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Prenatal diagnosis of β-thalassemia: 12 years' experience at a single laboratory in Pakistan

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Objective To evaluate the service for prenatal diagnosis of β-thalassemia in Pakistan.

Methods All prenatal diagnoses (PNDs) for β-thalassemia since the introduction of the service in 1994 were studied. PND was done by the Amplification Refractory Mutation System (ARMS), or linkage analysis, when required. The reported errors in PND were investigated for clerical mistakes, technical problems with PCR, maternal contamination and nonpaternity.

Results In the 12 years 2174 PNDs were done for β-thalassemia at the country's main referral center. The use of PND has increased from 26 in 1994 to 381 in 2006. Over 97% of the couples who requested PND already had an affected child. In over 97% of the cases PND was done by direct mutation analysis. The reported rate of misdiagnosis was 0.37%. The causes of misdiagnoses included one clerical mistake, three false positive PCR results, and two maternal contaminations in the chorionic villus sampling (CVS).

Conclusion PND for β-thalassemia is technically feasible by direct mutation analysis in most cases in Pakistan. The procedure is quick and cost effective. Strict quality assurance can achieve an acceptably low error rate. Copyright © 2007 John Wiley & Sons, Ltd.

KEY WORDS: prenatal diagnosis; β-thalassemia; Pakistan; misdiagnosis

INTRODUCTION

Thalassemia is the commonest single gene disorder in Pakistan. Approximately 5% of the population carries β-thalassemia trait and each year over 5000 new children are born with thalassemia major (Ahmed and Saleem, 1994). The cost of treatment of thalassemia is often beyond the reach of an average Pakistani family. The best possible solution for this problem is through prevention of birth of the affected child (Cao, 1987; Petrou, 1994). Married couples where both partners are carriers need to be offered prenatal diagnosis. A service for prenatal diagnoses (PNDs) of β-thalassemia was introduced in Pakistan for the first time in 1994 (Ahmed *et al*., 1994). The response of the couples to PND has been encouraging, and gradually, the service has gained popularity (Ahmed *et al*., 2000). During the past 12 years over 2000 PNDs have been done at the Armed Forces Institute of Pathology (AFIP), Rawalpindi. A retrospective analysis of the data was done to evaluate the overall performance of the service.

PATIENTS AND METHODS

Booking and counseling

The couples requesting PND of β-thalassemia were registered for chorionic villus sampling (CVS) after 10 weeks' gestation. They were counseled about the diagnostic procedure, possible outcome of the test and the chances of misdiagnosis. At the time of registration, blood samples were collected from each parent for hematology to confirm β-thalassemia and to perform the mutation analysis. The CVS were dissected under a stereo microscope at ×6 magnification. Maternal tissue in the sample, if any, was carefully separated and only the placental villi were collected for further DNA analysis. The couples who could not report in person to AFIP (outstation) were registered by their obstetricians and the parents' blood along with the dissected CVS in 2% SDS solution was sent to AFIP.

DNA analysis

The parents' blood samples were analyzed for the β-thalassemia mutations found in the Pakistani population by a Multiplex Amplification Refractory Mutation System (ARMS) (Ahmed *et al*., 1996). The CVS was tested for the parents' mutation(s) by the standard ARMS protocol (Ahmed *et al*., 2000). Each PND was carried out by including the parents' DNA, fetal DNA in duplicate for the mutation as well as the normal gene, appropriate negative and positive controls, and reagent blanks. When one or both of the parents' mutations could not be identified by ARMS, fetal diagnosis was done by linkage analysis using six polymorphic sites linked to the β-globin gene (Varawalla *et al*., 1992). The quality of the results was also assured by physical isolation of PCR reagents and products, autoclaving solutions, avoiding splashes, use of separate pipettes for pre- and post-PCR steps and aliquot reagents (Higuchi and Kwok, 1989).

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Follow-up

The couples with a fetal diagnosis of normal or β-thalassemia trait were advised to contact the lab if the child developed transfusion dependency after birth. In such cases the postnatal diagnosis was reconfirmed by DNA analysis. The prenatal diagnosis on the CVS was also repeated. The circumstances leading to the error in diagnosis were systematically investigated including clerical mistakes, technical problems with the PCR, maternal contamination in the CVS and nonpaternity. Maternal contamination and paternity were tested by short tandem repeat (STR) analysis at various loci (Antoniadi *et al*., 2002). The sensitivity of the STR test was established by using a serially diluted DNA sample.

RESULTS

During the 12 years (1994–2006) a total of 2174 PND were carried out for β-thalassemia at AFIP. The trend in the use of PND gradually increased with time as the service became known to more and more couples. It had increased from 26 in 1994 to 381 in 2006 (Figure 1). Most parents who requested PND already had an affected child. There were only 36 (1.7%) couples who did not have any affected child and even in most such cases an affected child was present in the extended family.

In most of the cases (97.2%), the diagnosis was possible by direct mutation analysis. However, in the remaining 61 (2.8%) cases linkage analysis was required. This included 23 (1.1%) cases where mutations in the genes of both the parents remained uncharacterized, and 38 (1.7%) cases in which one of the parents carried a previously known mutation. The uncharacterized β-thalassemia alleles have not yet been sequenced.

During the 12 years, 18 CVS samples were referred for PND of thalassemia because the couple had a transfusion-dependent child, and the parents were not found to have β-thalassemia. These samples were not processed any further.

The average reporting time from identification of the parents' mutation to the PND was 1 week. When a linkage analysis was required the time was extended up to 10 days. The cost of performing one test was US $65.

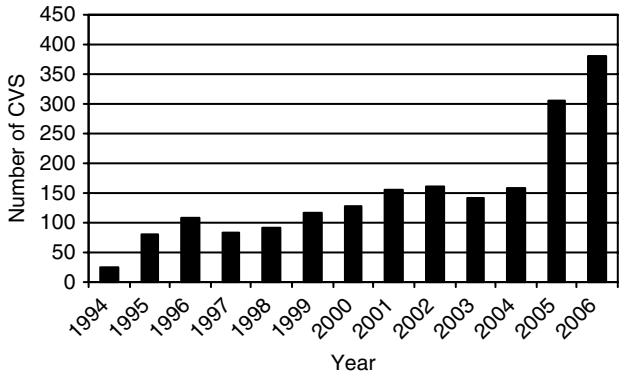

Figure 1—The annual number of PND for β-thalassemia in Pakistan

Out of the 2174 fetal diagnoses, thalassemia major was found in 542 (24.9%) of them, no β-thalassemia mutation in 526 (24.2%), and thalassemia trait in 1106 (50.9%). Table 1 describes the results of the parents' mutations and the fetal diagnoses. The frequency of fetal diagnoses as thalassemia major for Fr-8-9 (+G) and IVSI-5 (G-C) mutations was 24.6 and 24.4%, respectively.

In the 1632 nonthalassemia major fetal diagnoses a total of 6 children (0.37%) were reported to develop transfusion dependency after birth. Further investigations in the 6 misdiagnoses showed one clerical mistake in which a previously reported Cd15 mutation was misread as Cd5. In the remaining 5 cases false positive PCR results for the normal gene led to incorrect labeling of thalassemia major as thalassemia trait. In three such cases, the reason was a technical problem with the ARMS PCR (testing of the normal gene for Fr 8–9 (+G) mutation) (Figure 2). In the remaining two cases, maternal contamination in the CVS was proven on STR analysis (Figure 3). The lowest detection limit of the STR test by silver staining of the polyacrylamide gels was 20 ng (Figure 4).

DISCUSSION

A vast majority of the people in Pakistan are conservative Muslims. However, a clear religious verdict

Table 1—The frequency of parents' mutations and the fetal diagnoses in 2174 cases

	Fetal diagnosis			
Mutations	Major (%)	Normal (%)	Trait (%)	All (%)
Fr 8–9 (+G)	205 (24.6)	202 (24.3)	425 (51.1)	832 (38.3)
IVSI-5 (G-C)	130 (24.4)	135 (25.3)	268 (50.3)	533 (24.5)
Fr 41–42 (−TTCT)	56 (24.7)	49 (21.6)	122 (53.7)	227 (10.4)
Cd 15 (G-A)	31 (24.8)	35 (28.0)	59 (47.2)	125 (5.7)
Cd 5 (−CT)	29 (24.0)	29 (24.0)	63 (52.0)	121 (5.6)
Cd 30 (G-C & G-A)	19 (24.4)	19 (23.3)	40 (51.3)	78 (3.6)
IVSI-1 (G-T)	18 (26.1)	15 (21.7)	36 (52.2)	69 (3.2)
Fr 16 (−C)	17 (26.6)	13 (20.3)	34 (53.1)	64 (2.9)
Others	37 (29.6)	29 (23.2)	59 (47.2)	125 (5.7)
All	542 (24.9)	526 (24.2)	1106 (50.9)	2174 (100)

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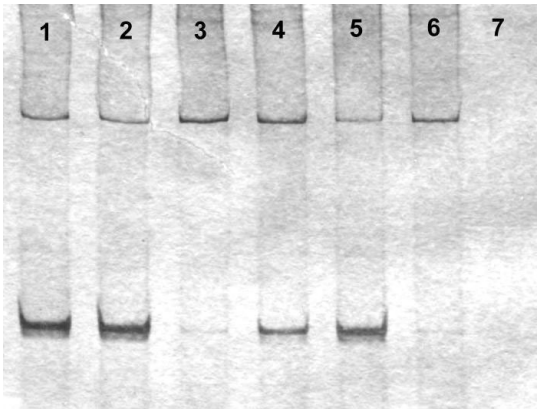

Figure 2—Silver-stained polyacrylamide gel electrophoresis of ARMS PCR. The lanes 1–6 show results of PCR for normal gene at Fr 8–9 (+G) locus. Lane 7 is a reagent blank. All lanes except the reagent blank also show 861 bp PCR internal control. In a normal PCR, the 215 bp fragment representing the normal gene is better amplified than the larger 861 bp internal control fragment. The lanes 1, 2, and 5 are positive, while lanes 3 and 6 are negative, indicating homozygous Fr 8–9 mutation. The 215 bp fragment in lane 4 is a false positive result because its intensity is significantly less than the positive results in lanes 1, 2, and 5, while its 861 bp internal control is comparable in intensity to that in the other lanes

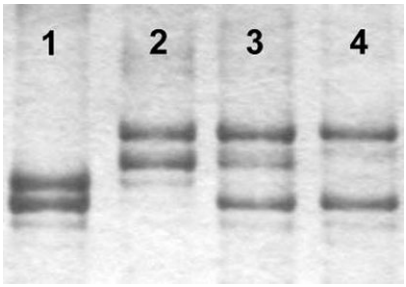

Figure 3—Sliver-stained polyacrylamide gel electrophoresis of amplified products at D21S11 STR locus. The lanes 1–4 show DNA of father, mother, and CVS, respectively. The sample in lane 3 shows one allele (bottom) inherited from the father and another allele (top) inherited from the mother. In addition, this sample also shows a third allele (middle), which has come from maternal contamination in the CVS. Lane 4 shows a second CVS sample, which is free from maternal contamination

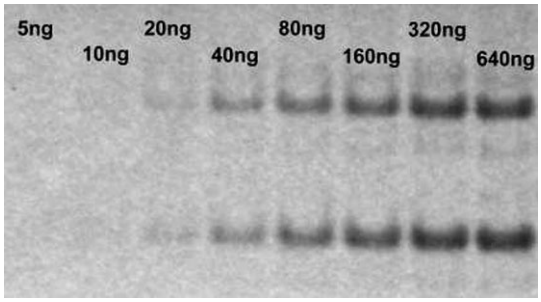

Figure 4—Sensitivity of the STR test at D21S11 locus after sliver staining of polyacrylamide gel electrophoresis. A serially diluted DNA sample shows that the lowest detectable concentration is 20 ng

allowing termination of pregnancy (TOP) for serious genetic disorders before 17 weeks of gestation has eased the way to offer PND and suggest TOP in Pakistan (Ahmed *et al*., 2000). The service has gained popularity among the affected couples and families during the past decade. Since then, a large number of couples, mostly with affected children, have used this facility. The estimated birthrate of babies born with β-thalassemia major in Pakistan is around 5000 per year (Ahmed and Saleem, 1994). This would mean a requirement of around 20 000 PND each year. AFIP is the major referral center for PND in Pakistan where more than 80% of the work on β-thalassemia is done. An average of around 300 PND a year at this center clearly indicates that only a very small fraction of the estimated 20 000 per year in Pakistan has been accomplished. The beginning in the right direction, however, has been made and it is hoped that with the passage of time PND would play a key role in the prevention of thalassemia in Pakistan.

Over 98% of the PND users in Pakistan already have an affected child. If thalassemia is to be controlled in the community, then the retrospective identification of the couples will have to be improved by prospective identification of at-risk couples (Alwan and Modell, 1997). In a country like Pakistan where consanguineous marriage is common, the answer lies in a targeted approach for prospective identification of the couples at risk of a recessive genetic disorder like thalassemia (Ahmed *et al*., 2002).

Prenatal diagnosis of β-thalassemia is technically feasible in Pakistan by direct mutation analysis in over 97% of the cases, and the remaining 3% may be helped by linkage analysis. The technique is quick and reliable with an error rate within an acceptable limit. Since it was not possible to check the postnatal diagnosis in every case the actual rate of misdiagnoses is not known. The overall proportion of cases diagnosed as thalassemia major (24.9%) is almost equal to the expected 25%, suggesting that not many thalassemia majors were missed. However, the proportion of thalassemia major fetuses in sufficient numbers of parents with the two most common mutations, that is, Fr-8-9 (24.6%) and IVSI-5 (24.4%) are just about 0.5% less than expected. These data support that the misdiagnosis rate for thalassemia major may actually be somewhere around 0.5%. The misdiagnosed cases quickly get noticed at the treatment centers that referred almost all of the couples who requested PND. These centers are regularly visited by the affected couples at least once a month for the treatment of their affected children. At the treatment centers, discussion among the parents about experiences of PND is a regular feature. A feedback from most couples who request PND shows that complications following CVS or misdiagnosis become a talk of the town, and such information quickly spreads among other parents (unpublished observations). It has also been observed that any such information also has a profound negative effect on the credibility of the service. Therefore, it is of utmost importance to maintain a very high standard of quality. It can only be achieved by following a strict protocol of DNA analysis with rigorous standards of quality assurance (Higuchi and Kwok, 1989). The error rate in this study (0.37%) is higher than the 0.1% by PCR-based

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methods in a longstanding PND service in the UK (Old *et al*., 2000).

In this study, misdiagnoses were mainly encountered due to technical problems with the PCR or maternal contamination in the CVS. False positive PCR results can be avoided by observing strict measures of quality assurance (Higuchi and Kwok, 1989). Maternal contamination in the CVS can be avoided by meticulous dissection of the CVS. The error rate can be reduced by taking additional steps like duplicate testing, or linkage analysis, in every case. But this would almost double the cost of testing. A useful step may be to perform STR analysis for maternal contamination when a fetal diagnosis of thalassemia trait is made. The STR test is sensitive with a low detection threshold (20 ng). Its sensitivity may be improved by using automated genetic analyzer (Antin *et al*., 2001).

As mentioned earlier, the current laboratory cost of one PND in Pakistan is about US $65. The present healthcare system in Pakistan does not provide free healthcare to the general population. Apart from a privileged section of the population employed in various Governmental or private organizations, the expenses on healthcare have to be borne by the individuals themselves. The per capita income in Pakistan is around US $800. At such a low level of income, an ordinary Pakistani finds it difficult even paying US $65 for a PND. Under these circumstances, it may not be possible to increase the cost to improve the quality of the results to a level that is only slightly better than what has already been achieved. It could also have a negative impact on the use of PND.

Old *et al*. (2000) have provided very useful guidelines for the good practice of PND for thalassemia. However, in the third-world countries, where cost of the test is a key factor, good quality of the results must be ensured while keeping the cost within an affordable range.

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