Effectiveness of one tube osmotic fragility screening in detecting β -thalassaemia trait

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SUMMARY The effectiveness of the one tube method of osmotic fragility with three buffered solutions (0.32% saline, 0.36% saline, and tyrode) as a screening test for β -thalassaemia trait was evaluated in several groups of subjects from Greece, Yugoslavia, and Thailand. The results clearly demonstrated that 0.36% saline is the most sensitive and effective solution since it could detect 96 to 100% of heterozygotes with β -thalassaemia, compared to about 80% with both 0.32% saline and tyrode. However, 0.36% saline gave false positive results in normal subjects and was also positive in haematological disorders which influence osmotic fragility. The screening test with 0.36% saline was applied more precisely in 1371 subjects. The test was false positive in 41 (9.1%) of 455 normal subjects while of 438 confirmed heterozygotes with β -thalassaemia it was positive in 431 (98%) and negative in only seven (2%). The test was also found to be positive in 80% of patients with iron deficiency anaemia and α -thalassaemia trait, in 68% of patients with Hb E trait, in 40% of patients with Hb S trait, and in 78% of heterozygotes with rare haemoglobin variants.

The increased sensitivity and effectiveness of 0.36% saline in detecting β -thalassaemia trait and other disorders influencing osmotic fragility as compared to 0.32% saline and tyrode solutions was also confirmed in a study of 384 unselected schoolchildren.

The β -thalassaemias have been described all over the world,^{1,2} but in certain countries the high prevalence of the abnormal gene causes serious problems in public health. In general, heterozygotes for the β -thalassaemias are characterised by hypochromic microcytic anaemia, decreased red cell osmotic fragility, and raised Hb A₂ or Hb F or both. In addition, there are some rare variants which can be detected only by chain synthesis.³⁻⁵

Complete haematological examination to establish the diagnosis of a heterozygote for β -thalassaemia is laborious and time consuming. For this reason a number of tests have been proposed for screening of heterozygotes with β -thalassaemia. These include osmotic fragility,^{3,6,7} MCV as automatically determined by electronic Coulter counter,^{8,9} and Hb A₂ determination by microcolumn chromatography.¹⁰ Each test has limitations and the precise diagnosis of β -thalassaemia trait in subjects at risk needs further investigation.⁶

A co-operative study was undertaken to evaluate the effectiveness of the simple and cheap one tube

method of osmotic fragility as a screening test to detect heterozygotes with β -thalassaemia, with the main objectives being to recommend it as a single screening test in areas where other methods are not easily applicable and also as a supplement to other screening methods.

Materials and methods

Experiments on the osmotic fragility screening test were carried out in three institutions: in Greece (series Gr), in Yugoslavia (series Y), and in Thailand (series T).

The test was applied to the following groups of subjects who were also investigated haematologically.

NORMAL SUBJECTS

These comprised 155 subjects who were assigned as normal with regard to abnormal haemoglobins and thalassaemia. All had normal haematological data including normal values of Hb A₂, Hb F, and serum iron. In addition, those of series T had no history of Hb Bart's, hydrops fetalis, or Hb H disease in their families.

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β -THALASSAEMIA TRAIT

This group comprised 213 subjects who were genetically designated as having β -thalassaemia trait which was confirmed by complete haematological investigation.

IRON DEFICIENCY ANAEMIA

This group comprised 39 patients with iron deficiency anaemia (series Gr), with Hb less than 10 g/dl and serum iron less than 30 μ g/dl.

 α -THALASSAEMIA TRAIT

This group comprised 15 subjects (series T), offspring or parents of patients with Hb H disease (α -thal 1/ α -thal 2 disease) who thus were obligatory cases of either α -thalassaemia 1 trait or α -thalassaemia 2 trait.

OTHER ABNORMAL HAEMOGLOBIN VARIANTS

This group comprised 40 subjects with abnormal Hb variants, for example, Hb E, Hb S, Hb O-Arab, Hb Lepore, Hb G, and Hb Strumica.

SCHOOLCHILDREN

A total of 384 unselected school children (series Y) was examined by the osmotic fragility screening test. Those who gave positive results in the test were investigated further for the presence of abnormal haemoglobins or thalassaemia.

NORMAL AND β -THALASSAEMIA TRAIT EVALUATED RETROSPECTIVELY

In 300 normal subjects and 225 genetically assigned and haematologically proven heterozygotes for β -thalassaemia from Greece, the results of screening with 0.36% buffered saline were evaluated retrospectively.

Methods

Three buffered saline solutions, 0.32%, 0.36% saline, and tyrode solution, were used in the screening test. For the preparation of the solutions see the appendix.

EXPERIMENT

A sample of 0.02 ml whole blood was pipetted into each of three tubes which contained 5 ml of the 0.32%, 0.36% saline, and tyrode solution, respectively. The pipette was rinsed 2 to 3 times with the buffered solution in the tube. After 5 minutes, the tube was mixed well and the results of the test were evaluated by visualisation as negative, suspicious, and positive.

INTERPRETATION

By visualisation, negative samples were characterised by a clear red haemoglobin solution indicating complete haemolysis of the red cells in the solution, and positive samples by a cloudy or smoky appearance because of incomplete haemolysis of the red cells. Suspicious samples were considered to be those few with a very fine cloudiness.

ESTIMATION OF PERCENTAGE HAEMOLYSIS IN THE SCREENING TEST

To estimate the percentage of haemolysis, the test tube was centrifuged and the haemoglobin (as Hb O₂) in the supernatant solution was measured by absorbance (optical density) in a photoelectric colorimeter at 540 nm. The absorbance corresponding to complete haemolysis of the blood sample was obtained from a haemoglobin solution of 0.02 ml of blood in 5 ml of distilled water. The approximate percentage of haemolysis of each sample was estimated as follows.

$$\% \text{ haemolysis} = \frac{A^{540} (\text{absorbance}) \text{ of sample in the buffered solution}}{A^{540} (\text{absorbance}) \text{ of complete haemolysis of the sample}}$$

The results of the visualised interpretation and percentage of haemolysis in the solution were as follows: negative corresponded to 91 to 100% haemolysis, suspicious to 86 to 90% haemolysis, and positive to less than 85% haemolysis.

HAEMATOLOGICAL DATA

Hb, RBC, PCV, MCV, and MCHC were automatically determined by an electronic Coulter counter, while Hb A₂ was estimated by cellulose acetate electrophoresis and elution and Hb F by alkaline denaturation.

Results**NORMAL SUBJECTS**

The results of the osmotic fragility screening test in 155 normal subjects are summarised in table 1. With the 0.32% saline and tyrode solutions, 99% of the normals gave negative results, with only 1% false positive. By contrast, with the 0.36% saline buffered solution, 9% (8.4% in series Gr and 11.4% in series T) of the normals gave false positive results. The difference in the incidence of the false positives in the two series is not statistically significant ($\chi^2=0.3$; $p<0.5$).

GENETICALLY PROVEN β -THALASSAEMIA TRAIT

The results of the screening are summarised in table 2. Up to 20% of heterozygotes escaped detection with both the 0.32% saline and tyrode solutions. By contrast, 96.7% of those with β -thalassaemia trait were detected with the 0.36%

TABLE 1 Results of osmotic fragility screening test in 155 normal subjects. Figures in parentheses indicate number of subjects

Buffered solution	Series Gr (120)			Series T (35)			Total (155)		
	Neg	Sus (%)	Pos	Neg	Sus (%)	Pos	Neg	Sus (%)	Pos
0.32% saline	99.2 (119)	0	0.8 (1)	100 (35)	0	0	99.4 (154)	0	0.6 (1)
0.36% saline	91.6 (110)	0	8.4 (10)	82.9 (29)	5.7 (2)	11.4 (4)	89.7 (139)	1.3 (2)	9.0 (14)
Tyrode	99.2 (119)	0	0.8 (1)	91.4 (32)	8.6 (3)	0	97.4 (151)	1.9 (3)	0.6 (1)

Neg = negative, Sus = suspicious, Pos = positive.

TABLE 2 Results of osmotic fragility screening test in 213 cases of genetically designated β -thalassaemia trait

Buffered solution	Series Gr (102)			Series Y (91)			Series T (20)			Total (213)		
	Neg	Sus (%)	Pos	Neg	Sus (%)	Pos	Neg	Sus (%)	Pos	Neg	Sus (%)	Pos
0.32% saline	14.7 (15)	0	85.3 (87)	18.7 (17)	5.5 (5)	75.8 (69)	5.0 (1)	10.0 (2)	85.0 (17)	81.2 (173)	3.3 (7)	15.5 (33)
0.36% saline	3.9 (4)	0	96.1 (98)	2.2 (2)	1.1 (1)	96.7 (88)	0	0	100.0 (20)	96.7 (206)	0.4 (1)	2.8 (6)
Tyrode	14.7 (15)	0	85.3 (87)	9.9 (9)	8.8 (8)	81.3 (74)	20.0 (4)	0	80.0 (16)	83.1 (177)	3.7 (8)	13.1 (28)

saline buffered solution, which appears to be quite satisfactory as a screening test. This was substantiated by the retrospective evaluation of the results of screening with the 0.36% saline buffered solution in 225 cases of genetically proven β -thalassaemia trait and in 300 normal subjects in Greece (table 3). Of those with β -thalassaemia

trait, 99% showed positive results in the test. However, the 0.36% saline screening test also gave false positive results in around 10% of the normal subjects. This figure is compatible with that in table 1.

These findings strongly suggest that by using 0.36% buffered solution as a screening test, almost all subjects with the β -thalassaemia trait can be detected.

TABLE 3 Retrospective evaluation of osmotic fragility screening with 0.36% saline buffered solution in 225 cases of genetically assigned β -thalassaemia trait and in 300 normal subjects in Greece (series Gr)

Buffered solution	Normal (300)		β -thalassaemia trait (225)	
	Neg	Pos	Neg	Pos
0.36% saline	89.6% (269)	10.4% (31)	0.9% (2)	99.1% (223)

IRON DEFICIENCY ANAEMIA AND OTHER ABNORMAL HAEMOGLOBINS

In addition to β -thalassaemia trait, it is of interest to know whether or not iron deficiency anaemia, other common abnormal haemoglobins, and α -thalassaemia can be detected by this screening test. Table 4 summarises the results of screening tests for these.

TABLE 4 Results of osmotic fragility screening test in iron deficiency anaemia, obligatory α -thalassaemia trait, and abnormal haemoglobin variants.

Groups of patients	Tyrode		0.32% saline		0.36% saline	
	Neg	Pos	Neg	Pos	Neg	Pos
Iron deficiency anaemia	43.6% (17)	56.4% (22)	46.1% (18)	53.8% (21)	20.5% (8)	79.4% (31)
α -thalassaemia trait	33.2% (5)	66.8% (10)	33.2% (5)	66.8% (10)	20.0% (3)	80% (12)
Hb E trait	56.2% (9)	43.8% (7)	69.7% (11)	31.3% (5)	31.2% (5)	68.8% (11)
Hb S trait	80% (12)	20% (3)	86.6% (13)	13.3% (2)	60% (9)	40% (6)
Rare abnormal Hb	33.3% (3)	66.7% (6)	32.2% (3)	77.8% (7)	22.2% (2)	77.8% (7)

As seen in table 4 the 0.36% saline solution generally gave more positive results than the 0.32% saline and the tyrode solutions did.

Thus of 15 obligatory cases of α -thalassaemia trait (either α -thalassaemia 1 trait or α -thalassaemia 2 trait), 12 (80%) of 15 cases were positive and three (20%) negative. The mean haematological values of the positive cases revealed definite hypochromia and microcytosis similar to those of the parents of cases of Hb Bart's hydrops fetalis,¹¹ while the mean haematological values of the three negative cases had milder red cell abnormalities. It is likely that those with the positive screening test represented α -thalassaemia 1 trait and those with the negative results the α -thalassaemia 2 trait.

The 0.36% saline test was also positive in 80% of patients with iron deficiency anaemia, in 69% of those with Hb E trait, in 40% of those with Hb S, and in seven (78%) of nine subjects with rare abnormal Hb variants, namely Hb Lepore trait (2) and Hb G trait (1).

SCHOOLCHILDREN

The results of the osmotic fragility screening test in 384 unselected school children in Yugoslavia are shown in table 5. With the 0.36% saline solution 9.6% were positive compared to only 2.3% and 4.1% with the 0.32% and tyrode solutions, respectively. All subjects with the positive screening test were studied further and the results are also listed in table 5.

The 0.32% saline and the 0.36% saline solution appeared to detect four cases of β -thalassaemia trait while the tyrode solution detected only two. Like the previous results the 0.36% saline seemed to be more sensitive than the 0.32% saline solution, since the former detected two more subjects with Hb O-Arab and four more with iron deficiency anaemia than the

latter. However, the remaining 22 children who were positive with the 0.36% saline had normal haematological data. Thus the positive screening test in the 22 (5.7%) children was believed to be false positive.

Discussion

Screening for β -thalassaemia is extremely difficult. This is mainly because of the heterogeneity of β -thalassaemias and the absence of a single pathognomonic finding to cover all β -thalassaemia variants.^{2 3 6} In spite of these difficulties, many attempts have been made to establish a screening test capable of detecting all β -thalassaemia variants. The proposed tests are osmotic fragility, MCV as automatically determined by electronic counter, and estimation of Hb A₂ by microcolumn chromatography. Apart from the osmotic fragility test the two others need well-equipped laboratories and technical experience. The osmotic fragility screening method has additional advantages as it is simple, cheap, and easy to apply. From the results of this study it was shown that the one tube method of osmotic fragility with 0.36% saline buffered solution was effective for screening of heterozygotes with β -thalassaemia. Although the test was effective in detecting almost 100% of subjects with β -thalassaemia trait, it was also positive in a number of subjects with iron deficiency anaemia, α -thalassaemia trait, Hb E, and Hb S trait, and false positive in nearly 10% of normal subjects. Similar results were reported by Cao *et al*¹² in Sardinia with 0.40% buffered NaCl. The test gave false negative results in 3.6% of heterozygotes with β -thalassaemia and false positive results in 6.5% of normal subjects. In these subjects other haematological disorders affecting osmotic fragility were not excluded.

On the other hand, screening of osmotic fragility with the two other solutions (0.32% saline and tyrode) was less effective.

It is well known that there are limitations to all screening tests proposed for the detection of heterozygotes for β -thalassaemia. However, all screening tests give valuable information by excluding normal subjects and thus restricting further investigation for the precise diagnosis to the small proportion of suspected subjects. To this end osmotic fragility seems to be a most valuable test to be used as a single screening test in areas with limited laboratory facilities and economic resources.

To increase the effectiveness of screening a combination of tests, which includes osmotic fragility, is used by a number of laboratories.^{6 11 12} Thus, the screening for thalassaemia includes complete blood count (MCV and MCH), osmotic

TABLE 5 Results of osmotic fragility screening in 384 unselected school children (series Y). Positive results for the test were investigated further for final diagnosis

Buffered solution	Osmotic fragility screening		
	Neg	Sus	Pos
0.32% saline	95.1% (365)	2.6% (10)	2.3% (9) A
Tyrode	84.3% (327)	10.6% (41)	4.1% (16) B
0.36% saline	84.1% (323)	6.2% (24)	9.6% (37) C

A. Of the 9 children, 4 had β -thalassaemia trait, 3 Hb Lepore trait, and 2 iron deficiency anaemia.

B. Of the 16 children, 2 had β -thalassaemia trait, 3 Hb Lepore trait, 2 iron deficiency anaemia, and 9 had normal haematological findings.

C. Of the 37 children, 4 had β -thalassaemia trait, 3 Hb Lepore trait, 2 Hb O-Arab trait, 6 iron deficiency anaemia, and 22 had normal haematological data.

fragility, and haemoglobin electrophoresis.⁶ Even with this combination, precise diagnosis is not easy and has to be established with appropriate additional laboratory investigations before any attempt at genetic counselling.

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APPENDIX Preparation of solutions

A 0.32% AND 0.36% SALINE BUFFERED SOLUTION¹³

Stock solution. 10% NaCl buffered solution is made as follows: 1.54 mol/l NaCl (90 g); 0.0961 mol/l Na_2HPO_4 (13.65 g); and 0.0156 mol/l $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (2.43 g) are dissolved in distilled water and the final volume is adjusted to 1 litre. The solution will keep in a well stoppered bottle.

1% NaCl buffered solution. In preparing the testing solutions, it is convenient to make first a 1% solution from the 10% stock solution by dilution with water.

Testing buffered solutions. The 0.32% and 0.36% saline buffered solutions are prepared by a dilution of 320 and 360 ml, respectively, of the 1% NaCl solution with distilled water to make 1 litre.

B TYRODE SOLUTION

Concentrated solution. The solution is made as follows: 0.6 mmol/l NaHCO_3 (0.05 g); 0.7 mmol/l $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (0.1 g); 0.1 mmol/l $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.2 g); 1.8 mmol/l CaCl_2 (0.2 g); 2.7 mmol/l KCl (0.2 g); 0.14 mol/l NaCl (8.2 g) are dissolved in that order in distilled water and the final volume is adjusted to 1 litre.

Testing solution. The tyrode solution is prepared by a dilution of 4 parts of the concentrated solution with 6 parts of distilled water.

Remarks. The chemicals must be pure and completely dried.

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