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Prenatal diagnosis of beta-thalassaemia in Pakistan: experience in a Muslim country

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A service for prenatal diagnosis of β-thalassaemia was introduced in Pakistan in May 1994. Two renowned Islamic scholars, consulted before the service was introduced, ruled that a pregnancy can be terminated if the fetus is affected by a serious genetic disorder, and if termination is before 120 days (17 weeks) of gestation. During the first 3½ years of the service 300 couples requested the test. Almost all the couples had been informed by their treating doctors. Most diagnoses were made between 10 and 16 weeks of gestation, and only 15 (5%) were reached after the 16th week. DNA analysis was by the amplification refractory mutation system (ARMS). A multiplex ARMS was developed in which three primer combinations identified the mutations in 91.5% of the couples. In 13 couples (4.3%) linkage analysis was required for the fetal diagnosis. In 47/53 (88.7%) women carrying an affected fetus the pregnancy was terminated. In six cases it was declined principally on religious grounds. Postnatal confirmation of the prenatal diagnosis was possible in 117 unaffected children. One year after the start of the service, interviews with 141 couples with an affected child showed that 72% knew of the availability of prenatal diagnosis. Thirty-two of the informed couples had had a pregnancy, but only 18 (56%) used prenatal diagnosis. The main reasons for non-utilization of prenatal diagnosis were the cost of the test and fear of undergoing the test, though some gave no clear explanation. This study demonstrates that prenatal diagnosis is feasible and acceptable in a Muslim country such as Pakistan. Copyright © 2000 John Wiley & Sons, Ltd.

KEY WORDS: prenatal diagnosis; thalassaemia; Pakistan

INTRODUCTION

At present, a regular service for prenatal diagnosis of thalassaemia does not exist in any Muslim country, and little is known about the response of Muslim communities to the availability of prenatal diagnosis and if termination of pregnancy (TOP). Data from the United Kingdom show that utilization amonst the British Pakistanis is very sensitive to gestational age at counselling, and uptake is greater when offered in the first trimester of pregnancy (Petrou, 1994).

Thalassaemia is the commonest single gene disorder in Pakistan, and each year there are more than 3500 new affected births in the country (WHO 1993, unpublished document EM/NCD/7-E/R/8.93/91). In May 1994 a service for prenatal diagnosis of β-thalassaemia was introduced in Pakistan. We report the process of introduction and the response of at risk couples to the availability of prenatal diagnosis.

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METHODS

Initial preparation

Prior to introducting the prenatal diagnosis service, two renowned Pakistani religious scholars (Mohammad Taqi Othmani and Dr Malik Ghulam Murtaza) were consulted on the Islamic view of TOP for a serious genetic disorder. Both scholars were of the opinion that the pregnancy can be terminated provided that the severe nature of the disorder is confirmed and the procedure is done before 120 days of gestation. They clearly prohibited termination after 120 days even if the fetus is affected.

The Fatimid Foundation, a charity organization treating thalassaemic patients, then started to inform the affected families. Information booklets in Urdu (the local language) were also distributed to the families. The service was advertised in the newspapers, and some programmes were put on television.

Booking a couple

At the time of booking the couples were counselled. The gestational age and the position of placenta were ascertained by ultrasound examination and the date for chorionic villus sampling (CVS) was booked. Blood samples (3–5 ml) from the parents, and an affected child if available, were collected for DNA

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analysis. Only three couples who requested prenatal diagnosis did not have an affected child. The diagnosis in the parents and the affected children were made in various laboratories in Pakistan. For cost implications we did not repeat carrier screening tests in the parents.

DNA analysis

The amplification refractory mutation system (ARMS) was used to screen for the β-thalassaemia mutations (Ahmed *et al*., 1996). In the first round of ARMS the five most common β-thalassaemia mutations were tested, followed by a second or a third round for uncommon or rare mutations, when the mutation was not identified. In 142 couples a multiplex ARMS PCR was used. Each couple was tested with three multiplex primer combinations (Table 1). The multiplexes AD-1, AD-2 and AD-3, were prepared as stock solutions with a final concentration of 5 pM/μl of each primer. The two control primers and the respective common primers (Ahmed *et al*., 1996) were also added to the primer mixture. The amplified products were of a sufficiently different size for resolution by polyacrylamide gel electrophoresis. However, the fragments generated by IVSI-1 and IVSI-5 differed by 5 bp and therefore were difficult to resolve. Similarly, there was no difference in the size of the fragments of Cd30 (G-C), Cd30 (G-A) and IVSI-1. The mutations IVSI-1 and IVSI-5 were differentiated by adding IVSI-1 primer to AD-1 and AD-2 multiplexes. IVSI-5 resulted in amplification with AD-1, but IVSI-1 resulted in amplification with AD-1 and AD-2. Amplification with AD-2 but not AD-1 indicated Cd30. The difference between the two mutations in Cd30 was not important because the same normal primer was required to differentiate between its homozygotes and heterozygotes. An allelic ladder for the various mutations was prepared by pooling the polymerase chain reaction products of separately amplified reactions. The amplified products were separated on 6% non-denaturing polyacrylamide gels using Mini-Protean apparatus (Bio-rad, USA). Two microlitres of the amplified product and 5 μl of the allelic ladder was run at 150 V for 40 min and the gels were stained with silver nitrate.

The chorionic villus sample was carefully dissected under a microscope and incubated overnight in a lysis buffer (SDS/EDTA/proteinase K). Fetal diagnosis was made by the ARMS reactions. When one or both parental mutations could not be identified by ARMS, the diagnosis was done by restriction fragment length polymorphisms (RFLP). Six polymorphic sites closely linked to the β-globin gene were used as linkage markers (Varawalla *et al*., 1992). Following a prenatal diagnosis information on pregnancy outcome was collected.

Follow up

Following prenatal diagnosis the information concerning complications in pregnancy (if any), TOP, and postnatal outcome was collected in as many cases as possible.

Response of at risk couples

One year after the introduction of the service, 141 randomly selected couples who visited the Fatimid Thalassaemia Centre, Lahore for treatment of their children were interviewed and asked: (1) if they had had any pregnancies since prenatal diagnosis became available; (2) if they had requested prenatal diagnosis; (3) if they were interested in using the test in any future pregnancies; and (4) about their attitude towards TOP.

RESULTS

Within 2–3 weeks from the announcement of the service the first couple came forward requesting prenatal diagnosis, and several other couples who were avoiding a pregnancy came for counselling. Thereafter, couples appeared at regular intervals and numbers increased steadily. During a period of 3½ years, 300 couples requested prenatal diagnosis. Almost all were informed of prenatal diagnosis by their doctors. In 31% one member, usually the mother, had watched a programme on television. A significant proportion (21%) were inspired by the experience of couples who had already used prenatal diagnosis, many of whom had healthy children after the test. The information booklets had not been delivered to all couples, and many couples were not able to read.

CVS

A total of 319 CVSs were done in 311 pregnancies (303 single pregnancies, eight twin pregnancies). Most were

Table 1—Combination of β-thalassaemia mutations screened by the multiplex ARMS PCR

AD-I		AD-II		AD-III	
Mutations	Fragment size	Mutations	Fragment Size	Mutations	Fragment size
Fr8–9 (+G)	215 bp	Cd5 (-CT)	205 bp	Cd15 (G-A)	500 bp
IVSI-5 (G-C)	285 bp	Fr16 (-C)	238 bp	Cap+1 (A-C)	567 bp
Fr41–42 (-TTCT)	439 bp	IVSI-1 (G-T)	280 bp		
IVSI-1 (G-T)	280 bp	Cd30 (G-C)	280 bp		
Del619 bp	242 bp	Cd30 (G-A)	280 bp		
		IVSII-1 (G-A)	634 bp		

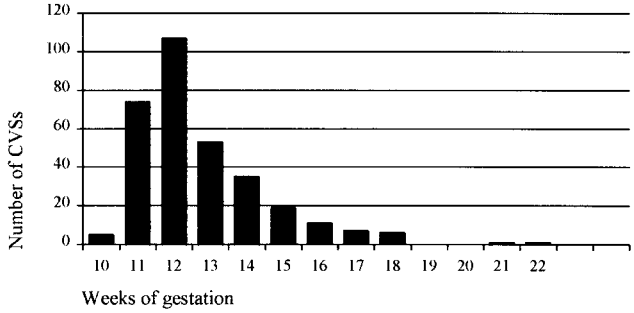

Figure 1—The gestation in 319 CVSs done for prenatal diagnosis of β-thalassaemia

done between 10 and 16 weeks of gestation, but 15 (4.7%) were done after the 16th week (Figure 1). Seventeen of the 300 couples (5.7%) used the test twice, and one couple used it three times. Fourteen of these couples had a previous prenatal diagnosis of an affected fetus.

DNA analysis

The first round of ARMS assigned a mutation to both of the parents in 115/158 (72.8%) couples. In the second round 153 (96.8%) couples, and after the final round 155 (98.1%) couples were assigned their mutations. The multiplex ARMS was technically feasible (Figure 2). The method was quick and simple. The other advantage was the use of only six reactions per couple to screen most of the mutations. This reduced the time and the cost of mutation analysis. AD-1 alone identified the mutations in 88/142 (62%) couples. AD-1 and 2 identified the mutations in 114 (80.3%) couples, while AD-1, 2 and 3 identified the mutations in 130 (91.5%) couples.

In fetal DNA testing the presence of the parental mutation and the absence of its normal allele indicated a homozygous fetus (Figure 3). The presence of mutant and normal alleles indicated a heterozygous fetus, while a normal allele but no mutation indicated a normal fetus. When the parents had different mutations and both were present in the fetus, a diagnosis of affected fetus was made, while the presence of one parental mutation indicated a heterozygous fetus. The results of 311 fetal diagnoses were: trait, 166 (53.4%); normal, 72 (23.1%); and major, 73 (23.5%).

RFLP analysis was necessary in 13/300 (4.3%) couples when one or both of the parental mutations was not found by ARMS. A prenatal diagnosis was successfully accomplished for all couples.

Follow-up

All the couples could not be followed up. Information on termination of pregnancy was available in 53/71 mothers with affected fetuses and in 47/53 (88.7%) the pregnancy was terminated (Figure 4). Six couples declined termination of pregnancy, mostly due to religious reasons. In 117 CVSs, diagnosed as normal or trait, the children have been born and no error in the prenatal diagnosis has been reported. 5.4% (17/311) had a spontaneous aborton within two weeks of the CVS. The complications were operator dependent

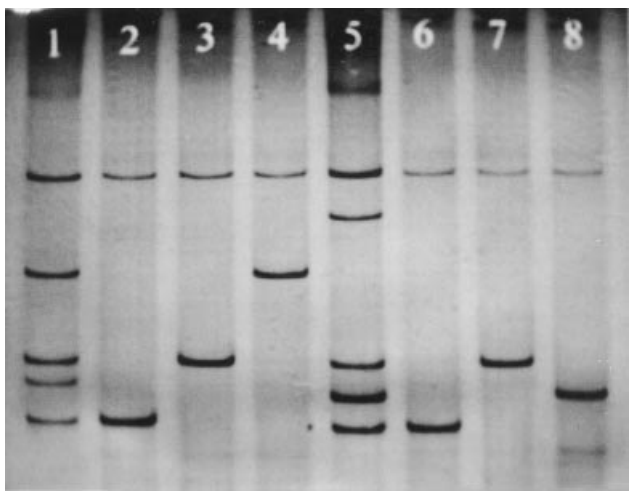

Figure 2—Silver stained polyacrylamide gel electrophoresis of multiplex ARMS PCR for β-thalassaemia mutations. Lanes 1 and 5 show allelic ladders for AD-1 and AD-2 respectively. Lanes 2, 3 and 4 show AD-1 multiplex reactions positive for Fr 8–9, IVSI-5, and Fr41–42 respectively. Lanes 6, 7 and 8 show AD-2 multiplex reactions positive for Cd5, Cd30, and Fr16 respectively. Control bands of 861 bp are visible in all lanes

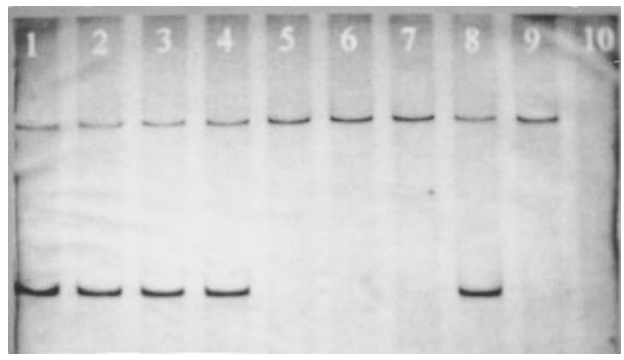

Figure 3—Silver stained polyacrylamide gel electrophoresis of ARMS PCR for prenatal diagnosis of thalassaemia. Lanes 1–9 show 861bp control bands. Lanes 1–4 show 285 bp bands of IVSI-5. Lanes 1 and 2 contain parent's samples while lanes; 3–4 contain fetal DNA in duplicate. Lane 5 is a negative control for IVSI-5. Lanes 6–7 show absence of bands for the normal allele of IVSI-5 in the fetal DNA. Lanes 8 and 9 are positive and negative controls for the normal allele of IVSI-5. Lane 10 is a reagent blank. The results indicate a homozygous fetus

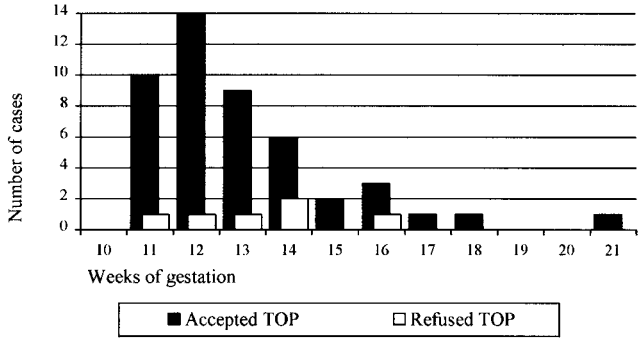

Figure 4—The response of the couples to TOP after fetal diagnosis of thalassaemia major

and varied at the two centres: 2.9% (2/136) at Lahore and 7.4% (13/175) at Rawalpindi.

Response of affected families to prenatal diagnosis

One hundred and one of the 141 randomly interviewed couples (72%) knew that prenatal diagnosis was available in Pakistan, while 38 (27%) were completely unaware. The remaining two couples had no interest because they had completed their families. During the period when prenatal diagnosis was available 62 couples had a pregnancy. Two women miscarried. Twenty-five of the 60 couples with a viable pregnancy (41.6%) were either unaware of prenatal diagnosis or came to know about it too late and therefore could not use it. Three couples were unaware of their risk. The remaining 32 couples were aware of the test facility: 16 (50%) requested prenatal diagnosis and two were waiting to use the test at the time of the study. Two couples chose to terminate the pregnancy without prenatal diagnosis. Twelve of the 32 couples (37.5%) knew about prenatal diagnosis but did not use it: six gave no clear explanation for this, two did not use it due to the cost, two due to the fear of undergoing the test and in two couples there was disagreement between the parents. The response to the enquiry to the possibility of using prenatal diagnosis in a future pregnancy showed that 29/141 (21%) had completed their families. In the remaining one hundred and twelve couples (91%) were in favour of prenatal diagnosis, four (3.6%) opposed it, and six were unsure. Of the 102 couples favouring prenatal diagnosis, 75 (73.5%) said they would request it unconditionally, while 27 (26.5%) would request it if free of cost. When asked about TOP, 124/141 (87.4%) couples felt they would terminate an affected pregnancy, 2% had a negative attitude, and the others were unsure.

DISCUSSION

Prenatal diagnosis has given a new dimension to thalassaemia prevention (Cao, 1987). First trimester fetal sampling methods combined with PCR-based diagnosis have also introduced this useful facility to third world countries where thalassaemia is an important public health problem (Saxena *et al.*, 1998). In our centre, most fetal diagnoses were done by direct mutation analysis, while in a minority of cases linkage analysis was used. Multiplex ARMS can substantially reduce the time and the cost of mutation analysis. In our hands multiplex ARMS was quick and efficient.

Inclusion of appropriate controls and reagent blanks has increased the accuracy of diagnosis (Higuchi and Kwok, 1989). The follow-up of a large number of children born after prenatal diagnosis shows the method adopted is accurate and reliable for large-scale application.

Reverse-dot blot offers an alternative method for screening a large number of mutations. The probes are filter bound and the non-labelled PCR product is hybridized to the immobilized allele-specific oligonucleotide probes (Cai *et al.*, 1994; Maggio *et al.*, 1993). This method is an alternative to multiplex PCR when screening beta-thalassaemia carriers in populations with a large number of beta-thalassaemia mutations. Denaturing gradient gel electrophoresis (Cai and Kan, 1990) also offers an alternative method for screening. However, the results are influenced by the presence of polymorphisms in the beta-globin gene and the same mutation gives different mobilities in the presence or absence of a polymorphism. For this reason we do not use this method for prenatal diagnosis, although this method would probably have avoided the necessity of RFLP analysis.

Our experience shows that prenatal diagnosis is technically feasible and is also accepted by many families who have children with thalassaemia major in Pakistan. However, its long-term role in prevention of thalassaemia in Pakistan would depend on how the community responds to the possibility of terminating pregnancies. Islam is the leading religion of Pakistan and it is the faith of over 95% of the population. The community would accept thalassaemia prevention if it is compatible with religious as well as cultural beliefs. The low rate of literacy makes it difficult for people to distinguish between their religious and traditional or cultural beliefs, and this can easily lead to misconceptions about the permissibility or prohibition of anything in religion. In the context of prenatal diagnosis, a fundamental question is therefore whether Islam

permits termination of pregnancy if the fetus is affected by a serious genetic disorder? The views of Islamic jurisprudence on the subject are unanimous in prohibiting abortion if it is carried out on the embryo after animation unless there is a valid reason. Views on abortion before animation differ widely, and range from permissibility to disfavour to prohibition (Makdur, 1974).

There is no legislation in Pakistan defining an upper limit for permissibility of termination, but it is important to work within a framework defined by consensus. Two renowned religious scholars in Pakistan, when asked to give an opinion on the subject, clearly considered termination is permissible provided it is done before 120 days (17 weeks) of pregnancy. We have not encountered criticism from any religious or social organization since introducing the service. However, if a debate on this subject should arise in future, the fact that we sought the opinion of prominent religious scholars, and that they consider early selective abortion permissable will be crucially important.

In our experience, couples who were hesitant in using prenatal diagnosis were relieved to learn that Islam permits termination of pregnancy under special circumstances. Since the introduction of the service several couples have requested the test after 17 weeks of pregnancy. After being counselled and informed about the Islamic view on terminating a pregnancy at this stage, most were disappointed but opted not to have prenatal diagnosis. The majority of couples with an affected fetus had little hesitation in terminating an affected pregnancy. Those who declined termination did so mostly on religious grounds.

The couples who had prenatal diagnosis are a selected group, as most had already made up their minds about terminating an affected pregnancy, while couples who had reservations about termination avoided the test. We found that 87% of a randomly selected sample of at-risk couples favoured termination of pregnancy in a future pregnancy if the fetus was affected,. Interestingly, though 91% of these couples also favoured prenatal diagnosis, only 56% had actually used it in their most recent pregnancy. We found that the cost of the test is an important barrier, as is to be expected in a third world country. The health budget of the country does not cater for thalassaemia. Treatment costs may be covered by a charitable organisation such as the Fatimid foundation, but families have to bear the cost of prenatal diagnosis themselves. This explains why 24% of the couples would (or could) use prenatal diagnosis only if it was provided free. Under the circumstances prevailing in Pakistan, the estimated cost of one prenatal diagnosis including CVS, laboratory diagnosis and termination in selected cases is approximately US$100. Even at such low cost, most couples are unable to afford the test. In a National prevention programme, the Government or the NGOs might be persuaded to subsidize the cost of prenatal diagnosis.

Almost all the couples in this study were identified retrospectively, when they already had at least one affected child. In communities where family size is small, retrospective identification of the couples and the offer of prenatal diagnosis is unlikely to reduce the incidence of thalassaemia major (Cao, 1987). By contrast, when final family size is large, retrospective counselling may lead to either cessation of reproduction or prenatal diagnosis and this can reduce the affected birth rate by up to 50% (Alwan and Modell, 1997). Further reduction in the birth rate of affected children would require prospective identification of at-risk couples. In a pilot study we have shown that extended screening of families identified by having an affected child can be a cost-effective approach for prospective identification of at-risk couples (Ahmed *et al*., submitted for publication). It may be advisable, at present, to offer screening to the extended families of over 7000 thalassaemic children registered at various treatment centres throughout the country, as they represent a sizeable proportion of the total at-risk families in the country. Such an approach may substantially reduce the birth rate of thalassaemia major in Pakistan.

At present, a regular service for prenatal diagnosis is not available in any of the Muslim countries. Therefore, their response to prenatal diagnosis is largely unknown. Our experience in an orthodox Muslim country clearly shows that prenatal diagnosis is accepted by the affected families provided at-risk pregnancies are identified in early pregnancy. Religious scholars also favour termination before 17 weeks' gestation if prenatal diagnosis indicated the fetus is affected by a serious genetic disorder. Socioeconomic conditions, education and organization of the health-care system varies considerably between the Muslim countries but their social cultural and religious background have several things in common. Our experience may be used as an example for developing countries with a similar background.

From our international contacts (M. Petrou, personal communication) it is clear that prenatal diagnosis is becoming more widely available in countries such as Jordan, Lebanon, Egypt, Tunisia and Malaysia (Petrou and Modell, 1995), and national prenatal diagnosis programmes are being considered by others such as Iran.

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