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Prenatal Diagnosis of Thalassaemias

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ABSTRACT In an aim to reduce the birth of children with β-thalassemia and other haemoglobinopathies with immediate effect a study was undertaken to screen the thalassemia carriers from a population of pregnant women attending the antenatal clinic of Ramakrishna Mission Seva Pratisthan hospital over a period of 5 years (1995-1999). From a total of 1962 female pregnant patients 7.04 % thalassemia carriers were identified by electrophoresis and HbA_2 estimation. NESTROFT test was also tried as another screening method as a simple and inexpensive method which showed comparable results. The husbands of these carrier mothers (138 in number) were offered carrier detection and 68.84% responded from which 15.8% carriers (15 in number) were identified. From these high risk couples only 6 agreed for prenatal diagnosis. 19 other couples were also identified as couples "at risk" from 133 referred antenatal cases who came for investigation of anaemia. Thus a total of 25 couples opted for prenatal diagnosis by DNA analysis. 26 pregnancies from 25 couples were investigated (one parent came twice). Prenatal diagnosis by DNA analysis showed 30.77% affected foetus having thalassemia mutations in homozygous or double heterozygous state while 69.23% were unaffected (foetus normal or having mutation in heterozygous state).

INTRODUCTION

Antenatal diagnosis especially during first pregnancy in high incidence areas was found to be very useful as short term program which includes identification of carriers, prenatal diagnosis for the couples "at risk" and termination of pregnancies if needed to prevent the birth of thalassemic children. Management becomes more easier and effective if the couple already know their carrier status and come for counselling in time i.e. at very early in pregnancy. Education and awareness among common people about the prevalence and adverse effect of the disease, cost of treatment etc has been strongly emphasized for effective thalassemia prevention. Thalassemia and other haemoglobinopathies, widespread globally cause a major public health problem in India. β-thalassemia is the commonest type of haemoglobinopathy seen in India characterized by reduced or absence of β-globin gene expression. Nearly 30 million people are carriers of beta thalassemia and 7000 babies with major β-thalassemia are born every year[5,19]. Carrier rate varies between 0-17% in different ethnic groups. Prevalence of thalassemia varies between 2.3 to 9.2% with an average of 5.5%. The expensive and long-term intensive medical care can place a burden on the afflicted families and society as a whole. Prevention and control of thalassemia involves awareness in the community, screening for detection of carriers, establishment of prenatal diagnostic facilities and optimal treatment of thalassemic children. The high incidence of β-thalassemia and abnormal haemoglobin E in normal populations of Eastern India including the heterogeneous population of Kolkata has already been reported in our previous studies[7-9]. Identification of the carriers or families can be initiated as a short-term programme to yield immediate results or as long term programme which will have its impact later[3].

The objectives of the present study was to reduce the births of thalassemic children with immediate effect. The strategy adopted for screening of thalassemia carriers was to target the population of pregnant women attending the hospitals/ health care centers so that antenatal diagnosis and counselling could be offered in the current pregnancy itself. The wife was tested first, and then the husband was asked to be tested if the wife was found to be a carrier. This reduces the screening load to 50%. If both the wife and husband are found carriers the couples at risk were offered prenatal diagnosis. This approach has been used with success in many other countries[1,2,11]. The couples identified late in pregnancy were counselled and offered prenatal diagnosis in subsequent pregnancies. Thus antenatal diagnosis of β-thalassemia is one of the useful programmes to combat this life threatening disease.

The Main Objectives of This Study were

1. To study the prevalence of thalassemia carriers in women of reproductive age group especially the prospective mothers attending the antenatal clinic of a general hospital.
2. To prevent the birth of first thalassemic child by screening pregnant women before 16 wks of gestation and offering prenatal diagnosis if needed.
3. To increase the awareness among general population about prevention of birth of genetically handicapped child.

MATERIALS AND METHODS

Three sets of pregnant women were screened

(i) Those attending Antenatal Clinic of our hospital (1962)

(ii) Those referred from other units – pregnant with anemia and

(iii) Those referred with history of one or more thalassaemic child (133). During the period of 1996 to 1999, a total of 1962 women—attending the antenatal clinic of Ramakrishna Mission Seva Pratisthan (RKMSP) were screened. The maternal age ranged from 14 to 46 years. All cases predominantly came from the State of West Bengal, particularly the district of South and North 24 Parganas. They were found to be mostly from Bengali Hindu population (97.4%). From group (i) Women of different gestational ages were screened irrespective of the feasibility of prenatal diagnosis. 138 husbands of carrier mothers were offered carrier testing. 95 of whom agreed counselling and prenatal diagnosis were offered to 15 couples where both parents were carriers (couples "at risk"). Only 6 of them agreed to go for prenatal diagnosis for their unborn baby. The gestational age for the mother of these couples was 14-16 weeks. Prenatal diagnosis and counselling were offered to another 19 couples from group (ii) and (iii).

Methods

The venous blood was taken in EDTA vials and following tests carried out:

(i) NESTROFT(Naked Eye Single Tube Red Cell Osmotic Fragility) test. It is a simple test based upon the osmotic fragility of red cells which has been applied widely with equal sensitivity and specificity[12,15,17].

(ii) Red cell indices by an electronic red cell counter (model no Sysmex K1000, Japan),

(iii) Haemoglobin electrophoresis on agar gel at pH 8.6[4]

(iv) HbA_2 estimation[1].

(v) Foetal haemoglobin was estimated by alkali denaturation method[6].

(vi) DNA analysis of couples were done before prenatal samples for each couple was collected. DNA was extracted using Qiagen blood extraction mini kit (Cat No 51104). If the couple have already an affected child, he or she was also considered for study especially when the mutations for one or both the parents of that particular family could not be characterized.

(vii) 10-15ml of amniotic fluid was subjected to centrifugation and the cells were collected in 200-400 µl of amniotic fluid while the rest of the amniotic fluid was discarded. DNA was extracted using the same kit following the same procedure as for whole blood.

(viii) PCR ARMS amplification

All samples were subjected to *in vitro* amplification by polymerase chain reaction (PCR) based amplification refractory mutation system (ARMS) technique using genomic DNA[13]. Screening for the presence of seven common β-thalassemia mutations of this region[8] was carried out. These were :IVS1nt5 (G->C), frarmeshift (FS) Codon 41-42 (-TCTT), Codon 30 (G->C), Codon 15 (G->A), FS Codon 8/9 (+G), 619 bp deletion and Hb E mutation at Codon 26 (G->A).

The mutations could be identified in 47 of the 50 individuals in 25 couples. The individuals whose β-mutations remained uncharacterized, DNA was subjected for identification of three rarer mutations, IVS 1 nt1 (G->T), cap+1 (A->C) and -88 (C->T) which were reported to be present in Indian populations[20].

When these mutations were also found to be absent, genomic DNA was subjected to identify the polymorphic sites on the β-globin gene using PCR technique followed by restriction enzyme digestion (RFLP)[10,14,16,18].

RESULTS AND DISCUSSION

A comparison between the use of NESTROFT test[12,17,15] and that of estimation of red cell indices and HbA_2 by electrophoresis for identification of carriers showed no significant difference in this present study. (Table I) Thus use of NESTROFT test useful for preliminary screening of (β -thalassemia carriers specially at places where there is problem of getting proper laboratory facilities with modern equipment. The percentage of carriers was found to be 7.13 and 7.04 respectively using these two methods. Only 4 Eβ patients were identified in this population (0.2%). The result is same using both the methods.

Table 1: Prevalence of thalassemia in pregnant women enrolled in the study identified by two different screening methods

	Screened by NESTROFT only	*Electrophoresis with HbA_2 Estimation*
Number Tested	1738	1960
Number of Thalassemia Carriers (β, D, E & S)	124	138
Eβ Thalassemia patients	4	4
% Normals	92.63	92.76
% Carriers	7.13	7.04
% Eβ patients	0.23	0.20

In the present study haematocrit levels were low in all cases compared to normal levels (Table 2). The lower values found for the lower limit of the normal range found in this group of study population may be due to anaemia during pregnancy or some other causes but these levels were found to be significantly higher than that of the carrier group or patients. A comparison between the MCV and MCH values of the normal and that in carrier groups showed significant differences between themselves except when normal group was compared with HbD carriers for these values.

The normal level of HbA_2 for this study population was found to have a range 1.2-3.4 Significantly higher HbA_2 or HbE values than normal were observed in carrier subjects.

The HbA_2 and HbE values were found to be much higher for HbE and HbD heterozygotes than β-thalassemia heterozygotes.

As the cut off limit of the percentage of HbA_2 is $\geq$ 3.5 for carrier detection[6], a comparative study was made for MCV and MCH values between the two groups ie. a) one group with <3.5 and b) another with $\geq$ 3. 5 HbA_2 %. It was observed that the highest percentage of 'normal' with <3.5 HbA_2% were found in the group with MCV $\geq$ 76 fI and MCH with $\geq$ 27 pg .It was noted that the most of the carriers (with HbA_2% $\geq$ 3.5) were found with MCV with normal range. MCH distribution was observed equally among the three groups ($\geq$ 27, 26-27, and <24 pg) (Table 3). It is clear that only red cell indices and haemoglobin estimation are not enough for confirmed identification of carriers. Estimation of HbA_2 is necessary for confirmation.

All 138 husbands of the patients identified as carriers were called for screening for carrier detection. Of them 95 husbands reported. 15 subjects were found to be carrier for either Hb E or β-thalassaemia. These couples were offered antenatal counselling and asked to come for prenatal diagnosis. From these 15 couples only 6 couples agreed for prenatal diagnosis.

A total of another 133 antenatal cases were also referred to our laboratory for investigation of thalassemia. A total of 47.36% carriers and 3.76% patients were identified. All 68 carrier/ patient cases were offered counselling and husbands were called for carrier testing. Only 41 husbands reported (60.3%) for testing.

From these 133 referred antenatal cases (32) couples came as referred cases after they had one or more abortions or MTP due to unknown causes or they had at least one or more affected thalassemia child.

This shows the poor awareness about thalassemia among common people which let them have children with thalassemia. This put them in both psychological and monitory burden. The painful experience of having a thalassemic child led them come for counselling for the future baby. All of them did not turn up for prenatal diagnosis for the pregnancy they were carrying due to late gestational age, ethical reasons or unknown cause.

Thus a total of 25 couples (6 couples were identified previously + 19 couples from the referred cases) agreed to go for prenatal diagnosis. Table 4 showed investigation of total 25 couples who agreed for prenatal diagnosis.

Table 2: Summary of data on thalassemia / haemoglobinopathy carrier/patient in comparison to that of normal (women only) in the study population

	Sample No.	*Hb(%)±SD (N. R. 11-16.5)*	*MCV (fl)±SD (N. R. 76-96)*	*MCH (pg)± SD (N. R. 27-32)*	*HbA_2(%)±SD (N. R. 0-3.5)*	*HbE (%)±SD (N. R. 0-0)*	*HbD/S(%)±SD (N. R. 0-0)*	*HbF(%)±SD (N. R. <1.0)*
Normal (a)	1820	11.05±1.21 (5.3-15.4)	99.91±9.94 (59.7-128.8)	30.00±3.52 (18.3-43.0)	2.77±0.39 (1.2-3.4)	-	-	0.63±0.15 (0.4-0.9)
β-carrier (b)	56	9.29±1.31 (6.3-12.7)	79.58±11.44 (63.6-115.8)	23.82±3.96 (18.4-39.1)	4.69±0.50 (40-5.9)	-	-	0.98±0.49 (0.4-2.3)
HbE carrier (c)	75	10.33±1.15 (7.1-12.7)	88.44±7.08 (73.3-100)	26.67±2.56 (19.5-33.4)	-	23.34±3.00 (117.2-31.4)	-	0.76±0.31 (0.4-1.0)
HbS carrier (d)	1	9.8	96.7	31.9	-	-	40.6	0.8
HbD carrier (e)	6	10.20±1.16 (8.3-11.6)	98.82±7.95 (84.6-110.1)	29.03±2.15 (25.6-31.7)	-	-	32.68±4.66 (23.6-38.1)	0.93±0.2 (0.6-1.2)
Eβ-patient (f)	4	6.83±0.94 (6.1-8.4)	72.70±9.02 (64.6-87.9)	21.03±2.91 (18.1-25.4)	-	54.55±10.46 (45.6-71.8)	-	15.48±6.11 (5-20.3)

Combination	*t-value*			
	Hb	*MCV*	*MCH*	*HbA_2 /E/D*
axb	9.62*	13.14*	11.44*	27.16*
axc	5.41*	13.62*	11.10*	50.17*
axd	1.81	0.34	1.10	15.74*
axf	8.96*	6.03*	6.19*	9.90*

*P<0.001; a-Normal, b-β-Carrier, c-E Carrier, d-D Carrier, f-Eβ thalassemia patient.

Table 3: Relationship between HbA_2 level and red cell indices in the study population (women only)

Group (with Hb A_2 Level in %)	*MCV (N. R.l 76-96 fl)*			*MCH (N. R. 27-32pg)*		
	≥ 76	75-70	<70	≥ 27	26-24	<24
a) <3.5 (Number present)	91.12% (1786)	1.27% (25)	0.36% (7)	79.69% (1562)	8.26% (162)	4.79% (94)
b) ≥3.5 (Number present)	5.61% (110)	1.07% (21)	0.56% (11)	2.24%(44)	2.70%(53)	2.29%(45)

N. R. - Normal range

Table 4: Frequency of different mutations identified by DNA analysis in the 25 couples investigated for prenatal diagnosis

Type of Mutation identified	*Mutation in father (No. of cases)*	*Mutation in mother (No. of cases)*	*Type of Mutant alleles (Total Number)*	*Total % of mutation observed*
For Carriers				
IVS 1nt5(G->C)/N	15	16	IVS 1nt5(G->C) (31)	59.62
Codon 26 (HbE)/N	6	5	Codon 26 (HbE) (14)	26.92
Codon 8/9(+G)/N	1	1	Codon 8/9 (+G) (2)	3.85
FS 41/42(-TCTT)/N	1	0	FS 41/42(-TCTT) (1)	1.92
Uncharacterized/N	1	2	Uncharacterized (3)	5.77
For patients			619bp del (1)	1.92
Codon 26/codon 26	1			
619bp del/codon 26		1		
Total	25	25		100.00

Table 5: DNA analysis for detection of mutations and diagnosis in 26 pregnancies investigated

Type of mutation identified	*Total no. in each case*	
Unaffected pregnancies		
IVS 1nt5(G->C)/N (Normal)	6	β – Carrier
619 bp/N	1	β - Carrier
Uncharacterized /N	1	β - Carrier
Codon 26/N	5	HbE- Carrier
N/N	5	
Affected Pregnancies		
Codon 26/IVS 1-5 (G->C)	3	E β-Thalassemia patient
IVS 1nt5(G->C)/IVS 1nt5(G->C)	3	β -Thalassemia patient
IVS 1nt5(G->C)/Codon 8/9	1	β-Thalassemia patient
IVS 1nt5(G->C)/Uncharacterized	1	β-Thalassemia patient

Most of the prenatal diagnosis were done at 2nd pregnancy (53.85%). Only one came at their 1st pregnancy as this couple was educated people. DNA analysis of all the parents (50 individuals) showed IVS 1-5 as the most prevalent mutation (59.62%) followed by Codon 26 (26.92%) (Table 4, 5) which supports our previous report of prevalence of β-thalassemia, mutation in Eastern India[7,8,9]. There were three uncharacterized mutations which we characterized with the help of RFLP sites for identification of transmission of mutations from parent to the foetus.

Overall the DNA analysis of foetus showed 30.77 % affected pregnancies (with 11.54 % β thal major and 19.23% Eβ thal) carrying both parent's mutation. 69.23 % were found to be unaffected. The most frequent mutation was again found to be IVS 1-5 (G-C) followed by Codon 26 in both affected and unaffected pregnancies (Table 5).

CONCLUSIONS

From the study the following conclusions can be made:

1) NESTROFT test was found to be effective in identification of carrier detection although there is much utility and need for further evaluation and use of Haemoglobin A_2 (Hb A_2) estimation is essential to confirm the carrier status. It is useful to screen large populations especially in the remote village areas and primary health care centers where proper laboratory facilities are not available. Finding out the red cell indices is also not enough for carrier

detection as in the present study we found a high incidence of carriers with $\geq$ 3.5 Hb A_2 among many of these antenatal female cases with normal MCV.

2) About 7.04% females attending the antenatal clinic were found to be carriers among this study population most of which were otherwise normal.
3) 15.8% couples were found "at risk" and genetic counselling was offered to these couples.
4) Due to poor awareness, social or superstitional obstacles, identification of carrier status for parents at late stage of pregnancy, not all the couples "at risk" came for prenatal diagnosis.
5) A high percentage of carriers/patients (47.36%) were detected in the referred antenatal cases.
6) Prenatal diagnosis were successfully done for 26 pregnancies from 25 couples referred to the lab as both parents were carriers. 69.23% foetuses were found to be unaffected and advised to carry on to term and follow up baby for more than 6 months. 30.77% found to be affected. Termination of pregnancies were suggested by the concerned couselling doctor. Most of the couples came forward for prenatal diagnosis only when they had already an affected child.
7) Antenatal screening during the first termination was found to be very useful as short term program which includes identification of carriers, prenatal diagnosis for the couples "at risk" and termination of pregnancies if needed to prevent the birth of thalassemic children.

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