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PRENATAL DIAGNOSIS OF β-THALASSAEMIA: EXPERIENCE IN A DEVELOPING COUNTRY

RENU SAXENA, PAWAN K. JAIN, ELIZABETH THOMAS AND ISHWAR C. VERMA*

Genetic Unit, Department of Pediatrics, World Health Organization Collaborating Center in Genetics, All India Institute of Medical Sciences, New Delhi, India

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SUMMARY

We present our experience with the amplification refractory mutation system (ARMS) for the prenatal diagnosis of β-thalassaemia in 415 pregnancies of 360 women. Five mutations of the β-thalassaemia gene common in Asian Indians accounted for 89·2 per cent and rare mutations for 7·2 per cent of all mutant chromosomes, while 3·3 per cent of chromosomes remained uncharacterized. Identical mutations were present in both parents in 43·2 per cent of cases, due to caste-based marriages in India. A confirmed diagnosis was given in 401 (98·3 per cent) cases, of which a complete diagnosis (whether the fetus was normal, a carrier, or homozygous) was possible in 391 (94·2 per cent) of the cases. In 15 couples, the mutation was identified in only one parent. In nine of these, the identified mutation was not present in the fetus, predicting normal/carrier status, while in five the identified mutation was present in the fetus, suggesting carrier/affected status. The abortion rate was 3·9 per cent. Pitfalls in diagnosis were failure of oligonucleotides to work, maternal contamination, and false paternity. The ARMS provides an inexpensive, robust and non-isotopic method for the prenatal diagnosis of β-thalassaemia in India. Recommendations are outlined for establishing a prenatal diagnostic service in developing countries. © 1998 John Wiley & Sons, Ltd.

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KEY WORDS: β-thalassaemia mutations; prenatal diagnosis; amplification refractory mutation system

INTRODUCTION

β-Thalassaemia is the commonest single gene disorder in many developing countries. In India, the frequency of carriers varies between 1 and 17 per cent in different regions, with a mean prevalence of 3·3 per cent (Modell and Bulyzhenkov, 1988). On the basis of current demographic data (UNICEF, 1996), it is estimated that there are 29·7 million carriers of β-thalassaemia in India and about 7000 infants with homozygous β-thalassaemia are born every year. The expense of therapy of a child with β-thalassaemia major in India is about US$3200 per child per year, which is beyond the reach of the majority of families. Prevention of the birth of affected infants is, therefore, a high priority to reduce the burden of the disease. At present, this can only be achieved by prenatal diagnosis, and the abortion of an affected fetus.

Prenatal diagnosis of β-thalassaemia has been available in developed countries for almost 20 years. Initially this was done by analysis of globin-chain synthesis by fetal blood cells obtained by placental aspiration (Kan *et al.*, 1975), and later by DNA analytical techniques such as Southern blotting and hybridization to synthetic oligonucleotide probes (Orkin *et al.*, 1983), dot blot (Saiki *et al.*, 1986), reverse dot blot (Saiki *et al.*, 1989; Cai *et al.*, 1994), and the PCR-based amplification refractory mutation system (ARMS) using allele-specific primers (Old *et al.*, 1990).

*Correspondence to: I. C. Verma, F.R.C.P., Department of Genetic Medicine, Sir Ganga Ram Hospital, Rajinder Nagar, New Delhi-110 060, India. E-mail: icverma@giasdl01.vsnl.net.in.

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In initial studies, fetal DNA was extracted from amniotic fluid cells (Kazazian *et al.*, 1980). The advent of chorionic villus sampling (CVS) propelled the application of prenatal diagnostic technology on a wider scale (Old and Higgs, 1982). An essential prerequisite for introducing this in developing countries is the delineation of mutations causing β-thalassaemia. For Asian Indians, this was achieved first by study of those who had migrated to western countries and then by study of subjects residing in India (Kazazian *et al.*, 1984; Wong *et al.*, 1986; Thein *et al.*, 1988; Varawalla *et al.*, 1991a,b; Jain *et al.*, 1994; Garewal *et al.*, 1994). We have also analysed the regional distribution of 1050 β-thalassaemia chromosomes in India (Verma *et al.*, 1997), which has helped immensely in establishing a prenatal diagnosis programe. We present our experience with prenatal diagnosis in 415 pregnant women at risk in India and provide guidelines for establishing similar programmes in other developing countries.

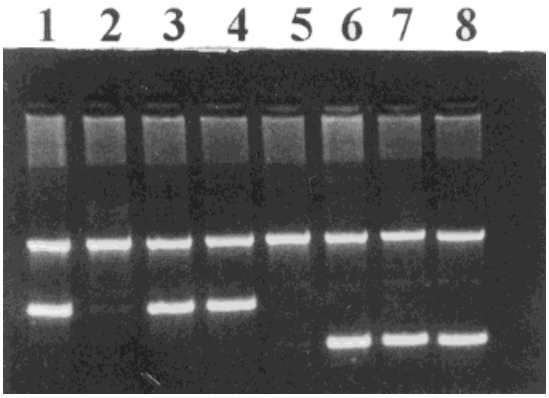

Fig. 1—Prenatal diagnosis of β-thalassaemia: parents with non-identical mutations. Lane 1: mother positive for codon 41/42; lane 2: father negative control for codon 41/42; lanes 3 and 4: CVS positive for mother's mutation; lane 5: mother's DNA negative for IVS-I-5; lane 6: father positive for IVS-I-5; lanes 7 and 8: CVS positive for father's mutation. The fetus is affected and will have β-thalassaemia

MATERIALS AND METHODS

From May 1992 through October 1996, 415 prenatal diagnoses were sought by 360 pregnant women. Of these, 349 (96·9 per cent) had a child with β-thalassaemia and desired prenatal diagnosis in a subsequent pregnancy. Eleven (3·1 per cent) couples had been identified to be carriers of the β-thalassaemia gene by haematological studies even before the birth of the first affected child.

Peripheral blood samples of the parents, the affected child, and normal children (if any) were collected in EDTA for delineation of the β-thalassaemia mutations. The chorionic villus (CV) samples were obtained by either transcervical or transabdominal placental biopsy at 8–20 weeks of gestation. The CV sample was carefully dissected from the maternal decidua under an inverted microscope. In four cases, a fetal blood sample was obtained at 18–20 weeks of gestation.

DNA was extracted from blood as well as the CV sample by standard sodium dodecyl-sulphate (SDS)/proteinase K digestion followed by phenol/chloroform extraction (Old and Higgs, 1982; Old, 1993), or salt precipitation (Miller *et al.*, 1988). The β-thalassaemia mutations in the parents and affected child (if available) were analysed using the polymerase chain reaction (PCR)-based ARMS technique (Newton *et al.*, 1989). The primers used were specific for a particular β-globin gene mutation and the PCR protocols were as previously described (Old *et al.*, 1990; Varawalla *et al.*, 1991a,b). Briefly, each reaction tube contained two sets of primers, one set to amplify a specific sequence as a 'control' and the other set for direct amplification of the mutation. The 'control' sequence of 861 bases lies at the 3′ end of the gene and stretches across the site of a 619 base-pair (bp) deletion, so that if the deletion is present, a shorter fragment of 323 bases is observed.

Each sample was initially screened for the five common mutations (IVS-I-5 G→C, IVS-I-1 G→T, codon 8/9 +G, codon 41/42 −CTTT, and 619-bp deletion). If no mutation was detected, the sample was analysed for 12 rare mutations reported in Asian Indians (codon 16 −C, Cap +1 A→C, codon 15 G→A, −88 C→T, codon 30 G→A, codon 30 G→C, codon 5 −CT, codon 47/48 +ATCT, IVS-II-837 G→T, IVS-I-110 G→A, IVS-I 25-bp deletion, and codon 88 +T). The presence of Hb E and sickle cell trait was also confirmed using allele-specific ARMS primers.

Prenatal diagnosis was done by looking for the parental mutations in fetal DNA. Appropriate samples of DNA (positive and negative for the particular mutation) were amplified along with the fetal DNA (Fig. 1). If the parental mutations were identical, the presence of the normal sequence at that site was examined by using appropriate allele-specific primers (Fig. 2). This allowed differentiation between homozygous and heterozygous

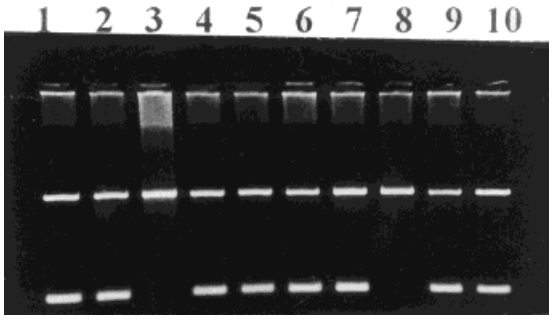

Fig. 2—Prenatal diagnosis of β-thalassaemia: parents with identical mutations. Lanes 1 and 2: mother and father positive for IVS-I-5; lane 3: negative control for IVS-I-5; lanes 4 and 5: CVS positive for IVS-I-5; lanes 6 and 7: mother and father positive for the normal sequence to IVS-I-5; lane 8: affected child (homozygous for IVS-I-5), negative for the normal sequence; lanes 9 and 10: CVS positive for the normal sequence. The fetus has β-thalassaemia trait

states. If the mutation was identified in one parent only, the presence or absence of this mutation in the CV sample was determined.

Restriction fragment length polymorphism (RFLP) markers linked to the β-globin gene were analysed to confirm the diagnosis in 31 (7·5 per cent) cases, in whom the parental mutations were unidentified or to confirm the ARMS results, especially in the presence of some of the rare mutations. The RFLPs studied were Hind II/ε, Hind III Gγ, Hind III/Aγ, Hind II/5′ψβ, Hind II/3′ψβ, Ava II/β, Hinf I/β (Bam HI/β), and Xmn I/Gγ, by previously described methods (Old and Ludlam, 1991; Varawalla *et al.*, 1992). Polymorphism of hypervariable region 3′ of the apolipoprotein B (apoB) gene was examined in 257 cases to rule out contamination of fetal DNA with DNA derived from maternal cells as described by Decorte *et al.* (1990).

RESULTS

Of the 415 prenatal diagnoses, 411 (99·0 per cent) were performed on CV samples and 4 (1·0 per cent) on fetal blood samples. The CV samples were obtained by the transcervical route in 245 (59·6 per cent) of the cases, and by the transabdominal route in 166 (40·4 per cent) of the cases. Figure 3 shows the gestation at which fetal tissue was sampled (mean 11·7 weeks).

In 314 (87·2 per cent) at-risk couples, only one pregnancy was monitored, while in 46 (12·8 per cent) women, two or more pregnancies were monitored (Table I).

In 642 (89·2 per cent) carrier parents, one of the five common Indian mutations was identified (Table II). Rare mutations were detected in 52 (7·2 per cent) parents, while 24 (3·3 per cent) parents were negative for the 17 mutations identified in Asian Indians. One couple presented in advanced pregnancy with a history of an infant who died at 3 months with a possible diagnosis of thalassaemia. This couple was found to be negative for all the known Indian mutations and a repeat analysis of their red cell indices and Hb A_2 proved them not to be carriers of β-thalassaemia.

In 391 (93·7 per cent) of 415 pregnancies, the mutations were identified in both parents and a

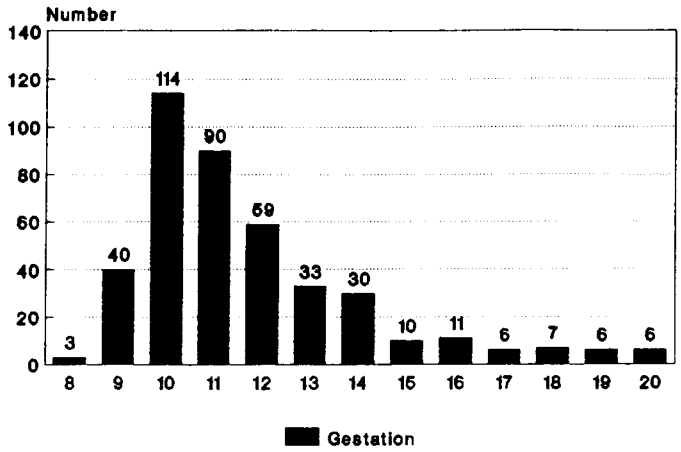

Fig. 3—Gestation at which the invasive procedure was carried out for prenatal diagnosis

Table I—Distribution of couples by the number of pregnancies monitored for prenatal diagnosis

No. of pregnancies	Couples		No. of fetal procedures
	No.	%	
One	314	87·2	314
Two (includes two twin pregnancies)	39	10·8	78
Three (includes one twin pregnancy)	5	1·4	15
Four	2	0·6	8
Total	360	100·0	415

Table II—Frequency distribution of β-thalassaemia mutations in couples opting for prenatal diagnosis ($n = 720$)

Mutation	Number	%
IVSI-5 G→C	217	30·0
619 bp del	165	22·9
IVSI-1 G→T	111	15·4
Codon 8/9 +G	89	12·4
Codon 41/42 −CTTT	60	8·3
Codon 16 −C	11	1·5
Hb E	8	1·1
Codon 47/48 +ATCT	7	1·0
Cap site+1 A→C	7	1·0
Sickle Hb	6	0·8
Codon 5 −CT	5	0·7
Codon 15 G→A	3	0·4
Codon 30 G→A	2	0·3
Codon 30 G→C	2	0·3
−88 C→T	1	0·1
Normal	2	0·3
Uncharacterized	24	3·3

Table III—Results in pregnancies where a precise diagnosis was achieved

Fetus	Parents with identical mutation	Parents with different mutations	Total	%
Normal	45	57	102	26·1
Trait	82	102	184	47·1
Affected	42	63	105	26·8
Total	169 (43·2%)	222 (56·8%)	391	

complete prenatal diagnosis was possible. Identical mutations were detected in 43·2 per cent of the couples, which required the use of primers for the normal sequence at the site of the mutation for accurate prenatal diagnosis (Fig. 2).

On molecular studies 26·1 per cent of fetuses were found to be normal, 47·1 per cent to be carriers, and 26·8 per cent to be affected with β-thalassaemia (Table III). In 15 fetuses, a complete diagnosis was not possible as the mutation remained unidentified in one parent, including two cases of false paternity (Table IV). Of these, the fetus was negative for the identified parental mutation in nine cases and therefore was either normal or a carrier with no risk of having β-thalassaemia major. In six cases, the fetus had inherited the identified mutation from the parent and was therefore at 50 per cent risk of being a carrier or affected. In these cases, either RFLP analysis was not informative for the uncharacterized parent's chromosome, or an affected/normal sib was not available for study. These cases were referred to another centre for prenatal diagnosis by globin-chain synthesis.

The diagnosis was not attempted in seven (1·7 per cent) cases, because of inadequate fetal tissue in four cases and abortion immediately after the CVS in three cases. In one case both parental mutations could not be identified. This couple underwent prenatal diagnosis based on globin-chain synthesis at 18 weeks and had an unaffected fetus.

In 16 (3·9 per cent) cases, abortion ensued, mostly in the early part of the study. These could be either procedure-induced or spontaneous, since there were three cases (not included in the present study) who had an appointment for CVS at 10–12 weeks of gestation but reported a spontaneous abortion before the invasive procedure itself.

Prenatal diagnosis by DNA extracted from fetal blood samples was done in four cases (Table V). Maternal blood contamination was ruled out in one case by study of apoB polymorphism, while in the other case this polymorphism was not informative. This couple continued the pregnancy and the carrier status of the fetus was confirmed after birth. In two cases, maternal blood contamination was excluded by the absence of the maternal mutation in the fetal DNA.

Table IV—Results in cases where prenatal diagnosis was carried out ($n=408$)*

		Number	% of diagnosis
(A) Precise diagnosis provided		401	98·3
(1)	Complete diagnosis	391	
(2)	Partial diagnosis —Not affected (normal/trait). One parent unidentifed (7). —False paternity (2)	9	
(3)	Both parents normal	1	
(B) Precise diagnosis not possible		7	1·7
(1)	50 per cent risk (trait/affected) —Positive for known parental mutations, other parent's mutation unidentified (5) —Both parents with same mutation, 'normal' sequence primer DNW (1)	6	
(2)	25 per cent risk —Both parent's mutation unidentified	1	
(C) Diagnosis not attempted		7	
	—Aborted before analysis	3	
	—Inadequate CVS	4	

*Excludes seven cases where the diagnosis was not attempted.

Table V—Prenatal diagnosis of β-thalassaemia by fetal blood sampling ($n=4$)

Sample No.	Maternal allele	Paternal allele	Fetus	Comment
1	IVS-I-5	IVS-I-5	Affected	No maternal blood contamination as negative for normal allele from mother
2	IVS-I-5	IVS-I-5	Trait	Confirmed after birth; apoB not informative for maternal contamination
3	IVS-I-5	IVS-I-5	Trait	ApoB informative; no maternal contamination
4	Codon 15	Codon 15	Normal	Confirmed by RFLP analysis; no maternal contamination as negative for maternal mutation

DISCUSSION

In the present study, CVS was carried out between 8 and 20 weeks of gestation but the majority (88·2 per cent) of women were tested during 9–14 weeks of gestation. Fetal tissue was collected by CVS by the transvaginal route if the pregnancy was between 8 and 12 weeks and by the transabdominal route in the later stages of gestation. In four cases, analysis was done on DNA from fetal blood samples because these women presented in advanced gestation (18–20 weeks). Contamination by maternal blood DNA was ruled out by study of apoB polymorphism.

The ARMS technique of allele-specific amplification used in the present study allowed precise identification of the mutations in both parents, making the prenatal diagnosis straightforward and easy in 98·3 per cent of the 408 fetuses at risk. In 15 (3·7 per cent) cases, the β-thalassaemia mutation was delineated in only one of the parents. However, in nine (60·7 per cent) of these cases a satisfactory diagnosis was possible, as the fetus was negative for the known parental mutation.

In 642 (89·2 per cent) of the carrier parents undergoing prenatal diagnosis, one of the five common mutations was identified. In 10·84 per cent of the pregnancies, the presence of the 'rare' Indian mutations had to be tested. In high-risk pregnancies involving β^+ mutations like Cap site +1 A→C and −88 C→T as well as cases of thalassaemia intermedia with two β-thalassaemia mutations (homozygotes or compound heterozygotes), the couples opted for prenatal diagnosis as they wanted completely healthy children without any reduced fitness. In 43·28 per cent of the cases, the parents had the same mutation. This requires testing for the normal sequence at the site of the mutation. Therefore, the cost for additional oligonucleotides for the identification of the rare mutations as well as the normal sequence at the site of the mutations is essential as it allows accurate diagnosis.

DNA diagnosis based on polymorphisms (RFLP) at different loci linked to the β-globin gene (Old and Ludlam, 1991; Varawalla *et al.*, 1992) was used only in cases where the CV sample was of doubtful quality and when β-thalassaemia mutations remained unidentified in either of the parents, provided a normal or an affected child was available for study.

The contamination of fetal tissue with maternal cells is a constant risk in tests on CV samples. If there is any doubt about the quality of fetal tissue separation, it is necessary to analyse using RFLP or other polymorphisms of hypervariable regions such as 3′ of the Ha-*ras* gene, 5′ to the joining segments of the heavy immunoglobulin gene, and 3′ of the apoB gene (Decorte *et al.*, 1990). We analysed the polymorphism of hypervariable region 3′ to the apoB gene because its heterozygosity among Indians is high (unpublished observations).

On the basis of the experience gained in the present study, we would recommend the following strategy for establishing a prenatal diagnostic service in developing countries: (1) The fetal sampling technique should be performed by a limited number of obstetricians, who should gain experience by performing CVS in cases undergoing medical termination of pregnancy. (2) Chorionic villi are the tissue of choice for obtaining fetal DNA. (3) Expertise in separating chorionic villus tissue from decidual (maternal) tissue must be acquired. (4) The mutations causing β-thalassaemia in a given region should be identified in at least 100 children with thalassaemia major or their parents (200 mutant chromosomes). (5) Any of the non-isotopic PCR-based mutation detection methods such as reverse dot blot or ARMS technique should be set up. Primers for the common and rare mutations, as well as for normal sequences at that site, should be procured. (6) Samples positive and negative for the mutation being analysed as well as a reaction with no DNA template must be tested at the same time, using the same reagents and chemicals. (7) Maternal contamination should be excluded by study of polymorphic genes like 3′ of the apoB gene. (8) If parental mutation(s) remain unidentified, diagnosis should be sought using linkage studies (RFLP), or DGGE or SSCP analysis. (9) Many laboratories obtain a fetal diagnosis by two separate techniques. This may not be feasible in developing countries and confirmation by a second technique should be restricted to special situations. Thus, by following the above strategy a prenatal diagnostic service can be established in all developing countries where β-thalassaemia is a major problem.

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