

## INTRODUCTION

Hemoglobin C (HbC) is one of the most prevalent abnormal hemoglobin mutations globally along with hemoglobin S and hemoglobin E.<sup>1</sup> HbC is the second most prevalent hemoglobinopathy in the United States and third most prevalent worldwide.<sup>2</sup> It is most prevalent in African countries, Haiti, India, and other populations of African descent.<sup>3</sup> The incidence of HbC gene is 17 to 28% in Western Africa, in the vicinities of Northern Ghana,<sup>4</sup> and 6.5% in Haiti.<sup>5</sup> Although HbC is widespread, the current distribution of HbC is poorly documented.<sup>1</sup>

HbC is a structural variant of normal hemoglobin A (HbA) that is caused by an amino acid substitution of lysine for glutamic acid at position six of the beta hemoglobin chain.<sup>6</sup> Due to electrostatic interactions between the positively charged B6-lysyl side chain and the negatively charged adjacent groups, HbC is less soluble than HbA in red blood cells.<sup>6,7</sup> In its oxygenated R (relaxed) state, HbC forms crystals inside red blood cells and these intraerythrocytic crystals contribute to clinical pathogenesis.<sup>8,9</sup> The crystallization of hemoglobin C occurs inside red blood cells of patients expressing  $\beta^C$ -globin and exhibiting the homozygous CC and heterozygous SC alleles.<sup>8</sup> It is thought that salt induced crystallization follows many of the thermodynamics and kinetic characteristics of crystal growth processes observed in vitro.<sup>10</sup> When the RBCs are dehydrated from the hypertonic salt solution, the electrostatic interactions become more sterically hindered causing the side chains and adjacent groups to interact more inducing the crystal formation. To classify a cell as containing a HbC crystal, the intracellular body must be very dense, have sharp, straight edges, and have partially or completely depleted the cytosolic content (Hb in solution) of the cell. This last feature was most apparent when a thin rim of RBC membrane was observed encircling the crystal inclusion(s).<sup>11</sup>

Homozygous hemoglobin C disease is manifested by mild hemolytic anemia, splenomegaly, and striking morphologic abnormalities of red cells<sup>12</sup> such as intraerythrocytic and extraerythrocytic rod shaped crystals, target cells, and nucleated red blood cells.<sup>13</sup> Red cells with crystals become rigid and are trapped and destroyed in the spleen, thereby reducing RBC life span to 30-55 days.<sup>13</sup> Due to this decreased red cell life span, patients with homozygous HbC can present with symptoms of mild anemia, while some patients occasionally experience joint and abdominal pain.<sup>13</sup> The prognosis for HbCC is good, but the quality of life may be disrupted with a mild anemia causing fatigue. Some HbCC patients are prescribed folic acid supplements to improve erythropoiesis to compensate for the anemia but for most no treatment is necessary.<sup>14</sup> In contrast, heterozygous HbAC patients are asymptomatic with no hematologic abnormality noted except target cells on blood smears.<sup>15, 13</sup>

When a patient co-expresses a HbC allele and hemoglobin S (HbS) allele, the patient exhibits HbSC disease. HbSC disease is considered a compound (double) heterozygous condition resulting from the genetic expression of two abnormal beta globin genes, HbS and HbC.<sup>16</sup> HbSC patients present most often with a mild to moderate hemolytic anemia; although there is a wide spectrum of disease severity in these patients.<sup>16</sup> Some patients exhibit symptoms similar to sickle

cell anemia to include frequent sickling crises, splenomegaly, hypoxia, fatigue, and shortness of breath.<sup>13</sup> Generally HbSC is less severe than HbSS, but HbSC still results in recurrent episodes of pain and progressive organ damage, resulting in a shortening of life-expectancy by 20–30 years in the northern hemisphere.<sup>17</sup> Some complications are more common in HbSC than HbSS, most notably proliferative retinopathy leading to visual loss.<sup>17</sup> The frequency of HbSC disease in African Americans is ~0.05%, which is less common than HbSS (0.16% of African Americans) and more common than HbCC (0.02% of African Americans) disease.<sup>18</sup>

In developed countries, the gold standard for diagnosing hemoglobinopathies is hemoglobin electrophoresis (HE).<sup>19</sup> Other methods to confirm HbS or HbC include Isoelectric Focusing (IEF), High Performance Liquid Chromatography (HPLC), and nucleic acid methods. HE, IEF and HPLC function by separating different hemoglobin types by electrical current and column chromatograph, respectively. HE, IEF, and HPLC are quick and efficient, but HE is more cost effective than IEF and HPLC. These methods are suitable for detecting large numbers of individual hemoglobinopathy mutations and offer improved resolution and identification of less common hemoglobin variants.<sup>20</sup> HPLC has the advantage of offering improved resolution and identification of certain hemoglobin variants over electrophoresis.<sup>13</sup> HPLC can also be useful in identifying hemoglobin variants with low oxygen affinity.<sup>13</sup>

By definition, underdeveloped countries do not have the same infrastructure standard, medical educational system, and financial resources as developed countries that are necessary to support modern laboratories. Resources like stable and continuous electricity, piped natural gas, clean water supply, refrigeration, and climate control are an expectation in developed countries. In addition, laboratory educational programs in many underdeveloped countries do not include adequate training on state-of-the-art instrumentation. Most importantly, high unemployment rates create an inability for many patients to pay for medical services thus reducing clinic revenues and the ability of clinics to purchase and support modern instruments.<sup>21</sup> HE, IEF, HPLC, and nucleic acid methods are expensive and require specialized reagents and instruments, consumable materials, stable electricity, refrigeration, and highly trained lab staff.<sup>20</sup> Although these tests are accurate and precise, in places where HbC is most prevalent these resources are largely unavailable making them impractical for use in underdeveloped countries.

Since current methods to diagnose HbC cannot overcome the challenges facing laboratories in impoverished countries, it is crucial to develop alternative methods that are cheap, simple, relatively fast, and accurate. It has been shown that HbC crystals can form intracellularly when blood from patients with HbCC or HbSC are exposed to a hypertonic salt solution.<sup>6</sup> It has been hypothesized that osmotic dehydration, produced by suspension of RBCs in 3% NaCl solution, results in formation of crystal inclusions.<sup>6</sup> Other published studies used phosphate buffer solutions at high molarity to induce the crystal formation<sup>8,22</sup> as well as 5% NaCl at 4-hour incubation periods that produced distinct crystals.<sup>11</sup> Our study seeks to develop a reproducible

method to identify HbC and possibly determine zygosity based on the number of intracellular HbC crystals that form in a hypertonic salt solution.

## **MATERIALS AND METHODS**

### Specimens

Whole blood samples were collected in EDTA by standard venipuncture technique from different members of the research team (N=5) to serve as a negative control (HbAA). Fifteen de-identified whole blood samples of HbSC collected in EDTA were obtained from Cardinal Glennon Children's Hospital, St. Louis, MO. Specimens were tested < 14 days from the date of collection and noted for hemolysis. Since samples are de-identified, St. Louis University Institutional Review Board considered the study to be non-human and, therefore, IRB waived.

### Basic Procedure

Eighty microliters (determined in the study) of HbSC or HbAA blood samples were washed three times in 0.85% saline to remove plasma and dry blotted to remove saline so not to dilute the salt solution. A variety of hypertonic salt solutions ranging in concentrations between 2%-10% were tested. A 1:3 ratio of cells to hypertonic salt solution (determined in the study) were incubated between 2-24 hours at temperatures between 22-40°C. Following incubation, the cells were stained using New Methylene Blue at a 1:2 dilution and slides were made. The number of RBCs containing HbC crystals were counted per 1000 red blood cells. Figure 1 illustrates HbC crystal morphology under a 100x oil immersion bright field microscope.

### Research Design

*Optimization of Salt Concentration:* Based on the literature and previous data from our lab, HbSC blood samples were tested with NaCl and Dulbecco's Phosphate Buffered Saline (DPBS) at concentrations ranging from 2%-10%.

*Optimization of Salt Type:* Four additional salt types were tested against the crystal formation of NaCl and DPBS. Calcium Chloride, Magnesium Chloride, Potassium Chloride, and Sodium Hydrogen Phosphate were tested at 5% (optimum) concentration.

*Optimization of Incubation Time:* Using optimal salt type and concentration, samples were incubated at 2 hours, 4 hours, 6 hours, 12 hours, and 24 hours.

*Optimization of Incubation Temperature:* Optimal temperature was obtained by comparing HbC crystal formation at temperatures of 22°C, 30°C, 37°C, and 40°C using optimized salt type, concentration, and incubation time.

### Data Analysis

Descriptive statistics (mean and standard deviation) were used to evaluate each salt type, salt concentration, incubation time, and incubation temperature tested. A one-way ANOVA test followed by a Tukey's HSD and Bonferroni post-hoc tests were performed to determine differences in mean HbC counts between each salt type, salt concentration, incubation time, and incubation temperature tested.

## RESULTS

Experiments testing various salt types, salt concentrations, incubation times, and incubation temperatures were performed to optimize the test method. Using the 3% NaCl concentration described in the literature as a target, NaCl and DPBS were tested in concentrations above and below this target, between 0.95% (Saline) and 10%, using HbSC blood samples. Using the optimized procedure and HbSC blood samples that are less than 14 days old, HbC crystal counts between 600-750/1,000 RBCs were consistently obtained. Negative control samples (non-HbC or AA genotype) never produced HbC crystals. HbC crystal numbers presented in Figure 2 are lower because counts were performed before the method was optimized.

HbC crystal formation in increasing salt concentration follows a Gaussian distribution. No HbC crystals form in saline, but crystal formation increases with rising salt concentration that peaks at 5% for both salt types and falls as salt concentration continues to rise. Counts were done over a several month period as samples became available. Data were statistically analyzed using ANOVA and the p-value was  $<0.0001$  for both the NaCl series and the DPBS series indicating statistical significance between some of the concentrations for both salt types. Post-hoc testing showed that 5% and 6% concentrations for both the NaCl and DPBS series were statistically different from the other concentrations tested (0.9%, 2%, 3.3%, and 10%) in the series. In both series the 5% concentration was statistically different from the 6% concentration ( $<0.0001$ ). Lastly, the 5% NaCl and 5% DPBS were close to reaching statistical significance from each other with a p-value of 0.068. Also, in both NaCl and DPBS series, post-hoc testing showed that 2%, 3% and 10% are all not significantly different from each other with a p-value of 1.000. The statistical analysis also showed that 6% NaCl was significantly different from the concentrations of 2% and 3% in NaCl and DPBS with a p-value of 0.001 and was significantly different from 6% DPBS with a p-value of 0.030.

Four other salts were tested (Calcium Chloride, Magnesium Chloride, Potassium Chloride, and Sodium Hydrogen Phosphate) and no HbC crystals were formed in any of these salt solutions at 5% concentration at 37°C for 24-hour incubation periods.

Previous work using an un-optimized procedure suggested a 24-hour incubation was optimal. However, the literature indicated crystal formation occurs at shorter times (3-4 hours).<sup>2,18</sup> Therefore, a range of incubation times was tested using the optimal salt type and concentration (Figure 3). Four-hour incubation produced the highest number of crystals and was chosen as the

optimal incubation time. ANOVA calculated a p-value of 0.0567. Post-hoc analysis showed that 4 hours was significantly different from the control of 24 hours with a p value of 0.042. No other temperature was significantly different from the others.

The literature consistently used 37°C for HbC crystal induction. However, testing was performed to ensure that body temperature was optimal for HbC crystal formation. Therefore, the assay was performed using the optimal salt type, salt concentration and incubation time but varied the incubation temperature from 23°C to 40°C. ANOVA p-value was 0.0197 indicating a statistical difference that justify performing the post-hoc testing. Temperatures of 37°C and 40°C showed the highest HbC crystal formation. Post-hoc testing showed a significant different between 22°C and 37°C (p-value=0.004) and between 22°C and 40°C (p=0.006). There was no statistical difference between 37°C and 40°C (p-value=1.000) or between 22°C and 30°C (p-value=0.509). Therefore, 37°C was chosen as the optimal temperature.

## DISCUSSION

This study has successfully developed a simple and low-cost method to detect the presence of HbC by inducing intracellular crystallization that can be visualized with a microscope. The optimized method uses 5% DPBS at 37°C for 4 hours counterstained with a 1:2 dilution of new methylene blue to provide a blue background to better visualize the natural orange colored HbC crystals. This method differs from previously published literature in that it optimizes and combines previously published variables into one method, producing HbC crystal counts greater than previously reported.<sup>4</sup> Crystal counts in the early experiments using varying salt concentrations were performed before optimizing for sample age, incubation time and temperature thus producing lower absolute crystal counts. Using the optimized method, counts of between 600-750/1,000 RBCs were observed using HbSC samples.

To optimize the procedure, it was necessary to determine the salt type and concentration for maximal crystal formation. Past studies reported that HbC crystals can form in 3% NaCl in virtually every HbCC cell,<sup>6,9</sup> in 5% NaCl after 4 hours,<sup>21</sup> and in a high molarity phosphate buffered saline.<sup>8,22</sup> Sodium chloride was tested first, because it was the most used salt reported in the literature. Other salts were tested against NaCl to include DPBS, calcium chloride, magnesium chloride, potassium chloride, and sodium hydrogen phosphate. It was found that a 5% DPBS was superior to all the salt types and concentrations tested.

HbC crystals in SC samples were also observed at various times between 2-24 hours. Optimal crystal formation was produced at 4-hour incubation time. The 4-hour incubation time was statistically different from 24 hours, but not statistically different from any other time points tested. However, 4-hour incubation time was chosen because it produced the most crystals in the shortest time. The 4-hour incubation time is sufficiently fast to be performed in clinics in underdeveloped countries where patients often wait for lab results to receive treatment before leaving the clinic to return home.

HbC crystals were observed in SC samples at different incubation temperatures, but 37°C was determined to be the optimal temperature to induce HbC crystal formation. Although most labs in underdeveloped countries do not have temperature-controlled incubators, they may not be necessary since HbC is often found in tropical or subtropical regions. Ambient temperatures in these areas range from approximately 20<sup>0</sup>C in the winter to 40<sup>0</sup>C in the summer. HbC crystal formation continues to occur to similar levels within this temperature range. In addition, most clinics labs in underdeveloped countries are constructed as “open air” and are not climate controlled. Therefore, indoor temperatures tend to be somewhat higher than outdoor temperatures ranging from approximately 30<sup>0</sup>C to 45<sup>0</sup>C. It was demonstrated that these temperatures optimize HbC crystal formation.

HbSC samples were used for these experiments for three reasons. First, patients with HbSC are usually symptomatic, so they seek medical intervention making their de-identified blood samples available for study. Individuals with HbAC (asymptomatic) and HbCC (mild anemia) do not require medical interventions so their samples were not readily available. Second, the literature indicated that HbSC blood samples produce higher HbC crystal formation in a 3% NaCl solution than HbAC individuals.<sup>4</sup> Third, HbSC genotype is more common than HbCC genotype.

It was observed during experimentation that sample age had a negative impact on the number of HbC crystals produced. Since the samples were de-identified and donated from the local children’s hospital, samples were not accessible until all diagnostic testing had been completed. After analyzing the blood sample age versus the number of crystals formed, data suggest that HbC crystal formation decreases rapidly when the blood sample reaches 14 days post-collection when stored at refrigerator temperatures (4<sup>0</sup>C). Therefore, it was decided to reject samples that were older than 14 days from collection. Early data used samples that were >14 days old. Although the data clearly indicated which procedural characteristics were optimal, actual crystal counts in older samples were less than ideal.

It is hypothesized that the mechanism of HbC crystal formation involves the loss of intracellular water that aggregates coalesced HbC molecules into patterns of tighter molecular packing with small regions of alignment that causes crystallization.<sup>23</sup> It was hypothesized that samples below 5% salt produced fewer crystals due to insufficient dehydration to push HbC molecules close enough to induce crystallization; and that salt concentrations above 5% excessively dehydrate the samples resulting in insufficient intracellular water to maintain the HbC molecules in solution.

This method has the potential to not only detect HbC but to determine genotype. This information is critical for quality patient care. Patients with HbSC need immediate and lifelong treatment. Patients with HbCC may have a mild anemia that can affect the quality of life. In addition, all patients with HbC must be aware of reproductive implications with an understanding of the risks involved. Assuming this method will differentiate HbC genotypes, it



can be used in conjunction with a simple and cost-effective method to detect HbS (Sickle Confirm)<sup>24</sup> to detect six different genotypes (AA, AC, CC, AS, SS, SC). This will provide physicians the ability to distinguish patients with serious illness (SS and SC), from patients with a mild anemia (CC), from carriers (AC and AS). Patients diagnosed with HbSS or HbSC can undergo life-saving treatment and the others can benefit from prenatal counseling.

The method is ideal in underdeveloped countries where finances and lack of infrastructure preclude the use of modern methods like electrophoresis, HPLC, and nucleic acid methods to identify and genotype hemoglobinopathies. The method is inexpensive, easy, relatively fast, and does not require instruments other than a microscope, which most labs in underdeveloped countries need to perform other laboratory procedures. DPBS and new methylene blue do not require refrigeration and can be stored at room temperatures for long periods of time. Stability experiments at ambient tropical temperatures are underway.

Table 1 shows that the cost per test for this method is <\$0.09 using the costs of the major consumable materials needed. Less than a dime makes this method much less costly than modern methods and in line with costs that can be absorbed and recovered from patient fees by clinics in underdeveloped countries. In addition to the consumables, labs would need to also provide 12 x 75mm plastic or glass test tubes, microscope slides, and a pipettor and pipette tips to perform the assay. However, tubes, microscope slides and pipette tips are inexpensive, already available in most labs, and reusable. Pipettors are expensive but many underdeveloped labs have a pipettor to perform other lab procedures.

A 4-hour incubation time for the procedure is not ideal, but reasonable to be performed in a single day. It is recommended that patients being tested for HbC using this method have blood collected first thing in the morning so data can be released during the same clinic visit. The method is also easy to perform and requires minimal training to identify intracellular HbC crystals. Testing involving microscopy is common in labs in underdeveloped countries, so microscopy skills by the laboratory staff are generally excellent.

The magnitude of HbC crystal formation in HbSC patients (Figure 4), about 60-75% of RBCs, suggests the potential for different HbC crystal counts between genotypes. IRB approval has been obtained to consent research subjects with four genotypes: AA (negative control), AC, CC, and SC to determine if HbC crystal counts can be used to distinguish these genotypes. Preliminary data show HbC crystal counts in HbAC samples of between 80-150/1,000 RBCs.

A method has been developed that optimizes HbC crystal formation using RBCs from patients with HbSC disease. The method is inexpensive and does not require instrumentation or resources to which underdeveloped countries do not have access. The method costs <\$0.09/test or \$12.90 for 150 tests and only requires a microscope. An incubator is not necessary to maintain the incubation around 37<sup>0</sup>C because the ambient temperature in most countries where HbC is

endemic is between 30°C and 40°C most of the year which is the temperature range of maximal crystal formation. This method is a relatively fast, simple and accurate way to test for HbC and has the potential to determine zygosity.

**Figure 1.** Shows HbC crystals under 100x Oil Immersion on a Bright Field Microscope. Two RBCs highlighted with the white box do not have HbC crystals.

**Figure 2.** Crystal Formation in Various Concentrations of Dulbecco’s Phosphate Buffer Saline (DPBS) versus Sodium Chloride.

\* = statistical significance

**Figure 3.** Crystal Formation at Different Incubation Times in Dulbecco’s Phosphate Buffer Saline.

\* = statistical significance

**Figure 4.** Crystal formation in Different Incubation Temperatures in Dulbecco’s Phosphate Buffer Saline.

\* = statistical significance

**Table 1.** Major consumable reagents and materials needed to perform the microscopic HbC method with costs/unit; costs/test; and cost/150 tests.

<b>Table 1: Cost Analysis for Microscopic HbC Method</b>				
<b>Product</b>	<b>Unit Size</b>	<b>Unit Cost</b>	<b>Cost/Test</b>	<b>Cost/150 Test</b>
Dulbecco’s Phosphate Buffer Saline (10X concentrate)	1 L	\$36.00	\$0.036	\$5.40
New Methylene Blue	250 mL	\$37.30	\$0.012	\$1.80
Parafilm	125’ x 4”	\$28.50	\$0.038	\$5.70
<b>Total Cost</b>		<b>\$101.80</b>	<b>\$0.086</b>	<b>\$12.90</b>









