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Original Paper

Biochemical, Cellular, and Proteomic Characterization of Hereditary Spherocytosis Among Tunisians

Nawel Trabelsi[a,b] Ghada Bouguerra[a,c] Faten Haddad[a,c] Monia Ouederni[d] Imen Darragi[a] Imen Boudrigua[a] Dorra Chaouachi[a] Mbarka Barmat[a] Chaker Fouzai[a] Mohamed Bejaoui[d] Samia Menif[a] Imen Kraiem[a] Salem Abbes[a,c]

[a]Laboratoire d'Hématologie Moléculaire et Cellulaire (LR16IPT07), Institut Pasteur de Tunis, Tunis, Tunisie, [b]Faculté des Sciences de Tunis, Université de Tunis El Manar, Tunis, Tunisie, [c]Faculté de Médecine de Tunis, Université de Tunis El Manar, Tunis, Tunisie, [d]Unité d'hématologie-immunologie pédiatrique, Centre National de Transplantation de la Moelle Osseuse, Tunis, Tunisie

Key Words
Hereditary Spherocytosis • Band 3 protein • EMA dye • Membrane proteins electrophoresis • Red cell fragility

Abstract
Background/Aims: Hereditary Spherocytosis (HS) is the most common erythrocyte membrane disorder causing hemolytic anemia. The wide heterogeneity of both clinical and laboratory manifestations of HS contributes to difficulties associated with the diagnosis of this disorder. Although massive data previously reported worldwide, there is yet no data on HS among the Tunisian population. Here we aim to characterize HS in Tunisian patients at biochemical and cellular levels, identify the membrane protein deficiency, and compare the accuracy of the diagnostic tests to identify the most appropriate assay for HS diagnosis. ***Methods:*** We investigated 81 patients with hemolytic anemia and 167 normal controls. The exploration of HS based on clinical and family history, physical examination, and the results of laboratory tests: blood smear, osmotic fragility test (OFT), cryohemolysis test (CT), pink test (PT), eosine-5'-maleimide (EMA) test, and erythrocyte membrane protein electrophoresis. ***Results:*** We identified 21 patients with HS, classified as severe (6/21;28.5%), moderate (10/21;47.6%), and mild (5/21;23.8%). The most prevalent protein deficiency was the band 3 protein detected in ten Tunisian HS patients. The EMA test showed a high specificity (97.5%) and sensitivity (94.7%) for HS diagnosis compared to the other screening tests. Interestingly, fourteen among sixteen patients presenting with homozygous sickle cells HbSS showed an increase of EMA fluorescence intensity compared to other anemic patients. ***Conclusion:*** Our study highlights the efficiency of the EMA dye for the detection of HS whatever the nature of the involved

Nawel Trabelsi
Laboratoire d'Hématologie Moléculaire et Cellulaire (LR16IPT07)
Institut Pasteur de Tunis (Tunisie)
E-Mail nawel.trabelsi@pasteur.utm.tn

protein deficiency. We report for the first time, the most prevalent protein deficiency among Tunisians with HS. Moreover, we found that the combination of the EMA-binding test with PT or incubated OFT improves the diagnosis sensitivity while maintaining a good specificity.

Introduction

Hereditary Spherocytosis (HS) is the most common of inherited hemolytic anemia due to erythrocyte membrane protein alterations. The clinical expression of HS ranges from clinically silent forms with well-compensated chronic hemolysis to severe transfusion-dependent anemia. Based on hemoglobin levels and reticulocyte counts, the clinical severity of HS is classified as mild, moderate, moderately severe, or severe [1-4]. According to the British Committee for Standards in Haematology guidelines, the confirmation criteria of HS diagnosis include: a family history of HS, clinical features consisting of jaundice and splenomegaly as well as laboratory results indicating the presence of spherocytes on the peripheral blood smear, increased mean corpuscular hemoglobin concentration (MCHC) and reticulocyte (Ret) values [5]. However, these characteristics overlap with other hereditary hemolytic anemia, thus complicating the diagnosis of HS. In these cases, more specific laboratory tests are needed. These tests are based on evaluating the degree of hemolysis induced either by a hypotonic solution (the osmotic fragility test and the pink test) [6, 7] or by a hypertonic solution (cryohemolysis test) [8, 9]. Yet, these tests do not detect a variable proportion of HS cases, particularly the mildest ones, and do not differentiate HS from secondary spherocytosis associated with other conditions such as autoimmune hemolytic anemias [6, 10, 11]. The flow cytometry test of eosin-5'-maleimide (EMA) binding shows high sensitivity and specificity for the HS diagnosis [12-14]. The current guidelines recommend the EMA-binding flow cytometry assay as a screening test for HS diagnosis [5, 15]. In the absence of genetic testing, the HS diagnosis confirmation requires the detection of a decrease in a specific protein (i.e. spectrin, ankyrin, band 3, and protein 4.2) by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [15]. Nevertheless, the SDS-PAGE is a laborious test and is not routinely carried out in most hematology laboratories.

Although massive data previously reported worldwide, there is yet no data on HS among the Tunisian population, even in most North Africa countries. Furthermore, the Tunisian population is known for a relatively high rate of consanguinity and endogamy that favor expression of genetic disorders [16]. In our study, we aim to characterize HS in Tunisian patients by evaluating the degree of hemolysis both in hypotonic and hypertonic solutions. Furthermore, we examine the EMA-binding to detect the band 3 complex abnormalities, and we identify the membrane protein deficiency in HS patients. Finally, we compare the accuracy of the diagnostic tests (EMA-binding test, osmotic fragility test, Cryohemolysis test, and pink test) to identify the most appropriate assay for HS diagnosis.

Materials and Methods

Subjects

For twenty-six months, we investigated twenty-one HS patients divided into nine unrelated individuals and twelve patients belonging to seven families. Additionally, sixty patients presenting with various forms of hereditary red blood cell disorders were recruited into the study. These subjects are divided into six Glucose-6-Phosphate Dehydrogenase deficient (G6PD) patients and fifty-four patients presenting with hemoglobinopathies. The latter group was comprised of sixteen patients presenting with homozygous sickle cell anemia (HbSS), eight composite heterozygotes for sickle cell anemia and β-thalassemia (HbSβ), thirteen patients presenting with heterozygous sickle cell anemia (HbAS), two patients presenting with hemoglobin C trait (HbAC) and fifteen β-thalassemia patients. A total of 167 healthy individuals, comprised of 73 males (43.7%) and 94 females (56.3%), were selected as controls. Blood samples were collected in

EDTA-containing tubes. For the sake of accuracy, samples were processed within 24 hours. The study was approved by the Ethics Committee of Pasteur Institute of Tunis, Tunisia, in accordance with the Declaration of Helsinki, and the blood samples were collected after obtaining informed consent.

Methods

For all subjects, complete blood counts were obtained using a Pentra 60C+ analyzer (ABX Diagnostics, Montpelier, France). The analysis of hemoglobin fractions was performed by automated capillary electrophoresis Capillaris TM2 Sebia FLEX Piercing. G6PD activity was assessed using Beultler's protocol. The diagnosis of HS was based on family history, the clinical and laboratory features of chronic hemolysis, the presence of spherocytes on blood smears, elevated reticulocytes count, and negative direct antiglobulin test. The explored laboratory diagnostic tests were the osmotic fragility test (OFT) executed both on fresh and incubated blood at 37°C/24h, the cryohemolysis test (CT), the pink test (PT), the EMA-binding test, and the erythrocyte membrane protein electrophoresis (EMPE test).

EMA-binding flow cytometry test (EMA test)

The EMA test was performed as described by King et al. [12, 13]. Red blood cells from each patient and their corresponding 6 controls were washed with a 0.5% PBS-BSA solution and then stained with 0.5 mg/mL EMA (Sigma-Aldrich Company, USA). Samples were incubated in the dark for one hour at room temperature and then washed three times with PBS/BSA. EMA fluorescence was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a logarithmic scale. Flow cytometry analysis was performed with a BD FACS Canto II (BD Biosciences 2350 Qume Drive San Jose, CA 95131-1807 USA). For each sample, 20 000 events were acquired. Forward scatter (FSC) and side scatter (SSC) were determined on a linear scale. Red cells with high FSC and SSC were gated and the EMA mean fluorescence intensity values (MFI) for these cells were determined. Results are expressed as a percentage of MFI of EMA according to the following equation:

$$\%\text{MFI of EMA} = \frac{\text{MFI patient} - \text{Mean MFI controls}}{\text{Mean MFI controls}} \text{x}100.$$

Erythrocyte Membrane protein electrophoresis SDS-PAGE (EMPE test)

Within 24 hours of blood collection, erythrocyte membranes were prepared using Dodge's method [17] with some modifications. Red cells were washed three times with 5 mM PBS and mixed with a lysis buffer containing a protease inhibitor cocktail (Biovision Incorporated, Milpitas CA, USA). After an incubation period of 10 minutes on ice, samples were centrifuged at 15000 rpm for 10 min at +4°C. Erythrocyte ghosts were washed at least three times until obtaining a white membrane. The protein quantification assay was performed by colorimetry according to Lowry's method [18]. The calibration range was carried out using 100 µg/mL albumin solution and the absorbance was measured at 750 nm. Then, the membrane proteins were solubilized in a solution containing 20 % SDS, 10 mM EDTA, 400 mM DTT, 47 % Glycerol, and 1 % Bromophenol Bleu and diluted in 5 mM PBS in order to obtain a final concentration of 1 mg/mL. Red cell membrane proteins were separated by SDS-PAGE. An 8µl of proteins of each sample was loaded in two different gels: the first gel was prepared using a 3.5% to 17% gradient of acrylamide according to Fairbanks [19], and the second gel using a 5% to 15% linear gradient of acrylamide according to Laemmli's method [20]. Gels were stained by Coomassie blue dye then scanned using a Bio-Rad GS-800 calibrated densitometer at a resolution of 36.3 X 36.3 microns. The gel images were analyzed by Bio-Rad's Quantity One software according to the manufacturer's instructions. The density of each protein band is expressed as a function of its optical density (OD) and its width in the gel after eliminating the background noise. To overcome possible errors in the amounts of proteins deposited in the gel, the density of each band is normalized as a function of the sum of the densities of all the bands detected in the track. For each patient, the relative quantity percentage (RQ%) of each membrane protein fraction was calculated and compared to the mean of four controls separated in the same gel and the same conditions.

Pink test (PT)

A volume of 10µl of each blood sample was mixed with a buffered hypotonic saline glycerol solution (70mM Bis-Tris, 25mM NaCl, 1.5mM NaN3, 135mM glycerol, HCl pH 6.66; and 2% TritonX as a lyses solution). After an incubation of 40 min at room temperature, followed by centrifugation at 1000x g for 5 min, the OD

of the supernatant was measured at 540nm using a spectrophotometer (Helios Gamma model 942, Thermo Electron Corporation, France). The results were expressed as hemolysis percentage and calculated using the following equation [7]:

$$\text{PT } (\%) = \frac{\text{OD supernatant}}{\text{OD 100\% lysis}} \text{x} 100.$$

Cryohemolysis test (CT)

Blood samples were washed three times with cold (kept at +4°C) 9 g/L NaCl solution. Then, red cells were mixed with a buffered sucrose solution (0.7 mol/L). Cells lysed in distilled water were used as a reference for 100% hemolysis. Where indicated, the supernatant corresponding to 100% hemolysis was diluted in 4 ml distilled water [9]. After a 10 min incubation at 37°C, the samples were incubated at 0°C for 10 min then centrifuged (6000 rpm for 5 min). The OD was measured at 540 nm. The extent of cryohemolysis was calculated using three different formulas as follows:

$$\text{CT1 } (\%) = \frac{\text{OD supernatant}}{\text{OD 100\% lysis}} \text{ x } 100 \text{ [21],}$$

$$\text{CT2 } (\%) = \frac{\text{OD supernatant}}{\text{OD 100\% lysis} * 5} \text{x } 100 \text{ [9, 22] and}$$

$$\text{CT3 } (\%) = \frac{\text{OD supernatant}}{\text{OD 100\% lysis} * 21} \text{x } 100 \text{ [23].}$$

Osmotic Fragility Test (OFT) on fresh and incubated blood

A series of hypotonic solutions ranging from 1 g/l to 9 g/l was prepared using a 100 g/l NaCl buffer. Then, 50 µl of fresh blood was added to each solution and incubated for 30 min at room temperature. After the centrifugation (2500 rpm for 5 min) of the fresh blood sample, the supernatant was decanted, and its OD was determined at 540 nm. The percentage of hemolysis was calculated as follows:

$$\text{OFT}(\%) = \frac{\text{OD supernatant}}{\text{OD 100\% lysis}} \text{x } 100.$$

The results were plotted against NaCl concentrations. The resulting OF curve was then compared to the one obtained from control samples and the curve features were determined using three indices: initial lysis (OFT-IL), complete lysis (OFT-CL), and median corpuscular fragility (OFT-MCF). Similarly, the same procedure was performed after a 24h incubation at 37°C of blood samples [23, 24].

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 5.00 (GraphPad Software, San Diego California USA). The reference ranges and the cutoff values for HS diagnosis of all tests (EMA test, PT, CT, OFT, and EMPE test) were established according to the European Federation of Clinical Chemistry and Laboratory Medicine recommendation for the review of biological reference intervals in medical laboratories [25]. Normally distributed data are expressed as means±SD [95% Confidence Interval] and compared pairwise by ANOVA test. The threshold of statistical significance was set at $p<0.05$. The receiver operating characteristic (ROC) curve analysis was used to calculate the area under the curve (AUC) values for each test to discriminate patients with HS from patients with other types of anemia, and hence to evaluate the diagnostic performance of the method.

Results

Clinical and hematological results

We report twenty-one HS patients comprised of eight males (38%) and thirteen females (62%). The median age at diagnosis was 5 years (6 months to 31 years). Six patients had a history of neonatal jaundice. According to hemoglobin (Hb) levels and reticulocyte (Ret) counts, HS was considered severe in six patients (28.5%; Hb < 8g/dl and Ret >10%), moderate in ten patients (47.6%; Hb >8g/dl and Ret >6%) and mild in five patients (23.8%;

near-normal Hb and Ret < 6%). The Hb/MCHC ratio showed a significant difference in moderate and severe groups compared to the mild HS group ($p<0.05$). The clinical, hematological, and biochemical features of HS patients are summarized in Table 1.

Table 1. Clinical, hematological, and biochemical features of HS patients

Parameters	Mild HS (N=5)	Moderate HS (N=10)	Severe HS (N=6)
Sex ratio (M/F)	2/3	3/7	3/3
Median age (range)	29 y (7 m -31 y)	5 y (6 m-26 y)	7 y (8 m -7 y)
Family history (N)	5	2	3
Icterus (N)	0	3	3
Splenomegaly (N)	0	5	5
Consanguinity (N)	-	4	1
Hb (g/dl, Mean±SD)	12.60 ± 0.38	10.51 ± 0.64	7.12 ± 0.80
MCHC (g/dl, Mean±SD)	35.44 ± 1.61	34.18 ± 1.59	32.38 ± 1.49
Hb/MCHC ratio (Mean±SD)	0.36 ± 0.02	0.31 ± 0.02	0.22 ± 0.03
Reticulocytes (%, Mean±SD)	3 ±1.50	12 ± 3	29 ± 5.50
Spherocytes on blood smear	+	++	++++

EMA test results

To assess the specificity of the EMA test and to establish the cutoff value, we investigated 60 patients presenting with other forms of hereditary hemolytic anemia and 167 healthy controls. Results of the flow cytometry analysis of EMA labeled erythrocytes from the patients and the control groups are shown in Fig. 1. The MFI percentage (%MFI) of EMA in the control group ranged from -7% to +7% [95% CI]. The established cutoff value for the EMA test was set at -20%, which was highly significant ($p<0.05$) and reflected a decrease of EMA fluorescence intensity in HS patients. This method showed a specificity of 97.5%, a sensitivity of 94.7%, and an AUC of the ROC curve of 98%. Interestingly, we found that 87.5% (14/16) of patients with homozygous sickle cell anemia HbSS showed a significant increase of EMA fluorescence intensity ($p <0.05$). Compared to the control group, the ranges of EMA intensity of patients presenting with β-thalassemia, HbAS, and HbSβ did not reach statistical significance. A 5-years-old male with G6PD deficiency showed a significant decrease in EMA fluorescence intensity (-32.3%) in whom spherocyte cells were observed in the blood smear. Similarly, one patient with HbAC showed a significant decrease in EMA intensity (-20.6%) compared to the control group.

EMPE test results

The EMPE was performed on samples from nineteen HS patients. A threshold at -13% was fixed to detect a protein deficiency. Isolated and combined protein deficiencies were detected in eighteen patients (95%). An isolated deficiency of band 3, ankyrin and spectrin were detected respectively in seven (39%), five (28%), and one (5%) of patients. A combined band 3-protein 4.2 and ankyrin-spectrin deficiencies were identified respectively in three (17%) and two (11%) of patients. Band 3 protein deficiency was the most frequent defect in HS patients (56%; 10/18), whereas ankyrin protein defect was found in 39% (7/18) of patients. Both spectrin and protein 4.2 deficiencies were detected in 17% (3/18) of cases, in the isolated and combined state (shown in Fig. 2). No association was detected between the type of protein defect and the decrease in fluorescence intensity of EMA-binding. Although the EMA test showed a significant fluorescence decrease (-30.2%) in one patient, the separation profile, as well as the densitometry results of the membrane protein analysis, did not show a significant statistical difference, compared to the control group. Inversely, a slight decrease in EMA fluorescence (-17.5%) was detected in another patient. Yet, this patient's membrane protein analysis showed a clear band 3 deficiency.

PT, CT, and OFT results

Both the PT and CT were performed in twenty HS patients, seventeen patients presenting with different forms of hemolytic anemia, and fifty controls. The calculated cutoff value of the percentage of hemolysis for PT was set at 28%. The corresponding sensitivity and specificity were 72.2% and 88.2% respectively, and the AUC of the ROC curve was 86%. For CT, three formulas were applied to calculate hemolysis percentage and the results are presented in Table 2. The 2.4% cutoff point yielded the best discriminatory value (70% sensitivity and 64.7% specificity) compared to the other cutoff values. To establish the reference ranges

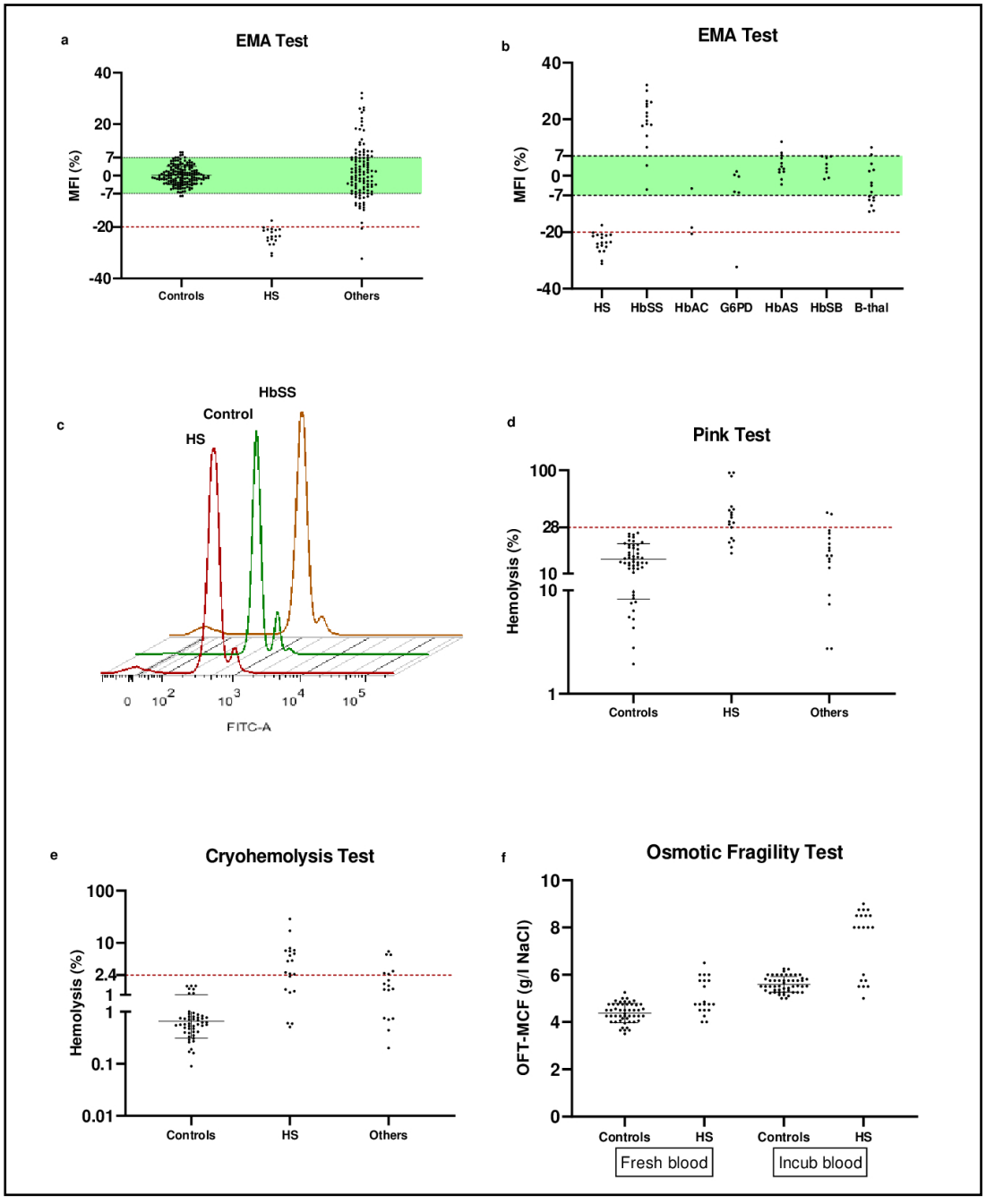

Fig. 1. Results of the flow cytometry analysis of EMA test and the red blood cell fragility tests (PT, CT, and OFT) from patients and controls. EMA: eosin-5'-maleimide. FITC: fluorescein isothiocyanate. FSC: forward scatter. SSC: side scatter. PT: pink test. CT: cryohemolysis test. OFT: osmotic fragility test. HbSS: homozygous sickle cell anemia. HbAS: heterozygous sickle cell anemia. HbSβ: heterozygotes for sickle cell anemia and β-thalassemia. HbAC: hemoglobin C trait. G6PD: Glucose-6-Phosphate Dehydrogenase. (a) Distribution of Mean Fluorescence Intensity (MFI) percentage of EMA dye in controls, HS patients, and patients with other forms of inherited hemolytic anemia. (b) Distribution of MFI% of EMA test in HS patients and the other patients classified according to the type of inherited hemolytic anemia: HbSS, HbAC, G6PD deficiency, HbAS, HbSβ, and β-thalassemia. (c) Histograms of EMA labeled erythrocytes from an HS patient (red), control (green), and HbSS patient (orange). (d, e) Distribution of hemolysis percentage of the PT and CT obtained from controls, HS patients, and other anemic patients. (f) Distribution of median corpuscular fragility (MCF) of OFT on fresh and incubated blood in controls and HS patients.

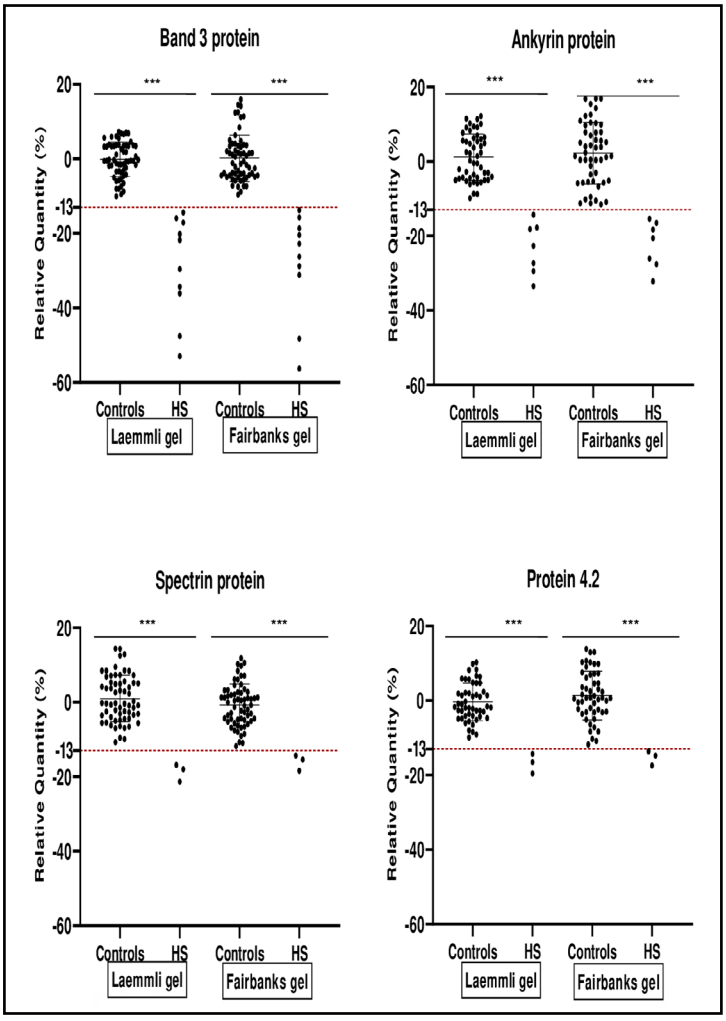

Fig. 2. Densitometry results of erythrocyte membrane protein electrophoresis test. The protein fractions in HS patients and normal controls expressed as a relative quantity percentage (RQ%) of each protein (band 3, ankyrin, spectrin, and protein 4.2) in both Laemmli gel 5%-15% and Fairbanks gel 3.5%-17%. Band 3-RQ% in controls was -0.08±4.63 and 0.22±6.22, respectively in Laemmli and Fairbanks gels. Ankyrin-RQ% in controls was 1.14±6.24 and 2.20±8.29, respectively in Laemmli and Fairbanks gels. Spectrin-RQ% in controls was 0.97±6.22 and -0.72±5.60, respectively in Laemmli and Fairbanks gels. Protein 4.2-RQ% in controls was -0.38±5.07 and 1.38±6.53, respectively in Laemmli and Fairbanks gels. ***: p <0.001.

in terms of initial lysis (OFT-IL), complete lysis (OFT-CL), and median corpuscular fragility (OFT-MCF), the OFT was performed on fifty control samples. The OFT-IL range was set at 5-5.5 g/l NaCl in fresh blood and at 7-7.5 g/l NaCl in incubated samples. Similarly, the OFT-CL range was set at 2-3 g/l NaCl in fresh blood and at 3-3.5 g/l NaCl in incubated samples. Finally, the OFT-MCF range was estimated at 3.65-4.85 g/l NaCl for fresh blood and 5-6.2 g/l NaCl for incubated blood. Among the tested samples, nine HS patients (45%) showed an increased OFT both in the fresh and incubated blood. However, eleven patients (55%) presented normal OFT in fresh blood but only five of them presented an increased OFT in the incubated blood. The diagnostic sensitivity of the OFT was estimated at 70%.

Table 2. Evaluation parameters of diagnostic tests: EMA test, PT, CT, and OFT. EMA: eosine-5'maleimide. PT: pink test. CT: cryohemolysis test. OFT: osmotic fragility test. MCF: median corpuscular fragility. PPV: positive predictive value. NPV: negative predictive value. AUC: area under curve

Tests	Cut-off (% †; g/l ‡)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	AUC (%)
EMA test	-20†	94.7	97.5	90	98.7	97	98
Pink test	28†	72.2	88.2	86.6	75	80	86
Cryo Test							
CT3	2.4†	70	64.7	70	64.7	67.5	67
CT2	7.4†	55	88.2	-	-	-	-
CT1	2.8†	60	64.7	-	-	-	-
OFT fresh-MCF	4.8‡	45	90	64.3	80.4	77.2	71
OFT incub-MCF	6.2‡	70	92	77.8	88.5	85.7	81

The results of PT, CT, and OFT are summarized in Fig. 1. Compared to fresh blood, the OFT in incubated blood was more indicative of HS. Overall, both OFTs and CT had comparable sensitivities (70%) which were slightly lower than that of PT (72.2%). Taken together, the EMA test and the three red cell fragility tests show that six patients were positive for four assays (EMA test, CT, PT, and incubated OFT) and only two patients were positive for all tests. The evaluation parameters vary between tests and are shown in Table 2. Compared to the other test, the EMA test showed the highest sensitivity (94.7%) and accuracy (97%). A combination of the EMA test with either the PT or the incubated OFT increased the sensitivity of the HS diagnosis to 100% and 94% respectively (shown in Fig. 1). However, in cases where a flow cytometer is not available, a combination of PT, CT, and incubated OFT reached the sensitivity to detect HS patients.

Discussion

To our knowledge, this is the first study characterizing Tunisian HS patients using the most common laboratory tests for the HS diagnosis. The systematic diagnosis of HS is based on a combination of the clinical and family history, and laboratory examinations [5]. In our study, the median age at diagnosis was about 5 years, ranging from 6 months to 31 years. Depending on the severity of hemolysis, HS may be diagnosed at any age, either in childhood or during adult life, as previously reported [5, 26]. In our data, 81% of patients are children and only 19% of patients are diagnosed in adult life. The age of onset is variable but often occurs between the ages of 3-7 years. Symptoms can develop in infancy, but some HS patients who have no symptoms or minor symptoms are diagnosed later in life. Furthermore, three patients with mild HS included in our study are fortuitously detected during a family exploration, except for two patients aged 7 months and 5 years. Most of the healthy controls included in our study are adults. Ideally, but not mandatorily, blood from healthy controls should be age-matched and drawn at the same time as the diagnostic blood sample [27]. Unfortunately, as we rely on control samples from anonymous blood donors, this approach is often not feasible. Finding normal age-matched controls, especially in children, can be a challenge [15, 28].

In our study, we evaluated the accuracy of the traditional and new assays in twenty-one HS patients and we identified the underlying membrane protein defects in these patients. Our results showed that the combination of EMA test with PT or incubated OFT seemed to improve the diagnosis sensitivity while maintaining a good specificity. The EMA test and PT appeared to be the most useful screening tests in our study, as opposed to the findings of Bolton-Maggs et al. [29] and Crisp et al. [10] who both reported that EMA test and CT have the highest percentage of positive results for HS diagnosis. From a procedural point of view, both the EMA test and PT require a small volume of blood samples (120µl for both assays) which makes them suitable for neonates and babies. Furthermore, they are timesaving (estimated time of preparation 3h). The only downside to the EMA test seems to be the necessity of using normal controls (6 samples) in each assay. The EMA-binding flow cytometry method measures the fluorescence intensity of erythrocytes labeled with the fluorescent probe EMA, and thus directly targets the structural lesion of the erythrocyte membrane proteins. The EMA probe interacts with transmembrane proteins band 3, Rh protein, Rh glycoprotein, and CD47 which are decreased in HS red cells. Other cytoskeletal proteins such as spectrin and protein 4.2 also induce a decrease in EMA fluorescence intensity, likely because they create a long-range modulation effect on the dye-binding site in band 3 protein [12]. Several studies [12, 30-33] reported a high specificity (96–99%) and sensitivity (89–96.6%) of the EMA test for HS diagnosis. Similarly, to these findings, our study showed a 97.5% specificity and a 94.7% sensitivity with a cutoff value fixed at -20%, proving thus the usefulness of this method for HS diagnosis. However, Crisp et al. [10] reported lower specificity and sensitivity of the EMA test. Their results were expressed in terms of decreased mean channel fluorescence (MCF) and increased coefficient of variation (CV) as a novel contribution to the interpretation of

the test. In this study, two parameters were set: a first cutoff point of 17% for MCF associated with 96% specificity and 70.2% sensitivity and a second cutoff point of 14% for CV showed 92% specificity and 67.7% sensitivity. In our study, the EMA test did not correlate with the clinical severity of HS. These results are similar to those of Arora et al. [34], Park et al. [35], and King et al. [12] who also reported the absence of correlation between MCF values and the need for transfusion in infancy or the decision to perform splenectomy.

In our study, the specificity of the EMA test to HS was evaluated by including a group of patients presenting with different forms of hereditary hemolytic anemia. Interestingly, 87.5% of patients with homozygous sickle cell disease (HbSS) showed a significant increase in EMA fluorescence intensity compared to patients presenting with HbAS, HbSβ, and β-thalassemia. Compared to the other groups, HbSS patients have the highest fractions of the sickled hemoglobin (HbS). Therefore, the significant increase in EMA fluorescence detected in SS cells could be explained by the HbS-mediated clusterization of the cytoplasmic domain of band 3 protein [16]. In fact, HbS has the propensity to polymerize under conditions of low oxygen saturation. This change leads to the instability of the hemoglobin, which in turn impacts the structure and function of band 3 protein. Unstable hemoglobin tends to autoxidize, denature and precipitate as hemichromes which have a high affinity for the cytoplasmic domain of band 3 and are prone to mediate the oxidative cross-linking through the formation of disulfide bonds [36]. Upon band 3 oxidation, tyrosine kinases activated by oxidative stress, proceed to phosphorylate the cytoplasmic domain of band 3 and thus resulting in band 3 clusterization and dissociation from cytoskeletal proteins through disruption of ankyrin binding [36-39].

The EMPE test revealed membrane protein abnormalities in eighteen patients and detected isolated (band 3, ankyrin or spectrin) and combined protein (band 3-protein 4.2 and ankyrin-spectrin) deficiencies. Band 3 defects were the most frequent protein deficiencies found in our population (56%). This result is supported by many studies reporting that band 3 protein, encoded by the *SLC4A1* gene, was the predominant deficiency in HS patients [11, 40-42]. Furthermore, mutations in the *SLC4A1* gene have been reported in association with isolated band 3 deficiency and combined deficiencies of band 3-protein 4.2 observed in SDS-PAGE [5, 15]. Ankyrin protein (*ANK1* gene) defects were found in 39% of Tunisian HS patients. Several studies have been reported that the *ANK1* gene mutations are associated with isolated ankyrin deficiency and combined deficiencies of ankyrin-spectrin, ankyrin-spectrin-protein 4.2, ankyrin-protein 4.2, and spectrin-protein 4.2 detected by SDS-PAGE [5, 15]. Both spectrin and protein 4.2 abnormalities were found in 17% of HS cases. The spectrin protein is composed of two antiparallel heterodimers of α-spectrin and β-spectrin that interact head-to-head to form a tetramer. The α-spectrin and β-spectrin units are encoded by the *SPTA1* and *SPTB* genes, respectively. Mutations in *SPTA1* and *SPTB* genes are associated with an isolated α-spectrin and/or β-spectrin protein deficiency in SDS-PAGE. Furthermore, mutations in the *EPB42* gene encoding the protein 4.2 are reported with a complete protein 4.2 deficiency detected by SDS-PAGE [5, 15]. Ankyrin and spectrin defects were described as the most frequent red cell membrane protein abnormalities in Argentinian, Italian, Mexican, and Spanish populations [10, 43-45], compared to the protein 4.2 deficiency which is more predominant in a Japanese population [5]. This could be explained by the small number of recruited HS patients in our study as well as ethnic differences between the studied populations. The EMPE was normal in one HS patient. This result could be explained by the increased level of reticulocytes (25%), which have a high ankyrin content that could mask an ankyrin deficiency [46]. According to the study of 300 Milan HS patients, between 8% to 11% of patients with HS had no detectable membrane protein reduction. The sensitivity of SDS-PAGE in detecting partial ankyrin deficiency is improved by the splenectomy [15, 39].

In our data, the cutoff value of the PT in HS patients was set at 28%, which was slightly lower than the study of Vettore et al. (28.5%). We reported 72.2% sensitivity and 88.2% specificity with 86% AUC of the ROC curve, as opposed to Vettore et al. who reported a sensitivity of 100% and a specificity of 95% studied in 42 HS patients [7]. Similar to our results, Mariani et al. reported a sensitivity of 78% [40]. Bianchi et al. [47] compared

various HS laboratory tests in 150 HS patients and showed that the EMA test was the best in terms of disease specificity (98%) with a sensitivity (93%) that was comparable to PT and acidified glycerol lysis test (91%; 95%). Along those lines, Bucx et al. [48] demonstrated that a 24h incubation period increased the sensitivity of the PT to 100% while simultaneously decreasing the specificity (72.1%) compared to the same test executed on fresh blood samples (97.4% sensitivity and 94.7% specificity). Albeit suggested as one of a reference test in the guidelines for HS diagnosis [3], the data concerning routine utilization of CT are controversial [8, 9, 21, 29, 40, 49, 50]. Using the CT3 formula, the 2.4% cutoff yielded the best discriminatory value (70% sensitivity and 64.7% specificity) compared to other cutoff values.

The OFT is the most traditional method used for the diagnosis of HS. The test is performed on both fresh and incubated blood which increases the sensitivity of the test. In our study, the sensitivity of the OFT in fresh blood was 45% and in incubated blood was 70% which is lower than those reported in earlier studies [34, 35, 40, 47, 51, 52]. The sensitivity of incubated OFT was higher than that of fresh blood because the stress induced by incubation at 37°C causes an increase in osmotic lysis to a greater extent in the spherocytes than the normal red cells. Normal OFT in fresh or incubated blood does not rule out the possibility of HS, as 10% to 20% of HS patients lack circulating spherocytes and could give off a false negative result even with incubated OFT [5, 52]. Moreover, both tests are labor-intensive, time-consuming, and lack specificity seeing as they can give positive results for HS in other congenital or acquired red cell defects [5, 12, 53].

Conclusion

Our study emphasizes that the EMA test is an efficient tool for HS diagnosis, notwithstanding the nature of the protein deficiency. A combination of the EMA test and the PT gives better results than any single test alone. The EMA test is highly recommended as a diagnostic tool for HS with the availability of a flow cytometer and should be always performed with 6 normal controls. In cases where a flow cytometer is not available, a combination of PT, CT, and incubated OFT are recommended and reached the sensitivity to detect HS. A predominant band 3 protein deficiency in HS Tunisian patients was detected using the EMPE test. We present a set of primary data of HS among Tunisians. Increasing the number of samples is required to strengthen the characterization of HS and related red blood cell membrane diseases at a cellular and molecular level.

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Author Contributions

Conception: Salem Abbes. Acquisition, Analysis, and interpretation of data: Nawel Trabelsi, Ghada Bouguerra, Faten Haddad, Imen Darragi, Imen Boudrigua, Dorra Chaouachi, Mbarka Barmat, Chaker Fouzai. Writing-original draft: Nawel Trabelsi. Writing-review and editing: Nawel Trabelsi, Ghada Bouguerra, Imen Kraiem. Agreement: Salem Abbes, Samia Menif, Monia Ouederni, Mohamed Bejaoui.

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Statement of Ethics

The study was approved by the Ethics Committee of Pasteur Institute of Tunis, Tunisia, in accordance with the Declaration of Helsinki, and the blood samples were collected after obtaining informed consent.

Disclosure Statement

The authors have no conflicts of interest to declare.

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