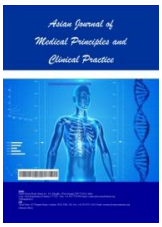

Asian Journal of Medical Principles and Clinical Practice

4(4): 21-28, 2021; Article no.AJMPCP.71416

The Diagnostic Accuracy of Cryohaemolysis in Hereditary Spherocytosis

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Article Information

Editor(s):
(1) Prof. Suprakash Chaudhury, Dr. D. Y. Patil Medical College, India.
Reviewers:
(1) Cees Th. Smit Sibinga, University of Groningen, Netherlands.
(2) Nataly Apollonsky, Drexel University College of Medicine, USA.
(3) Jean-Luc Wautier, Paris Diderot University, France.
Complete Peer review History: https://www.sdiarticle4.com/review-history/71416

Original Research Article

Received 20 May 2021
Accepted 25 July 2021
Published 13 August 2021

ABSTRACT

Introduction: Hereditary spherocytosis (HS), a heterogenous disorder, requires a screening test with high predictive value in the absence of a family history and when diagnosis is equivocal. This study evaluates the validity and reliability of cryohaemolysis test (CHT) as a cost-effective, sensitive, and specific method in the diagnosis.
Methods: An analytical cross-sectional study was done with a sample size of 62 divided between two groups. One group had diagnosed patients and their relatives with HS and the other group had spherocytes due to other causes.
Results: The sensitivity and specificity of the CHT were 100% and 93.5% respectively. The predictive value of a positive and negative test was 93.9% and 100% respectively. The false negative and positive percentages were zero and 6.45, respectively. The CHT is affected neither by the number of reticulocytes nor spherocytes. The percentage of cryohaemolysis varied among the affected individuals of the same family.
Conclusion: Our study showed that CHT is ideal in identifying patients and silent carriers as a quick, simple and economical method. The test is highly sensitive and specific making it the right choice for developing countries.

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Keywords: Hereditary spherocytosis; cryohaemolysis test; Haemolytic anaemia; spherocytes; reticulocyte count.

1. INTRODUCTION

Hereditary spherocytosis is heterogeneous in respect to clinical presentation, membrane protein abnormalities and the protein gene mutations. The diagnosis of HS is often made in childhood and young adult life. However, it may be diagnosed at any time, even in the seventh to ninth decades of life [1]. The primary lesion in hereditary spherocytosis is a defect in the membrane proteins ankyrin, band 3, beta or alpha spectrin, or protein 4.2 leading to reduced stability of the lipid bilayer.

There is no diagnostic challenge in a patient presenting with typical HS as they have a family history of HS, and presents with a haemolytic anaemia, spherocytes, reticulocytosis, and a negative antiglobulin test. Three quarters of HS cases have a dominant mode of inheritance, and the remainder is either recessive or non-dominant.

Diagnosis becomes difficult in the latter group when there is no family history particularly due to its clinical heterogeneity which spans from an asymptomatic carrier state to severe haemolysis. Though the typical spheroidal and hyperdense red cell in the peripheral blood is the laboratory hallmark of the disorder, its presence is an uncertain diagnostic criterion and is non-specific. This is due to the presence of spherocytes in several other conditions such as congenital dyserythropoietic anemia type II (CDA II), autoimmne haemolytic anaemia, distal renal tubular acidosis and other rare conditions such as cryohydrocytosis. Tests done in these conditions such as osmotic fragility test would be positive in any condition giving rise to spherocytes and these are not membrane specific. In the absence of spherocytes in the blood film with no abnormalities in the red cell indices and normal reticulocyte count, a 'carrier' state cannot be completely excluded1 [1]. The co-inheritance of other haematological disorders such as beta thalassemia trait can lead to confusion in the diagnosis. In illnesses, such as obstructive jaundice that alters the lipid composition of the red cell membrane which reduces haemolysis, it is essential to perform confirmatory tests for the diagnosis. In patients with obstructive jaundice, red cells accumulate cholesterol and increase their surface area, thereby acquiring a flattened shape and an increased resistance to osmotic lysis [2].

Several other tests such as osmotic fragility test (OFT), glycerol lysis test and its modified versions such as acidified glycerol lysis test (AGLT) have been used to diagnose HS, however they lack specificity in the diagnosis. Performance of eosin-5´-maleimide (EMA) binding test, fragility test by flow cytometry and SDS – polyacrylamide gel electrophoresis (PAGE) analysis of red cell membrane proteins is restricted to very few specialized laboratories and not practical for routine use in an under resourced laboratory. CHT is suggested a screening test with a high predictive value for HS [1]. Therefore, performance of CHT is considered as a single parameter that identifies all cases of HS including the less severe cases, and its low cost makes it very suitable for an under resourced set up. The aim of this study is to evaluate the diagnostic accuracy of CHT in HS in relation to other conditions associated with spherocytes.

2. MATERIAL AND METHODS

2.1 Design, Sample and Techniques

An analytical cross-sectional descriptive study was done with a sample size of 62 divided between two groups. One group had diagnosed patients and their relatives with HS (n=31) and the other group had spherocytes due autoimmune hemolytic anemia (n=9), induced spherocytes by heat (n=11) and outdated packed cells (n=11).

The study was carried out among the patients in the Colombo south teaching hospital haematology clinic during the period of 2012 to 2015.

Samples from normal healthy subjects were obtained to define the normal control.

Group 1(G -1) – Spherocytes with HS (n=31)

Patients who were already diagnosed as HS. In the absence of a family history (only one), diagnosis was made by a positive clinical history along with blood picture findings, red cell indices and a negative direct antiglobulin test. The participants with hereditary spherocytosis

included 16 males and 15 females. None of the patients had been transfused blood within three months preceding blood sampling.

Group 2(G-2) – Spherocytes with other conditions. (n=31) Samples were taken from 3 types of settings.

G-2.a)Autoimmune haemolytic anaemia (n= 9) - These patients had the typical blood picture of warm autoimmune haemolytic anamiea(AIHA) with a positive direct antiglobulin test and a negative family screening.

G-2.b)Artificial red cells (n= 11). - samples of normal healthy adults whose blood samples were pretreated with heat to produce spherocytes .The red cells were washed and heated to 50°C for 15 minutes to induce spherocyte formation as explained by Streichman, Gesheidt and Tatarsky (3).

G-2.c)Stored red cells (n= 11) – They contained outdated red cells comprising of numerous spherocytes

We also analyzed family members of 9 affected individuals (already in Group 1) with HS. A total of 20 were studied in this group and 11 of them were asymptomatic. They had mild reticulocytosis, negative DAT, few or no spherocytes and they were clinically normal with no anaemia.

The demographic data and the required clinical history were obtained using a questionnaire. Two samples of 2ml of blood in K2EDTA were drawn, one for the CHT and the other sample for routine tests (Red cell indices, and reticulocyte count), from each category. Blood samples were also obtained from voluntary healthy subjects as normal controls in order to decrease intra-assay variation. Universal precautions were followed when drawing blood and the biohazard materials were discarded appropriately.

The test was performed within 24 hr of collection to avoid storage lesions. Complete blood count using a Beckman Coulter analyzer, examination of blood smears, reticulocyte count and direct antiglobulin test were performed on all the selected subjects. Following that, CHT and OFT were performed on all the samples of group 1 and group 2 and the control samples of normal healthy adults. CHT was done with the method which is described below.

2.2 Method of CHT

The test was performed on all the samples and interpreted according to Dacie and Lewis's Practical Haematology4. The centrifuged blood and washed red cells in cold NaCl were kept on ice till tested. A reagent of buffered 0.7mmol/l sucrose was mixed with 50 microliters of cell suspension followed by incubation for 10 mints at 37°C and transferring to an ice bath for another 10 mints. Absorbance of the supernatant was checked after centrifuging and diluting the sample. The result was interpreted from an equation. The normal range was considered as 3%-15% while HS was considered when the percentage of haemolysis was more than 20.

3. RESULTS AND DISCUSSION

3.1 Results

All the samples of group 1 and group 2 had a positive OFT. The control samples from the normal healthy adult population had negative results for OFT.

The mean value for CHT of the control sample from the healthy adults was 9.8%. The normal range reported by Streichman et al is 3-15% [2]. However, it is recommended that individual laboratories establish their own reference values for the method. We have found that most normal samples give <3% (Fig. 1).

The mean value of the CHT in HS was 68.0+/-18.6 and there were no false negative results as all the patients with HS had a positive CHT.

In patients with autoimmune haemolytic anaemia (G-2.a), the mean value of the CHT was 15.2+/-15.9 . However, there were 2 patients who had false positive results for CHT. When they were excluded, the mean of the cryohaemolysis level for the patients with true positives for autoimmune haemolytic anaemia was 9.2+/-3.9 [3].

In artificial red cells (G-2.b), none of the samples tested positive CHT and the mean was 11+/-2.9 [4].

Stored red cells (G-2.c) also gave a negative result with a mean of 10.43+/-5.50.

Both the groups G-2.b and G-2.c did not give any false positive results.

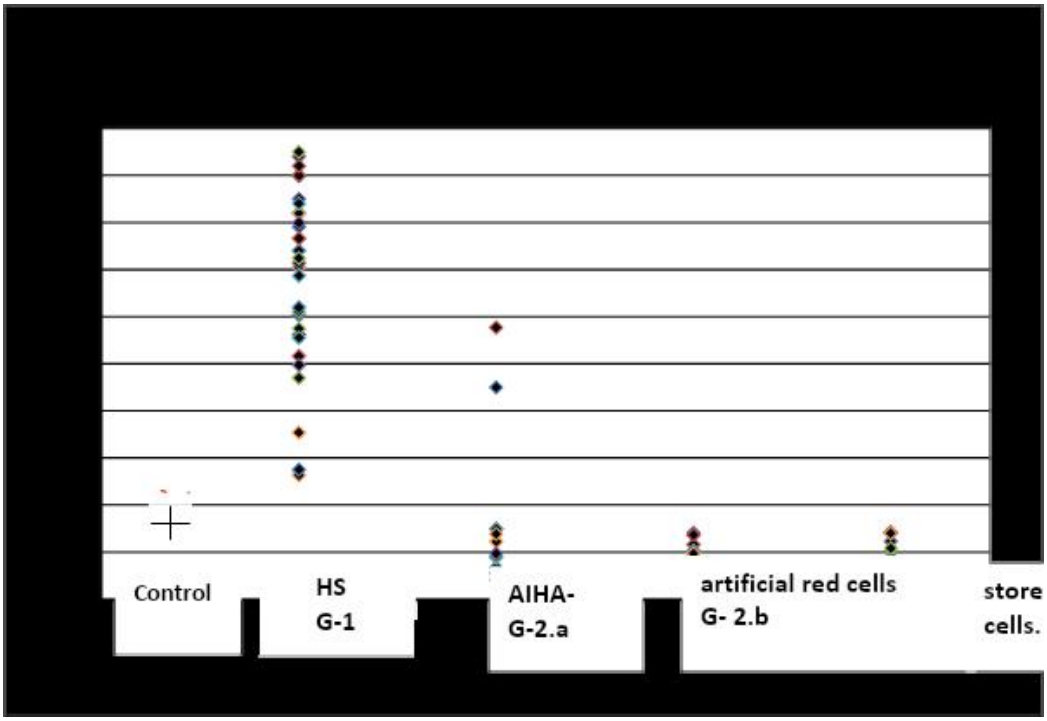

Fig. 1. Comparison of the percentage of cryohaemolysis in hereditary spherocytosis, autoimmune haemolytic anaemia, artificial red cells and stored red cells: (G-2.a, G-2.b. and G-2.c) and the control

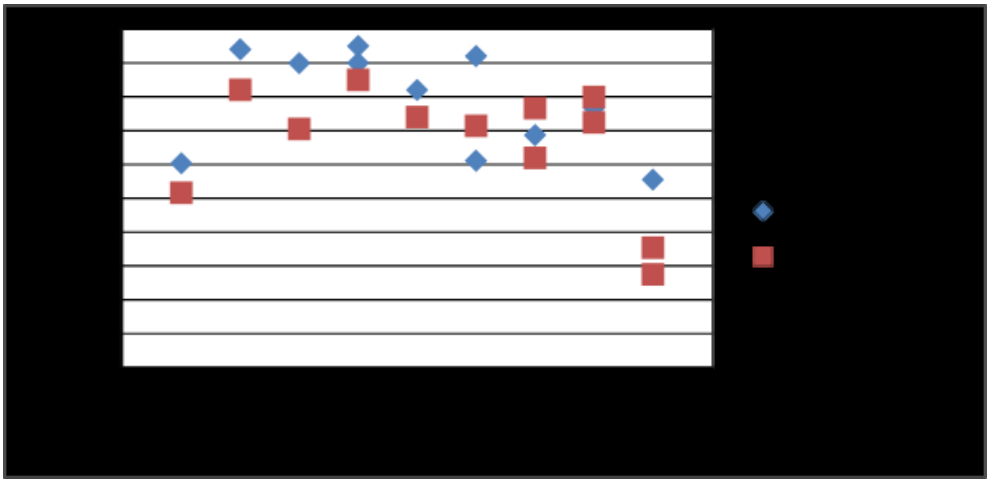

Fig. 2. Comparison of the level of cryohaemolysis between symptomatic and the asymptomatic family members of HS by percentage

Results were assessed in the 9 families with their asymptomatic family members. The percentage of cryohaemolysis varied among the affected family members of HS [Fig. 2]. In some families, the symptomatic as well as the asymptomatic family members had similarly high values of cryohaemolysis. The mean value of the symptomatic patients was 70.8+/-19.2 whereas the mean of the silent carriers (asymptomatic) of family members was 65.0+/-18.3 and there was no marked difference in the value between the two groups.

Table 1. Statistics comparing the symptomatic and asymptomatic individuals of HS

Category	n	Mean value of CHT	Standard Deviation	Standard Error mean
symptomatic	11	78.6%	15	4.5
asymptomatic	12	65.7%	18.4	5.3

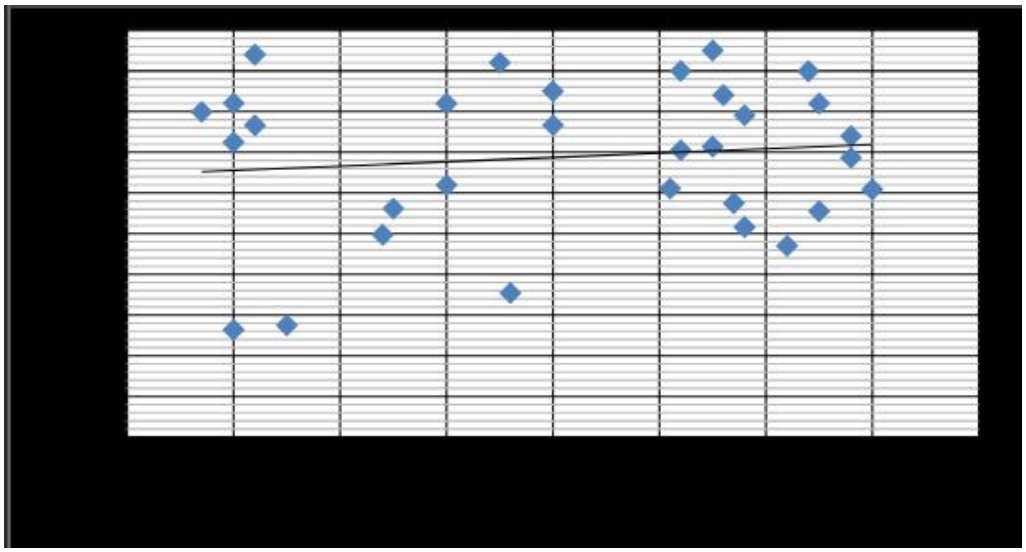

Fig. 3. Correlation between the percentage of spherocytes and the percentage of cryohaemolysis

Table 1 shows the calculated 'p' value is 0.079 which showed no significant statistical difference of the level of the cryohaemolysis between symptomatic and the asymptomatic.

The correlation between the percentage of spherocytes and the percentage of cryohaemolysis was assessed [Fig. 3]. Some patients who had a few numbers of spherocytes had a high percentage of cryohaemolysis. The pearson correlation coefficient (r) is 0.12 (-0.257 to 0.497). Since the confidence interval includes the null value (0) there is no statistically significant correlation between the percentage of spherocytes and the percentage of cryohaemolysis. We detected two families who had spherocytes <15% but had a level of cryohaemolysis of >70%. This could probably be due to the underlying molecular defect which could not be detected due to unavailable resources.

3.1.1 The time consumption and labor intensiveness to perform the CHT

When comparing the time, CHT requires only about 30 minutes whereas about two hours is spent on OFT. OFT is also labor intensive and the procedure is complicated involving 12 different tubes with different concentrations of the reagent along with the controls. CHT requires only one tube with the reagent with another tube with distilled water to prepare the 100% lysis along with a control. Further processing of the sample had to be done within two hours for the osmotic fragility test whereas the CHT could be performed even on the following day (within 24 hours).

3.2 Discussion

In our study, we wanted to evaluate the validity and the reliability of CHT in identifying HS and thereby to assess the sensitivity and the specificity as well as the positive and the negative predictive value of it.

In HS, red cells are sensitive to the temperature changes in CHT. Cold induced haemolysis in hypertonic medium has been described by many authors (Lovelock 1953, 1954,1955 1957; Morris 1975: Meryman 1974: Meryman, Williams & Douglas, 1977; Green and Jung 1977; Jung and Green 1978; Zade-Oppen, 1968) [5].

Phase transition of membrane lipids take place with the change in the temperature from 37°C to 4°C and the normal membrane undergoes some accommodation to this phase transition resulting in decreased fluidity. Alteration in the membrane protein framework due to hypertonicity prevents normal adaptation of the membrane to the change in temperature,

resulting in rupture of the membrane. This was initially described by Green and Jung 5. The hypertonic cryohaemolysis of pathologic red cells was studied by Streichman, Gesheidt and Tatarsky in 1990 and it was found that the red cells in HS are uniquely sensitive to these conditions and this concept was proposed for the detection of Hereditary Spherocytosis3. This was further confirmed by Romero et al in 1997 [6].

The extent of haemolysis is highly dependent on the tonicity of the medium, temperature and the time duration of the incubation period. When comparing the sucrose and sodium chloride on an osmolar basis, it was found that sucrose produced a comparable effect at a lower tonicity [5].

The extent of cryohaemolysis at 0.7M sucrose was found to be a good measure of sensing pathologic cells and is not dependent on the clinical or haematological states of the patient [7]. Streichman, Gesheidt and Tatarsky artificially induced sphericity by pretreatment of red cells with heat (50°C) and chemicals. At 50°C an irreversible change in spectrin takes place and this change *per se* does not cause haemolysis. It was found that these cells were osmotically fragile and reached 50% haemolysis after shorter time intervals than HS samples but none of them had increased levels of cryohaemolysis (3).

This fact emphasizes that reduction in the ratio between surface area and volume *per se* is not sufficient to affect cryohaemolysis. Though it was recommended that the test should be performed in fresh samples or samples less than 1 day old, it was further reviewed and shown that there is no significant alteration up to 4 days at 4°C [8]. However, we could not elaborate on this aspect due to inadequate sample, as blood was drawn during routine testing.

Based on the results, the calculated sensitivity and specificity of the cryohaemolysis test were 100% and 93.54% respectively (Table 2).

The predictive value of a positive test was 93.9% and the predictive value of a negative test was 100%. The percentage of false negative was zero percent, and the percentage of false positive was 6.45%. We detected three patients with no family history of HS and 2 more families who had a few numbers of spherocytes (<15%). However, they also had other features to confirm the diagnosis. These findings demonstrate that in situations where the diagnosis is equivocal with few spherocytes on the blood film with no other laboratory, evidence of clinical or positive family history, the CHT would be important as a screening test. In our population two patients with autoimmune haemolytic anaemia were positive for CHT. Co-existence of HS and AHAI cannot be ruled out with the methodology that was used by our method. In such instances, direct antiglobulin test should be helpful to confirm the diagnosis.

The CHT can also be positive in the presence of aberrant band 3 proteins which are seen in Melanesian elliptocytosis and in CDA type II. Unfortunately, this is a limitation specially in CDA type II where blood picture findings can overlap particularly when there is acute haemolysis with no family history of HS, where other specialized tests will be necessary.

The study also proved that the CHT is neither affected by a high reticulocyte count nor by low number of spherocytes. The levels of cryohaemolysis varied among the affected individuals of the same family which could be due to underlying molecular defect with variable expression. The silent carriers also had similar elevated levels of cryohaemolysis showing no significant statistical difference of the level of cryohaemolysis between the two groups. Absence of any significant statistical difference of the level of cryohaemolysis between the symptomatic and the asymptomatic could also be due to a small sample size. However, this can be further studied by analyzing a large sample by performing a multicentre study. In future, it would be best to recruit other types of conditions which would also have red cell membrane anomalies such as patients with haemolytic anaemias, CDA type II, Southeast Asian ovalocytosis, distal renal tubular acidosis, Thalassemia, and enzymopathies. This allows a more precise determination of the specificity and sensitivity of CHT in detecting HS.

Table 2. Screening test results

	Disease positive	Disease Negative	Total
Test positive	31	02	33
Test negative	00	29	29
Total	31	31	62

These data reveal that the CHT is a superior test in identifying the patients as well as the silent carriers with HS. It has been proven that in the silent carriers the surface area to volume ratio is not adequately reduced to be detected by osmotic fragility test [9].

Correct diagnosis with differentiation from the other haemolytic anaemias is very important in order to make correct therapeutic decisions. Since this test can be performed even with a small volume of blood this would be useful in testing the neonates as well. The severity of anaemia showed no correlation with the test results or neonatal jaundice [10]. Another study by Crisp RL et al. also concluded that CHT and flow cytometry are the easy methods with the highest diagnostic accuracy. CHT showed a specificity of 95.2% in this study [11].

4. CONCLUSION

Compared to the OFT, for developing countries, the low cost of the CHT with a shorter turn over-time, utilizing less manpower and a higher degree of sensitivity and specificity makes it the right choice for screening Hereditary Spherocytosis.

CONSENT

Consent was obtained from all the parties, including the patients and their families for the study.

ETHICAL APPROVAL

Ethical clearance was obtained from the ethical review committee of Colombo South Teaching hospital, Kalubowila, Sri Lanka. Informed and written consent was obtained by all the patients prior to the study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle4.com/review-history/71416