Research Article

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Comparison of classical and flowcytometric osmotic fragility and flowcytometric eosin-5-maleimide binding tests in diagnosis of hereditary spherocytosis

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Abstract

Objectives: Hereditary spherocytosis (HS) is the most common congenital hemolytic anemia in Northern Europe and North America. Damaged or deficient proteins in the erythrocyte membrane cause the condition, leading to a decrease in the surface area and volume of erythrocytes. Specialists traditionally use the osmotic fragility test (C-OF) for diagnosis. Researchers have developed new methods for flow cytometry-based tests that are the eosin-5-maleimide binding (EMA) and osmotic fragility (FC-OF) tests. In this study, we aimed to determine and compare the power of discrimination of FC-OF, EMA, and C-OF tests. Another purpose is to investigate the effect of incubation.

Methods: We performed both real-time and incubated C-OF, EMA, and FC-OF on 20 patients diagnosed with HS and 30 healthy controls. We diagnosed HS based on family history, spherocytes, and clinical and lab findings.

Results: We found that the success of all tests in the classification was statistically significant ($p<0.001$). The discriminatory power of C-OF was not different from that of EMA and FC-OF. FC-OF discriminated better than EMA. Incubation increased C-OF performance and decreased EMA and FC-OF performance.

Conclusions: We think FC-OF should be preferred. Because it has higher discrimination power, specificity, and sensitivity. It gives faster results, costs less, and needs less labor. The lack of flow cytometer devices in every center is the biggest handicap of FC-OF and EMA. But we think that the FC-OF method can be adapted to hemogram devices available in every center in the future.

Keywords: hereditary spherocytosis; flow cytometry; flow cytometric osmotic fragility test; eosin-5-maleimide binding test; eosin-5-maleimide; osmotic fragility

Introduction

Hereditary spherocytosis (HS) is the most common congenital hemolytic anemia in Northern Europe and North America. It occurs in about 1 in 2,000 people. Damaged or deficient proteins in the erythrocyte membrane cause the condition, leading to a decrease in the surface area and volume of erythrocytes. This anomaly also leads to an increase in spherocytes trapped in the spleen. This condition is caused by several gene mutations. In summary ankyrin 1 (ANK1) mutations (50 %), spectrin beta erythrocytic (SPTB) mutations (20 %), band 3 (solute carrier family four member 1 (SLC4A1)) mutations (15 %), erythrocyte membrane protein band 4.2 (EPB42) mutations (10 %), spectrin alpha erythrocytic 1 (SPTA1) mutations (5 %). The net result is loss of membrane and membrane proteins, notably band 3 (SLC4A1) [1–6].

HS can be mild or severe. It can be asymptomatic or cause life-threatening anemia. The most common clinical findings are anemia, jaundice, and splenic enlargement. The presence of spherical erythrocytes in the peripheral smear is a key diagnostic indicator. In mild cases of HS, it may be hard to detect spherocytes in the smear. In 20–25 % of patients, the

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smear may appear normal. In immune hemolytic anemia, too, we observe spherocytes. So it requires more tests for differential diagnosis [7, 8].

The Turkish Hematology Society recommends these lab tests to diagnose HS: complete blood count (CBC), peripheral smear, reticulocyte count, direct antiglobulin, lactate dehydrogenase (LDH), bilirubin, and osmotic fragility tests. In the CBC, mean cell volume (MCV) and hemoglobin (Hb) are normal or low. Mean red cell hemoglobin concentration (MCHC) and red cell distribution width (RDW) are usually high. The direct antiglobulin test is negative. Reticulocyte counts rise as the bone marrow responds to the hemolysis. There is an increase in the levels of indirect bilirubin and LDH in the serum of the patient. The weakening of the erythrocyte membrane reduces its resistance to hypotonic saline (increased OF) [8].

The classical OF test (C-OF) measures erythrocyte resistance to varying concentrations of hypotonic salt solutions. C-OF is in common use, but it can't separate HS from other hemolytic anemias that cause spherocyte formation. It may give normal results in HS patients with iron deficiency, obstructive jaundice, and high reticulocyte levels. But researchers reported that samples incubated 24 h at 37 °C yield better results [8–10].

Flow cytometry (FC) based eosin-5-maleimide binding test (EMA) uses a fluorescent dye, eosin-5-maleimide. It binds to transmembrane proteins on the erythrocyte membrane. These are band 3, CD47, and Rh-related glycoproteins. The main transmembrane protein that eosin-5-maleimide interacts with is band 3. If the band three protein is missing or defective, there will be a decrease in maleimidine binding compared to normal erythrocytes. The FC detects this decrease by measuring the fluorescence intensity which is in correlation with the amount of EMA binded to the red blood cells (RBCs) [11].

Flow cytometric OF test (FC-OF) tests erythrocytes' osmotic fragility. In this method, the cells are hemolyzed with deionized water. Then, it counts the robust erythrocytes, called "residual erythrocytes," (RRBC) in the environment using FC. The count reflects the strength and degree of osmotic fragility of erythrocytes [12].

This study aimed to determine the power of C-OF, FC-OF, and EMA in detecting HS. It also aimed to find their cut-off values, sensitivity, and specificity. Finally, we evaluated the effects of 24 h incubation to the test performances.

Materials and methods

This study included 20 patients admitted to a local university hospital in 2017–2018, diagnosed with HS. It also included 30 healthy individuals without HS.

Hematologists diagnosed the HS patients based on a family history, the presence of spherocytes, clinical findings, and various lab tests (CBC, peripheral smear, reticulocyte count, direct antiglobulin test, LDH, bilirubin, and OF). The differential diagnosis was made with the presence of spherocytes, increased C-OF or incubated C-OF, and a positive family history. Increased C-OF or incubated C-OF, family history, and spherocytes were present in all patients. Hematologists detected spherocytes by evaluating peripheral blood smears stained with Giemsa.

The medical history of the individuals included in the control group was reviewed. CBC and biochemistry values were examined. Individuals who did not have any chronic disease, and did not have iron deficiency and whose vitamin B12 and Hb values were within normal values were included in the control group. This study excluded pregnant and breastfeeding women, children under 1 year, the elderly over 68 years, and patients transfused in the last 90 days. Written informed consent was collected from all participants. We used peripheral blood samples with EDTA of participants. We ran samples without waiting. Hb concentration, MCHC, MCV, and RDW assays were analyzed by CBC analyzer (XN-1000, Sysmex, Japan) with the electrical impedance method. This research was approved by the local Ethics Committee (13/04/2017-104).

Classic osmotic fragility test (C-OF)

We performed the test based on the method described by Parpart et al. [13]. This method measures hemolysis that occurs after the addition of blood to 16 different concentrations of hypotonic sodium chloride (NaCl) solution (0.9–0.0 %). The resulting supernatant was scanned at a wavelength of 540 nm by a spectrophotometer (UVMini1240, Shimadzu, Japan). Tube 1 with 0.9 % NaCl was used blindly. The rate of hemolysis in tube 16 (including only distilled water) was considered 100 %, and the rate of hemolysis in tube 1 (0.9 % NaCl) was considered 0 %. A graph (NaCl concentration (%)-hemolysis (%)) was created (Figure 1). The salt concentration at which hemolysis started was determined with the help of the graph. The salt concentration at which 5 % hemolysis occurred was accepted as the onset of hemolysis.

Flowcytometric eosin-5-maleimide binding test (EMA)

The test was performed based on the method described by King et al. [14]. We received eosin-5-maleimide dye (Sigma-Aldrich, St. Louis, USA) as a lyophilized powder and stored it

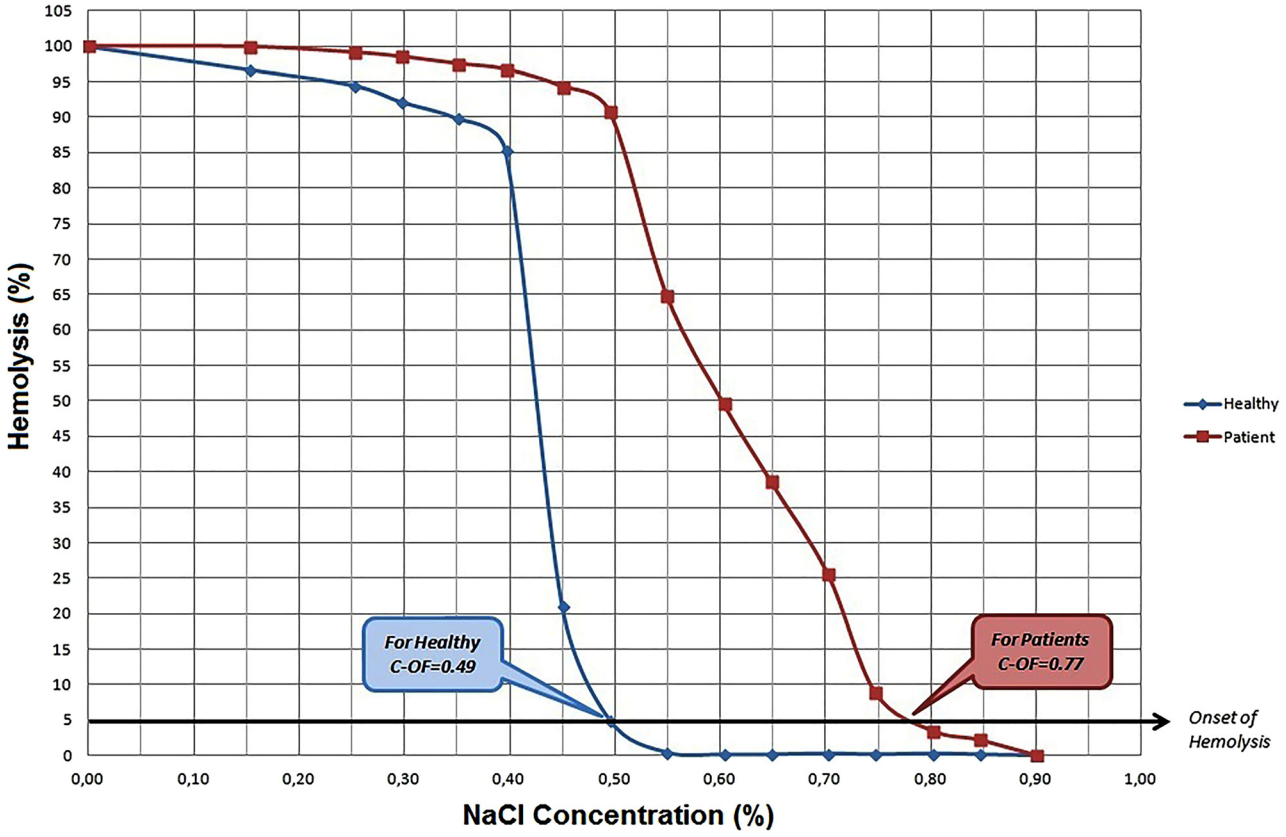

Figure 1: Results representing NaCl concentration (%) vs. hemolysis (%) in a healthy and a patient individual (C-OF test).

at 2–8 °C. The dye was diluted with phosphate buffered saline (PBS) to obtain a 0.5 g/L concentration and than aliquoted the stocks into 0.1 mL portions and stored them at −80 °C to use one in each set of run.

The blood sample was washed twice with PBS. Then 5 µL washed blood sample and 25 µL working dye were added into microcentrifuge tube. The mixture incubated in the dark at room temperature for 1 h, then centrifuged at 12,000 g for 1 min. The precipitate was washed twice with 500 µL of a 0.5 % bovine serum albumin (BSA)/PBS mixture. Then 500 µL of the 0.5 % BSA/PBS mixture was added to the precipitate. 100 µL of this RBC suspension was taken into a tube and then 1.4 mL of a 0.5 % BSA/PBS mixture was added. The final RBC suspension obtained was subjected to the FC (FACS Calibur, Becton Dickinson, San Jose, USA). 15,000 RBCs were counted. RBCs were gated by setting the forward scatter (FSC) and side scatter (SSC) graph to logarithmic scale (Figure 2A). Fluorescent channel 1 (494–520 nm) was used to show eosin-5-maleimide binding. The mean channel fluorescence (MCF) value indicates eosin-5-maleimide binding (Figure 2B).

MCF ratio was calculated with the following formula; MCF ratio=mean MCF of patients/mean MCF of normal controls.

Flowcytometric osmotic fragility test (FC-OF)

Test was performed based on the method described by Won and Suh [12]. Whole blood samples were examined within 3 h. RBC suspensions with identical erythrocyte counts were prepared for each patient. The formula suggested by Won and Suh [12] was used to determine the blood volume. Then the calculated blood volume was diluted with 1 mL of PBS. A 10 µL of the sample was taken from this suspension and added to a 5 mL polyethylene tube containing 1.1 mL of PBS. The final RBC suspension obtained was immediately analyzed by FC (FACSCalibur, Becton Dickinson, San Jose, USA). The FSC/time (204.80 s) graph was created. We created 11 gate in this graph, each lasting approximately 15 s (Figure 3). After the first region was passed, the tube was removed without ending the analysis, and 0.9 mL of distilled water was added to the tube to induce osmotic hemolysis. The tube was reinserted into the injection section, and the measurement was continued up to the eleventh region. The number of events for each region before and after mixing distilled water in the FSC/time plot (Figure 3) was selected as a parameter that reflects the number of erythrocytes. The percentage of RRBC was calculated following formula

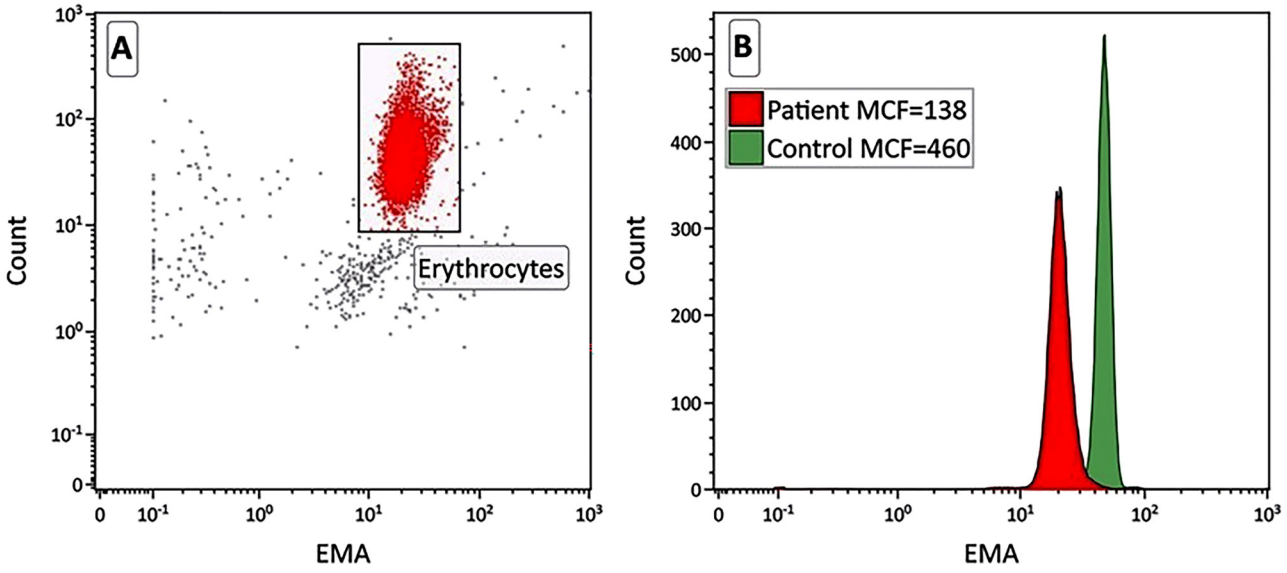

Figure 2: Eosin-5-maleimide test. A) Basic side scatter (SSC)/forward scatter (FSC) histogram of erythrocytes. B) Results representing eosin-5-maleimide labeled erythrocytes in a healthy and a patient individual (EMA test).

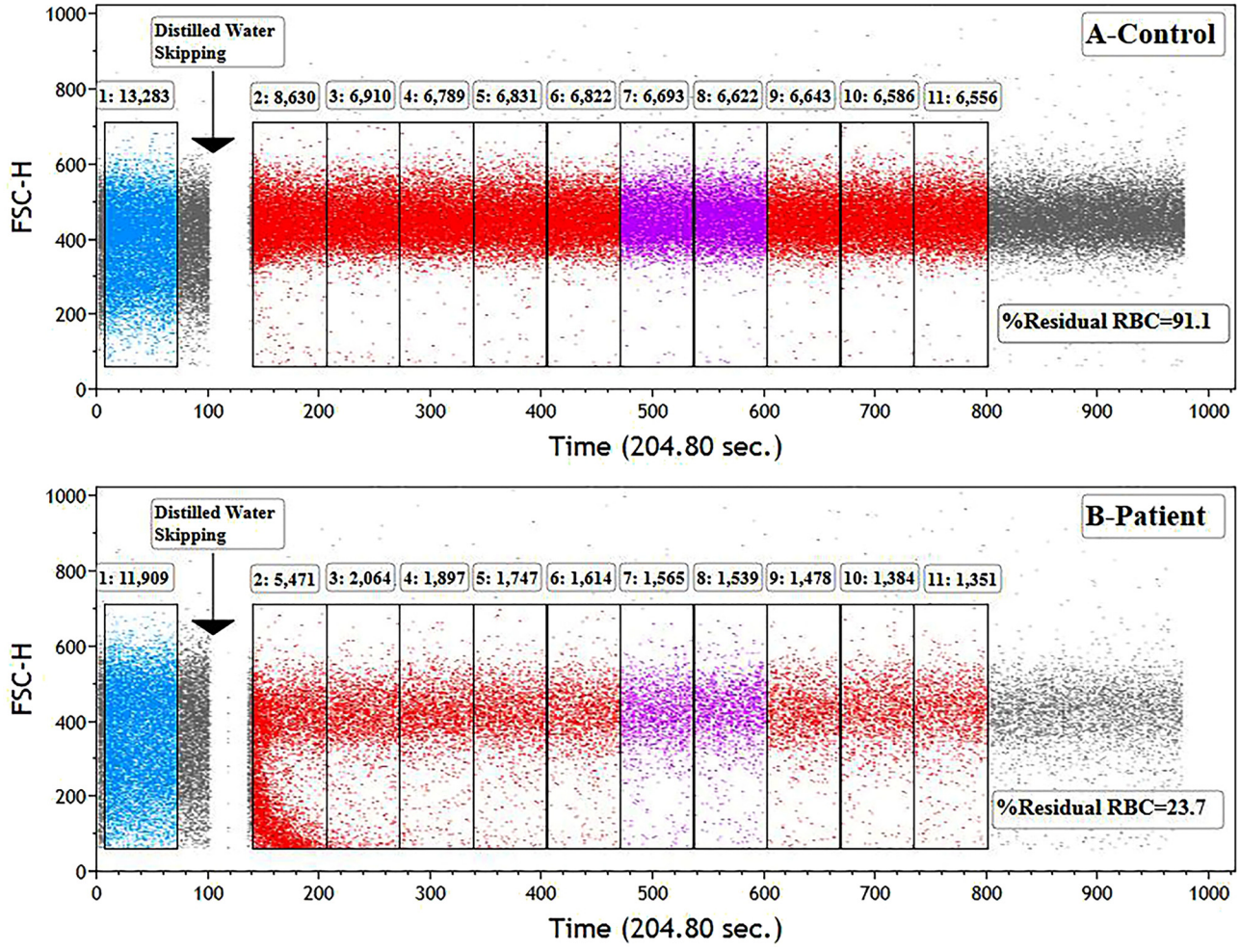

Figure 3: Residue erythrocyte numbers before and after the addition of distilled water erythrocytes in a healthy and a patient individual (FC-OF test). When calculating the % residual RBC, for erythrocyte count before hemolysis we used RBC count in gate 1 (blue), for erythrocyte count after hemolysis we used the average of the erythrocyte counts in gates 7 and 8 (purple). Additionally, we multiplyed the RBC count by the dilution factor in the first gate to eliminate the dilution effect.

suggested by Won and Suh [12] to determine the degree of osmotic hemolysis.

$$\text{RRBC \%} = \frac{\text{Mean event counts of 7st and 8st regions}}{\text{Event count of first region}*\left(\frac{1.1}{2}\right)}*100\,(\%) \quad (1)$$

The percentage of RRBC can be defined as the ratio of the RBC count remaining after inducing hemolysis of the sample with distilled water to the RBC count before hemolysis. Won and Suh recommend taking the average of the RBC count in gates 7 and 8 for the RBC count after hemolysis and taking the RBC count in gates 1 for the RBC count before hemolysis. Additionally, they recommend the number of events in the first region multiply by the dilution factor to eliminate the effect of dilution.

Testing of the samples after a 24 h incubation

After keeping whole blood samples in a water bath at 37 °C for 24 h, we processed the samples according to the test methods described above.

Statistical methods

Continuous variables were expressed as mean, standard deviation (SD), and median with range. Extreme values were checked with the box-plot graphic method and were removed from the data set. Normality control was performed using the Shapiro–Wilk test. An independent sample *t*-test was used to compare continuous variables. Receiver operating curve (ROC) analyses were used to determine an optimum cut-off value of parameters and the separation power on the discrimination of HS and control. A pairwise comparison of the parameters was made by finding the difference in the area under the ROC curve (AUC) of the parameters. Descriptive statistics were performed using a demo version of SPSS 17 for Windows (SPSS, Chicago, IL). For ROC and AUC calculations, the data were analyzed using the demo version of the MedCalc package program. The degree of significance between groups (p-value) <0.05 is considered statistically significant.

Results

Twenty patients with HS and 30 healthy individuals who did not have any chronic disease, and did not have iron deficiency and whose B12 and Hb values were within normal values were included in our study. The mean age of HS patients and control group were 22.1 ± 13.9 and 28.2 ± 9.9, respectively. No statistically significant difference was found in mean age between two groups (p=0.077). There were nine male (45 %) and 11 female (55 %) in patients group and 18 male (60 %) and 12 female (40 %) in control group. There was no statistically significant difference between the two groups in terms of gender (p=0.732). Hb concentration, MCHC, MCV, and RDW parameters of the patient and control group were compared. There was a statistically significant difference in terms of Hb concentration, MCHC, and RDW levels (p=0.009, p=0.001, p<0.002, respectively). There was no significant difference between the two groups' MCV values (p=0.894) (Table 1).

According to the results of the control group, the cut-off value of parameters was dedected by ROC analyse. For C-OF, the cut-off value was detected as ≤0.5 % NaCl concentration. Values <0.5 % NaCl concentration was accepted as normal. Values >0.5 % NaCl concentration was accepted as abnormal. The sensitivity and specificity were 96.6 and 80 %, respectively and AUC was 0.935, p<0.001. For incubated C-OF (inc.C-OF), the cut-off value was ≤0.7 %. Values <0.7 % NaCl concentration was accepted as normal. Values >0.7 % NaCl concentration was accepted as abnormal. Both sensitivity and specificity were 100 %, and the AUC was 1.0, p<0.001. For EMA, the cut-off value was dedected as >222.6. MCF values <222.6 was accepted as abnormal. MCF values >222.6 was accepted as normal. The sensitivity and specificity were 90 and 85 %, respectively and the AUC was 0.897, p<0.001. For incubated EMA (inc.EMA), the cut-off value was dedected as >193.4. MCF values <193.4 was accepted as abnormal. MCF values>193.4 was accepted as normal. The sensitivity and specificity were 93.3 and 75 %, respectively and the AUC was 0.853, p <0.001. For FC-OF, the cut-off value was dedected >67.2 %. The percentage of RRBC >67.2 % was accepted as normal. The percentage of RRBC <67.2 % was accepted as abnormal. Both sensitivity and specificity were 100 %, and the AUC was 1.0, p<0.001. For incubated FC-OF (inc.FC-OF), the cut-off value was dedected >7.95 %. The percentage of RRBC >7.95 % was accepted as normal. The percentage of RRBC <7.95 % was accepted as abnormal. The sensitivity and specificity were 80 and 95 %, respectively and the AUC was 0.930, p<0.001 (Table 2A and Figure 4).

To show the superiority of the parameters, we compared the parameters in pairs. We calculated the differences in the AUC using SPSS. The AUC differences show the advantages of the parameters over each other. C-OF-inc.C-OF AUC difference=0.065 (p=0.064), C-OF-EMA AUC difference=0.038 (p=0.55), C-OF-inc.EMA AUC difference=0.081 (p=0.29), C-OF-FC-OF AUC difference=0.065

Table 1: Blood count parameters and demographic findings.

Parameters	Patient (n=20)	Healthy control (n=30)	p-Value
Gender (male/female)	9/11	12/18	0.732
Age, year	22.1 (5.0–51.0)	28.2 (5.0–47.0)	0.077
Hemoglobin, g/dL	11.3 ± 2.9 (6.0–15.0)	13.2 ± 1.6 (11.0–17.0)	0.009
MCV, fL	84.2 ± 4.7 (74.0–92.0)	84.3 ± 3.6 (77.0–92.0)	0.894
MCHC, g/dL	34.8 ± 1.5 (31.0–37.0)	33.4 ± 0.98 (32.0–35.0)	0.001
RDW, %	17.9 ± 4.9 (12.0–30.0)	12.3 ± 0.7 (11.0–14.0)	<0.001

MCV, mean red cell volume; MCHC, mean red cell hemoglobin concentration; RDW, red cell distribution width; SD, standard deviation; p-value, degree of significance between groups (p<0.05). Data are given as mean or mean ± SD. Range values of the data are shown in parentheses.

Table 2A: Performance results of the classic osmotic fragility test, eosin-5-maleimide binding test, and flow cytometric osmotic fragility test.

Parameters	Cut-off value	AUC [95 % CI]	p-Value	Sensitivity [95 % CI]	Specificity [95 % CI]
C-OF, %	≤0.5	0.935 [0.828–0.985]	**<0.001**	96.6 [82.8–99.9]	80[56.3–94.3]
inc.C-OF, %	≤0.7	1.00 [0.929–1.00]	**<0.001**	100 [88.4–100.0]	100 [83.2–100.0]
EMA, MCF	>222.6	0.897 [0.778–0.965]	**<0.001**	90 [73.5–97.9]	85 [62.1–96.8]
inc.EMA, MCF	>193.4	0.853 [0.725–0.937]	**<0.001**	93.3 [77.9–99.2]	75 [50.9–91.3]
FC-OF (% RRBC)	>67.2	1 [0.929–1.000]	**<0.001**	10,0 [88.4–100.0]	100 [83.2–100.0]
inc.FC-OF (% RRBC)	>7.95	0.930 [0.821–0.983]	**<0.001**	80 [61.4–92.3]	95 [75.1–99.9]

AUC, area under the curve; C-OF, classic osmotic fragility test; inc.C-OF, incubated classic osmotic fragility test; EMA, eosin-5-maleimide binding test; inc.EMA, incubated eosin-5-maleimide binding test; MCF, mean channel fluorescence; FC-OF, flow cytometric osmotic fragility test; inc.FC-OF, incubated flow cytometric osmotic fragility test; % RRBC, residue percentage of erythrocytes; CI, confidence interval; p-value, degree of significance between groups (p<0.05). Bold numbers indicate significant difference.

(p=0.064), C-OF-inc.FC-OF AUC difference=0.005 (p=0.92), inc.C-OF-EMA AUC difference=0.103 (p=0.039), inc.C-OF-inc.EMA AUC difference=0.147 (p=0.021), inc.C-OF-FC-OF AUC difference 0 (p=1), inc.C-OF-inc.FC-OF AUC difference=0.07 (p=0.057). EMA-inc.EMA AUC difference=0.043 (p=0.53), EMA-FC-OF AUC difference=0.103 (p=0.039), EMA-inc.FC-OF AUC difference=0.033 (p=0.61), inc.EMA-FC-OF AUC difference=0.147 (p=0.021), inc.EMA-inc.FC-OF AUC difference=0.076 (p=0.28), FC-OF-inc.FC-OF AUC difference=0.07 (p=0.057) (Table 2B).

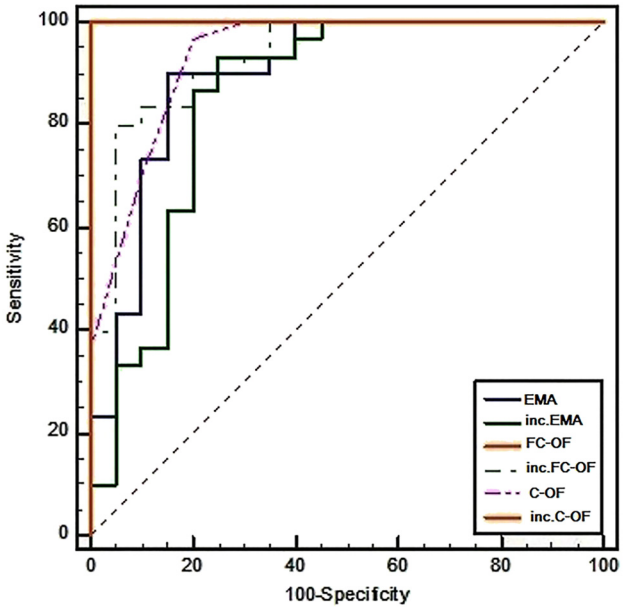

Figure 4: Receiver operating characteristic (ROC) curves of classic osmotic fragility test (C-OF), flow cytometric eosin-5-maleimide binding test (EMA) and flow cytometric osmotic fragility test (FC-OF).

Discussions

C-OF is a traditionally used test for the diagnosis of HS. There are opinions that the test has low reliability and cannot distinguish HS from other hemolytic anemias. These concerns have led to the development of new testing methods. In this study, we investigated the role of C-OF, EMA, and FC-OF in the diagnosis of HS. ROC analysis assessed the discriminatory power of the tests. We determined the AUC and cut-off values and investigated which test was superior and how incubation affected test performance.

In this study, samples were collected from 20 HS patients and 30 non-HS healthy controls. All HS patients had a positive family history, spherocytes, or increased osmotic fragility. HS patients consisted of 16 different families. There was no statistical difference between the gender and age

Table 2B: Pairwise comparisons of the classic osmotic fragility test, eosin-5-maleimide binding test, and flow cytometric osmotic fragility test.

Parameters	C-OF	inc.C-OF	EMA	inc.EMA	FC-OF	inc.FC-OF
C-OF (Difference AUC, p)	n.a.	0.065 (p=0.064)	0.038 (p=0.55)	0.081 (p=0.29)	0.065 (p=0.064)	0.005 (p=0.92)
inc.C-OF (Difference AUC, p)		n.a.	**0.103 (p=0.039)**	**0.147 (p=0.021)**	0.00 (p=1.00)	0.07 (p=0.057)
EMA (Difference AUC, p)			n.a.	0.043 (p=0.53)	**0.103 (p=0.039)**	0.033 (p=0.61)
inc.EMA (Difference AUC, p)				n.a.	**0.147 (p=0.021)**	0.076 (p=0.28)
FC-OF (Difference AUC, p)					n.a.	0.07 (p=0.057)
inc.FC-OF (Difference AUC, p)						n.a.

AUC, area under the curve; C-OF, classic osmotic fragility test; inc.C-OF, incubated classic osmotic fragility test; EMA, eosin-5-maleimide binding test; inc.EMA, incubated eosin-5-maleimide binding test, FC-OF, flow cytometric osmotic fragility test, inc.FC-OF, incubated flow cytometric osmotic fragility test; CI, confidence interval; n.a., not applicated; p, degree of significance between groups (p<0.05). Difference AUC, was calculated for pairwise comparison of the parameters. Bold numbers indicate significant difference.

distributions of patients and controls (p=0.077 and p=0.732, respectively) (Table 1).

The cut-off value of C-OF was≤0.5 % saline. This value is consistent with the literature (Trabelsi et al. [15] reported a cut-off value of 0.48 % saline, Crisp et al. [16] reported 0.445 % saline). While Crisp et al. [16] reported the discrimination power of C-OF as AUC=0.827, we found this value to be AUC=0.935. While our specificity is 80.0 % and our sensitivity is 96.6 %, Arora et al. [9] reported specificity and sensitivity of test as 62.1 %, 86.3 %, Shim and Won [17] as 73.6 %, 86.7 %, Trabelsi et al. [15] as 45 %, 90 %, respectively. Our AUC, sensitivity, and specificity values of the C-OF test were higher than those of the other studies. We think that varied inclusion criteria may explain the differing outcomes across studies. For instance, Tarabelsi et al. included 9 patients with increased C-OF, 6 patients with normal C-OF, and 5 patients with increased inc.C-OF in their study. Parpart's criteria included positive EMA, FC-OF, C-OF, and two inc.C-OF tests without specifying C-OF results [13]. Shim and Won incorporated both normal and high C-OF results, diagnosing HS via SDS-PAGE and EMA when C-OF was normal [17]. In this study, all patients had increased C-OF or increased inc.C-OF results.

EMA's diagnostic power for HS showed an AUC of 0.897, with a >222.6 MCF cut-off value, 85 % specificity, and 90 % sensitivity. Previous studies reported varying results: Stoya et al. [5] found a 0.99 AUC, 99.1 % specificity, and 96.6 % sensitivity at 400 MCF. Warang et al. [18] achieved perfect results at 1,076 MCF (AUC=1, both specificity and sensitivity are 100 %). Joshi et al. [19] reported 83.0 % specificity and 80.4 % sensitivity at 4230 MCF. These disparate cut-off values are quite interesting. Also, other researchers have pointed out this situation [19, 20]. Altough researchers [21] have suggested that the device may be responsible for the differences, different cut-off values have been reported in the literature by other researchers using our device (Table 3). Also, Joshi et al.'s [19] MCF values fluctuated greatly from day to day, despite identical conditions. As a solution, researchers suggest studying 4–6 healthy controls with the patient. Accordingly, they predict that the decrease in EMA binding can be more accurately measured if the results are shown as the percentage decrease in MCF compared to the control, or the MCF ratio (patient/control). Table 3 shows the EMA results of some researchers and the devices they used. As shown in Table 3, we and other researchers have found the MCF ratio to be in the range of 0.68–0.79. Researchers may consider presenting the results as the MCF ratio. However, studying 4–6 controls with the patient is a negative situation that increases costs. The cut-off value, specificity, and sensitivity values of the FC-OF were >67.2 , 100.0, and 100.0 %, respectively. We found that FC-OF has the ability to discriminate HS with an AUC of 1.00. Arora et al. [9] determined the cut-off value as 25.7 %, Warang et al. [18] as 23.6 %, Won and Suh [12] as 23 %, Shim and Won [17] as 61.9 %. Arora et al. [9] reported the specificity, and sensitivity of the test as 98.6 %, and 96.6 %, Warang et al. [18] as 98.0 %, and 100.0 %, Won and Suh [12] as 96.0 and 100.0 %, Shim and Won [17] as 87.5 and 91.3 %, respectively. Arora et al. [9] reported the AUC as 0.98, Warang et al. [18] as 1.00. Table 4 shows the FC-OF results of some researchers and the devices they used. Our study found that the FC-OF cut-off value was higher than the cut-off value reported by Arora, Warang, Won and Suh. We found it to be close to the cut-off value reported by Shim and Won. All researchers reported high values for the test's discrimination, specificity, and sensitivity. Reports suggest that clinical severity or ethnic origin may be the reason for this difference. Also, researchers have noted that it takes time to remove the sample from the device for hemolysis and then put it back into the device. Reports show that a delay in this step reduces the percentage of RRBCs.

Table 3: Comparison of various studies evaluating the eosin-5-maleimide binding test.

Parameters	Kar et al. [21]	Stoya et al. [5]	Joshi et al. [19]	Warang et al. [18]	El Gendy et al. [22]	Our study
Cut-off value, MCF	10,126	400	4,230	1,076	170	222.6
AUC [95 % CI]	0.99	0.99	NA	1.00	0.988	0.897
Sensitivity [95 % CI]	96.4	96.6	80.4	100	91.4	90
Specificity [95 % CI]	94.2	99.1	83	100	100	85
HS MCF	8,082.2	340.4	3,102	831.7	150.9	195.4
Normal MCF	11,861.5	478.3	4,638	1,176.9	207.3	286.6
HS MCF/Normal MCF	0.68	NA	0.79	NA	0.727	0.68
Device	FACS Canto	FACSort	FACS Canto II	FACS Calibur	FACS Calibur	FACS Calibur

AUC, area under the curve; CI, confidence interval; MCF, mean channel floresans; HS, hereditary spherocytosis; NA, not aplicated.

Table 4: Comparison of various studies evaluating the flow cytometric osmotic fragility test (FC-OF).

Parameters	Arora et al. [9]	Won and Suh [12]	Shim and Won [17]	Warang et al. [18]	Manivannan et al. [23]	Tao et al. [24]	Our study
Cut-off value (% RRBC)	25.7	23	61.9	23.6	16.2	23.6	67.2
AUC [95 % CI]	0.98	NA	0.904	1.00	NA	0.921	1.000
Sensitivity [95 % CI]	96.6	100	91.3	100	97.5	85.7	100
Specificity [95 % CI]	98.6	96	87.5	98	93.3	97.2	100
HS mean % RRBC	12.4	11	NA	9.31	8.82	20.9	29.3
Mean % RRBC (healthy control)	66.5	66.1	NA	46.2	34.5	48.3	92.2
Device	FACS Canto II	FACS Calibur	FACS Calibur	FACS Calibur	FACS Canto II	FACS Calibur	FACS Calibur

AUC, area under the curve; HS, hereditary spherocytosis; % RRBC, percentage of residual erythrocytes; CI, confidence interval.

We compared the parameters pairwise by determining the difference in the area under the ROC curve of the parameters. Contrary to popular opinion, we found that the discriminatory power of the C-OF test was not statistically different from the discriminatory power of the EMA (p=0.55) and FC-OF (p=0.064) tests. There was a statistical difference between the discriminatory power of the FC-OF and the EMA (p=0.039). This indicates that the FC-OF performed better than the EMA (Table 2B). Arora et al. [9] found that the discriminatory power of the FC-OF (AUC=0.98) and the discriminatory power of the EMA (AUC=0.99) was close to each other. Warang and colleagues [12] found that the discriminatory power of FC-OF (AUC=1.00) and EMA (AUC=1.00) was close to each other too.

Studies on traditional OF show that incubation of the sample at 37 °C for 24 h improves the performance of the test. Arora et al. [9] reported specificity and sensitivity of the test as 79.3 %, 87.7 %, Trabelsi et al. [15] as 70 %, 92 %, Shim and Won [17] as 72.3 %, 81.8 %, respectively. Our incubated test results (specificity=100 %, sensitivity=100 %) seem to be much better than the literature, as in the fresh sample. There was no statistical difference between fresh and incubated samples (p=0.064).

We investigated the effect of incubation on the performance of the tests. Our work reveals that incubation has no statistical impact on C-OF's discriminatory power (p=0.064). It did not increase the discriminatory power of the EMA and FC-OF and, in fact, decreased it (for EMA-inc.EMA p=0.53) (for FC-OF-inc.FC-OF p=0.057). Incubation had no effect on the test's discriminatory capacity (Table 2B). Won and Suh [12] found, similarly, a decline in FC-OF's discriminatory capacity after incubation.

In conclusion, despite the C-OF requiring more labor and time, its ability to effectively differentiate HS and be performed with devices and materials found in almost every laboratory suggest that C-OF test will continue to be widely used in the diagnosis of HS. The EMA is more costly than other tests due to the eosin-5-maleimide stain used and the need to run 5–6 control samples with the patient. Besides, the reporting format of the EMA needs to be standardized. The lack of flow cytometer devices in every center is the biggest handicap of FC-OF and EMA. However, the steady increase in the widespread use of flow cytometers will enable greater use of EMA and FC-OF. At this point, based on our findings, we should prefer FC-OF for centres with flow cytometers. Reasons for this recommendation are FC-OF has high AUC,

specificity and sensitivity. It gives results much faster than EMA and C-OF. It is very cheap and requires less labour.

Modern hematological analyzers that perform CBC use two methods that are electrical resistance or impedance (Coulter method) and optical analysis. These hematological analyzers combine the efficiency of the Coulter principle with the sensitivity of flow cytometry. At this point, Warang et al. [18] state that FC-OF can be adapted to devices performing CBC. Thus, HS and other hemolytic anemias can be detected easily. We value and support this suggestion of Warang et al. If researcher can integrate the FC-OF technique into CBC devices, it will enable broader screening for erythrocyte disorders that cause hemolytic anemia because almost every hospital has these devices.

Limitations of the study

The study's limitations are its small sample size, despite using statistical methods to guide selection. Lack of polymorphism analysis in HS patients narrows the scope of research and limits the applicability of the findings.

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Research ethics: The study was conducted in accordance with the Declaration of Helsinki, as revised in 2013. Ethical approval was obtained from the Mersin University, Clinical Research Ethics Committee on 13.04.2017 and numbered 2017/104.

Informed consent: Informed consent was obtained from all individuals included in this study.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission. P.E. and C.G reviewed the reports and conducted the study. C.G. performed the laboratory analyses. S.Ü., A.T. did the clinical evaluation. P.E., C.G., G.T. contributed to the evaluation of the results and revised the manuscript.

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