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Research Article

Evaluating the role of indirect bilirubin, urobilinogen and Shine & Lal index as an alternative screening tool for beta thalassemia minor

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ABSTRACT

Background: Beta thalassemia continues to be a significant burden to Western India particularly Saurashtra region of Gujarat. Since cost of treatment is high emphasis must be shifted from treatment to prevention that includes mass screening as most effective tool including RBC indices & peripheral blood smear. These tests have limited availability, re quire sophisticated equipments and are expensive. Thus, there is need for simple, low cost and reliable test which can be used in absence of sophisticated equipments. The present study has evaluated the validity of such test: indirect bilirubin and urine urobilinogen. Study had two objectives: 1) Estimation of indirect bilirubin, urobilinogen and Shine & Lal Index. 2) Comparing specificity and sensitivity of above test with HbA2 electrophoresis.
Methods: The present study was conducted on 100 (n=100) subjects in blood bank, department of pathology, government medical college Rajkot, Gujarat, India. In first group 50 subjects (Thalassemia minor) were selected while in second group 50 (n2=50) normal individuals from hospital staff were selected. Complete-haemogram, serum-direct, indirect and total bilirubin, urine urobilinogen and their sensitivity and specificity were calculated.
Results: Of the 50 cases in test group, 41 had higher Indirect Bilirubin level (>0.7 mg/dl), 35 had high urobilinogen level (>1 mg/dl). In control group out of 50 cases, 3 had high indirect bilirubin levels, 4 had high urobilinogen levels. Indirect-bilirubin had sensitivity of 82%, specificity of 94%. Urobilinogen showed sensitivity of 70% & specificity of 92%.
Conclusion: Indirect bilirubin and urine-urobilinogen is a valuable, cost-effective screening test for beta-thalassemia-trait with sensitivity & specificity comparable to RBC indices.

Keywords: Indirect-bilirubin, Screening test, Thalassemia, Urobilinogen

INTRODUCTION

The Inherited haemoglobin disorders are the most common single gene defects in human beings.[1] Among them, the thalassemia syndromes particularly the beta thalassemia are major causes of morbidity throughout the world.[2] According to WHO data, it is estimated that 1.5% of world's population is carrier of beta Thalassemia while in India, carrier frequency varies from 3-17% in different ethnic groups with nearly 30 million people affected and >8000 children born every year with beta thalassemia minor.[3] Only 10-15% of these children receive optimal treatment.[4] The Western part of India, comprising of Maharashtra, Gujarat, Rajasthan and Goa accounts for 50.7% of all Beta Thalassemia cases in whole India.[5] Among them, in Gujarat, maximum number of beta thalassemia minor cases are seen in Saurashtra region (46.9%), making it endemic for beta thalassemia disease.[6] Thus, the Saurashtra region is in grave need of active surveillance for detecting maximum number of cases and dispensing appropriate preventive measures and treatment. The cost of ideal treatment of 1 thalassemia

major child (regular blood transfusions, iron chelation, blood tests) is nearly Rs. 100000/annum.[7] The only curative treatment currently relevant is Bone-marrow transplantation which is not affordable by most of patients.[8] Thus, the birth of a Thalassemia child places considerable physical and economic strain not only on its family but also on community and nation. With these limitations, emphasis must be shifted from treatment to prevention, which includes–health education, mass-screening, genetic counselling and prenatal-diagnosis.[9] Various screening parameters are peripheral blood smear-examination, red-cell indices (by automated cell counter) & osmotic fragility tests.[10] All these tests are time consuming, have limited availability, less accurate, require sophisticated equipments and expensive.[11] Thus, there is a need for a simple, low cost, rapid and reliable test which can be used in absence of sophisticated equipments (like automated-cell counters) and within the reach of the rural population where it is required the most. The present study has evaluated the specificity and sensitivity of such test - indirect bilirubin, urine urobilinogen and Shine & Lal index in comparison with HbA2 electrophoresis.

Aims & objectives

The present study had two objectives:

1) Estimation of indirect bilirubin, urobilinogen and Shine & Lal Index among the prediagnosed beta thalassemia minor patients.
2) Comparing the specificity and sensitivity of bilirubin, urobilinogen and Shine & Lal index individually and in combination in comparison to HbA2 electrophoresis.

METHODS

Study area

The study was conducted in "Pandit Deendayal Upadhyay medical college" Rajkot and its associated civil hospital. It is a sentinel area for the thalassemia affected patients in Saurashtra region and served as a good study area for thalassemia screening tests.

Study type

The hospital based cross-sectional study was conducted among 50 beta thalassemia minor individuals and 50 normal individuals as a control. Estimation of indirect bilirubin, urobilinogen and Shine & Lal Index was done in total 100 individuals and compared with that of HbA2 electrophoresis. The cross sectional study is chosen as it is most cost-effective and gives rapid results.[12]

Study population and study design

The study population for thalassemia minor was taken from among the relatives of the thalassemia major patients coming to Blood Bank for regular blood transfusions. Those thalassemia minor patients were confirmed by HbA2 electrophoresis method at the institutional laboratory. The 50 normal individuals were taken from the hospital staff. The study was conducted after obtaining the necessary clearance from the ethical committee of the institution and informed consent of the patient. The confidentiality of patient information was maintained. The estimation of indirect bilirubin, urobilinogen and Shine & Lal index was done.

Selection criteria

The selection criteria consisted of

Inclusion criteria

1) Patient confirmed as beta thalassemia minor by HbA2 electrophoresis.
2) Patients of all ages.
3) Patients of both sexes.
4) Patient that is well oriented, stable and cooperative were included.

Exclusion criteria

1) Patient with any hepatobiliary disease.
2) Any renal disease.
3) Any blood coagulative disorder was excluded from the study.
4) Patients of Iron deficiency anemia.

Data collection procedure

The laboratory tests performed were serum bilirubin, urine urobilinogen and complete blood count (for Shine & Lal index) on the beta thalassemia minor patients. For this about 4 ml of blood was collected from the median cubital vein of the patient by taking aseptic precautions. Out of this, 2 ml of blood was taken in plain bulb for serum bilirubin estimation and 2 ml of blood was taken into the EDTA (ethylene diamine tetra acetic acid) bulb for complete blood count. A freshly voided mid-stream urine sample of about 5 ml was collected in a sterile container for urine urobilinogen test and sent for performing these tests at the institutional laboratory.

Laboratory methods

Indirect and Total Bilirubin was detected by - "Easy bilirubin kit, Modified Jendrassik & Groff method" by monochromatic method at room temperature and read absorbance at 546 nm against distilled water.

Urine urobilinogen was detected by Dipstick method with the ten parameter strip based on the principle of; stable diazonium salt producing a reddish azo compound with urobilinogen.

Electrophoresis was done by the method of "Agarose gel" at alkaline pH of 8.4-8.8 at 100 volts for 15-20 minutes.

Haemoglobin and RBC indices were calculated by automated cell counter Sysmex K-21 at the institutional laboratory.

Normal range for serum indirect bilirubin is taken as 0.2-0.9 mg/dl.

Any sample greater than >0.9 mg/dl is considered as hyperbilirubinemia.[13]

Urine urobilinogen normal level is 0-1 mg/dl if its value comes out >1 mg/dl then it is considered as abnormal.[14]

Normal values of RBC count is taken as 4.3-5.6 x 106/mm^3 for adult males and 4.0-5.2 x 106/mm^3 for adult females; MCV (mean corpuscular volume): 79-93.3 fL (femtolitre), MCH (mean corpuscular haemoglobin): 26.7-31.9 picogram/cell; PCV (packed cell volume) is taken as 38.9-46.4% for adult males and 35.4-44.4% for adult females.[15]

Shine & Lal Index (MCV2X MCH/100): Thalassemia <1530 and iron deficiency anemia: >1530.[16]

Data analysis

The laboratory results of all four tests were collected and statistically analyzed and interpreted for their accuracy and comparison with each other. The data was analyzed with help of Microsoft excel (2007) and Epi Info 7 and following values were calculated: Specificity, sensitivity, positive predictive value, negative predictive value, false positive value and false negative value. The values of indirect bilirubin, urobilinogen and Shine & Lal index were compared with that of HbA2 electrophoresis. From this comparison conclusion was drawn whether the aforementioned screening tests were statistically significant or not. The combined sensitivity and specificity was calculated by the following formula:

Combined sensitivity: 1-(1-Sensitivity of Test-1) x (1-Sensitivity of Test-2)[17]

Combined Specificity: 1-(1-Specificity of Test-1) x (1-Specificity of Test-2)[17]

Thus the combined sensitivity and specificity was calculated and compared with the confirmatory tests.

RESULTS

The present study had maximum number of patients belonging to 31-40 years age-group (33 patients; 66%) with the range from 21 to 60 years. From these, majority of patients studied were males. The patients had no complaints in carrying out daily activities but some complaint of fatigue and weakness (14 patients; 28%).

The following table gives information regarding the 50 patients of beta Thalassemia selected from the relatives of beta Thalassemia major patients which were confirmed by HbA2 electrophoresis.

The different haematological investigations carried out were indirect bilirubin, urine urobilinogen, haemoglobin, mean corpuscular volume, mean corpuscular haemoglobin concentration, packed cell volume and Shine & Lal index (MCV2 x MCH/100). The investigations revealed that majority of 1st group patients had haemoglobin ranging from 10.8 to 7.1 g% with mean of 8.90 ± 1.28 g%. While the 2nd group had Haemoglobin level ranging from 11.0 to 12.8 with mean of 11.19 ± 1.11; which is substantially higher than the 1st group. Shine & Lal index in 1st group is 1077 ± 354 and in 2nd group it is 744 ± 361 which is also significantly lower than in 1st group. The indirect bilirubin and urine urobilinogen in 1st group patients were 0.9 ± 0.3 and 1.3 ± 0.4 while in 2nd group they were 0.4 ± 0.3 and 0.8 ± 0.3 respectively which are much lower than in 1st group.

Of the 50 cases in first group, 41 had higher indirect bilirubin level (>0.7 mg/dl), 35 had high urine urobilinogen level (>1 mg/dl), Shine & Lal index was lower (<1530) in 49 cases while all 50 had HbA2 level >3.5%. In second group out of 50 cases, 3 had high indirect bilirubin levels, 4 had high urobilinogen levels, Shine & Lal index was low (<1530) in only 2 while no one had HbA2 level >3.5% in second group. The following table indicates the different haematological parameters.

Table 1: Age related comparison in thalassemia minor group (Group-1).

Age group (year)	Total No. of patients			Percentage (%)		
	Male	Female	Total	Male	Female	Total
21-30	3	1	4	6	2	8
31-40	18	15	33	36	30	66
41-50	6	4	10	12	8	20
>50	1	2	3	2	4	6
Total	28	22	50	56	44	100

Table 2: Age related comparison in Control group (Group-2).

Age group (year)	Total No. of individuals as control			Percentage (%)		
	Male	Female	Total	Male	Female	Total
21-30	6	2	8	12	4	16
31-40	15	11	26	30	22	52
41-50	3	4	7	6	8	14
>50	8	1	9	16	2	18
Total	32	18	50	64	36	100

Table 3: Different Biochemical values in the study in group-1 (Beta thalassemia minor) and group 2 (Control group).

Parameter	Study group	Control group
Indirect bilirubin (mg/dl)	0.9 ± 0.1	0.4 ± 0.1
Urine urobilinogen (mg/dl)	1.3 ± 0.2	0.4 ± 0.1
Hb (g/dl)	9.90 ± 1.43	11.19 ± 1.31
MCV (fl)	71.145 ± 3.30	97.08 ± 7.96
MCH (pg)	20.67 ± 1.34	28.4 ± 2.15
MCHC (g/dl)	28.40 ± 1.48	29.02 ± 1.99
Shine & Lal index	1077 ± 354	1744 ± 361
PCV	34.56 ± 4.48	38.69 ± 3.89
RBC count (million/cumm)	4.32 ± 0.53	6.36 ± 0.76

Table 4: Results of bilirubin and urobilinogen in Shine& Lal index in both groups.

	Study group (Thalassemia minor)	Control group	Total
	HbA2 >3.5%	HbA2<3.5%	
Indirect bilirubin present	41	3	44
Indirect bilirubin absent	9	47	56
Total	50	50	100
Urine urobilinogen present	35	4	39
Urine urobilinogen absent	15	46	61
Total	50	50	100
Shine & Lal index <1530	49	2	51
Shine & Lal index >1530	1	48	49
Total	50	50	100
Combine bilirubin and urobilinogen present	47	1	48
Combine bilirubin and urobilinogen absent	3	49	52
Total	50	50	100

Indirect-bilirubin showed a sensitivity of 82%, specificity of 94%. Urobilinogen showed sensitivity of 70% & specificity of 92%. Shine & Lal index showed sensitivity of 98% & specificity of 96%.

Combined sensitivity & specificity of bilirubin & urobilinogen was found to be 94% & 98% respectively.

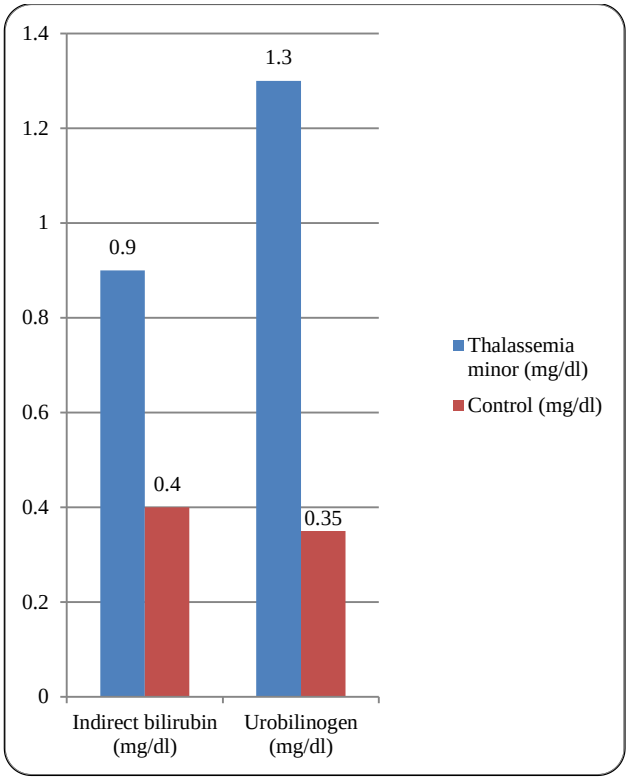

Figure 1: Values of indirect bilirubin and urobilinogen in group-1 (Beta thalassemia minor) and group-2 (Control).

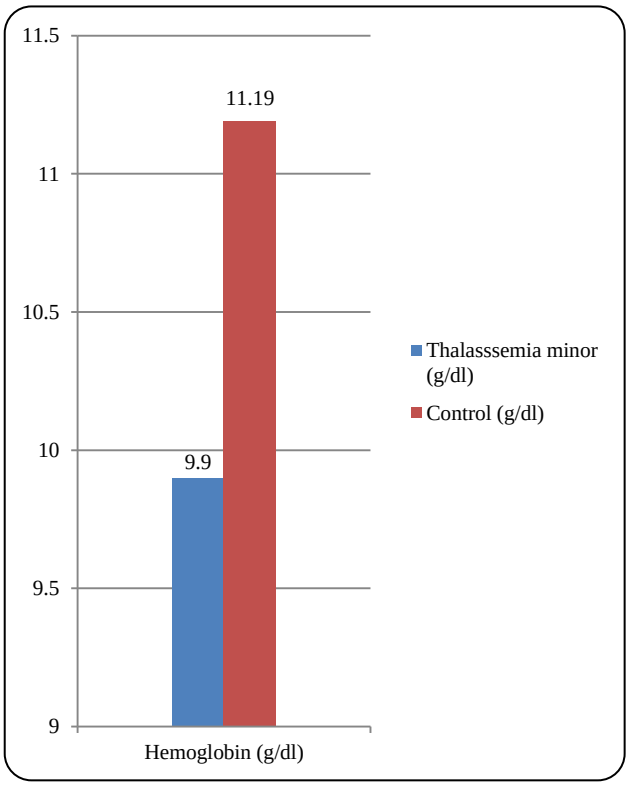

Figure 2: Values of haemoglobin in group-1 (Thalassemia minor) and group-2 (Control group).

Table 5: Sensitivity, specificity, PPV, NPV, FP and FN values in bilirubin, urobilinogen, Shine & Lal index and combined bilirubin and urobilinogen.

Sensitivity, specificity, PPV, NPV, FP and FN	
Indirect bilirubin	
Sensitivity	82%
Specificity	94%
Positive predictive value	93.18%
Negative predictive value	83.92%
False positive	6%
False negative	18%
Urine urobilinogen	
Sensitivity	70%
Specificity	92%
Positive predictive value	89.74%
Negative predictive value	75.4%
False positive	8%
False negative	30%
Shine & Lal index	
Sensitivity	98%
Specificity	96%
Positive predictive value	96.07%
Negative predictive value	97.95%
False positive	4%
False negative	2%
Combined indirect bilirubin and urobilinogen	
Sensitivity	94%
Specificity	98%

DISCUSSION

The high prevalence of beta thalassemia minor in the region of Saurashtra, Gujarat (46.9%), among the Western India warrants the use of an "Effective preventive strategy" for the thalassemia disease.[6]

Preventive strategies for beta thalassemia minor: There are many preventive strategies including Health education, Mass-screening, Genetic-counselling and Prenatal diagnosis.

Among them mass screening tests appears to be most effective owing to its cost effectiveness as well as efficacy in a developing country like India. Various screening parameters include peripheral blood smear-examination, red-cell indices (by automated cell counter) and osmotic fragility tests. Among all of these there is a requirement of the sophisticated equipments like automated cell counter and also the skilled work force for the effective implementation on mass scale in rural areas. Thus, these are neither cost effective nor applicable on mass population in rural areas. Thus there is an utter need to develop a screening test which is already an established test in any laboratory set-up; particularly in rural part of India. So the present study has evaluated the validity and efficacy of such test- indirect bilirubin and urine urobilinogen.

Role of indirect bilirubin and urobilinogen in beta thalassemia minor

Owing to the chronic haemolytic condition of the beta thalassemia minor individuals, there is rise in the level of indirect bilirubin as well as urine urobilinogen. As mentioned in the "Results section"; the higher value of the indirect bilirubin and urobilinogen in thalassemia minor individuals as compared to the normal individuals is statistically significant (P value <0.05). The detection of indirect bilirubin and urobilinogen may play an important role as a screening tool but its validity increases when both the tests are combined. The combined sensitivity and specificity was calculated by the following formula:[17]

Combined sensitivity: 1-(1-Sensitivity of Test 1) x (1-Sensitivity of Test 2)

Combined specificity: 1-(1-Specificity of Test 1) x (1-Specificity of Test 2)

The combined test has the sensitivity and specificity of 94% and 98% respectively .While Shine & Lal index has sensitivity and specificity of 98% and 96%. Thus both the tests seem to be comparable in regards to validity and efficacy. The difference in the values compared with control is statistically significant (P value <0.05) as per the statistical analysis done in Epi Info (CDC Atlanta 2007).

The Likelihood ratio of these tests is calculated by:

Positive likelihood: LR+ = Sensitivity/1-Specificity[18]

Negative likelihood: LR- = 1-Sensitivity/Specificity[18]

From the Table 7 it is clear that the likelihood of the combined test is maximum about 47. It indicates the likelihood that the test result would be positive in a patient with the beta thalassemia minor compared to the control. And this value is higher than Shine & Lal index. This states that this test is more likely to be positive in beta thalassemia trait than the red blood cell indices. While the negative likelihood ratios are less in Shine & Lal index - 0.02 while in the combined test it is 0.06. Indirect bilirubin & urobilinogen have positive likelihood ratio of 13.7 and 8.75 respectively.

Table 6: Comparison between bilirubin plus urobilinogen and Shine & Lal index.

	Bilirubin + Urobilinogen	Shine & Lal index
Sensitivity	94%	98%
Specificity	98%	96%

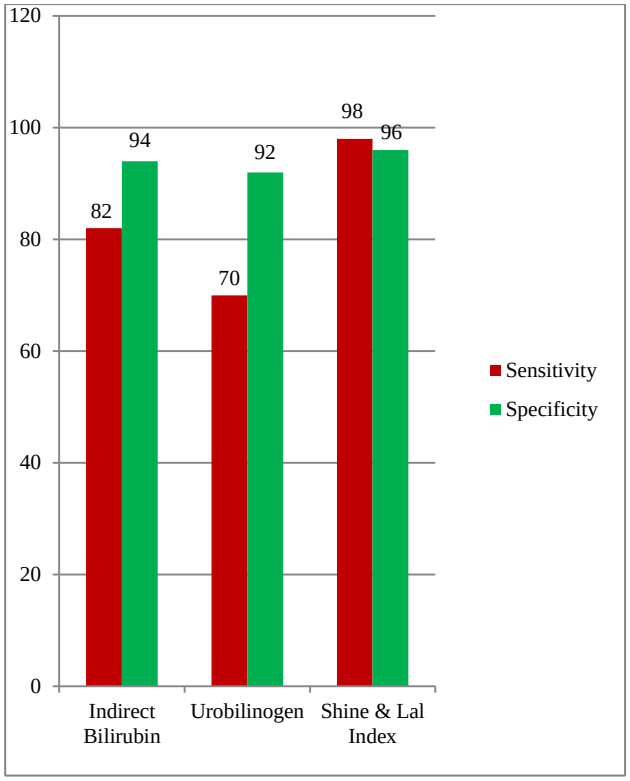

Figure 3: Showing sensitivity and specificity of bilirubin, urobilinogen and Shine & Lal index.

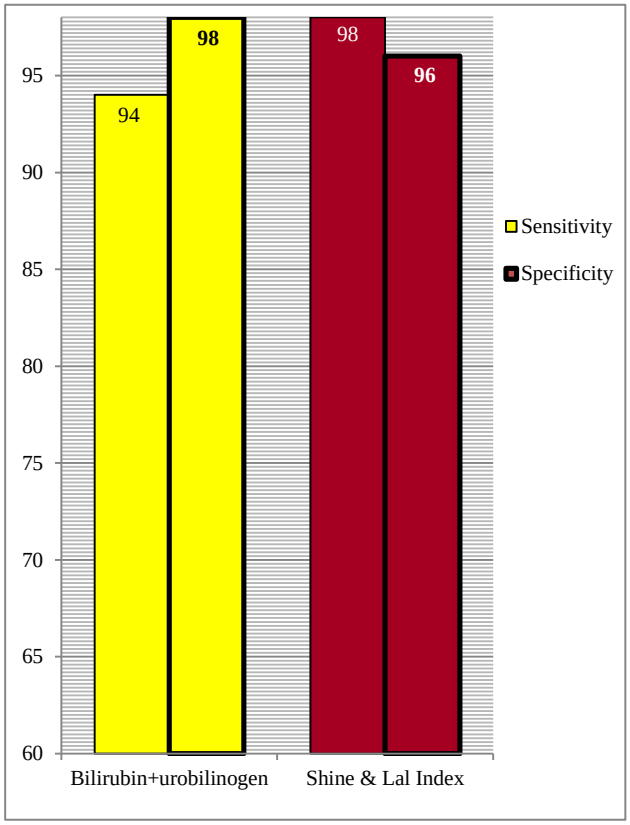

Figure 4: Comparison bar diagram between bilirubin + urobilinogen and Shine & Lal index for sensitivity and specificity.

Table 7: Likelihood ratios of the bilirubin, urobilinogen, Shine & Lal index.

	Indirect bilirubin	Urobilinogen	Combined test	Shine & Lal index
Positive LR+	13.7	8.75	47	24.5
Negative LR-	0.19	0.32	0.06	0.02

Beta thalassemia minor and other haemolytic conditions

The validity of the test in other haemolytic conditions needs to be reviewed before applying this test as a standard screening tool.

In other haemolytic conditions like - hereditary spherocytosis, sickle cell disease, G6PD deficiency; there may be an abnormal rise in the indirect bilirubin and urobilinogen.

Thus it may lead to a decrease in the sensitivity of the test. But as compared to beta thalassemia minor, these diseases are less common so the benefits of this test far outweigh its disadvantages. Even in iron deficiency state, there could be changes in the red blood cell indices but no any change in the indirect bilirubin and urobilinogen.

Future scope

The future scope of the test would be marvellous if the test is applied on a mass population and we are able to get higher detection rates.

The high sensitivity and specificity of this test clearly states the ability of the test to detect maximum number of cases as possible in the rural areas of Saurashtra region. The test must also be supplemented by the health education, prenatal diagnosis and genetic counselling.

Combining all of these efforts will truly serve the purpose of preventing the occurrence of Thalassemia major cases in the succeeding generations.

Limitation of the study

As the study has been done on the suspects of Thalassemia minor there is also a chance of any other co-morbidities like the haemolytic anemia, liver pathology, iron deficiency anemia and other hemoglobinopathies which may lead to false-positive test and so may lead to reduced sensitivity of the test. So it is implied that this test must be applied or considered relevant only after ruling out haemolytic conditions and liver pathologies like Gilbert's disease and others.

Though it can be applied as a cost effective screening test in rural settings it may further be confirmed using electrophoresis test.

CONCLUSION

1) The use of indirect bilirubin & urobilinogen as a screening test will benefit in terms of its cost effectiveness and its availability in rural areas that are devoid of sophisticated instruments like automated cell counters and skilled expertise required for finding red cell indices (like Shine & Lal index).

2) Another added advantage of this test is that it will help in differentiating thalassemia from iron deficiency anemia, as in iron deficiency anemia no rise in serum bilirubin level is seen.

3) It also rules out the possibility of having any liver disease.

Suggestions

1) A short term plan: An utter need to design an action plan to curb the disease effectively by doing genetic counselling among the already diagnosed thalassemia minor patients to avoid consanguious marriages and opt for prenatal diagnosis if already married.

2) A long term plan: A national health programme that encourages people to undergo screening tests at a particular time in the year in the endemic region.

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Conflict of interest: None declared
Ethical approval: The study was approved by the institutional ethics committee

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