PROTOCOL TITLE

Validation of a point-of-care screening tool for children with sickle cell disease

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ABSTRACT

Background: Sickle cell disease (SCD) is the commonest genetic disorder in Nigeria, affecting up to 150,000 newborns per year. Given the high frequency of the HbS gene and the large population of affected children, high infant and under-5 mortality, it is estimated that SCD contributes at least 6% of all childhood mortality and of those affected, only 10% in sub Saharan Africa (SSA) are likely to reach adolescence. The clinical course of SCD is characterized by acute and chronic complications, which may be severe, leading to significant morbidity and mortality. Timely diagnosis is key to prevent or manage these complications. However, current diagnostic methods rely on laboratory systems that are expensive and time consuming or may even be unavailable.

General Aim: The primary study objective is to validate the HemeChip technology as a rapid, point-of care (POC) platform for screening SCD.

Methodology: This will be a single site cross-sectional study.

Expected Outcome: The validation of the HemeChip technology as an affordable, robust, easy-to-use POC platform for screening of sickle cell disease (SCD).

1. BACKGROUND

1.1 Introduction

Sickle cell disease (SCD) is a group of inherited disorders of haemoglobin (Hb) synthesis, first described in the medical literature by James Herrick in 1910. Each year about 300,000 infants are born with SCD, including more than 200,000 cases in sub-Saharan Africa alone.[1] In Nigeria, alone, there are over 150,000 of these children born annually and it is estimated that between 50-90% of these children die before their fifth birthday.[2] Overall, in the region, 6% of all childhood mortality in children less than 5 years of age is due to SCD complications and infections. Vaso occlusive crisis and anemia are serious complications of SCD, with infection often being the major cause of hospitalizations, crisis and death.

SCD is caused by a point mutation in the sixth codon of the beta globin chain that produces normal Hb (HbA). This substitution of hydrophilic glutamic acid with hydrophobic valine produces sickle Hb (HbS), which is abnormally polymerized at low oxygen conditions causing sickling. Abnormal polymerization of HbS affects red cell membrane properties, shape, and density, and subsequent critical changes in inflammatory cell and endothelial cell function.[3] The clinical consequences of SCD include painful crises, widespread organ damage, and early mortality.

1.2 Problem statement

Current standard practices for diagnosing SCD are high performance liquid chromatography (HPLC) and bench-top Hb electrophoresis. These two approaches, however, require trained personnel and state-of-the-art facilities, both of which may be lacking in many parts of sub-Saharan Africa where the disease is most prevalent.[4] These laboratory methods also carry significant costs which may be unaffordable for most patients.

1.3 Relevance and Innovation

HemeChip diagnostic system offers an original and innovative solution, leveraging a novel engineering approach, to point of care (POC) diagnosis of SCD. HemeChip

separates haemoglobin protein types in a miniscule volume of blood (1µL) on a piece of cellulose acetate paper that is housed in a micro-engineered chip with a controlled environment and electric field. Differences in Hb mobilities allow separation to occur within the cellulose acetate paper. A micro-engineered design and multiple layer lamination approach are utilized in fabricating the HemeChip. The design allows rapid manual assembly and results are available within a few minutes of performing the test. HemeChip can also integrate with a mobile user interface (e.g. IPhone, IPod), which shows the test result quantitatively and objectively on the screen. HemeChip can be used by anyone after a short (30 minute) training, eliminating the need for highly skilled personnel.

2. AIMS

2.1 General objective

The primary study objective is to validate the HemeChip technology as a novel, point-of care (POC) platform for screening SCD. The results obtained using the HemeChip will be compared to High Performance Liquid Chromatography (HPLC), and the sensitivity and specificity of the HemeChip will be determined.

2.2 Secondary objective

i. To determine the acceptability of using the HemeChip for POC screening of SCD during clinical assessments.

3. LITERATURE REVIEW

Despite its recognition as a global public health problem, SCD remains a neglected health priority in many low- and middle-income countries. Ironically, the burden of haemoglobinopathies is projected to increase in the coming decades. By 2050, it is estimated that the proportion of sickle cell anaemia births in sub-Saharan Africa will be about 88% of the worldwide total, compared to 79% in 2010. This increase will be largely as a result of population growth and public health transition.[5]

The pathophysiology of SCD includes premature red cell haemolysis, vascular obstruction, endothelial damage, chronic inflammation and infarction. These result in acute and chronic complications such as severe anaemia, bone pain, stroke and end organ damage.[6,7] Complications may begin during infancy particularly in individuals with homozygous disease. Newborn screening for SCD has been shown to be highly effective in reducing the morbidity and mortality associated with SCD, when linked to comprehensive healthcare services.[8,9] National programs for routine, universal newborn screening for SCD are lacking in many African countries, Nigeria inclusive.[10] Consequently, most children are diagnosed opportunistically, when they present to health facilities with clinical symptoms and signs suggestive of SCD.

Standard diagnostic testing for sickle cell disease include cellulose acetate or citrate agar electrophoresis (bench top electrophoresis), IEF, high performance liquid chromatography (HPLC), capillary electrophoresis and molecular analysis.[11]

Bench top Hb electrophoresis: This is a laboratory method that can be done under alkaline or acidic conditions and with a variety of sieving materials such as gel or paper.[12] Under alkaline conditions, Hb types C, A2, S, F, and A have net negative charges and migrate towards the positively charged electrode forming visible bands that can be used to identify various Hb disorders. Although useful for the rapid screening of a small number of samples, its results may suffer from inaccuracies at low concentrations. Moreover, alkaline Hb electrophoresis has the disadvantage of having poor resolution between HbS and HbF, particularly in neonates who have high HbF levels, which may result in inaccurate identification of overlapping bands.

Isoelectric Focusing (IEF): IEF exploits the fact that the net charge of a protein varies with the pH of the surrounding medium. Utilizing this variation, proteins are separated based on their isoelectric points (pI), which can be defined as the point at which a protein possesses zero net charge. The technique uses an applied electric field across a gel medium with a fixed pH gradient, each Hb type becomes immobilized once it reaches its pI. IEF exhibits higher resolution than Hb electrophoresis, thus it is capable of distinguishing between a larger number of Hb variants. However, due to the larger number of bands that this higher resolution results in, IEF results are harder to interpret.[12,13] IEF is also expensive and results can be inaccurate especially at low Hb concentrations. Despite these disadvantages, IEF is considered suitable for newborn screening since diagnosis is possible with very small sample volume or even an eluate from a dried blood spot.[12]

High Performance Liquid Chromatography (HPLC): HPLC separates a fluid into its components based on size and charge using cation exchange chromatography to identify the various Hb types in a blood sample. Unique aspects of this test are full automation and accurate quantification of the Hb levels. These machines are relatively expensive and are not readily available in developing countries.[4] In resource rich countries like the US, HPLC has largely replaced Hb electrophoresis and IEF as the gold standard.

DNA Analysis: DNA-based assays can be used to detect the mutations in β globin that produce abnormal Hb.[14] However, it is generally more expensive than the previously described methods and is most often used for prenatal testing rather than postpartum.[15]

HemeChip Technology: HemeChip technology offers an original and innovative solution, leveraging a novel engineering approach, to point-of-care diagnosis of SCD. The basis of HemeChip technology lies in Hb electrophoresis, in which Hb types C, A2, S, F, and A0 have net negative charges in an alkaline solution. Differences in Hb mobilities allow separation to occur within the sieving medium, cellulose acetate paper.

There is no POC device for SCD or any other Hb disorder screening on the market. Currently available tests require skilled technicians in central labs. In Africa, HPLC costs up to $50 and takes 2-3 weeks to get the results. The National Institutes of Health in the United States has awarded a handful of innovation research grants in the past year to six small businesses to develop affordable POC diagnostic technologies for SCD (Correlia Bio., Rockland Immun., Daktari Diag., Silver Lake Res., Halcyon Biom., and Qoolabs). The vast majority of these companies rely on lateral flow immunoassays, which embody specific antibodies. Unlike the HemeChip technology, antibody based devices are specific to only a single disease, and would not work for different Hb disorders. Furthermore, the performance and reliability of lateral flow devices quickly degrade under uncontrolled environmental conditions in resource-limited countries.[16]

HemeChip technology is a low-cost, point-of-care translation of the electrophoresis method that has been well established, readily accepted by clinicians, and widely applied in SCD diagnosis for well over 40 years. HemeChip was clinically tested and successfully benchmarked against available standard methods (i.e., HPLC, bench-top electrophoresis) using 82 blood samples from 27 patients at University Hospitals, Cleveland, USA. HemeChip offers a $2 screening test for SCD (and other Hb disorders), which takes 10 minutes to run. It is mobile and easy-to-use; it can be performed by anyone after a short (30 minute) training. The $2 HemeChip based newborn screening can be overlaid on existing successful (WHO, UNICEF data) primary vaccination programs in sub-Saharan Africa, as a time-for-screening. Primary customers would be the front-line health care workers in government-established immunization programs and pediatric clinics in sub-Saharan Africa.

4. METHODOLOGY

4.1 Study design

This is a single site, cross-sectional study.

4.2 Study site

Study participants will be identified through the Community-Acquired Pneumonia and Invasive Bacteremia Disease study (CAPIBD) currently enrolling at three government hospitals in Kano, Nigeria to include Amino Kano Teaching Hospital, Murtala Mohammed Specialist Hospital and Hasiya Bayero Pediatric Hospital.

Aminu Kano Teaching Hospital (AKTH) is one of the pioneer medical schools in northern Nigeria and provides tertiary referral service for several neighboring states. The 500 bed tertiary facility is located in the state of Kano and lies within the Sudan savannah belt. AKTH has a staff strength of 1,984 and serves as the teaching hospital for Bayero University Kano. The hospital provides health care for Kano and 5 neighboring states, which has a combined population of over 25 million people. AKTH receives regular power supply from the public grid and has both generator and battery backups. Murtala Muhammed Specialist Hospital (MMSH) and Hasiya Bayero Children's Hospital are affiliated government hospitals where faculty, residents and students from AKTH rotate to gain pediatric experience.

Murtala Mohammad Specialist Hospital is one of the pioneer hospitals in Nigeria. Established in 1924, it is reputed to be the second largest hospital in West Africa. It is located in the central city of Kano State and as such, caters for a majority of the estimated 10 million people in the State. The hospital has a staff strength of 1,917 out of which, there are about 97 medical doctors and 415 nursing staff. The outpatient clinics cater for an average of 30,000 patients monthly.

4.3 Subjects/study population

Subjects eligible for this study include children age 6 weeks to 60 months enrolled in CAPIBD from one of the three enrollment sites in Kano, Nigeria.

Inclusion/Exclusion criteria

Inclusion criteria:

Age 6 weeks to 60 months with one of the following true:

1. fever or hypothermia (Temp ≥38□C or ≤36□C) Plus one of the following (prostration, excessive crying, poor feeding, altered consciousness, convulsion, difficulty breathing, profuse vomiting, diarrhea)
2. Rapid breathing (0-2months>60 breaths/min, 3-12months >50 breaths/min, 13-59 months > 40 breaths /min)

AND

3. Provision of signed and dated written informed consent by parent or guardian

Exclusion criteria:

1. Parent of child chooses to opt out of the study after initial consent.
2. Blood transfusion within 3 months of study enrollment.
3. Presence of condition or abnormality that in the opinion of the investigator would compromise the safety of the child or the quality of the data.

4.4 Sample size estimate

Sample size is based on the known prevalence of SCD in Nigeria (SCD prevalence of 2.39% and a carrier rate of about 23%)[19], using the following formula and a 95% confidence interval.[17]

1. Specify clinically acceptable values for the width of 95% confidence interval (CI), sensitivity (SN), specificity (SP), and the disease prevalence (P).

2. Calculate the number of patients with disease, defined as the sum of true positive cases (TP) and false negative cases (FN) using:

$$TP + FN = Z^2_{\alpha/2} \frac{SN(1-SN)}{W^2}$$

3. Calculate the sample size required to obtain the desired sensitivity as:

$$N1 = \frac{TP + FN}{P}$$

4. Calculate the number of patients without disease, defined as the sum of true negative cases (TN) and false positive cases (FP) using:

$$TN + FP = Z^2_{\alpha/2} \frac{SP(1-SP)}{W^2}$$

5. Calculate the sample size required to obtain the desired specificity as:

$$N2 = \frac{TN + FP}{P}$$

Where $Z_{\alpha/2}$ is a statistical constant having a value of 1.96 for 95% CI.

6. Select the required sample size as the greater of N1 and N2.

The calculations made for the HemeChip test sample size are summarized in Table 1 below:

Prevalence estimate	P	0.024
Width of the 95% CI: 10%	W	0.10
Sensitivity expected: 98%	SN	0.97
Specificity expected: 98%	SP	0.97
Statistical constant, 1.96 for 95% CI	Zalpha	1.96
Number with disease, True Positive + False Negative	TP+FN	11
Sample size required for sensitivity	**N1**	**468**
Number without disease, False Positive + True Negative	FP+TN	11
Sample size required for specificity	**N2**	**11**
FINAL SAMPLE SIZE (greater of N1 and N2)		**468**

Total maximum enrollment of 5,000 patients, age 6 weeks to 60 months identified through CAPIBD.

Procedure

The CAPIBD medical officer will introduce the study and ask if parent/caregiver is willing to participate. If yes, signed informed consent will be obtained and the subject assigned a unique identification number through CAPIBD. The clinical research assistant (CRA) assigned to the study will complete a questionnaire including demographic data and family information, and perform a heel or finger prick to obtain a blood sample for HemeChip and HPLC (page 12).

HemeChip Test

a. Finger-stick or heel-stick blood sample (20 µL) is mixed with deionized water for red blood cell lysis and the mixture allowed to set for 3 minutes.
b. 65 µL of a buffer solution is used to wet the paper inside the HemeChip.
c. A disposable metal applicator is used to stamp 1 µL of the mixture onto the paper strip inside the HemeChip through the sample application port.
d. 185 µL of the buffer solution is pipetted into two buffer chambers through buffer loading inlets on the HemeChip.
e. The HemeChip is loaded into the reader and the power supply in the reader turned on. The test runs for five minutes and diagnosis is obtained on a computer connected to the reader.
f. Data is stored on computer and results delivered by fax delivery and secured email to a dedicated port for the attention of study PI.
g. Study CRA contacts caregivers of screen-positive children via phone and all who screen positive will be referred to the local SCD clinic for follow up care.

HPLC Test

a. All blood samples collected via heel or finger prick on dot-blot paper will be shipped to the IFAIN lab in Abuja on a monthly basis.

b. HPLC confirmatory results will be obtained from the IFAIN lab on a monthly basis.

c. Study CRA contacts caregivers of screen-positive babies via phone following confirmatory results.

d. All subjects with abnormal HPLC results will be enrolled in the pediatric sickle cell clinic, for parental education, penicillin prophylaxis, pneumococcal vaccination, malaria prevention, fever and pain management protocol, splenic palpation and contact with SCD team.

4.4 Data handling and quality assurance

The PI will ensure the accuracy, completeness, and timeliness of the data recorded. Only the investigators and designated study personnel will have access to these data. Identified patients will be given a unique study number through CAPIBD and only the study number will be included in the dataset. Once a complete dataset has been gathered on all patients, the identifiers will be discarded, and only the de-identified data will be maintained. Study personnel will enter data from source documents corresponding to a subject's visit into the protocol-specific electronic Case Report Form (eCRF). All data collected during the course of this study will be reviewed and verified for completeness and accuracy by the PI.

The data will be entered into a validated database, which will allow safe and secure storage. After data have been entered into the study database, a system of computerized data validation checks will be implemented and applied to the database on a regular basis. Any changes to the study database will be documented.

4.5 Statistical analysis

Data will be analyzed and reported based on established methods in the literature.[18] Statistical significance will be set at 95% confidence level for all tests ($p<0.05$). Receiver-operating curves will be utilized to assess sensitivity and specificity of

HemeChip in comparison to HPLC as the standard test. Sensitivity will be calculated as # true positives / (# true positives + # false negatives) and specificity will be calculated as # true negatives / (# true negatives + # false positives). Positive predictive value (PPV) will be calculated as # true positives / (# true positives + # false positives) and negative predictive value (NPV) will be calculated as # true negatives / (# true negatives + # false negatives).

5 Dissemination of results

Study results will be disseminated at a clinical meeting in the Department of Child Health at Case Western Reserve University, published in peer-reviewed scientific journals and presented at relevant scientific conferences.

6 ETHICAL ISSUES

6.4 Study conduct

The study will be performed in accordance with ethical principles that have their origin in the Declaration of Helsinki and are consistent with the International Conference on Harmonization (ICH)/Good Clinical Practice (GCP).

Informed consent

All study participants must have a legal representative that is in competent mental condition to provide written, informed consent on behalf of the study subject before entering the study. A copy of the signed informed consent form will be given to the parent or legal guardian. Each subject, parent or legal guardian will be notified that they are free to withdraw from the study at any time.

6.5 IRB of record

There will be no research activity performed or data collection at UNMC. All research activity and data collection will be performed at the Nigeria site. The CWRU team will take responsibility for data collection, storage, submitting continuing reviews and

amendments for the study. CWRU team will also give the approved documents to UNMC in a timely manner and make sure to keep track of everything that occurs at that site.

University of Nebraska Medical Center (UNMC) has developed several microbiology diagnostic laboratories for invasive bacterial infections in Nigeria children. UNMC supports the International Foundation Against Infectious Disease (IFAIN),a registered non-profit in Nigeria, which manages local research activities. The Co-I from UNMC, (Stephen Obaro) is founding member and Trustee of IFAIN and the PI on several rants currently in execution by UNMC through IFAIN in Nigeria. Some of the services IFAIN provides include microbiology and molecular diagnostic services, such as sickle cell disease screening through HPLC. UNMC also has a strong network with multiple researchers across Africa who work with hemoglobinopathies and vaccine preventable infections. UNMC provides essential expertise and support to fulfill the activities for our proposal.

6.6 **Safety**

The study poses minimal risk to participants. There will be the minor discomfort of a heel (or finger) prick. Bruising at the site of skin puncture may occur. For parents/caregivers, there is a small, time inconvenience for administration of the study questionnaire.

Timeline/Work schedule

Timeline:

- IRB approval: 2 months (FEB/MAR 2017)
- Recruitment and training of study staff: 1 month (MAR 2017)

- Recruitment: 3 months (APR 2017 to JULY 2017)
- Data entry, cleaning and analysis: APR 2017 to AUG 2017
- Write up and dissemination: AUG 2017 to OCT 2017

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