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Challenges and Developments in Prenatal Diagnosis of Beta-Thalassemia: A Study on Diagnostic Accuracy

Bijan Keikhaei MD[1], Nozar Kalantarfard MD[1], Mohammad Reza Mahmoudian-Sani PhD[*1]

1. Thalassemia and Hemoglobinopathy Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
*Corresponding author: Dr. Mohammad-Reza Mahmoudian-Sani, Thalassemia and Hemoglobinopathy Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. Email: mohamadsani495@gmail.com. ORCID ID: 0000-0002-2500-8629.

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Abstract

Background: Beta-thalassemia major is prevalent in areas like Khuzestan, Iran, and causes severe anemia requiring lifelong treatment. Despite successful prevention programs, diagnostic errors in Prenatal Diagnosis (PND) persist, leading to affected births. This study evaluates the accuracy of PND, identifies the causes of errors, and suggests improvements to diagnostic protocols.

Materials and Methods: A retrospective descriptive cross-sectional study (2012–2018) with 202 beta-thalassemia carrier couples from Shafa Hospital, Ahvaz, Iran. Fetal DNA analysis was conducted via Chorionic Villus Sampling (CVS) and amniotic fluid sampling using Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS-PCR), Gap Polymerase Chain Reaction (Gap-PCR), and sequencing. Statistical analysis results revealed factors influencing diagnostic errors.

Results: Affected infants were diagnosed with beta-thalassemia major at an average age of five months. The results showed six diagnostic errors (2.14%), primarily associated with point mutations. Errors occurred more frequently in amniotic fluid sampling (4.05%) than in CVS sampling (1.45%). CD 36/37, IVS-II-1, and Fr8–9 were the most common detected mutations.

Conclusion: Improving the accuracy of PND for beta-thalassemia is crucial, particularly in regions with high prevalence, such as Khuzestan, Iran. Although the overall diagnostic error rate was low (2.14%), the consequences of such errors are significant. The diagnostic error can lead to the birth of affected children and an added burden on families and healthcare systems. Most errors were linked to point mutations and were more frequent in amniotic fluid sampling than in CVS. To minimize such diagnostic mistakes, advancements in molecular diagnostic techniques—especially for detecting rare mutations—are necessary.

Keywords: Beta-Thalassemia, Chorionic Villus Sampling, Diagnostic Error, Mutation, Prenatal Diagnosis

Introduction

Beta-thalassemia is one of the most prevalent hereditary genetic disorders, particularly in populations across the Mediterranean, the Middle East, and South and East Asia. This autosomal recessive disorder is caused by mutations in the beta-globin gene, resulting in reduced or absent production of beta-globin chains, which are essential components of hemoglobin (1). The severe form of the disease, beta-thalassemia major, is characterized by severe anemia that requires lifelong blood transfusions and iron chelation therapy.

Without proper management, patients face numerous complications, including growth retardation, severe bone deformities, and organ damage, particularly to the heart and liver, due to iron overload and oxidative stress. These complications often result in a significant reduction in the quality of life and premature mortality (2, 3). In the global thalassemia belt, Iran experiences a high prevalence of this disorder, particularly in southern and western provinces such as Khuzestan. Factors such as consanguineous marriages, genetic predisposition, and limited awareness in past decades have contributed to the increased incidence of thalassemia in these

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regions. Iran has implemented comprehensive prevention programs, including premarital screening, genetic counseling, and prenatal diagnostic (PND) testing (4, 5). Among these strategies, PND has played a pivotal role in reducing the incidence rate of births of children affected by beta-thalassemia major. PND testing often benefits from advanced molecular techniques, such as Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS-PCR), Gap Polymerase Chain Reaction (Gap-PCR), and sequencing-based approaches, and serves as a cornerstone for identifying mutations in the beta-globin gene. These techniques offer high sensitivity and specificity, enabling the detection of single-gene mutations and molecular anomalies that are associated with thalassemia (6, 7). Despite these advancements, diagnostic errors continue to be a significant concern of healthcare systems. Even minor inaccuracies in these tests can result in the birth of infants with beta-thalassemia major, impose profound psychological and financial burdens on families, and place considerable strains on healthcare systems (1, 8). This raises two crucial research questions: How accurate and reliable are current PND methods for detecting beta-thalassemia in real-world practice? and What are the contributing factors to diagnostic errors in this context? Khuzestan Province, one of the regions in Iran with the highest prevalence of thalassemia, faces these challenges(9). Previous studies have emphasized that employing advanced diagnostic methods and optimizing protocols can significantly reduce errors and improve diagnostic accuracy. Furthermore, enhancing education and genetic counseling in high-risk regions can play a vital role in preventing new cases (10). The present study aims to evaluate the accuracy and reliability of PND testing for beta-thalassemia in Khuzestan Province. The present research aims to provide practical recommendations for enhancing diagnostic processes and prevention strategies by identifying the primary sources of diagnostic errors and assessing their consequences. The findings of this study could serve as a foundation for developing new policies aimed at reducing the incidence of beta-thalassemia major and improving the quality of genetic and public health services.

Materials and Methods

Study Population, Sampling, and Research Design

This retrospective descriptive cross-sectional study evaluated data from individuals referred to the genetics laboratory at Shafa Hospital in Ahvaz, Iran, between 2012 and 2018 for beta-thalassemia evaluation. The study focused on couples referred to the Jundishapur Thalassemia Center for pre-pregnancy evaluations to assess the presence of beta-thalassemia-related mutations. If both parents were identified as carriers of thalassemia, they were advised to undergo PND during pregnancy. PND was performed using chorionic villus sampling (CVS) between weeks 10 and 12 of gestation or amniotic fluid sampling at week 16. CVS samples were examined under an inverted microscope to separate maternal and fetal tissue. The isolated fetal tissue underwent DNA extraction and purification for molecular analysis. Molecular abnormalities were identified using PCR-based techniques, including PCR-ARMS and Gap-PCR. Beta-globin gene haplotypes were further assessed using Restriction Fragment Length Polymorphism PCR (RFLP-PCR). Diagnosis confirmation was performed through direct sequencing to ensure accuracy and reliability.

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Inclusion and Exclusion Criteria

The study included parents identified as carriers of the beta-thalassemia gene. Parents who did not carry the beta-thalassemia gene, those identified as carriers of the alpha-thalassemia gene, and individuals unwilling to participate in this research were excluded from the study.

Data Analysis and Statistical Methods

The sample size for this study was determined using the census method, and the sample included patients evaluated at the Thalassemia Center between 2012 and 2018. Descriptive statistics, including mean, median, standard deviation, and frequency distributions, were used for examining all variables. The Shapiro-Wilk test was used to assess the normality of continuous data. Since the data were not normally distributed, the Kruskal-Wallis test was used for data analysis. A *p*-value of less than 0.05 was considered statistically significant. Data analysis was conducted using SPSS software, version 22 (IBM Corp., Armonk, NY, USA).

Ethical Approval

This study was conducted following the ethical guidelines approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (Ethics No: IR.AJUMS.REC.1397.957). Patient data was anonymized through using codes instead of personal identifiers and for ensuring protecting patients' confidentiality and privacy. All ethical and legal standards were strictly adhered to throughout this study. The study imposed no financial burden on participants, and their standard treatment for the condition was maintained throughout, ensuring no impact on their care.

Results

Demographic Characteristics of Participants

In this study, 202 couples (404 individuals) from marriage candidates who referred to Ahvaz Thalassemia Centers were evaluated. Affected infants were diagnosed with beta-thalassemia major at an average age of five months. The mean maternal age at the time of PND was 30.2 years. Considering the study population, the average age of women was 28.74 ± 8.17 years, and that of men was 33.04 ± 11.65 years.

There was no statistically significant difference in age between the two genders ($P > 0.05$).

Results of Fetal Beta-Thalassemia Mutations

The most common mutations observed in parents and fetuses were CD36/37, IVS-II-1, and Fr8–9. (Table I)

Errors in Prenatal Detection of Beta-Thalassemia

Between 2012 and 2018, six instances of erroneous prenatal diagnosis resulted in the unintended delivery of infants with beta-thalassemia major. Due to technical limitations or interpretation errors in direct sequencing, particularly for rare point mutations, three diagnostic errors were documented among the 74 amniocentesis procedures, corresponding to a 4.05% error rate. An equal number of mistakes were found among the 207 CVS procedures, yielding a 1.45% error rate. Details for these misclassified cases are provided in Table II. Although Table II presents genotypic data on misdiagnosed cases, further analysis can suggest that several technical and biological factors may have contributed to these diagnostic errors. For instance, misinterpretation of sequencing results, especially for rare point mutations, such as IVSI.1 or Cd5 may have occurred due to limited reference databases or operator's lack of experience. Additionally, allele dropout

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during PCR amplification could be responsible for falsely classifying heterozygous fetuses as homozygous. In amniotic fluid samples, which are associated with a higher error rate (4.05%), maternal DNA contamination remains a probable source of misdiagnosis, particularly in cases where fetal-maternal tissue separation is not thoroughly ensured. Classification of errors as 'technical limitation', 'interpretational error', or 'sample contamination' may enhance future quality assurance measures in molecular diagnostic protocols.

Table III compares the diagnostic performance of amniocentesis and CVS. Although the absolute number of errors was the same with regard to both techniques, amniocentesis exhibited a higher proportion of errors. The overall diagnostic error rate across all tested cases was observed to be 2.14%, indicating that CVS is a more dependable technique for prenatal detection of beta-thalassemia.

Table I: Distribution of beta-thalassemia mutations observed in parents and fetuses

Mutation Type (Frequency)	Number of Parents	% of Parents	Number of Fetuses	% of Fetuses
CD 36/37	176	45.01%	65	31.55%
IVS- II-1	72	18.41%	28	13.59%
Fr8–9	45	11.51%	33	16.02%
IVS I-110	18	4.60%	11	5.34%
CD8/9	11	2.81%	6	2.91%
IVSI 16	4	1.02%	9	4.37%
IVS II-745	9	2.30%	4	1.94%
ATG-ACG	8	2.05%	1	0.49%
CD 44	7	1.79%	1	0.49%
-57 A>T	4	1.02%	0	0.00%
Fr 8-9 (+G)	3	0.77%	2	0.97%
28 (C-A)	2	0.51%	0	0.00%
88 (C-A)	1	0.26%	0	0.00%
IVSI-S	1	0.26%	0	0.00%
IVSI-b (C-T)	1	0.26%	0	0.00%
CDS (-CT)	1	0.26%	0	0.00%
CD1S(TGG-TGA)	1	0.26%	2	0.97%
IVS II 848	1	0.26%	1	0.49%
Cd5/Cd5	1	0.26%	0	0.00%
Unavailable Information	26	6.65%	43	20.87%

Table II: Characteristics of cases leading to misdiagnosis

Prenatal Diagnosis	Postnatal Diagnosis	Fetal Sample	Mother Genotype	Father Genotype	Gestational Age	Mother's Age	Probable Cause of Error
Fr8-9/Fr8-9	N/N	Amnion	Fr8-9/N	Fr8-9/N	18	31	Misinterpretation of a rare mutation
Cd5/Cd5	N/N	Amnion	Cd5/N	Cd5/N	15	36	Allele dropout during PCR
IVSIL1/IVSI-110	N/N	Amnion	IVSIL1/N	IVSI-110/N	16	17	Maternal contamination or allele dropout
IVSI-110/IVSI-110	IVSI-110/N	CVS	IVSI-110/N	IVSI-110/N	11	31	Interpretation error
IVSI.1/Cd54(-T)	N/N	CVS	IVSI.1/N	Cd54/N	11	35	Misclassification of complex mutation
Cd36-37/Cd36-37	N/N	CVS	Cd36-37/N	Cd36-37/N	11	24	Sequencing artifact or interpretation error

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Table III: Comparison of diagnostic error rates between amniocentesis and cvs procedures

Sampling Method	Number of Procedures	Number of Errors	Error Rate (%)
Amniocentesis	74	3	4.05%
CVS	207	3	1.45%
Total	281	6	2.14%

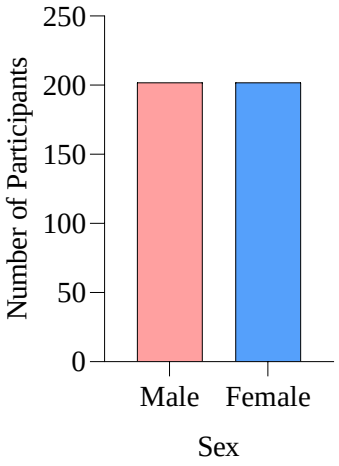

Figure 1. The gender distribution among 404 participants

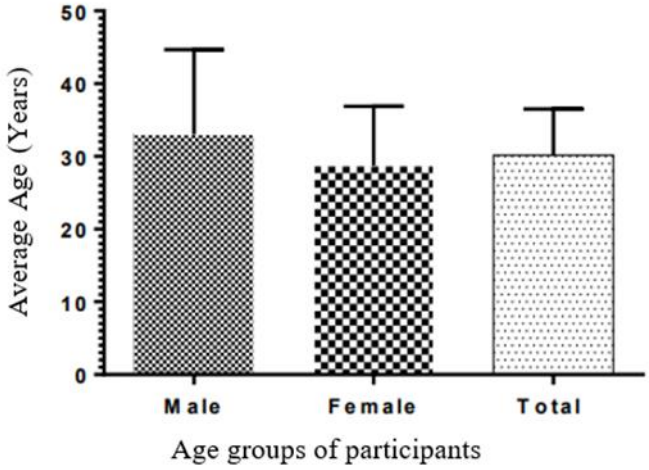

Figure 2. The age distribution of participants by gender

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Discussion

Beta-thalassemia with over 70 known mutations, including large deletions and more than 50 distinct point mutations is reported globally, including in Iran, as a genetically heterogeneous disorder. The study was conducted on 202 carrier couples who referred to a PND center in Khuzestan Province. The results revealed, despite the application of established molecular techniques, critical challenges in the accuracy of PND. Concerning prenatal diagnosis of beta-thalassemia, the regional carrier rate of approximately 10%, particularly in southern provinces such as Khuzestan, underscores the need to optimize diagnostic protocols and prevention strategies in high-prevalence areas (4, 11-14). Consistent with the previously reported regional patterns, the most common mutations observed in the study were CD36/37, IVS- II-1, and Fr8–9In Iran, mutation prevalence varies by province; IVS II-1 is more frequent in the north, whereas IVS I-5 is predominantly found in the south (15-19). International comparisons support this heterogeneity. For example, Ahmed et al. identified Fr8–9, IVS I-5, and C5 as frequent mutations in Pakistan (20), and Murad et al. reported IVS I-1, C39, and IVS I-5 in Syria (21). Despite the use of molecular tools, such as ARMS-PCR and Gap-PCR, for the diagnosis of beta-thalassemia in this study, the findings revealed six diagnostic errors (2.14%), of which all involved point mutations. These errors resulted in the birth of six infants with beta-thalassemia major, illustrating the limitations of standard techniques in detecting rare or complex mutations. Notably, diagnostic errors were significantly more frequent in amniotic fluid samples (4.05%) than in CVS samples (1.45%). The higher reliability of CVS is likely attributable to earlier sampling (weeks 10–12 vs. 16), superior DNA quality, and lower risks of maternal contamination. These observations are in line with the findings of Moghadam et al., who reported a 0.4% error rate using CVS in relation to 1,016 pregnancies in the Fars Province, with 99% of affected cases electively terminated (22). Li et al. reported zero errors in 545 high-risk Chinese pregnancies using CVS combined with High-Performance Liquid Chromatography (HPLC) evaluation, demonstrating the potential for near-perfect accuracy with an appropriate methodology (23). The study confirms that point mutations are particularly susceptible to diagnostic failure when only ARMS-PCR and Gap-PCR are applied. These methods, though accessible and cost-effective, lack the resolution to identify uncommon or structurally complex mutations. In contrast, advanced technologies, such as multiplex ligation-dependent probe amplification (MLPA), long-read sequencing, and next-generation sequencing (NGS) offer broader detection capabilities and significantly improve sensitivity in identifying rare variants (24-26). Furthermore, lack of comprehensive mutation databases, insufficient technical infrastructure, and limited availability of reference samples for re-testing may have led to errors. These factors underscore the urgent need for national-level standardization and investment in quality control systems across diagnostic laboratories. Emerging non-invasive diagnostic technologies present promising alternatives. Although CVS and amniocentesis remain gold standards for fetal DNA sampling, they pose such risks as miscarriage and maternal DNA contamination. To mitigate these risks, short tandem repeat (STR) typing is recommended to confirm fetal DNA origin (24-28). Meanwhile, non-invasive prenatal testing (NIPT) using cell-free fetal DNA (cffDNA) from maternal plasma is gaining momentum. Techniques such as NGS, combined with relative haplotype dosage (RHDO), have demonstrated 100% sensitivity and specificity in early pilot

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studies (29). Nevertheless, limitations such as low fetal fraction and maternal DNA interference persist (30). Promising results have also been reported using droplet digital PCR (ddPCR) and cell-free Biomarker Enrichment and Separation Technique (cfBEST). A study from Cyprus that used ddPCR with relative variant dosage (RVD) analysis achieved 97.06% diagnostic accuracy, 100% sensitivity, and 95% specificity (31). Similarly, cfBEST demonstrated 99.19% sensitivity and 99.92% specificity across 143 pregnancies, suggesting clinical-grade reliability of the technique (32). Another study achieved early and accurate detection of paternal alleles in 79% of pregnancies by targeting highly heterozygous β-globin Single Nucleotide Polymorphisms (SNPs) (33). In summary, while current invasive techniques remain standard practice, integrating advanced molecular technologies and transitioning toward safe non-invasive methods could significantly reduce diagnostic errors. Establishing centralized mutation databases, enhancing personnel training, improving pre- and post-test counseling, and revising insurance policies to cover emerging diagnostic techniques are essential steps forward. These combined efforts will contribute to reducing the birth of affected infants and strengthening the national thalassemia prevention program. To clarify, improving diagnostic protocols and optimizing molecular techniques should encompass several practical measures. First, CVS is recommended over amniocentesis, as it was associated with a lower error rate in this study and provided higher-quality fetal DNA at an earlier gestational stage. Second, it is necessary to go beyond conventional ARMS-PCR and Sanger sequencing and incorporate advanced platforms, such as NGS or MLPA to improve detection of rare or complex mutations. Third, strengthening laboratory quality control systems, ensuring access to updated mutation databases, and enhancing technical training for laboratory staff and genetic counselors are essential to minimize errors and ensure consistent diagnostic accuracy in prenatal testing.

This study was limited by its sample size, potential under detection of rare mutations due to conventional methods, and restricted access to advanced techniques. DNA quality, particularly in amniotic samples, can impact accuracy. Future research should apply regression analysis to assess clinical and molecular predictors of diagnostic errors.

Conclusion

This study revealed an overall 2.14% diagnostic error rate in prenatal beta-thalassemia testing, with errors occurring more frequently in amniotic fluid samples compared to CVS. While both methods are standard, amniocentesis carries a higher risk of maternal DNA contamination and generally yields lower-quality fetal DNA, primarily when performed in the second trimester. Therefore, whenever possible, CVS should be prioritized due to its earlier timing, greater diagnostic accuracy, and reduced contamination risk. To further enhance molecular diagnostic precision, it is crucial to implement advanced techniques, such as NGS and MLPA, strengthen laboratory quality control measures, and provide comprehensive training programs for personnel. Emphasizing these improvements will help minimize diagnostic errors and enhance the effectiveness of national thalassemia prevention efforts, particularly in regions with high prevalence, such as Khuzestan. These findings underscore the importance of continuous innovation in prenatal diagnostics to ensure early and reliable detection of beta-thalassemia, thereby supporting the long-term success of national prevention programs.

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Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical Considerations

The Ethics Committee for Research at Ahvaz Jundishapur University of Medical Sciences approved the study (IR.AJUMS.REC.1397.957). This study was conducted in accordance with the Declaration of Helsinki.

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Authors' Contributions

Concerning the present study, B.K. contributed to the conceptualization, methodology, and data analysis. N.K. was responsible for data collection, writing the original draft, and reviewing and editing the manuscript. M.R.M. was responsible for writing the manuscript, as well as performing the analysis and editing the manuscript. All authors have reviewed and approved the manuscript for publication.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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