

Development of a Microscopic Method to Identify Hemoglobin C Conditions for Use in Developing Countries

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ABSTRACT

Hemoglobin C (HbC) is one of the most prevalent hemoglobinopathies worldwide along with sickle cell hemoglobin (HbS) and hemoglobin E. Hemoglobin C disease produces mild symptoms but is a life-threatening disease if inherited with HbS (HbSC). HbS and HbC are most prevalent in sub-Saharan countries under-equipped to identify its presence through gold standard methods like electrophoresis, high-performance liquid chromatograph, or isoelectric focusing. This study aims to create a simple and inexpensive method to identify the presence of HbC in human blood using limited resources with the potential to determine zygosity. HbC crystals that form when blood is incubated in a hypertonic salt solution become visible microscopically when stained with new methylene blue. The method was optimized by modifying the salt type, salt concentration, incubation time, and incubation temperature. The optimized method incubated red blood cells (RBCs) in a 5x Dulbecco's phosphate buffered saline at 37°C for 4 hours. Blood samples with the HbSC genotype yielded between 600–750 HbC crystals for every 1,000 RBCs counted while negative control samples (AA) did not produce HbC crystals. Preliminary results indicate that AS samples produce between 80–150 HbC crystals/1,000 RBCs. Future studies will include testing of blood samples of AC, CC, SC, and AA genotypes to determine if the number of crystals that form using this method will differentiate genotype. If so, this inexpensive, simple, and relatively rapid method can be used to evaluate patients with HbC and determine genotype in underdeveloped countries where HbC is prevalent.

ABBREVIATIONS: ANOVA - analysis of variance, DPBS - Dulbecco's phosphate buffered saline, HbA - hemoglobin A, HbAA - hemoglobin AA, HbAC - hemoglobin A disease, HbC - hemoglobin C, HbCC - hemoglobin C disease, HbS - sickle cell hemoglobin, HbSC - hemoglobin SC, HbSS - sickle cell anemia, HE - hemoglobin electrophoresis, HPLC - high-performance liquid chromatography, IEF - isoelectric focusing, IRB - institutional review board, NaCl - sodium chloride, RBC - red blood cell.

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INTRODUCTION

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

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 6 of the β -hemoglobin chain.⁶ Because of electrostatic interactions among the positively charged B6-lysyl-side chain and the negatively charged adjacent groups, HbC is less soluble than HbA in red blood cells (RBCs).^{6,7} In its oxygenated-relaxed state, HbC forms crystals inside RBCs, and these intraerythrocytic crystals contribute to clinical pathogenesis.^{8,9} The crystallization of HbC occurs inside the RBCs of patients, which expresses β^C -globin and exhibits the homozygous CC and heterozygous SC alleles.⁸ It is thought that salt-induced crystallization follows many of the thermodynamics and the kinetic characteristics of crystal growth processes observed *in vitro*.¹⁰ When the RBCs are dehydrated from the hypertonic salt solution, the electrostatic interactions become more sterically hindered, which causes the side chains and adjacent groups to interact, further inducing the crystal formation. To classify a cell as containing a HbC crystal, the intracellular body must be very dense, have sharp and straight edges, and have partially or completely depleted the cytosolic content (hemoglobin 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).¹¹

Homozygous HbC disease is manifested by mild hemolytic anemia, splenomegaly, and striking morphologic abnormalities of red cells,¹² such as intraerythrocytic and extraerythrocytic rod shaped crystals, target cells, and nucleated RBCs.¹³ Red cells with crystals become rigid and are trapped and destroyed in the spleen, thereby reducing RBC life span to 30–55 days.¹³ Because of 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.¹³ The prognosis for hemoglobin C disease (HbCC) is good, but the quality of life may be disrupted with a mild anemia that causes fatigue. Some HbCC patients are prescribed folic acid supplements to improve erythropoiesis and compensate for the anemia, but for most no treatment is necessary.¹⁴ In contrast, heterozygous hemoglobin A disease (HbAC) patients are asymptomatic with no hematologic abnormality noted except for target cells on blood smears.^{13,15}

When a patient coexpresses a HbC allele and a sickle cell hemoglobin (HbS) allele, the patient exhibits hemoglobin SC (HbSC) disease. HbSC disease is considered a compound (double) heterozygous condition that results from the genetic expression of 2 abnormal β -globin genes: HbS and HbC.¹⁶ HbSC patients present most often with a mild to moderate hemolytic anemia; however, there is a wide spectrum of disease severity in these patients.¹⁶ Some patients exhibit symptoms similar to sickle cell anemia (HbSS) to include frequent sickling crises, splenomegaly, hypoxia, fatigue, and shortness of breath.¹³ Generally HbSC is less severe than HbSS, but HbSC still results in recurrent episodes of pain and progressive organ damage, which can result in a shortened life expectancy by 20–30 years in the northern hemisphere.¹⁷ Some complications are more common in HbSC than HbSS, most notably proliferative retinopathy that leads to visual loss.¹⁷ 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).¹⁸

In developed countries, the gold standard for identifying hemoglobinopathies is hemoglobin electrophoresis (HE).¹⁹ 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 electric 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.²⁰ HPLC has the advantage of offering improved resolution and identification of certain hemoglobin variants over electrophoresis.¹³ HPLC can also be useful in identifying hemoglobin variants with low-oxygen affinity.¹³

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.²¹ HE, IEF, HPLC, and nucleic acid methods are expensive and require specialized reagents and instruments, consumable materials, stable electricity, refrigeration, and highly-trained laboratory staff.²⁰ Although these tests are accurate and precise, these resources are largely unavailable in places where HbC is most prevalent, which makes them impractical for use in underdeveloped countries.

Because current methods to identify HbC cannot overcome the challenges that face 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 is exposed to a hypertonic salt solution.⁶ It has been hypothesized that osmotic dehydration, produced by suspension of RBCs in 3% sodium chloride (NaCl) solution, results in formation of crystal inclusions.⁶ Other published studies used phosphate buffer solutions at high molarity to induce the crystal formation^{8,22} as well as 5% NaCl at 4-hour incubation periods that produced distinct crystals.¹¹ This 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 hemoglobin negative control (hemoglobin AA [HbAA]). Fifteen de-identified whole blood samples of HbSC collected in EDTA were obtained from Cardinal Glennon Children's Hospital in St. Louis, Missouri. Specimens were tested <14 days from the date of collection and were noted for hemolysis. Because samples were de-identified, St. Louis University Institutional Review Board (IRB) considered the study to be nonhuman and, therefore, IRB waived the study.

Basic Procedure

Eighty microliters (determined in the study) of HbSC or HbAA blood samples were washed 3 times in 0.85% saline to remove plasma and were dry blotted to remove saline so as 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

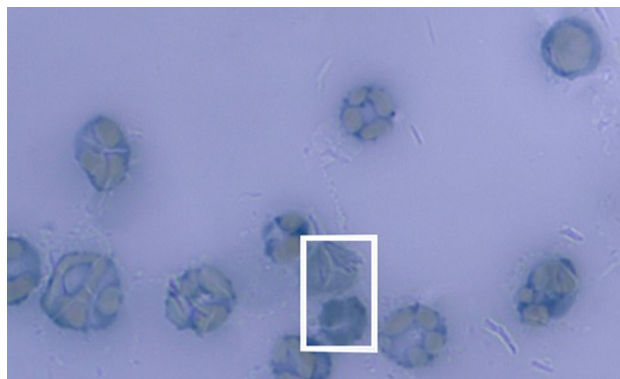


Figure 1. Shows HbC crystals under 100x oil immersion on a bright field microscope. Two RBCs highlighted with the white box do not have HbC crystals.

of RBCs containing HbC crystals were counted per 1,000 RBCs. 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 laboratory, 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 SD) were used to evaluate each salt type, salt concentration, incubation time, and incubation temperature tested. A 1-way analysis of variance (ANOVA) test followed by a Tukey's honest significance test and Bonferroni *post hoc* tests were performed to determine differences in mean HbC counts among 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 were 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, which indicated statistical significance among 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 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

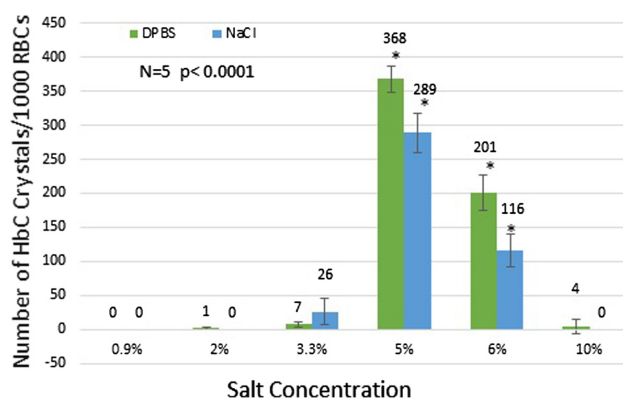


Figure 2. Crystal formation in various concentrations of DPBS versus NaCl. * = statistical significance.

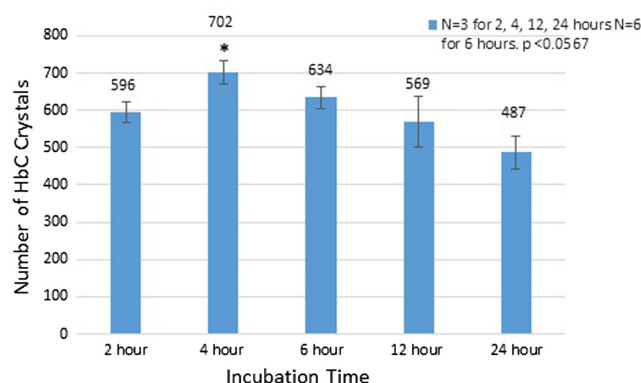


Figure 3. Crystal formation at different incubation times in DPBS. * = statistical significance.

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 that used an unoptimized procedure suggested a 24-hour incubation was optimal. However, the literature indicated crystal formation occurs at shorter times (3–4 hours).^{2,18} 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, which indicated a statistical difference that justified performing the *post hoc* testing. Temperatures of 37°C and 40°C showed the highest HbC-crystal formation. *Post hoc* testing showed a significant difference between 22°C and 37°C (*P* = 0.004) and between 22°C and 40°C (*P* = 0.006). There was no statistical difference between 37°C and 40°C (*P* = 1.000) or between 22°C and 30°C (*P* = 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, which produces HbC-crystal counts greater than previously reported.⁴ 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,^{6,9} in 5% NaCl after 4 hours,²¹ and in a high molarity phosphate buffered saline.^{8,22} NaCl 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 laboratory 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 laboratories in underdeveloped countries do not have temperature-controlled incubators, they may not be necessary because HbC is often found in tropical or subtropical regions. Ambient temperatures in these areas range from approximately 20°C in the winter to 40°C in the summer. HbC-crystal formation continues to occur to similar levels within this temperature range. In addition, most clinical laboratories 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°C to 45°C. It was demonstrated that these temperatures optimize HbC-crystal formation.

HbSC samples were used for these experiments for 3 reasons. First, patients with HbSC are usually symptomatic; they seek medical intervention, which makes 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.⁴ Third, HbSC genotype is more common than HbCC genotype.

It was observed during the experiment that sample age had a negative impact on the number of HbC crystals produced. Because 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 postcollection when stored at refrigerator temperatures (4°C). Therefore, it was decided to reject samples that were older than 14 days from collection, including 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.²³ It was hypothesized that samples below 5% salt produced fewer crystals because of insufficient dehydration to push HbC molecules close enough to induce crystallization. It was also hypothesized 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 also 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)²⁴ and to detect 6 different genotypes (AA, AC, CC, AS, SS, SC). Detection will provide physicians the ability to distinguish patients with serious illness (SS and SC) from patients with a mild anemia (CC) or 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 laboratories 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

Table 1. Cost analysis for microscopic HbC method: major consumable reagents and materials needed to perform the microscopic HbC method with costs/unit, costs/test, and cost/150 tests

Product	Unit Size	Unit Cost	Cost/ Test	Cost/150 Test
DPBS (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
Total cost		\$101.80	\$0.086	\$12.90

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, laboratories would need to also provide 12 x 75 mm 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 laboratories, and reusable. Pipettors are expensive but many underdeveloped laboratories have a pipettor to perform other laboratory 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 that 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 laboratories 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 among genotypes. IRB approval has been obtained to consent research

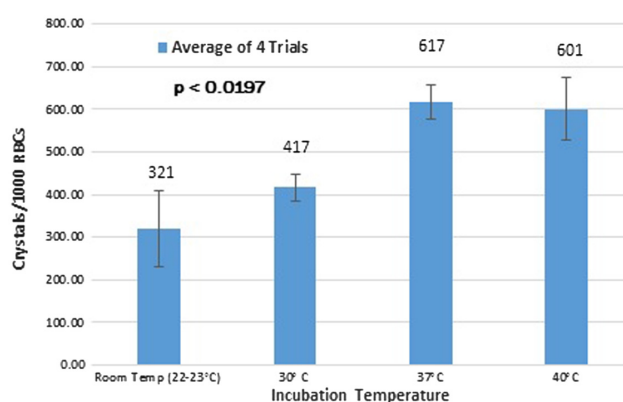


Figure 4. Crystal formation in different incubation temperatures in DPBS. * = statistical significance.

participants with 4 genotypes—AA, 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°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.

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