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Original Research Article

Experience with NESTROFT for screening for thalassemia trait/ minor: evaluation against CBC and HPLC in a high prevalence region in Saurashtra, Gujarat, India

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ABSTRACT

Background: Hemoglobinopathies pose a significant health burden in India. Prevention programmes can significantly reduce this burden. Although sophisticated methods of screening for β thalassemia trait are available, a cheap and simple method is beneficial for population screening. Although the Naked Eye Single Tube Red Cell Osmotic Fragility Test (NESTROFT) has been evaluated in many studies, sample sizes were small in some and many earlier studies have not done complete blood count (CBC) and High-Performance Liquid Chromatography (HPLC) in all the cases. We evaluate the suitability of NESTROFT for detection of β-thalassemia trait in a high prevalence region in Saurashtra, Gujarat.
Methods: Here, 1000 unrelated individuals were studied. NESTROFT, CBC and estimation of HbA2 and HbF or other hemoglobin variants were done by HPLC.
Results: Prevalence of β thalassemia trait was 7.8% in this population. NESTROFT showed an overall sensitivity and specificity of 94.87 and 85.38 respectively for the detection of β thalassaemia trait. Using red cell indices (MCH <27 pg and MCV <80 fl), One β thalassemia trait with normal indices would have been missed. Among twelve individuals with other hemoglobinopathies (HbS, HbD, HbE, δβ thalassemia trait or HPFH), seven had a positive NESTROFT while three had normal MCV & MCH values.
Conclusions: NESTROFT is a cost-effective sensitive test which does not require any equipment and can be done in remote areas. It remains a useful first line screening test when large populations have to be screened.

Keywords: Beta thalassemia trait, Carrier screening, Complete blood count, High performance liquid chromatography, Naked eye single tube red cell osmotic fragility test

INTRODUCTION

The thalassemia syndromes are a heterogeneous group of single gene disorders inherited in an autosomal recessive manner. Approximately 7% of the global population are carriers of hemoglobin disorders including the β and α thalassemia's, sickle cell disorders and other hemoglobinopathies. These disorders being common mainly in the Mediterranean region, Africa, Southeast Asia and the Indian subcontinent.[1] β Thalassemia is the commonest single gene disorder in India. With the average prevalence of carriers being 3-4% in the country,

it is estimated that there would be 35-45 million heterozygotes of β thalassemia in the Indian population.[2,3] The Saurashtra region in Gujarat has been shown to have much higher carrier rates than other districts in this state.[4] In communities like the Kutchi Bhanushalis and Lohanas the prevalence of carriers varies from 5 to 17%.[2-5] It is thus important to intensify screening programs to identify β thalassemia carriers and offer them genetic counseling before marriage or identify couples at-risk before reproduction.

In this study authors have evaluated the simple and inexpensive Naked Eye Single Tube Red Cell Osmotic Fragility Test (NESTROFT) as a preliminary screening test against using the red cell indices and the more sophisticated High Performance Liquid Chromatography (HPLC) for picking up β thalassemia traits in this high prevalent region in and around Rajkot city.

METHODS

Inclusion criteria

- Only unrelated individuals of 18 to 35 years of age were included.

Exclusion criteria

- Individuals less than 18 years & over 35 years of age. Family relatives were also not included in this study.

Study Period was January 2018 to June 2018 (6 months). A total of 1000 subjects were screened, majority in the age group of 18 to 35 years. They included 491 (49%) individuals from thalassemia screening camps in colleges, 213 (21%) antenatal women coming to two government hospitals, 155 (16%) individuals who voluntarily came for screening and 141 (14%) individuals referred by others. The study was approved by the Institutional Review Board.

Blood samples were collected in EDTA after an informed consent. NESTROFT, Complete Blood Count (CBC) and HPLC analysis were done in all individuals. NESTROFT visually assesses osmotic fragility of red cells in a single tube using 0.36% buffered saline. The working solution (0.36% buffered saline) was prepared freshly by diluting the 10% stock solution with distilled water. 20 µl blood was added to 2 ml of the working solution, the tube mixed and stood for 30 minutes at room temperature. The tube was held against a white paper with a thin black line.[6,7] If the line was visible the test was negative and if it was not visible it was positive. A fresh blood sample of a known β thalassemia trait served as a positive control and a blood sample taken in distilled water as a negative control.

CBC was done using a three-part cell counter (Sysmex XP100, Transasia, Japan). Mentzer index was calculated using the formula (MCV/RBC). A value of <13 was used as a cut off for picking up beta thalassemia traits.

The percentage of HbA2, HbF and other hemoglobin variants were measured by HPLC using the Variant II analyzer (Bio-Rad Laboratories, Hercules, USA). The cut off value of HbA2 taken to indicate a β thalassemia carrier was ≥4.0%.

Statistical analysis

Sensitivity, specificity, positive and negative predictive values of NESTROFT, MCV, MCH and Mentzer index were calculated taking the HPLC findings as the gold standard.

RESULTS

Among the 1000 individuals screened, 78 individuals (7.8%) were diagnosed as β thalassemia trait using HPLC as the gold standard. 12 individuals had other hemoglobinopathies which included Hb S trait- 4; Hb D Punjab trait- 6; Hb E trait- 1 and δβ thalassemia trait/HPFH trait-1. This group of individuals with other hemoglobinopathies were analysed separately.

The findings of NESTROFT, MCV, MCH and the Mentzer index in the β thalassemia traits (HbA2 ≥4.0%) vs individuals not having β thalassemia trait (HbA2 <4.0%). NESTROFT was positive in 74 out of the 78 β thalassemia carriers as well as in 133 individuals who did not have β thalassemia trait. MCV was normal (≥80 fl) in one β thalassemia trait and MCH was also normal (≥27 pg) in one trait. The Mentzer index was suggestive of β thalassemia trait (<13) in 61 individuals.

Among the 133 individuals who were NESTROFT positive but did not have β thalassemia trait, 58 individuals had MCV<80 fl, MCH<27 pg and RBC count ≥4.5 million/cmm. These 58 individuals (5.8 %) could possibly having other hemoglobinopathies (Table 1).

Sensitivity, specificity, positive and negative predictive values of NESTROFT, MCV, MCH and Mentzer index for picking up β thalassemia traits.

The highest sensitivity was seen with MCV and MCH while Mentzer index showed the highest specificity. All the four parameters had a high negative predictive value (98.12-99.83) but the positive predictive value varied from 19.74 to 75.3 for the different parameters (Table 2).

The results of NESTROFT and the haematological findings in the 12 individuals with other hemoglobinopathies. In this group, 7 individuals were NESTROFT positive and 9 had reduced MCV and MCH levels (MCV<80 fl, MCH<27 pg). 3 Hb D Punjab traits, 1 HbS trait and 1 Hb E trait were missed by NESTROFT, while using red cell indices, 2 Hb D Punjab traits and 1 Hb S trait were missed (Table 3).

Table 1: NESTROFT, MCH, MCV and Mentzer index in β thalassemia carriers (Hba2 ≥4.0%) and non-carriers of β thalassemia (Hba2 <4.0%).

HPLC findings	No. of individuals	NESTROFT		MCH		MCV		Mentzer index	
		Positive	Negative	<27 PG	≥27PG	<80 FL	≥80 FL	<13	≥13
HBA_2 ≥4%	78	74	4	77	1	77	1	61	17
HBA_2 <4%	910	133	777	313	597	313	597	21	889

Table 2: Sensitivity and specificity of different parameters for picking up β thalassemia carriers.

	NESTROFT	MCH	MCV	Mentzer index
Sensitivity (%)	94.87	98.71	98.71	78.2
Specificity (%)	85.38	65.6	65.6	97.8
PPV (%)	35.75	19.74	19.74	75.3
NPV (%)	99.49	99.83	99.83	98.12

PPV=Positive predictive value, NPV=Negative predictive value

Comparison of sensitivity and specificity of NESTROFT in the present study with earlier reports where apart from NESTROFT, CBC and Hb analysis either by HPLC or by cellulose acetate electrophoresis and elution was done in all the individuals studied. The sensitivity and specificity have varied from 78.48-100% and 63.38-100% respectively in the different studies. These findings are comparable to these studies. Although authors negative predictive value was very high (99.49) the positive predictive value was low (35.75). 3 of the 12 earlier studies with high negative predictive values also had a lower positive predictive value using NESTROFT (Table 4).

DISCUSSION

The purpose of this study was to evaluate the effectiveness of NESTROFT for preliminary screening for beta thalassaemia traits in a region in western India where the prevalence of carriers is high. An earlier study on the prevalence of beta thalassemia carriers in different districts of Gujarat had shown that the highest prevalence was seen in the Saurashtra region varying from 3.3-9.5% in different districts and it was 5.2% in Rajkot. The population of Gujarat is 314,912,60 and the population of Rajkot is 14,67,607 as per the Census of India 2011. Hence screening for beta thalassemia trait is very important in Rajkot where authors found the prevalence of beta thalassemia carriers to be 7.8% in the present study.

Authors found the sensitivity and specificity of NESTROFT to be 94.87% and 85.32% respectively for picking up β thalassemia carriers. Different studies undertaken between 1981 to 2016 where NESTROFT, CBC & Hb analysis was done in all cases have shown a variable sensitivity of NESTROFT ranging from 78.48 to 100% and specificity ranging from 63.38 to 100% (Table 4). Different target populations were studied in these reports. Kattamis et al, first described the one tube osmotic fragility test for screening to detect β thalassemia carriers with different saline gradations and found the concentration of 0.36% to be the most suitable.[6] Gorakshaker et al, found a sensitivity of 98.1-100% and specificity of 83.5-84.8% when the results were recorded by three different observers.[7] Hence there were no significant variations when different persons visually recorded the results.

Table 3: NESTROFT, CBC and Hemoglobin analysis in the other hemoglobinopathies detected.

HPLC	NEST ROFT	HB (G/DL)	RBC (X 10^6 /UL)	HCT (%)	MCV (FL)	MCH (PG)	MCHC (G/DL)	RDW	HB A_2 (%)	HB F (%)	HB S/HB D/HB E (%)
Hb D Punjab trait	Positive	11.2	5.09	34.3	67.4	22.0	32.7	15.7	3.0	0.2	Hb D 30.6
Sickle cell trait	Negative	15.5	5.36	43.1	80.4	28.9	36.0	12.1	3.0	1.6	Hb S 37.8
Hb E trait	Negative	12.0	4.62	34.5	74.7	26.0	34.8	14.9	0.0	0.4	Hb E 25.1
Hb D Punjab trait	Negative	12.3	4.76	34.8	73.1	25.8	35.3	13.7	3.2	0.3	Hb D 35.1
HPFH or delta beta thalassemia trait	Positive	11.8	4.69	34.7	74.0	25.2	34.0	15.8	2.7	14.8	
Sickle cell trait	Positive	9.9	5.27	28.2	53.5	18.7	35.1	14.4	3.9	0.3	Hb S 25.5
Sickle cell trait	Positive	12.1	4.62	34.9	75.5	26.2	34.7	13.6	3.3	0.4	Hb S 35.4
Hb D Punjab trait	Negative	15.6	5.30	43.2	81.5	29.4	36.1	12.6	2.4	0.3	Hb D 39.0
Hb D Punjab trait	Negative	11.9	4.56	34.0	74.6	26.1	35.0	15.2	2.3	0.2	Hb D 37.7
Sickle cell trait	Positive	11.4	4.37	33.5	76.7	26.1	34.0	14.0	2.6	1.0	Hb S 35.8
Hb D Punjab trait	Positive	12.1	4.21	36.6	86.9	28.7	33.1	13.6	1.7	1.9	Hb D 31.4
Hb D Punjab trait	Positive	12.1	4.66	36.4	78.1	26.0	33.2	12.9	0.6	0.2	HB D 37.5

Table 4: Sensitivity, specificity and predictive values of NESTROFT in different studies.

Reference	Number of individuals screened	Target population	Methods used	Sensitivity %	Specificity %	PPV %	NPV %
Kattamis et al[6]	1371	Several groups of subjects from Greece, yugoslavia, Thailand	NESTROFT (0.32% saline, 0.36% buffered saline, tyrode); CBC; cellulose acetate electrophoresis	98.4	91	91.3	98.3
Gorakshake R et al[7]	380	Gujaratis, Sindhis of Mumbai	NESTROFT (results recorded by 3 observers), CBC, cellulose acetate electrophoresis	98.1-100	83.5-84.8	48.6 - 50.9	99.6 - 100
Thomas et al[10]	137	Patients with suspected haemoglobin disorders	NESTROFT, HPLC	98.7	66.6	87	96.5
Gomber et al[11]	253	Group i-relatives of thalassaemia major patients, group ii-normal children	NESTROFT, CBC, cellulose acetate electrophoresis	Group I 95.59 group II 85.71	Group I 84.1 Group II 81.7		99.21
Bobhate et al[12]	110	Persons suspected for thalassemia, persons from high risk community, parents and siblir of known thalassaemic major patients	NESTROFT, Hba_2 estimation by electrophoresis	97.1	100	100	98
Chow et al[13]	148	Randomly selected individuals	NESTROFT (0.32%,0.34%,0.36% buffered saline), CBC, HPLC	95	86	94	88
Singh et al[14]	124	Group I-normal persons, grou II-persons with genetically proven beta thalassemia trait, group ii-persons with proven iron deficiency anaemia	NESTROFT (0.35%,0.36%,0.37%, 0.38%, 0.39% buffered saline), red cell indices and Hba_2 estimation	97.7	83.3	95.5	90.9
Sumera et al[15]	503	Group I-normal persons, grou II-microcytosis with normal ferritin and Hba2 >3.5, group iii-person with proven iron deficiency anaemia	NESTROFT, CBC, serum ferritin, HB electrophoresis	93	88	74	97
Chakrabarti et al[16]	500	Antenatal women at a rural tertiary care hospital	NESTROFT, CBC, HPLC	94.12	95.23	41.02	99.78
Prasad et al[17]	84	Suspected cases of beta thalassemia and other haemoglobinopathy	NESTROFT (0.35%,0.36%,0.37%, 0.38%, 0.39% buffered saline), red cell indices and HPLC	92.31	63.38		
Mendiratta et al[18]	1000	Antenatal women	NESTROFT, CBC, HPLC	78.48	94.14	53.45	98.08
Ismail et al[19]	1702	Family members of 130 thalassemia major patients	NESTROFT (0.32%, 0.34%, 0.36% buffered saline) CBC, HPLC	93.6	89.1	84.4	95.7
Sharma et al[20]	121	Normal siblings of thalassemia major patients	NESTROFT, CBC, HPLC	93.22	88.70	88.7	93.22
Present study	1000	Group I-individuals from thalassemia screening camps in colleges, Group II-antenatal women coming to two government hospitals, Group III-individuals who voluntarily came to our department for screening of thalassemia, Group IV-Individuals referred by physicians and gynaecologists or other laboratories for thalassemia screening	NESTROFT, CBC, HPLC	94.87	88.14	35.75	99.5

Bhukhanvala et al, determined the cut off values for red cell indices by screening obligate carriers of β thalassemia who were parents of affected children and found that using MCV <80 fl and MCH <27 pg, they missed 5 out of 179 carriers.[8] In their population in south Gujarat, they suggested using a higher cut off value of MCH (MCV ≤78 fl and MCH ≤28 pg) for picking up β thalassemia carriers.

Using NESTROFT we missed 4 β thalassemia carriers while using MCH and MCV as a first line screen we would have missed only one β thalassemia trait. 58% of cases with other hemoglobinopathies were picked up by NESTROFT while 75% of these cases had reduced MCV and MCH values.

Based on this result of NESTROFT, CBC and HbA2 estimation, we suspected 58 of the individuals screened to be having other hemoglobinopathies. This would give a prevalence of other hemoglobinopathies to be 5.8%. At present, about 250 million people (4.5% of the world population) carry a potentially pathological haemoglobinopathy gene. Each year about 300 000 infants are born with major haemoglobinopathies.[9]

CONCLUSION

Large scale population screening in a region like Saurashtra is required for genetic counselling and control programmes in view of the high prevalence of β thalassemia trait detected here. When resources are limited, NESTROFT could still be used as a primary screening tool, however if the CBC can also be done simultaneously, chances of missing β thalassemia carriers would be negligible.

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