Flow Cytometric Eosin-5'-Maleimide Test is a Sensitive Screen for Hereditary Spherocytosis

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Abstract Hereditary spherocytosis (HS) is a clinically heterogeneous disease characterized by mild to moderate hemolysis resulting from red cell membrane protein defects. Diagnostic tests include hemogram, reticulocyte count and blood smear evaluation, osmotic fragility, cryohemolysis, SDS-PAGE, flow cytometry using eosin-5'-maleimide (EMA) and genetic studies. We evaluated the flow cytometric EMA-binding test and compared it with osmotic fragility in 51 consecutive cases of suspected HS aged between 10 days and 62 years. In addition, 4 cases suspected on blood smears underwent EMA testing alone. The 16 EMA-positive cases who were determined to have HS had overlapping hemoglobin levels and reticulocyte counts with the 35 patients with normal EMA results, highlighting the importance of the flow cytometric test in providing a definitive diagnosis. Flow cytometric EMA binding test was thus a simple and relatively faster method to confirm HS in our experience.

Keywords Hereditary spherocytosis · Flow cytometry · Eosin 5 maleimide · Osmotic fragility

Introduction

Hereditary spherocytosis (HS) is an inherited red cell membrane disorder that affects 1 in 2000 Caucasians and less commonly in African American and southeast Asians [1]. Though clinically heterogeneous, the patients usually present with mild anemia, intermittent jaundice and sometimes splenomegaly. Routine laboratory investigations often show raised mean corpuscular hemoglobin concentration (MCHC), elevated bilirubin levels, reticulocytosis and spherocytes on blood smear. Though the clinical history and red blood cell indices are helpful in diagnosing of HS, screening and confirmatory tests are required to establish the same. Osmotic fragility (OF) test is the most widely used screening test. Various other methods including cryohemolysis and SDS-polyacrylamide gel electrophoresis are also commonly used [2]. In the present study, we have evaluated cases for screening HS using eosin 5 maleimide (EMA) by flow cytometry and compared the results with classic osmotic fragility.

Materials and Methods

The study consists of 51 consecutive cases which we received at our facility for OF test, over a period of 5 months, between November 2016 and March 2017. All cases were suspected of having HS. The grounds for clinical suspicion included recurrent jaundice, splenomegaly, presence of spherocytes on smear and negative direct Coombs test.

Both ethylenediaminetetraacetate (EDTA) and sodium heparin whole venous blood samples, 2 ml each were collected for each case. EDTA sample was used for flow cytometry analysis using EMA. Heparin sample was used

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Fig. 1 **a** Control sample: Left—forward and side scatter used to gate the red blood cell population, highlighted in green. Right: histogram of EMA tagged red cells on FL1. **b** Patient sample positive for HS: decrease in MFI (right) as shown by shift of EMA tagged red cells to the left as compared with that of control

for OF test. For control, a similar set of samples were collected from age matched healthy donors. The tests were done within 4 h from time of sample collection.

Age range was from 10 days to 62 years. The male to female ratio was 1:1.

For each case a complete blood count, reticulocyte count, peripheral blood smear prepared by automatic smearing and stained with May Grunwald Giemsa, direct and indirect bilirubin, OF and EMA by flow cytometry were done. Complete blood counts and corrected reticulocyte counts were done on Sysmex 5-part cell particle analyzer using flow cytometry and bilirubin was detected by diazotized sulphanilic acid method.

In addition to the above, we encountered 4 cases that were sent to us for routine peripheral blood analysis where spherocytes were found on smear. For those cases, all tests mentioned above, except for osmotic fragility (due to lack of heparin sample) were done after appropriate counselling of the patients and clinicians and receiving verbal consent for carrying out the investigations.

Osmotic fragility (OF) test: Standard method that involves mixing blood with large excess of buffered saline of varying concentrations. The fraction of red cells lysed at each saline concentration was determined colorimetrically. For all cases that yielded normal results, further testing after an extended incubation for 24 h was done. Reference range of mean corpuscular fragility (MCF) for OF control: 4–4.5.

Procedure for EMA binding (flow cytometry): Hundred microliter EDTA blood added to tubes with 2 ml phosphate buffer saline and washed. Twenty-five microliters of 0.5 mg/mL EMA (Sigma-Aldrich Company) was added to each tube and incubated in dark for 1 h at room temperature. This was washed with PBS. 100 μ l RBC suspension from each tube and 1.4 ml PBS made into a final suspension for flow cytometric analysis. The analysis performed on flow cytometer, FC500 Beckman Coulter. Minimum 50,000 events were acquired. Forward (FSC) and side scatter (SSC) plots were used. RBCs with high FSC and SSC gated and Mean Fluorescence Intensity (MFI) of the

Table 1 A comparison of demographic and laboratory data in patients with and without hereditary spherocytosis

	Hereditary spherocytosis (n = 16)	Patients with normal EMA study (n = 35)
Median age and age range	23 years (2–56 years)	21 years (10 days–62 years)
Gender (male: female)	1:1	1:1
Hemoglobin (g/dl) mean ± SD and (range)	11.8 ± 2.7 (7.6–17)	10.4 ± 3.4 (5.4–18.2)
Hematocrit (%) mean ± SD and (range)	38 ± 3.2 (34–42)	40 ± 3.6 (36–44)
Mean corpuscular volume (fL) mean ± SD and (range)	85 ± 5.5 (70–88)	89 ± 4.8 (75–90)
Mean corpuscular hemoglobin (pg) mean ± SD and (range)	26 ± 2.2 (22–29)	29 ± 2.0 (24–32)
Mean corpuscular hemoglobin concentration (g/dl) mean ± SD and (range)	34.3 ± 1.77 (30–37.1)	31.8 ± 2.2 (24.4–36.5)
Corrected reticulocyte count (%) mean ± SD and (range)	10.8 ± 4.9 (4.09–24.28)	5.3 ± 1.2 (0.4–40)
%nRBCs (Nucleated red blood cells/100 white blood cells) (mean) and (range)	2 ± 0.5 (0–3)	4 ± 1.0 (0–7)
Spherocytes on peripheral blood smear (%) mean ± SD and (range)	37 ± 18.34 (2–50)	0.0
Serum indirect bilirubin (mean) and (range)	4.2 ± 2.2 (1.38–6.69)	0.8 ± 0.3 (0.14–38.57)
EMA binding test result (%) (mean MFI) mean ± SD and (range)	29.1 ± 7.2 (13–37.9)	1.53 ± 2.97 (0–11)

red blood cells was obtained in Fluorescein isothiocyanate (FITC) (FL1) channel. The RBCs emit green fluorescence when bound to EMA (Fig. 1).

For each case, two age matched controls using from healthy donors were run along with the test and the mean was used for calculation. MFI of the control and MFI of the patient's sample were used in the following formula to obtain a percentage reduction in MFI of patient:

$$\frac{\text{MFI}(\text{control}) - \text{MFI}(\text{patient})}{\text{MFI}(\text{control})} \times 100$$

Reference range/result interpretation: $\geq 21\%$ reduction in MCF: HS, 15–20% reduction in MCF: most likely to be HS, $< 15\%$ reduction in MCF: unlikely to be HS [3].

Results

Of the 51 cases for which both OF test and flow cytometry using EMA was done, 10 cases (@) (Fig. 1) were positive for HS by flow cytometry as well as OF by manual method. Two cases (¥) were positive for HS by flow cytometry and showed normal result by classic osmotic fragility test

(Table S1). Both these cases continued to show normal OF result even after incubation for 24 h. The mean MFI of control samples was 90. The average time taken to perform the EMA assay was 2 h compared with 5 h for OF.

For the four patients (*) where peripheral blood spherocytosis was noted, only flow cytometry using EMA was done and results showed a significant decrease in the MFI, highly suggesting HS (Table S1). Demographic and laboratory data of patients with and without HS is compared in Table 1.

Discussion

Osmotic fragility test was the investigation of choice in most centers till the advent of the specific EMA test. The test involves assessment of resistance of red cells to hemolysis by subjecting the cells to osmotic stress using varying concentrations of hypotonic sodium chloride solution. When performed accurately, the test gives good results. However, the method is laborious and highly depends on well trained technical skill resulting in decreased reproducibility of results. Another disadvantage

of OF is the lack of uniformity in establishing cutoff for positive cases [4, 5]. Moreover, literature states that the test is sensitive to detect abnormalities only when at least 2% of red blood cells are spherocytes [1]. Therefore, for milder or atypical forms of HS where the number of spherocytes are fewer, the test may fail to show an abnormality. This method also lacks specificity as it may show positive results in immune or other hemolytic anemias [6].

Band 3 protein exists as a dimer and tetramers in the red cells and has multiple protein binding sites. It interacts with spectrin and Ankyrin molecules as well as protein band 4.2, all of which play a pivotal role in maintaining the structure and stability of red blood cell cytoskeleton. Eosin 5 maleimide dye binds to lysine 430 in the first extracellular loop of band 3 protein on the red cells. In hereditary spherocytosis, there is a decrease in MFI compared with that of control which is attributed to reduced binding of the dye to the quantitative or functionally defective band 3 protein [7, 8]. Though guidelines recommend using four or five controls for each case, we have used only two, which is a limitation of this study.

Our experience though this study, not only showed a high degree of concurrence between OF and EMA binding test, but the latter clearly highlighted a higher sensitivity and specificity in screening for HS [9–11, 13]. However, further investigation by confirmatory tests was recommended.

Flow cytometric analysis using the dye, EMA has been a well-studied and validated in screening and diagnosis of HS. The test is simple, cost effective, highly reproducible, less labor intensive and far more sensitive in diagnosis of HS [12, 13]. There is also published data on its utility in diagnosing HS in post splenectomy status with efficacy [14]. Though flow cytometer may not be available in under resourced facilities, our experience has proved without doubt that where a flow cytometer is available, this method of testing helps us deliver accurate reports to the patients and clinicians at a much shorter time.

Compliance with Ethical Standards

Conflict of interest Authors Preethi S. Chari and Sujay Prasad have no conflict of interest.

Ethical Approval We request waiver of the requirement for ethical approval since no experimentation was performed on the human participants. Only the results of the additional EMA testing done on the routine blood samples sent for clinically indicated laboratory

testing (that would otherwise have been discarded after the OFT and hemogram tests were performed) are presented in an anonymized manner.

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