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Mean corpuscular volume/mean corpuscular hemoglobin values are not reliable predictors of the β-thalassemia carrier status among healthy diverse populations of Himachal Pradesh, India

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Abstract:

BACKGROUND: Himachal Pradesh is a hill state in North India in the Western Himalayas. β-thalassemia is a genetic disorder of hemoglobin inherited in an autosomal recessive manner that results in defective globin production leading to the early destruction of red blood cells. β-thalassemia has long been neglected in Himachal Pradesh due to popular belief that it runs along "Lahore-Gujarat-Punjab" belt in India. Therefore, there is no β-thalassemia testing facility currently in the state.

METHODS: To estimate the prevalence of β-thalassemia carriers, we calculated the sample size based on probability proportional to size self-weighing design. In each of 20 selected colleges, 111 students having an age of 18–25 were tested for high-performance liquid chromatography (HPLC) and complete blood count. Some were further tested for the mutations. We computed sensitivity, specificity, positive predictive value (PPV) and negative predictive value, and receiver operating characteristic curve for mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) red cell parameters.

RESULTS: Of the 2220 students, 57 were found to be β-thalassemia carrier by HPLC. The overall prevalence rate was 2.6% which translates to probable 180,000 β-thalassemia carriers in Himachal Pradesh. Six districts bordering highly endemic Punjab had a higher prevalence. Hemoglobin D-Punjab, Heterozygous-Iran Trait, and raised fetal hemoglobin were found. Thalassemia major and sickle cell disease were not found. Anemic status or MCV/MCH parameters were not found to be reliable predictors of thalassemia carrier status among the healthy populations of HP. The predominant mutation found was IVS 1–5 G > C.

CONCLUSION: Popular ongoing strategy for screening with MCV and MCH has low-PPV and can miss upto 37% of true thalassemia carriers. HPLC is better strategy for screening carriers and reduces further spread of thalassemia.

Keywords:

Complete blood count, hematological parameters, high-performance liquid chromatography, prevalence, β-thalassemia carriers

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Introduction

β-thalassemia is a genetic disorder of hemoglobin dysfunction inherited in an autosomal recessive manner that results in defective globin production leading to the early destruction of red blood cells (RBCs) and consequent microcytic hypochromic anemia. It is estimated that 1.5% of the

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global population, i.e., 80–90 million people, are the carriers of β-thalassemia, and the South-East Asian region (India, Thailand, and Indonesia) account for approximately 50% of the world's carriers– approximately 40 million people and approximately half of the affected births.[1] β-globin gene (HBB) is located in the short arm of chromosome 11, and >200 HBB gene mutations are known to be associated with β–thalassemia globally and of these about 28 mutations have been documented in Indian patients.[2] It is estimated that there are 30 million β-thalassemia carriers in India only, but no population-based survey has been done in any state till date. It is also estimated that every year >7000 children are born β-thalassemic major in India and require frequent blood transfusions besides iron-chelating therapy. There are seven common β-thalassemia mutations found in India, and carrier rate may go up to 9.5%–15% in different regions in the general population in India.[3,4] Thalassemia has long been neglected in Himachal Pradesh due to popular belief that it runs along "Lahore-Gujarat-Punjab" belt in India. Therefore, no thalassemia testing facility is currently available in the state.

Himachal Pradesh is a hilly state of North India in Western Himalayan belt and has better health indicators than national averages, but there was no population-based data on genetic disorders in the state. The author while working in the State Blood Bank, Shimla, realized the threat of thalassemia from affected children who used to come for blood transfusion and submitted a project proposal of estimating the prevalence of β-thalassemia trait in the state of Himachal to the National Rural Health Mission. The project was approved with the objective of estimating the prevalence of thalassemia trait in Himachal Pradesh and to find suitable red cell based parameters for the establishment of a low-cost screening program in the state.

Methods

Since, there is no prevalence study done in any state, a multicentric study[5] done in six cities of six states in India was taken as reference, and higher limit of prevalence of 2.94% was taken to calculate the sample size. Using public domain Epi Info software version 7.1.2.0 developed by the Centers for Disease Control and Prevention, Atlanta, USA with a cluster sample technique with a confidence limit of 1% and confidence interval of 95% with design effect of 2, with 20 clusters we got a sample size of 2220, i.e., 111 students in each cluster. To account for nonresponse, sample loses and deterioration during the transport we proposed to take additional 10% samples and total target sample size came out to be 2442. To maximize the benefits, we decided to take college-going young marriageable population of 18–25 years of age as the target population. The selection of colleges was done by probability proportional to size self-weighing design. All the colleges including private ones were line listed, and a random number was selected using MS Excel, the sampling interval was added to this number to finalize 20 colleges comprising a population of 39,710 students. Before the screening, we sensitized the students of all 20 selected colleges through poster competitions and debates over the issue. A total of 45 Senior Secondary schools and one university were also involved in the awareness and stakeholder engagement. An awareness poster in the Hindi language, commonly used in this state, focusing on knowing the thalassemia carrier status before marriage was developed by the authors for sensitization, [Figure 1]. Interactive "Hello Doctor" radio talks were also organized. After sufficient sensitization over 2 years, blood sampling camps were conducted in consultation with director of higher education. The ethical clearance was taken from the Institutional Ethics Committee of J. P. University-wide "IEC/Project No-27-2015", Dated 27-11-2015. Every student was asked to give informed consent in the form of written signed consent statement, whereas right to participate was voluntary. In each selected college, students were asked to pick slips for inclusion or exclusion and those included were tested for high-performance liquid chromatography (HPLC) and complete blood count (CBC) parameters. No one was denied the right for test, but only first 111 samples were included for analysis as per the sample size. Most of the colleges were Rural Government Colleges, and most of the participants were girls. The collected samples were analyzed locally same day or within 24 h for CBC by the Hematology Counter "Act Diff 5 CP Hematology Analyzer-Beckman Coulter Act Diff 5", and the second samples were sent to Gurgaon for HPLC analysis by Bio-Rad variant II HPLC system by β-thalassemia short program. Seven type of Hb variants were tested with the help of HPLC, i.e., HbA, HbA2, fetal hemoglobin (HbF), hemoglobin (Hb D), HbS, hemoglobin D-Punjab (Hb D) Iran Trait, HbC, and unknown peaks with a disclaimer that this screening test does not rule out any Alpha-thalassemia/Hb variants that elute at similar retention times on HPLC. Receiver operating characteristic (ROC) curves were plotted for both mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) values using MedCalc easy to use the statistical software (Atlanta, Georgia). The methodology to prepare the ROC curve using the MedCalc software trial version was DeLong *et al*.[6] Confidence intervals and *P* values were calculated using Open Epi, Score (Wilson) software.[7]

Three common family counseling sessions were organized with the help of professional counselors to inform the thalassemia carrier students, the status they have and precautions they need before or after marriage. With some funds left from the project, we went ahead with the genetic analysis of some small samples that

Figure 1: Thalassemia poster for general awareness

were collected during counseling sessions after the due consent of the participants. Those who had HPLC done were tested for the five common genetic mutations and that sibling accompanying affected students were tested for HPLC and CBC to detect any carrier status.

Results

A total of 2469 students volunteered against the requirement for 2220 students. Among 2220 samples, 57 were found to be a β-thalassemia carrier. The overall prevalence rate was found to be 2.57% (confidence interval 2–3.3; $P \leq 0.0000001$) that translates to probable 180,000 β-thalassemia carriers in Himachal Pradesh. Nearly, 72% of the study population were female, 97% were Hindus, and 67% were from the general category [Table 1]. Prevalence of β-thalassemia carrier rate was similar across religion, cast, and sex [Table 2]. Districts bordering Punjab state that has 1.5 million carriers with beta-thalassemia,[8] were found to be more affected [Table 3]. Thalassemia major or hemoglobin sickle cell, HBS, were not found, details of Hb variants found are given in Table 4. Anemic status or hematological markers were not found to be sensitive predictors of thalassemia carriers. A total of 50 out of 57 β-thalassemia carriers did not have anemia [Table 5] contrary to the widespread belief that β-thalassemia carriers are anemic.[9] Distribution of HbA2 is described in Table 6. Overall hematological markers both MCH <27 pg and MCV <80fl were not found to be sensitive predictors of β-thalassemia carrier status [Tables 7 and 8]. ROC curves were plotted for testing the ability of both MCV/MCH to detect the β-thalassemia carrier status [Figures 2-4]. The ROC curve

Table 1: Sample characteristics (*n*=2220)

	n (%)
Sex	
Female	1596 (72)
Male	624 (28)
Religion	
Hindu	2156 (97.1)
Muslim	27 (1.2)
Sikh	26 (1.2)
Buddhist	10 (0.5)
Christian	1 (0.0)
Caste	
General	1381 (62)
OBC	327 (15)
SC	418 (19)
ST	94 (4)

provided an area under the curve (AUC) value of 0.764 for MCV and 0.781 for MCH, suggesting only fair and not very good discrimination ability.[10]

A total of 26 samples were tested during counseling sessions out of that 15 were tested for five common genetic mutations through polymerase chain reaction , and the results are at Table 9. The most common mutation found was IVS 1–5 G > C, 41/42 TCTT, Codon 8/9+G, and no mutation was found for 619 bp-deletion and IVS 1-1 G > T. Interestingly, sample NPR-170 had HbA2 value 5.3 and HbF value 3.6 and reduced MCV/MCH values pointing out that it represents uncommon mutation beyond the five tested by us. Sample DHR-049 Hb variant analysis shows borderline high normal HBA2 window of 3.5 and rest of the values including MCV/MCH are

Table 2: Prevalence of thalassemia trait (*n*=57)

	n	*N*	Prevalence (CI with *P*)
Sex (%)			
Female	44	1596	2.8 (CI: 2-3.7, <0.0000001)
Male	13	624	2.1 (CI: 1.2-3.5, <0.0000001)
Religion (%)			
Hindu	55	2156	2.6 (CI: 2-3.3, <0.0000001)
Muslim	1	27	3.7 (CI: 0.6-18.2, <0.0000001)
Sikh	1	26	3.8
Buddhist	0	10	0.0
Christian	0	1	0.0
Caste (%)			
General	36	1381	2.6 (CI: 2-3.6, <0.0000001)
OBC	9	327	2.8 (CI: 1.5-5.1, <0.0000001)
SC	11	418	2.6 (CI: 1.5-4.7, <0.0000001)
ST	1	94	1.1 (CI: 0.2-5.8, <0.0000001)
Total	57	2220	2.6 (CI: 2-3.3, <0.0000001)

CI=Confidence interval

Table 3: District bordering Punjab having a higher prevalence

District of birth	Carrier	Healthy	Total (%)
Bilaspur	4	62	66 (6.1)
Una	11	259	270 (4.1)
Kangra	17	444	461 (3.7)
Hamirpur	5	143	148 (3.4)
Mandi	11	329	340 (3.2)
Sirmour	3	116	119 (2.5)
Solan	2	142	144 (1.4)
Kullu	2	271	273 (0.7)
Shimla	2	355	357 (0.6)
Chamba	0	8	8 (0.0)
Kinnaur	0	15	15 (0.0)
L and S	0	10	10 (0.0)
New Delhi	0	1	1 (0.0)
Pathankot	0	3	3 (0.0)
Punjab	0	1	1 (0.0)
Roper	0	1	1 (0.0)
Saharanpur	0	2	2 (0.0)
Haryana	0	1	1 (0.0)
Grand total	57	2163	2220 (2.6)

in normal range, therefore, further genetic analysis is required for the final diagnosis. Out of 11 siblings of the carriers who volunteered for testing, two brothers were found positive for thalassemia trait, i.e., KSG-118 B (HbA2 = 4.6) and HMR-084B (HbD = 37.0) [Table 9].

Discussion

We found 57 samples positive for β-thalassemia trait, but there are borderline HbA2 between 3 and 3.9 that may be a carrier and may have lead us to underestimate the actual burden of the disease. Rosnah *et al*. found that 36/117 borderline samples (30%) were positive for thalassemia trait.[11] We had 610 such samples having borderline HbA2 between 3 and 3.9, and 23 were detected as a carrier by HPLC against expected 183 carriers as per the study referenced above. Another study also flags the limitation of HPLC in borderline HbA2 values to predict β-thalassemia trait and suggest to combine

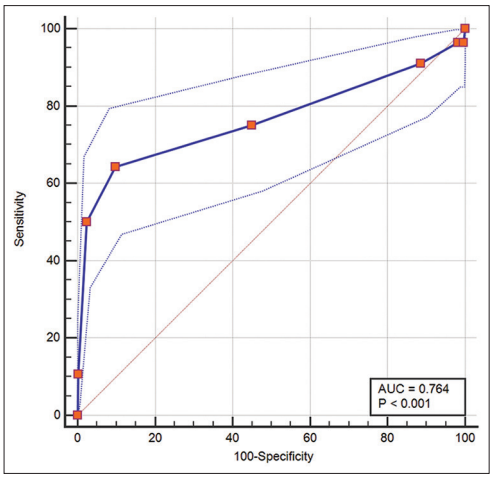

Figure 2: Receiver operating characteristic curve for the ability of mean corpuscular volume to detect the β-Thalassemia carrier

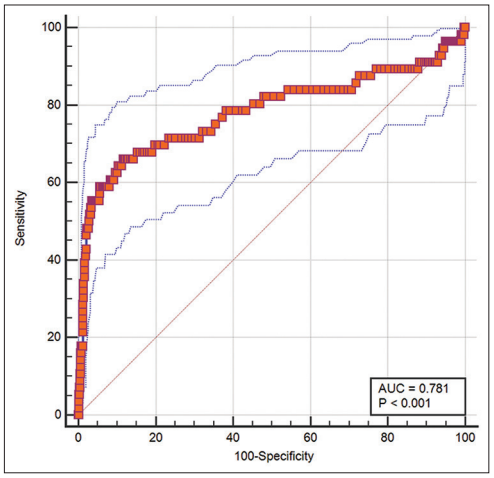

Figure 3: Receiver operating characteristic curves for the ability of mean corpuscular hemoglobin to identify the β-thalassemia carrier

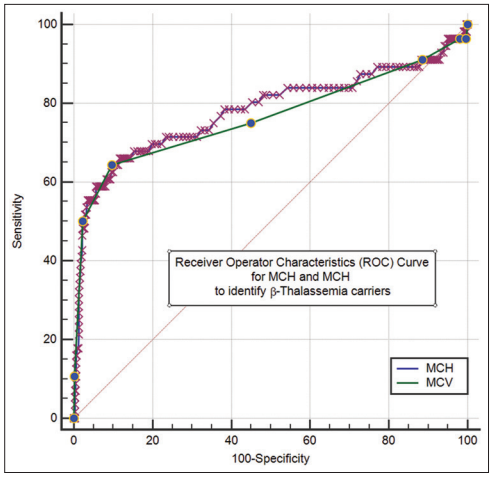

Figure 4: Receiver operating characteristic curve for mean corpuscular volume/mean corpuscular hemoglobin to identify the β- thalassemia carriers

borderline HbA2 values with MCH <27 pg for better estimation.[12] Moreover, with this value, we found 23 possible carriers with HPLC than expected 31 out of 610 having borderline HbA2 values. However, sensitivity analysis we did for hematological markers in our study shows that although MCH <27 pg was found to be capturing more β-thalassemia carriers, but the positive predictive value (PPV) was low for both MCH <27 pg and MCV <80fl separately or collectively [Table 7]. Therefore, both MCH <27 pg and MCV <80fl are not reliable predictors of detecting β-thalassemia trait in mass screening camps as they can miss upto 37% true β-thalassemia carriers. The ROC curve provided an AUC value of 0.764 for MCV and 0.781 for MCH, suggesting only fair and not very good discrimination ability of these parameters to detect β-thalassemia carrier status. However, we can say that an HPLC value of HbA2 >4 is a fairly good indicator for β-thalassemia carrier detection and borderline values (3–3.9) may need to be combined with the cell parameters (MCH <27) as detailed above. Another study observes that it appears difficult to differentiate the thalassemia major and intermedia by red cell morphology alone or with absolute values. Therefore, blood findings must be correlated with a clinical picture for an accurate diagnosis.[13] Most of the studies done to calculate the sensitivity of hematological markers as cut off for the detection of β-thalassemia trait have a small sample and are not population-based random sampling studies and, therefore, give higher sensitivity than observed by this study. Some of the other studies showing the sensitivity of cell indices, the reliability of cell indices was lost in averages that blurred the real picture or some of the studies were carried out were laboratory/hospital based.[14-16] A study[17] from Thailand advocated MCH <26.5 as cut off to get PPV of 40.4% but in this population-based study, this value could capture a total of 314 persons as possible carriers but only 37 actual carriers out of 57 carriers. Higher PPV values than we observed in this study for MCV, i.e., 30.85% were found in a study[18] on pregnant women in India, but cell indicators still are not reliable as they may still declare 70% potential carriers as noncarriers. In this study, on pregnant women, the PPV of the naked eye single tube red cell osmotic fragility test (NESTROFT) was found to be more (53.45%) than those of RBC count, however, we found making NESTROFT solution complicated and full of flaws and difficult to maintain uniformity/osmolality[19] in low-ionic strength solution over long durations. In another study,[20] the PPV of NESTROFT was calculated to be 35.3% while those of cell indices it was 32.5% for MCV <80 fl and 30.6% for MCV <75 fl. Out of 25.2% NESTROFT positive, actual positive captured was only 9.8%. In another study, Shewale *et al*. used readymade 0.36% buffered saline for NESTROFT and found that it missed 46 persons out of 1000 known β-thalassemia carriers and picked up 179 persons as β-thalassemia carriers out of 1000 healthy persons which has cost implications as well for getting HPLC or genetic evaluation for confirmation. False positivity in this study was attributed to the possible anemic status of the persons.[21]

In this study, anemia was no longer a factor in nondiagnosis of β-thalassemia trait, as 12% of anemic carriers were identified by the HPLC, this is in tune

Table 4: Hemoglobin variants in the carriers as reported by the laboratory

Type Hb variant (*n*=57)	Carrier, *n*	Noncarrier
HB variant analysis shows BTT HBA2 window of ≥3.9	36	
HB variant analysis is suggestive of HBD Punjab heterozygous	8	
HB variant analysis shows borderline high normal HBA2 window of 3.3	5	43
BTT with slightly raised HBF window	3	
HBD Iran trait	2	
HB variant analysis shows borderline high normal HBA2 window of 3.5	1	
HB variant analysis shows borderline high normal HBA2 window of 3.7	1	
HB variant analysis show borderline raised HBA2 window of 3.8%	1	
HB variant analysis appears to be normal with slightly raised HBF noted acquired elevation		2
Normal		2118
Total	57	2163

HB=Hemoglobin, HBF=Fetal hemoglobin, HBD=Hemoglobin D variant Punjab, BTT=β-thalassemia trait, HBA2=Hemoglobin A_2

Table 5: Prevalence of thalassemia trait by anemia status

	Carrier	Healthy	Total	Prevalence (%), *P*
Anemia (<11 g/dl)	7	140	147	4.8 (CI: 2-9.5, <0.0000001)
No anemia (>11 g/dl)	50	2023	2073	2.4 (CI: 1.8-3.1, <0.0000001)
Total	57	2163	2220	2.6 (CI: 2-3.3, <0.0000001)

CI=Confidence interval

Table 6: Distribution of level of hemoglobin A_2 among screened population

HBA2	Carrier by HPLC	Healthy	Total population	Prevalence (%), *P*
<3.0	11*	1565	1576	0.7 (CI: 0.39-1.28, <0.0000001)
3.0-3.4	2	591	593	0.33 (CI: 0.9-1.2, <0.0000001)
3.5-3.9	10	7	17	58.8 (CI: 36.1-78.39, 0.0000001)
≥4	34	0	34	100

*Mostly HbD >34.4 (as mutation seen in 34.4) and raised HBF window. HBF=Fetal hemoglobin, HBD=Hemoglobin D variant Punjab, HBA2=Hemoglobin A_2, HPLC=High-performance liquid chromatography, CI=Confidence interval

Table 7: Distribution of level of hemoglobin A_2 among screened population with respect to mean corpuscular volume/mean corpuscular hemoglobin values

HBA2	The total screened population sample	Carrier by HPLC	Carrier by MCV ≤80	Carrier by MCH ≤27
<3.0	1576	11	200	332
3.0-3.4	593	2	7	25
3.5-3.9	17	10	4	6
≥4	34	34	33 except HBD 38.1	34
<3.0-≥4	2220	57	244	397

HBA2=Hemoglobin A_2, HPLC=High-performance liquid chromatography, HBD=Hemoglobin D variant Punjab, MCV=Mean corpuscular volume, MCH=Mean corpuscular hemoglobin

Table 8: Sensitivity and specificity of mean corpuscular volume/mean corpuscular hemoglobin to predict thalassemia carrier status

	Carrier	Healthy	Total	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
MCV <80	36	200	236	63.1	90.7	15.2	98.9
MCV ≥80	21	1963	1984				
Total	57	2163	2220				
MCH <27	39	343	382	68.4	84.1	10.2	99.0
MCH ≥27	18	1820	1838				
Total	57	2163	2220				
MCH ≤30	46	1020	1066	80.7	52.8	4.3	99.0
MCH >30	11	1143	1154				
Total	57	2163	2220				
MCH <27 and MCV <80	36	195	231	63.1			

PPV=Positive predictive value, NPV=Negative predictive value, MCV=Mean corpuscular volume, MCH=Mean corpuscular hemoglobin

Table 9: Detected mutations in some of the samples we could get voluntarily during counseling

Code	HPLC	Genetic mutation
CM-002	4.5 value for HBA2	IVS 1-5 G>C mutation (β+)
KUL-137	4.4 value for HBA2	IVS 1-5 G>C mutation (β+)
KSG-118	4.5 value for HBA2	IVS 1-5 G>C mutation (β+)
SL-016	3.7 value for HBA2	IVS 1-5 G>C mutation (β+)
KSG-120	4.8 value for HBA2	IVS 1-5 G>C mutation (β+)
HMR-84	34.4 value for HBD	IVS 1-5 G>C mutation (β+)
DHR-091	5.9 value for HBA2	41/42 TCTT mutation (β+)
NPR-048	5.7 value for HBA2	Codon 8/9+G mutation (β+)
NPR-170	5.3 value for HBA2 3.6 value for HbF	Common five mutations not detected, need to be tested for other mutations
DHR-049	3.5 value for HBA2	Common five mutations not detected, need to be tested for other mutations
JHN-025	3.3 value for HBA2	Not detected
SJP-047	3.3 value for HBA2	Not detected
KSG-159	3.3 value for HBA2	Not detected
NPR-029	3.2 value for HBA2	Not detected
NPR-152	3.0 Value for HBA2	Not detected

HPLC=High-performance liquid chromatography, HBA2=Hemoglobin A_2, HBF=Fetal hemoglobin, HBD=Hemoglobin D variant Punjab

with another study[22] done by P Sharma *et al*. that reported that iron deficiency is not a barrier for detecting β-thalassemia trait with HPLC.

Overall 6.3% of healthy students, mostly girls of marriageable age were found to be anemic that has implications for the maternal mortality rate and infant mortality rate. Severely, anemic girls were also counseled. Anemia was defined as per the National Family Health Survey-4 Manual.

National guidelines on for the prevention and control of hemoglobinopathies in India available on online[23] prescribe a screening protocol based on four steps, i.e., NESTROFT followed by MCV/MCH values <80/27 followed by HPLC followed by the DNA studies. We attempted to look for more sensitive red blood cell values for low-cost mass screening, but to our surprise, these cell indicators missed 37% of actual carriers despite nearly 99% of negative predictive value that the national policy refers to [Table 8] which is unacceptable. If we want to control thalassemia, we should start compulsory screening of college-going students and pregnant women with HPLC without thinking of cost implications to halt increasing carriers and birth of thalassemic children or children with other hemoglobinopathies. New genetic-based super kits can detect up to 20 types of genetic mutation and can be customized to suit local requirements. Such kits need to be developed in-house for better cost-effectiveness. A popular ongoing strategy for screening with MCV and MCH has low PPV and miss many true thalassemia carriers and has subsequent repercussions for the parents and the nation regarding costs[24] and to the thalassemic children, regarding clinical burdens.[25]

Conclusion

Popular ongoing strategy for screening with MCV and MCH parameters has low-PPV and can miss upto 37% of true thalassemia carriers. HPLC is better and cost effective[26,27] strategy for screening carriers that would reduce further spread of thalassemia.

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Conflicts of interest

There are no conflicts of interest.

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