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Effectiveness of red cell osmotic fragility test with varying degrees of saline concentration in detecting beta-thalassaemia trait

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ABSTRACT

Introduction: This study was performed with the objective of determining the efficacy of naked eye single tube red cell osmotic fragility test (NESTROFT) as a screening test for beta-thalassaemia trait, and to standardise a saline concentration which could give best results with minimum error and maximum sensitivity and specificity.

Methods: Five concentrations (0.35 percent, 0.36 percent, 0.37 percent, 0.38 percent and 0.39 percent) of buffered saline solutions were used. NESTROFT was applied to three groups of subjects: 24 normal individuals, 87 subjects with genetically-proven beta-thalassaemia trait and 13 patients with proven iron-deficiency anaemia.

Results: The results demonstrated that 0.36 percent was the best saline concentration for NESTROFT. It could detect 97.7 percent of heterozygous beta-thalassaemia patients, compared to 94.25 percent, 91.95 percent, 88.51 percent and 82.76 percent detection rates obtained with 0.35 percent, 0.37 percent, 0.38 percent and 0.39 percent saline concentrations, respectively. Specificity of NESTROFT with 0.36 percent saline was also higher at 83.3 percent, whereas that of 0.35 percent, 0.37 percent, 0.38 percent and 0.39 percent was 79.17 percent, 79.17 percent, 70.83 percent and 62.5 percent, respectively. This test with 0.36 percent saline concentration was also positive for three (23.08 percent) patients with iron-deficiency anaemia.

Conclusion: NESTROFT done with 0.36 percent buffered saline solution provides more accurate results compared to the other concentrations tested. Since the test is sensitive, cost-effective, rapid and reliable, it may be considered as the single screening test to be used in areas with limited laboratory facilities and economic resources.

Keywords: anaemia, beta-thalassaemia trait, naked eye single tube red cell osmotic fragility test (NESTROFT), red blood cell osmotic fragility, thalassaemia

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INTRODUCTION

The incidence of beta-thalassaemia in different regions of India varies from 3% to 17%,[1] with a mean prevalence of 4%.[2] It is estimated that there are about 45 million carriers of the beta-thalassaemia gene in India, while about 15,000 affected infants are born every year, contributing to about 10% of the total thalassaemics born all over the world.[3] However, only 10%–15% of these children receive optimal treatment.[4] Treatment may extend the life of the thalassaemic individual into early adulthood, but it is very cumbersome and costly, mainly consisting of blood transfusions and expensive iron chelation therapy. The only cure available today is bone marrow transplantation, which is risky and too costly for most of the patients. Thus, the birth of a thalassaemic child places considerable strain, not only on the affected child and its family, but also on the community and the nation at large. With these limitations, emphasis must shift from treatment to prevention of such births in the near future. Various screening tests for carrier screening have been developed, such as determination of red cell indices, haemoglobin A (HbA), haemoglobin A_2 (HbA_2), and foetal haemoglobin (HbF) level estimation. However, all these techniques are time consuming and expensive for population screening. Therefore, there is a need for a simple, low cost, rapid and reliable technique for the screening of the masses. The present study evaluates the efficacy of naked eye single tube red cell osmotic fragility test (NESTROFT) as a screening test for detecting heterozygous beta-thalassaemia.

METHODS

A total of 124 individuals were registered for the present study and the subjects were divided into three groups. Group I comprised 24 normal individuals with normal haematological parameters, group II consisted of 87 genetically-proven carriers, and group III included 13 individuals with proven iron-deficiency anaemia. Blood

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Table I. Results of NESTROFT with different dilutions of saline in normal, beta-thalassaemia trait and iron-deficiency subjects.

Buffered saline solution concentration (%)	Positive result			Negative result			Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Efficiency of test (%)
	Normal (n = 24) (FP)	β-thal (n = 87) (TP)	Iron def. (n = 13)	Normal (n = 24) (TN)	β-thal (n = 87) (FN)	Iron def. (n = 13)					
0.35	5	82	-	19	5	13	94.25	79.17	94.25	79.17	90.9
0.36	4	85	3	20	2	10	97.7	83.3	95.5	90.9	94.6
0.37	5	80	-	19	7	13	91.95	79.17	94.12	73.08	89.2
0.38	7	77	-	17	10	13	88.51	70.83	91.67	62.96	84.7
0.39	9	72	-	15	15	13	82.76	62.5	88.89	50.0	78.4

samples of each individual were drawn and subjected to a battery of tests, including NESTROFT, complete haemogram and HbA_2 estimation. $HbA_2 > 3.5\%$ was treated as the gold standard for the diagnosis of the thalassaemia trait. The procedures followed were in accordance with the ethical standards of the regional committee on human experimentation.

A 10% stock solution of buffered saline was prepared by dissolving 90 g NaCl, 13.655 g Na_2PO_4 and 2.4 g $NaH_2PO_4.2H_2O$ in 1,000 ml of distilled water. From this 10% stock solution, 1% buffered saline was prepared by 1:10 dilution with distilled water. Five buffered saline solutions with concentrations 0.35%, 0.36%, 0.37%, 0.38% and 0.39%, were prepared by further diluting 35 ml, 36 ml, 37 ml, 38 ml and 39 ml of 1% buffered saline solution with 65 ml, 64 ml, 63 ml, 62 ml, and 61 ml of distilled water, respectively, to make 100 ml of each solution. The test was performed using different concentrations of buffered saline solution. 2 ml of each of five concentrations of buffered saline was taken in five separate test tubes (10 × 1 cm diameter) each and 2 ml of distilled water was taken in another tube, so that there was a set of six test tubes. EDTA blood (20 μL) was added to each of these six tubes. The tubes were shaken well and left undisturbed for half an hour at room temperature. After 30 minutes, the contents of all these tubes were shaken and the tubes were held against a white paper on which a thin black line was already drawn.

The line was clearly visible through the contents of the tube containing distilled water (control). If the line was similarly visible through the contents of the tubes with buffered saline, then the test was considered as negative; if the line was not visible, then the test was considered positive. In the case of a blurred line, the test was considered doubtful. The interpretation of this doubtful result was taken as being positive. In the case of carriers, if the test was positive, it was called true positive (TP), and if negative, it was called false negative (FN). On the other hand, in case of normal samples, if the test was negative, it was called true negative (TN), and if positive, it was called false positive (FP). The principle of NESTROFT is based on the limit of hypotonicity which the red cells can withstand. There is a pronounced decrease in osmotic fragility of red cells in beta-thalassaemia.

Sensitivity, specificity, positive and negative predictive values and efficiency of the test were calculated as validity statistics by the following formulae:

Sensitivity:	$100 \times TP \div (TP+FN)$
Specificity:	$100 \times TN \div (TN+FP)$
Positive predictive value:	$100 \times TP \div (TP+FP)$
Negative predictive value:	$100 \times TN \div (TN+FN)$
Efficiency of test:	$100 \times (TN+TP) \div (TP+FP+TN+FN)$

RESULTS

The results are summarised in Table I. With 0.35%, 0.36%, 0.37%, 0.38% and 0.39% buffered saline solutions, 19, 20, 19, 17 and 15 of the normal individuals gave negative results corresponding to a specificity of 79.17%, 83.3%, 79.17%, 70.83%, and 62.5%, respectively. The rates of FP results among normal individuals were 5.8%, 4.6%, 5.8%, 8.05% and 10.3% with five different dilutions of saline (0.35%, 0.36%, 0.37%, 0.38% and 0.39%, respectively). The rates of FN results among those with beta-thalassaemia minor patients were 5.75%, 2.29%, 8.05%, 11.5% and 17.24% for 0.35%, 0.36%, 0.37%, 0.38% and 0.39% buffered saline, respectively. By contrast, 97.7% of those with beta-thalassaemia trait were detected with 0.36% buffered saline, which is quite satisfactory for a screening test. The sensitivity of the other saline concentrations for detecting beta-thalassaemia trait was comparatively low with 94.25%, 91.95%, 88.51% and 82.76% for 0.35%, 0.37%, 0.38% and 0.39% saline solutions, respectively.

With 0.36% buffered saline, the positive predictive

Table II. Usefulness of NESTROFT in the detection of heterozygous beta-thalassaemia: comparison of data with previous studies.

	Present study	Kattamis et al.[9] (1981)	Mehta et al.[6] (1988)	Gorakshaker et al.[7] (1990)	Raghavan et al.[8] (1991)	Thomas et al.[10] (1996)	Manglani et al.[13] (1997)	Maheshwari et al.[11] (1999)	Sirichotiyakul et al.[12] (2004)
Sensitivity (%)	97.7	98.4	95.0	98–100	95.5	98.4	94.4	91.0	97.6
Specificity (%)	83.3	91.0	82.1	82–84	87.0	66.6	64.2	95.0	72.9
Positive predictive value (%)	95.5	91.3	73.1	50.0	70.5	81.5	97.6	55.0	33.6
Negative predictive value (%)	90.9	98.3	97.0	99–100	98.3	96.5	35.3	99.0	99.5

value for the test was 95.5%, which is higher than those for other dilutions; 94.25% for 0.35%, 94.12% for 0.37%, 91.67% for 0.38% and 88.89% for 0.39% saline, respectively. Negative predictive value of the test for 0.36% saline (90.9%) was also higher than that of 0.35% saline (79.17%), 0.37% saline (73.08%), 0.38% saline (62.96%) and 0.39% saline (50%). With 0.36% buffered saline, the efficiency of the test was 94.6%, indicating that only 5.4% (error rate of the test) of all the subjects were misclassified. The error rates for 0.35%, 0.37%, 0.38% and 0.39% saline solutions were 9.1%, 10.8%, 15.3% and 21.6%, respectively. The osmotic fragility test with 0.36% saline in 13 subjects with iron-deficiency anaemia was positive for 3 (23.08%) patients. No other saline concentration gave a positive test for iron-deficiency anaemia.

DISCUSSION

Any programme for the prevention of Cooley's anaemia requires, as a preliminary step, the reliable identification of young people with thalassaemia. Screening for beta-thalassaemia trait is extremely difficult. This is mainly because of the heterogeneity of beta-thalassaemias and the absence of a single test to unravel all beta-thalassaemia variants.[5] In spite of these difficulties, many attempts have been made to establish a screening test capable of detecting all beta-thalassaemia variants. Various screening tests for carrier screening have been developed, viz. determination of red cell indices, HbA, HbA_2, and HbF level estimation; however, all these techniques are time consuming and expensive for population screening. This study showed that the concentration of 0.36% buffered saline was more efficient in detecting heterozygous beta-thalassaemia patients than the four other saline strengths (i.e. 0.35%, 0.37%, 0.38% and 0.39%). Similar concentration of buffered saline (i.e. 0.36%) has been used by various workers to study its effect on the osmotic fragility of red cells and working out the reliability of this concentration in detecting the beta-thalassaemia trait.

Table II compares the sensitivity, specificity, positive and negative predictive values of the NESTROFT using 0.36% buffered saline in the present study with those of other similar studies. Though all the previous reports have shown a sensitivity that is above 91%, the specificity has varied from 64.2% to 95%. The sensitivity of NESTROFT in the present study was 97.7%, which is quite comparable with the sensitivity reported in other studies (Table II). The specificity in the present study was 83.3%, which is comparable to results obtained by Mehta et al,[6] Gorakshakar et al[7] and Raghavan et al.[8] The negative predictive value of the test in carriers during the present study was 90.9%. The result is low but comparable with the studies of Kattamis et al,[9] Mehta et al,[6] Gorakshakar et al,[7] Raghavan et al,[8] Thomas et al,[10] Maheshwari et al[11] and Sirichotiyakul et al,[12] who reported values of 98.3%, 97.0%, 99%–100%, 98.3%, 96.5%, 99.0% and 99.5%, respectively.

In the present study, the test with 0.36% buffered saline led to both high sensitivity and specificity; this is a desirable factor for judging the effectiveness of a screening test. Calculation of the negative predictive value in the test almost rules out the possibility of beta-thalassaemia trait in the general population. The application of this test for screening the cases before further investigations would reduce financial implications faced in performing other costly tests on the general population. The positive predictive value of the test has significance in a particular population with high prevalence of the disease. The positive predictive value was quite high (95.5%) and comparable to the studies conducted by Kattamis et al,[9] and Manglani et al,[13] who reported values of 91.3% and 97.6%, respectively, and higher than 73.1% reported by Mehta et al,[6] 50% reported by Gorakshaker et al,[7] 70.5% reported by Raghavan et al,[8] 81.5% reported by Thomas et al,[10] 55% reported by Maheshwari et al,[11] and 33.6% reported by Sirichotiyakul et al.[12]

Although screening of the thalassaemia trait using 0.36% buffered saline was successful in detecting 97.7% of subjects with this trait, it was also positive in 23.08% of subjects with severe iron-deficiency anaemia and

gave a FP test in 16.7% of the normal individuals, which is quite comparable with 18.5% reported by Gomber et al.[14] Although this test is easy to perform, fast, cheap and does not require sophisticated equipment, there are certain limitations to this test. As observed during the study, it gives FP results in the case of patients with iron-deficiency anaemia. This would affect the specificity of the test in a population with a high incidence of iron-deficiency anaemia. Therefore, subjects positive with NESTROFT need to undergo further investigations to confirm the diagnosis. The test also needs careful standardisation.

The prevalence of beta-thalassaemia in India varies in different regions. The estimated predictive values for negative and positive NESTROFT will vary depending upon the prevalence of other diseases among the patients evaluated in this study. NESTROFT with 0.36% buffered saline still showed a very high negative predictive value. The present data therefore confirms that a negative NESTROFT is very useful in ruling out beta-thalassaemia. NESTROFT has thus emerged as an inexpensive, most sensitive and specific test of population screening for the beta-thalassaemia trait, and is considered suitable for large scale use in developing countries like India, which has limited financial and technical resources.

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