Flow cytometric analysis of patients with hereditary spherocytosis – an Indian scenario

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ABSTRACT

Objectives: Flow cytometry osmotic fragility test (FC-OFT) was a recently introduced screening test for hereditary spherocytosis (HS). This study was conducted to evaluate the utility of FC-OFT in all newly diagnosed cases of HS, to compare its diagnostic value with conventional OFT and to correlate with clinical disease severity.

Methods: In this study, the percentage of residual red cells (%RRC) was measured using flow cytometer after creating a red cell suspension. Subsequently, this was spiked with deionized water for FC-OFT in all cases of HS (n = 40), healthy subjects (n = 40) and beta-thalassemia traits (BTT) (n = 20).

Results: The receiver operator curve analysis defined the optimal cut-offs for FC-OFT-derived indices, such as %RRC value (≤16.29%) and %RRC ratio (>1.72), for HS cases when compared with healthy subjects and BTT (p < 0.05). The FC-OFT (96%) achieved higher test efficiency than the conventional OF test (68.9%). A significant positive and a negative correlation were found between number of spherocytes/hpf and %RRC ratio (p = 0.001) and %RRC values (p = 0.0486). No significant correlation was observed between %RRC value (p = 0.8934), %RRC ratio (p = 0.6348) and HS disease severity score.

Conclusion: Our results suggest that FC-OFT could be the better screening test for HS cases in developing countries if flow cytometer is available.

KEYWORDS
Hereditary spherocytosis; flow cytometry; osmotic fragility test; residual red cells

Introduction

Hereditary spherocytosis (HS) is one of the most common inherited hemolytic anemias. It is due to the deficiency or dysfunction of one or more of red blood cell (RBC) membrane proteins such as α spectrin, β spectrin, ankyrin, an anion channel protein (band-3 protein) and protein 4.2. The hallmarks of the disease are anemia, intermittent jaundice, splenomegaly and responsiveness to splenectomy [1,2]. It is also characterized by phenotypic and genotypic heterogeneity [3]. Various tests such as red cell osmotic fragility test (OFT), acidified glycerol lysis time (AGLT) test, osmotic gradient ektacytometry, hypertonic cryohemolysis test, eosin-5-maleimide (EMA) binding test and flow cytometric osmotic fragility test (FC-OFT) are available for making diagnosis [4–7]. Still in most of the centers, red cell OFT is considered as the gold standard test because it is available easily and simpler to perform [8]. More expensive and cumbersome molecular tests, such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) test, are available to accurately diagnose the protein defects. However, each one has its advantages and disadvantages [6]. More recently, FC-OFT was recently introduced as a simple, quantitative, more sensitive and cost-effective technique for effectively screening the HS patients. It also correlates better with clinical disease severity [7,9]. This prompted us to use this newer screening technique at our center in all newly diagnosed HS patients and to compare its result with those of conventional OFT as well as the clinical disease severity.

Materials and methods

Subjects

This was a prospective observational study conducted from June 2014 to December 2015. Forty healthy subjects were selected as the normal control group (Group 1). Forty newly diagnosed typical cases of HS were included in the study (Group 2). Twenty cases of β thalassemia trait (BTT) were also analyzed (Group 3). Patients who underwent splenectomy for HS, received blood transfusion within last 3 weeks, autoimmune hemolytic anemias (AIHA), membranopathies other than HS and enzymopathies were excluded from the study. This study was approved by Institute Ethics Committee, and is in accordance with the current version of the Helsinki Declaration.

Laboratory investigations

Blood samples were collected in ethylene diamine tetra acetic acid (EDTA) after informed consent and were
analyzed within 2 hours of blood collection. Red cell morphology was evaluated on Jenner–Giemsa-stained peripheral smear. Hematological indices were measured on a Sysmex automated cell counter (Sysmex XT-1000i; Sysmex Corporation, Kobe, Japan). Variant or abnormal hemoglobin (Hb) analysis was done using cation exchange high performance liquid chromatography (HPLC) on the VARIANT™ Hemo-globin Testing System (Bio-Rad Laboratories, Hercules, CA, U.S.A.), wherever required. Reticulocyte staining was done using 1% new methylene blue. All the patients and control groups were also tested by incubated OFT (iOFT).

**Flow cytometric osmotic fragility test**

**Preparation of a red cell suspension**

It involves two steps. In the first step, the specific volume (µl) of EDTA blood was diluted in tube 1 (micro-centrifuge tube) containing 1 ml of normal saline (NS). To keep the fixed number of RBC to be diluted in tube 1 across the entire patient and control groups, the volume of the blood to be diluted depends on the RBC count obtained from the Sysmex counter XT-1800i; we used the formula according to Won and Suh [7] and Shim and Won [9]

\[
\text{Blood volume (µl)} = \frac{130}{(\text{number of red cells/µl})/10^6}
\]

In the first step, this calculated volume of blood was diluted just prior to testing, mixed and processed to the next step as soon as possible. In the second step, 10 µl from tube 1 was transferred to tube 2 (FACS tube) containing 1.1 ml of NS. This second diluted blood will be the final red cell suspension for flow cytometric acquisition and analysis. Similarly, two tubes were prepared from healthy controls to run simultaneously. We also analyzed FC-OFT from the known BTT patients.

**Acquisition**

In the BD FACS Canto II flow cytometer using the Diva software, template was created with time on the x-axis against forward scatter (FSC-H) on the y-axis and eight regions with equal time split (R1–R8) each spanning for 11 seconds. The thoroughly mixed red cell suspension from tube 2 was installed at the acquisition port. After the first region elapsed during acquisition (R1), the tube was removed using the stop acquiring option and 0.9 ml distilled water (DW) was added and acquisition continued up to the eighth region (R8) using the append option to have all the events in the same plot. It took approximately two minutes to run each sample (Figure 1).

**Analysis**

The event count per region (R1–R8) was used for calculating the percentage of residual cells. The percentage of residual red cells (%RRC value) was calculated from

![Figure 1. Red color – events before DW spiking; green color – events after DW spiking. % residual red cells (%RRC value) are 32.14% (healthy control) (A), 8.32% (HS patient) (B) and 67.27% (BTT patient) (C).](image-url)
region R7 and R8 events (Figure 1). Increased osmotic fragility is indicated by low %RRC value.

The degree of osmotic hemolysis was expressed as ‘%RRC value’ and was calculated based on the following formula:

\[
\text{% residual red cells (%RRC) value} = \frac{\text{Mean event count of last two regions}}{\text{Event count of first region} \times 1.1/2.0 \times 100 (\%)}
\]

1.1/2.0 is the multiplying correction factor, because the first region is also in a diluted state for comparison with the remaining regions which are diluted by spiking with 0.9 ml DW.

The other index, which was calculated based on FC-OFT findings, includes %RRC ratio as follows:

\[
\text{% RRC ratio} = \frac{(\% \text{RRC of a normal control})}{(\% \text{RRC of a patient})}
\]

Thus, a low %RRC value and a high %RRC ratio indicated increased FC-OFT.

**Conventional iOFT**

All these three groups were tested for iOFT. Fifty microliter of heparinized peripheral blood incubated at 37°C for 24 hours were added to each of the 13 test tubes containing 5 ml of sodium chloride (NaCl) at varying concentrations such as 0.9, 0.85, 0.8, 0.75, 0.7, 0.65, 0.6, 0.55, 0.5, 0.45, 0.4, 0.35 and 0.3% and also to the 14th tube containing 5 ml of DW. This test was performed at room temperature and these tubes were gently mixed and the suspensions were left at room temperature for half an hour. These tubes were centrifuged at 1500 rpm for 10 minutes and the degree of hemolysis was noted as optical density (OD) values using a yellow-green filter at 540 nm in photoelectric calorimeter from the supernatant portion transferred to the cuvette without disturbing the pellet below. OD value of the 14th tube is taken as 100% hemolysis, OD values of the other tubes were noted and percentage of hemolysis was calculated. Median corpuscular fragility was noted from the graph with percentage of NaCl in the x-axis and percentage of hemolysis in the y-axis.

In this study, a diagnosis of HS was made based on (i) clinical manifestations and (ii) the exclusion of other causes of hemolytic anemia or secondary spherocytosis was based on negative direct Coomb’s test and autoimmune disease work-up, normal G6PD deficiency screening by methylene blue reduction test, normal Hb HPLC and bone marrow morphology, if clinically indicated. Even if the results of an iOFT were negative, other laboratory findings, such as increased MCHC, spherocytosis in the peripheral smear, and reticulocytosis together with a family history were used to confirm a diagnosis of HS [4,5]. The clinical disease severity of HS patients was done using the scoring system according to Shim and Won [9]. Keeping the clinical diagnosis of HS as gold standard according to Tao et al. [10], FC-OFT and iOFT were evaluated.

**Statistical analysis**

The data were presented as mean ± standard deviation. Student ‘t’ test was used to compare the data of the different groups. Receiver operator curves (ROC) were used to define cut-off of FC-OFT test. Pearson’s correlation analysis (for parametric and normally distributed data) or Spearman’s correlation analysis (for nonparametric or non-normally distributed data) was used to define the correlation between FC-OFT indices and clinical severity of HS. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated for both conventional iOFT and FC-OFT. Test efficiencies of both the test were calculated and defined as the fraction of cases correctly classified, that is (true positives + true negatives)/total cases. Absolute correlation coefficient values (r) of >0.3 and ‘p’ values of <0.05 were taken to indicate statistical significance. Statistical analyses were performed using the MEDCALC software (http://www.medcalc.be).

**Results**

**Cut-off definitions and the test efficiency of the FC-OFT indices**

Using the non-HS group (healthy subjects and BTT patients) as disease controls, optimal cut-off values for %RRC ratio and %RRC value obtained from the ROC curve (Figure 2) are presented in Tables 1 and 2 (>1.72 for %RRC ratio and ≤16.29% for %RRC value). The discriminatory power of these values among the three groups is shown in Figure 3.

**Comparison of the test efficiencies of the FC-OFT and conventional iOFT**

The FC-OFT was considered positive when %RRC value of ≤16.29% and %RRC ratio of >1.72 according to the cut-off definitions and the conventional iOFT of patient was positive when the OD value of patient was increased as compared to the healthy control. The evaluation of FC-OFT based on %RRC value ≤16.29% and %RRC ratio of >1.72 showed sensitivity, specificity, PPV and NPV of 92.5, 98.3, 97.3 and 95.1%, respectively. The test efficiency of FC-OFT was 96% with significant p < 0.00001 (Table 3). Similarly, the diagnosis of HS based on increased iOFT had sensitivity, specificity, PPV and NPV of 82.5, 93.3, 89.1 and 88.8%, respectively. The test efficiency of iOFT was 89% with significant p < 0.00001 (Table 4).
Correlation between HS severity scores and FC-OFT indices

A significant negative correlation was found between number of spherocytes per high power field and %RRC values ($p = 0.0486$). However, no significant correlation was found in Hb, corrected reticulocyte count, total bilirubin, family history and transfusion history ($p = 0.4408$, $p = 0.1530$, $p = 0.4464$, $p = 0.7989$ and $p = 0.6675$, respectively). Similarly, a significant positive correlation was found between spherocytosis grade in peripheral blood smear and %RRC ratio ($p = 0.001$). In the case of hemoglobin, corrected reticulocyte count, total bilirubin, family history and transfusion history, no significant correlation was found ($p = 0.4408$, $p = 0.1530$, $p = 0.4464$, $p = 0.7989$ and $p = 0.6675$, respectively). No significant correlation was observed between %RRC value ($p = 0.8934$), %RRC ratio ($p = 0.6348$) and HS severity score.

**Table 1.** FC-OFT for the healthy controls, HS and BTT groups based on %RRC value.

| Groups     | % RRC value ROC cut-off ($>16.29\%$) ($n$) | % RRC value ROC cut-off ($\leq 16.29\%$) ($n$) | Mean ± SD %RRC value |
|------------|---------------------------------------------|-----------------------------------------------|----------------------|
| Healthy controls | 39                                          | 1                                            | 34.55 ± 14.98        |
| HS          | 3                                           | 37                                           | 8.82 ± 5.29          |
| BTT         | 20                                          | 0                                            | 66.73 ± 13.39        |

Average %RRC value had significant ‘$p$’ value <0.05 among the three groups.

**Table 2.** FC-OFT for the healthy controls, HS and BTT groups based on %RRC ratio.

| Groups     | % RRC ratio ROC cut-off ($<1.72$) ($n$) | % RRC ratio ROC cut-off ($>1.72$) ($n$) | Mean ± SD %RRC ratio |
|------------|----------------------------------------|----------------------------------------|----------------------|
| Healthy controls | 36                                          | 4                                      | 1.20 ± 0.51          |
| HS          | 1                                       | 39                                     | 7.64 ± 10.53         |
| BTT         | 19                                      | 1                                      | 0.54 ± 0.10          |

Average %RRC ratio had significant ‘$p$’ value <0.05 among the three groups.

**Correlation between HS severity scores and FC-OFT indices**

**Figure 2.** ROC analysis for %RRC value and %RRC ratio.

**Figure 3.** Discriminatory power of the FC-OFT based on %RRC value and %RRC ratio among the three groups.
Table 3. The diagnosis of HS by FC-OFT based on %RRC value and %RRC ratio.

| Diagnosis by FC-OFT* | Gold standard |
|----------------------|---------------|
|                      | HS group | Non-HS group | Total |
| Positive (%RRC value ≤16.29% and %RRC ratio >1.72) | 37 | 1 | 38 |
| Negative (%RRC value >16.29% and %RRC ratio <1.72) | 3 | 59 | 62 |
| Total | 40 | 60 | 100 |

*Sensitivity, specificity, PPV, NPV and test efficiency were calculated from the above values.

Table 4. The diagnosis of HS by conventional iOFT.

| Diagnosis by iOFT* | Gold standard |
|-------------------|---------------|
|                   | HS group | Non-HS group | Total |
| Positive | 33 | 4 | 37 |
| Negative | 7 | 56 | 63 |
| Total | 40 | 60 | 100 |

*Sensitivity, specificity, PPV, NPV and test efficiency were calculated from the above values.

Discussion

Hereditary spherocytosis (HS) is one of the most common inherited red cell membranopathies characterized by anemia, intermittent jaundice, splenomegaly and response to splenectomy [1–3]. It is very well known for its phenotypic and genotypic diversity [3]. Bolton-Maggs et al. in HS guidelines (2004) [4], later updated in 2011 [5] recommended that diagnosis of HS should be made based on the combination of typical clinical features, laboratory investigations and family history. The laboratory investigations include finding of spherocytes in the peripheral blood with increased reticulocyte count, increased MCHC, raised OFT and ruling out other causes of spherocytes such as AIHA by negative Coombs’ test. The positive family history was usually obtained in 75% of the cases. In such a typical HS case, even if the OFT is negative, no further testing is required [4,5]. We kept the clinical diagnosis of HS as the gold standard according to Tao et al. [10] and compared the test efficiency of FC-OFT and conventional iOFT in screening the typical HS patients.

The conventional iOFT is used as a first-line screening test in most of the laboratories in India because of its easy availability. However, it is not without limitations such as labor intensive, need for 24 hour incubation, known to give false positive and false negative results [4–6,11]. In this study, conventional iOFT was positive in 33 cases yielding the sensitivity, specificity, PPV and NPV of 82.5, 93.3, 89.1 and 88.8%, respectively. Therefore, screening test with much higher sensitivity such as FC-OFT may be required especially if there is strong clinical suspicion but with equivocal laboratory findings [7,9,12–14].

The FC-OFT was added to the diagnostic test armamentarium recently and each studies recommended different cut-off values which were actually based on the FACS caliber flow cytometer [7,9,12–14]. However, we used BD FACS Canto II (Becton Dickinson, San Jose, CA, U.S.A.) with the Diva Software for analysis. We need to run the sample till R1 tube needs to be removed using stop acquiring option and 0.9 ml of DW was added and then to continue the test run till R8 using the append option to show all the events in the same plot. This is the technical modification used in this study which yielded comparative results to the other studies. Another technical issue is that the delay between after the addition of DW into final red cell suspension and injection into the acquisition port of flow cytometer causes further reduction in %RRC values [9].

Shim et al. [9] recommend that %RRC ratio to be calculated in order to reduce the false-positive results. We used both the parameters (%RRC value and %RRC ratio) and established the cut-off in Indian patients based on the ROC analysis. The sensitivity, specificity and test efficiency of FC-OFT at cut-off of ≤16.29% (%RRC value) were 97.5%, 93.3% and 96%, respectively. Similarly, at cut-off of >1.72 (%RRC ratio), these value were 92.5%, 98.3% and 94%, respectively.

The FC-OFT had a significant discriminatory power for both %RRC value and %RRC ratio when compared among healthy subjects, HS and BTT groups (p < 0.05) similar to other studies [9,13,14]. This newly established cut-off helped us in picking up four typical HS cases which were negative by conventional iOFT. Only one non-HS patient had positivity with this cut-off and found to have increased MCV of 112 FL. Hence the sensitivity, specificity, PPV and NPV of FC-OFT (%RRC value cut-off of ≤16.29%) for making the diagnosis of HS were 92.5%, 98.3%, 97.3% and 95.1%, respectively.

We also analyzed the reasons for the false-negative FC-OFT results in three typical HS cases. In two cases, there were a significant number of microcytic hypochromic red cells, later found to have Hb E trait on Hb HPLC with normal ferritin levels. The third case had normal ferritin and Hb HPLC which was actually attributed to random error.

We adopted the clinical disease severity scoring system from Shim et al study [9] which was actually a conglomerate of Bolton-Maggs et al. [4,5] and Perrotta et al. [1] scoring system. Shim Won [9] claimed that FC-OFT does reflect the clinical severity of HS. We also analyzed similarly such individual parameters in this study and found that %RRC value and %RRC ratio had negative (p = 0.0486) and positive correlation (p = 0.001) with number of spherocytes/hpf in the peripheral smear. However unlike these studies, other individual parameters as well as overall HS severity scoring did not show significant correlation with either of these FC-OFT indices. This could be explained by the fact...
that there were no cases of trait/mild cases and moderate cases in our study.

The recommended screening tests are the cryohemolysis test and EMA binding test in atypical cases because of its high predictive value [4, 5, 8]. The limitation of our study is that these tests were not performed. Although EMA binding test is having higher sensitivity and specificity in diagnosing HS, the disadvantages with this test include lack of universal reference ranges for normal and HS cases, higher cost of this reagent particularly in developing countries, difficulty in preserving this reagent in aliquots at −20°C and light sensitivity were well documented [4–6, 15]. Moreover, atypical cases and HS cases that underwent splenectomy were excluded from the study. According to the clinical disease severity, no cases of mild-to-moderate degree were obtained during the study period. This could be explained by the fact that mild-to-moderate cases might not seek early medical attention in our country. Another possible explanation as reported in recent guidelines, the sensitivity of conventional IOFT drop down particularly in cases with milder phenotype and smaller number of spherocytes. Moreover, not always the MCHC is increased in all cases of spherocytosis particularly if not corrected for reticulocytosis [15]. Nevertheless, this study is important for establishing FC-OFT cutoff for Indian population as well as comparing the two different types of OFT. Much larger studies are required to establish its role in assessing disease severity by including mild HS, atypical HS and splenectomized patients as well as in cases that could present problems in differential diagnosis of HS (i.e. RBC enzymopathies, congenital dyserythropoietic anemias and AIHA)

To conclude, the FC-OFT showed higher efficiency than conventional IOFT. Both %RRC value and %RRC ratio had greater test efficiency (96 and 94%, respectively) in diagnosing HS cases. Moreover, %RRC value and %RRC ratio had negative and positive correlation with number of spherocytes in the peripheral blood. We recommend that FC-OFT being rapid, sensitive, more specific and cost-effective test modality for HS and should replace the conventional IOFT.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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