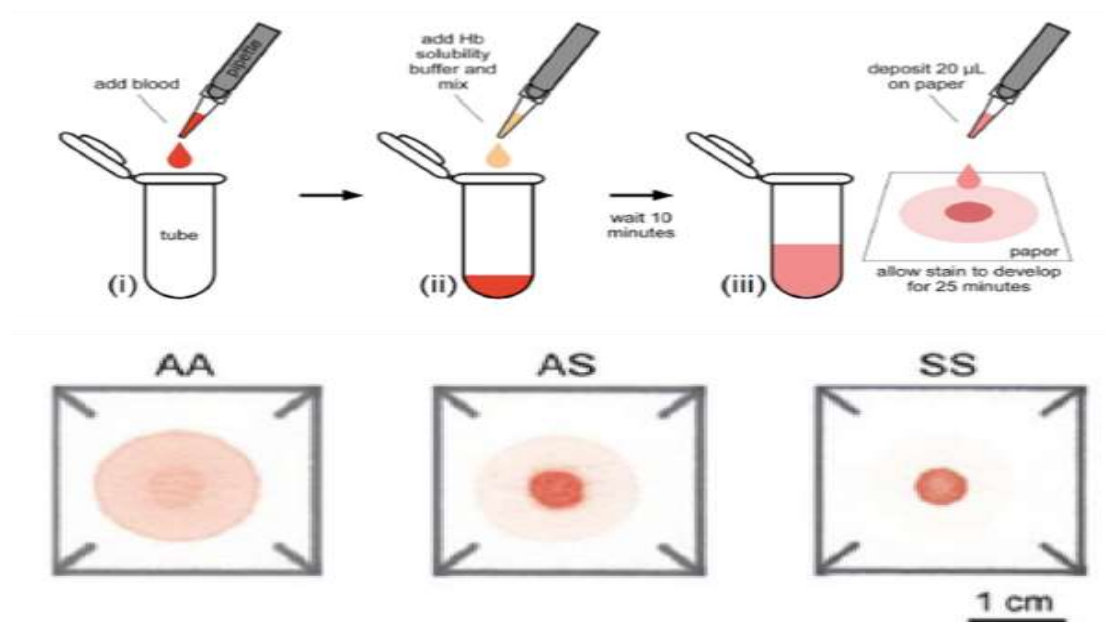


Figure 2 Paper-based Hemoglobin Solubility Assay: The figure shows stepwise illustration of the above-mentioned assay (upper panel). The pattern and the color intensity profile are studied to determine the type of hemoglobin present in an individual (Shevkoplyas)

2.22 Lateral Flow Immunoassays:

Lateral flow immunoassay also known as Sickle SCAN, can be used to detect HbA, HbS and HbC (Fig 3A). Different polyclonal antibodies are conjugated with colored nanoparticles and this combination is immobilized in a test strip on four different test lines, in which each line corresponds to a hemoglobin type (HbA, HbS, HbC, and Control). The control line ensures that no error has occurred during the procedure. Five microliter of sample blood is mixed with hemoglobin solubility buffer in 200:1 ratio and applied to the strip. The sample moves along the paper strip and once it reaches the lines corresponding antigen-antibody complexes are formed. Formation of a colored

line indicates the presence of the corresponding hemoglobin in the blood sample. The entire procedure takes only 2 minutes and has a sensitivity of ~99% (Kanter *et al.* 2015). A recent study reported the sensitivities of the different hemoglobin in the blood to be 98.3%, 99.5% & 100% for HbA, HbS, and HbC respectively. As low as 2% of hemoglobin concentration was found to be sufficient to properly conduct the assay. This study also found out that presence of high percentage of HbF did not affect the detection of HbS or HbC (McGann *et al.* 2016). The specificity and sensitivity of this methodology are shown in the table 1 below. The above study also investigated the shelf-life of this device and found that the device properly functioned even after being stored at 37° C for 30 days. The assay has few disadvantages. The assay relies on visual confirmation that can sometimes lead to misinterpretation of actual result. The antibody conjugation with colored nanoparticle makes the device expensive due to increase in fabrication complexity. The presence of antibody requires the device to be ideally stored at low temperatures or under refrigeration.

HemoTypeSC is a new type of lateral flow assay that uses monoclonal antibodies against HbA, HbS and HbC. This assay consists of the following parts: a) Sample pads laminated with fiberglass. b) A Nitrocellulose membrane with antibodies to different types of hemoglobin at four different locations of the membrane (antibodies corresponding to three hemoglobin and one control) and c) A cellulose wick (Fig. 3B). One microliter of blood is diluted to 1mL with distilled water followed by addition of 15µL of this diluted blood to the sample pad. This is then immersed in a 150µL assay buffer solution containing red-colored colloidal gold nanoparticles and the solution is allowed to be absorbed in the strip for 10 minutes before taking the strip out of the solution. The test strip is then visually detected for the line on the strip where the antibodies are deposited, which indicates the presence of the specific hemoglobin type.

It takes around 20 minutes to obtain result by this assay (Quinn *et al.* .2016). The HemoTypeSC assay achieved 100% sensitivity and at the same time had a low operating cost. The assay was able to distinguish between HbAA, HbAS, HbAC, HbSS, HbSC, and HbCC but it could not detect HbF and HbA₂ Hb types.

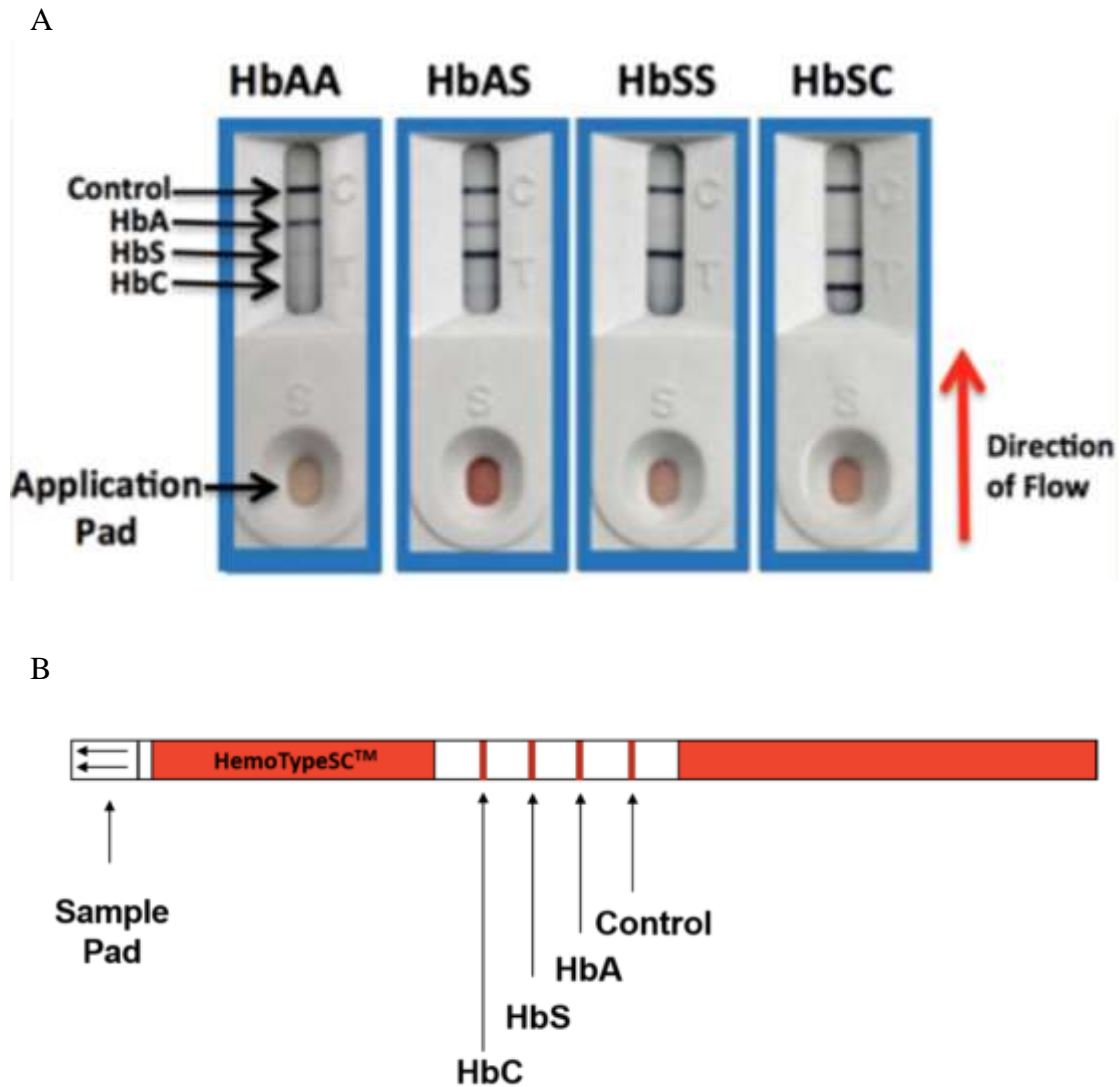
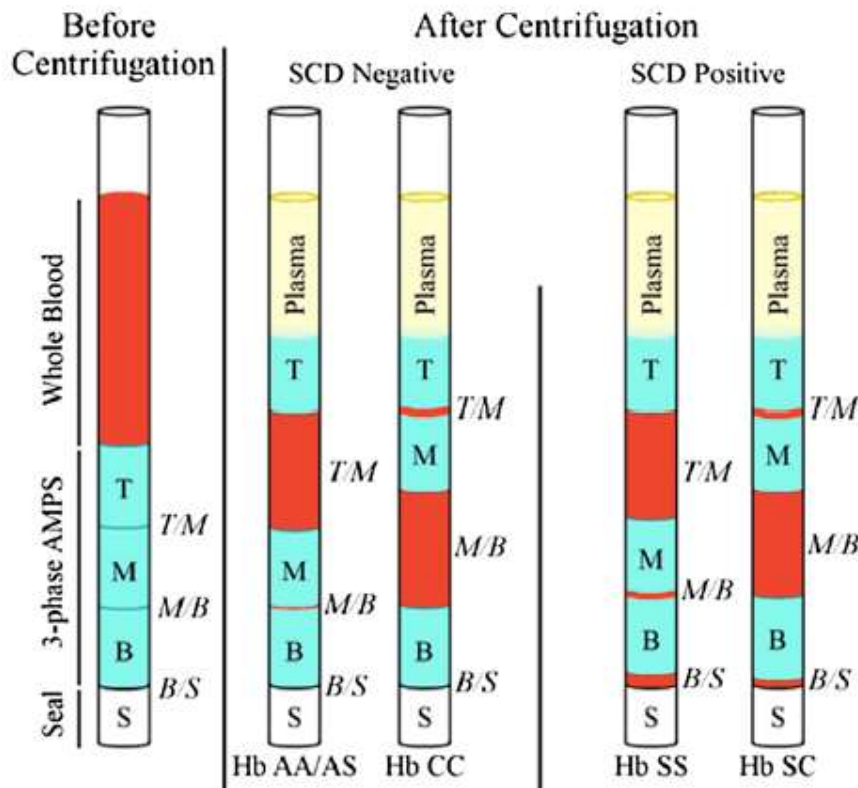


Figure 3 Lateral Flow Immunoassay: (A) The sample is added in the application pad, which then moves via capillary action to the test lines conjugated with different polyclonal antibodies as shown. The blue line that appears notifies the type of hemoglobin present.

2.23 Density-Based Separation:

This technique uses the difference in cell densities to separate SCD cells from normal cells. SCD cells are relatively denser than the normal cells. Aqueous multiphase systems (AMPS) have been developed by Kumar *et al.* It consists of two or three-phase AMPS that uses cell density measurement to detect RBC. The above methodology has

a sensitivity as high 90-91% and specificity ranging from 88-97% (Kumar *et al.* 2014). Five microliters of blood are mixed with aqueous polymeric solution and then loaded into capillary tubes (Fig. 4). The tubes are then centrifuged for 10 minutes that causes dense RBC of SCD patients to settle. This method has several limitations that include the higher cost due to inclusion of the centrifugation step. Centrifugation also makes it difficult to conduct this test at POC settings. Besides samples need to be run in batches increasing the turnaround time. Density based separation method cannot differentiate between HbAA and HbAS. Also, newborn screening is not possible with this method due to the presence of high % of HbF that restricts the occurrence of dense RBC. Other factors like medication, health conditions, treatment processes and genetic factors affect the density of RBC and hence the outcome of test result of this assay (see Table 1



below).

Figure 4 The figure shows the different outcome of the density-based assay. The sample blood moves through the different phases i.e. top, middle, and bottom and then accumulates at the interphases depending on the type of hemoglobin present (Kumar *et al.* 2014)

2.24 Micro engineered Electrophoresis:

Micro engineered electrophoresis (HemeChip) is a SCD screening method recently developed and can be used to differentiate between HbA, HbA₂, HbS, HbF and HbC. The HemeChip device consists of a cellulose acetate paper for electrophoresis that is housed in a microfabricated Polymethyl Methacrylate (PMMA) chamber. An electric field is applied, and the types of hemoglobin are separated. An application software that can be downloaded and used in the mobile phone for image processing purpose at the POC has also been developed (Fig. 5). This software is used to quantitate the hemoglobin amount in the HemeChip (Ung *et al.* 2015).

Less than 5 μ L of blood is first mixed with deionized (DI) water to lyse the RBC and release Hb content into the solution. Less than 1 μ L of this is then stamped onto the cellulose acetate paper and an electric field is applied that causes the hemoglobin types to travel different distances across the paper strip. The result can be achieved in as little time as 10 minutes with high sensitivity and specificity among the different hemoglobin types. It is a low-cost assay that has relatively good accuracy and takes very less time to get result. This methodology can be compared in terms of detection and quantification quality with standard HPLC assay and electrophoresis screening methods used in laboratories. The inclusion of mobile devices to analyze the result also makes this device quite practical in terms of its usage at the POC. This device requires a very low power supply, which can be easily substituted with a rechargeable battery for field applications. However, detection of high percentage of HbF in newborns that usually overshadows other Hb types is still a challenge (see Table 1 below).

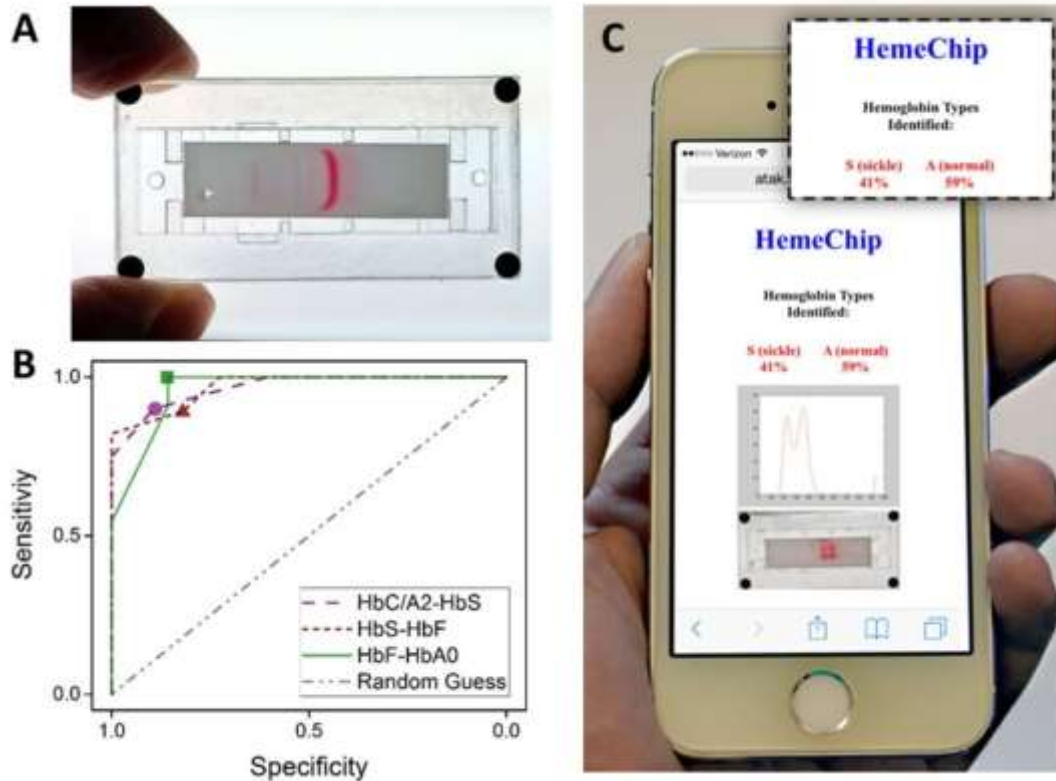


Figure 5 A) The HemeChip with blood sample being separated into respective bands different hemoglobin type B) Graph showing Sensitivity and specificity C) Mobile Software for image processing and quantification of HemeChip results at the POC (Ung et al. 2015).

Table 1 Summary of methodology for screening SCD at Point of Care Testing.

Technique	Advantages	Disadvantages	Cost per test	Equipment cost	Turn around time	Sensitivity	Specificity	Ref.
Paper-based hemoglobin solubility	Easy to use, no batching required	Cannot distinguish HbSC and HbAS, Blood clotting issue; result interpretation affected by human error	\$0.7	\$300-500 (for automated detection)	20 mins	94.2%	97.7%	Yang et al. 2013
Lateral flow immunoassay (Sickle SCAN)	Easy to use, result can have obtained rapidly, no other additional	Cannot quantitate Hb; result interpretation affected by human error	\$5	NA	2 mins	99%	99%	McGann et al. 2016

	equipment required							
Lateral flow immunoassay (HemoTypeSC)	Easy to use, no other additional equipment required	No support for quantification; result interpretation affected by human error	\$0.25	NA	20 mins	100%	100%	Quinn et al. 2016
Density-based separation	Results obtained rapidly, Simple to operate	Bulky centrifuge requirement makes this less practical to be used at POC; Batching needed to run centrifuge; cannot differentiate between HbAA and HbAS; high HbF levels can give inaccurate results; health and treatment conditions can alter RBC density	\$0.5	\$150-1600	10 mins	90-91%	88-97%	Kumar et al. 2014
Micro engineered Electrophoresis (HemeChip)	Easy to use, low cost, robustness, results can be obtained rapidly and comparable with la-based electrophoresis assays, can be annexed with a mobile device;	High HbF concentration in newborns can make it difficult to interpret the result	\$0.9	\$500 for automated detection	<10 mins	89-100%	82-89%	Ung R et al. 2015

3. Discussion and Conclusions:

Proper screening and early diagnosis of SCD individuals is vital to their management and treatment. Improper screening or a delay results in higher mortality rates among SCD patients. Several SCD screening techniques were developed in the early 1970s. Unfortunately, many such methodologies proved to be unreliable since they gave false positive or negative results that led to wrong treatment and confusion. In order to standardize laboratory techniques and interpretation for SCD screening a Hemoglobinopathy Reference Laboratory (HRL) was created at the Centers of Disease Control (CDC) (Naik *et al.* 2015).

This review focused on the different methodologies that are currently used to screen SCD individuals that include both newborns and adults. The most common screening methodologies currently used comprise of sickle solubility testing, electrophoresis, HPLC, CZE and IEF. Sickle solubility-based screening assays usually have lower cost and they are based on the principle of insolubility of HbS in a reducing agent that causes turbidity due to lysis of HbS- containing RBC in solution. The solubility test however cannot differentiate between SCD and SCT individuals and gives false negative result in newborns with higher HbF content. Hence additional tests that include hemoglobin electrophoresis, HPLC and IEF are also used that serve the purpose of either being primary identification of sickle hemoglobin types or as confirmatory tests. These techniques can provide quantification of hemoglobin and clearly differentiate between SCT and SCD individuals. Hemoglobin electrophoresis method is based on the principles of gel electrophoresis and is an inexpensive method that separates types of hemoglobin present in the blood based on their relative size and charge.

IEF is based on the principle that the net charge in the proteins varies based upon the surrounding pH and at their respective isoelectric point (pI) all proteins carry zero

charge. Hemoglobin molecules present in the blood samples are thus run in a gel having pH gradient under an electric field and they stop as they reach their respective pI points. IEF methodology has relatively low cost and has high throughput abilities and thus is the assay-of-choice in many clinical laboratories. In this context a pilot study conducted in Tunisia, Africa has been mentioned here that uses a low cost SCD screening technique by collecting blood samples on blotting paper printed in a common office printer and then analyzing the samples in a laboratory prepared gel for IEF assay (Hajer *et al.* 2012).

HPLC although being an expensive method for hemoglobinopathy screening is used in many clinical laboratories across developed nations for its accuracy in detection and less time to get result.

An alternative methodology for screening SCD individuals is LC-MS/MS, which is able to detect Hb peptides following digestion of blood spots with trypsin. This is also an extremely accurate and fast method that can be applied for population screening for identification of clinically important globin mutations.

SCD screening can also be achieved by DNA analysis, one of the methodology in this category include RFLP assay, in which β -globin gene is first PCR amplified and then cut with the restriction enzyme *DdeI*. Absence of this restriction site indicates the presence of mutated β -globin gene since the point mutation eliminates the above restriction site. Whole exome genome sequencing (WES) to look for SNVs in the β -globin gene is also currently followed by several laboratories although sequencing is an expensive technology and only developed nations can afford it.

The review also explains the emerging POC methodologies that are developed considering the cost factor at a certain location, trained technicians available and accessibility. Four such emerging methodologies have been discussed here that are as

follows, a) paper-based hemoglobin assays, b) lateral flow immunoassays, c) density-based separation, and d) micro engineered electrophoresis (HemeChip).

The paper-based hemoglobin assay takes utilizes capillary action of blood sample when applied to a paper-based device called μ PADs that causes different hemoglobin types of form unique patterns that can be easily distinguished from one another.

The lateral flow immunoassay comprises of a test strip that has polyclonal antibodies against different hemoglobin types conjugated with colored nanoparticles. An antigen-antibody complex is formed between respective hemoglobins once the blood sample reaches the lines in the strip.

Density-based separation has an AMPS comprising of two and three phases that uses cell density measurements to detect RBC. Blood samples are centrifuged in capillary tubes and SCD cells that are of higher density settle at the lowest interphase.

The micro engineered electrophoresis (HemeChip) consists of a cellulose acetate paper housed inside a PMMA chamber where sample blood electrophoresis is conducted to separate different hemoglobin types. A mobile-based application software can be used that is compatible with this assay to process image.

Most of these emerging methodologies are able to distinguish between HbA, HbA₂, HbS, HbF and HbF hemoglobin types. These emerging methodologies are based on resources that are easily available at the POC of even economically challenged nations.

The advantages and disadvantages of each methodology for screening of SCD have been discussed in this review. However, considering every factor that includes overall cost, practicality of using an assay in both developed and developing nations, available resources to perform the test, sensitivity, specificity, different Hb variants that can be identified with certainty, the methodology-of-choice would be Lateral Flow immunoassay (Sickle SCAN and HemoTypeSC). This methodology is categorized as a

POC assay because of its low cost and easy-of-use even in remote areas of developing nations. As can be seen in Table 1, the lateral flow assay has both sensitivity and specificity ranging between 99-100%. The cost of running the assay varies from \$0.25-\$5, which is low compared to more expensive assays like HPLC and WES. A recent study has been conducted in order to select the best methodology based on the low cost of use, portability, easy-to-use diagnostic tests especially in resource-poor countries (Nwegbu *et al.* 2017). In this study the performance characteristics of Lateral flow assay (Sickle SCAN) was compared with cellulose acetate electrophoresis (CAE) and high-performance liquid chromatography (HPLC) by evaluating several subjects for HbSS, HbSC and HbAS. The SickleSCAN™ showed diagnostic sensitivity, specificity and test efficiency of 100.0, 98.2 and 98.2%, respectively for sickle cell disease (Hb SS and Hb SC) that was comparable to the other two methods but at a much lower cost and easy-of-use at remote locations.

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