

## ACARRIER SCREENING AND PRENATAL DIAGNOSIS OF FAMILIES WITH $\beta$ -THALASSEMI

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### ABSTRACT

The contribution of inherited disorders among human beings to congenital abnormalities and childhood mortality is also a significant factor globally. These conditions are mostly untreatable but can only be prevented and treated on an asymptomatic basis. It is more deplorable in inbred populations such as the one experienced in Pakistan, with higher rates (70%) of consanguinity marriages. Consanguinity enhances the chance of an individual having a variant that is inherited in a homozygous fashion, thereby leading to a recessive illness. However, prenatal diagnosis is an effective approach to regulate the frequency of carriers of genetic disorders with an autosomal recessive mechanism of inheritance. This study examined eight families that had a history of  $\beta$ -thalassemia being screened against the most frequent mutations that cause the condition. Genotyping was done by Amplification Refractory Mutation System (ARMS), an adaptation of PCR, with allele-specific primers used to detect mutation successfully in all of these families. The results showed that the most common mutation of the investigated families was IVS-I-5 and F508del. The results, thus, elucidate the value of national carrier screening programs, premarital screening, and genetic counselling, showing great potential to significantly reduce the incidence of at-risk births by  $\beta$ -thalassemia major. This study provides valuable genomic information and thus forms a strong foundation for the development of policy measures and a contribution to a future where  $\beta$ -thalassemia can successfully be prevented through early diagnosis, community awareness, and informed reproductive decision-making.

**Keywords:** Autosomal recessive disorders, carrier screening, consanguineous marriages, HBB gene mutations, prenatal diagnosis.

### INTRODUCTION

One of the most widespread inherited blood diseases in the world is Thalassemia, a genetic disorder which is a result of inheritance mutations that impact the production of globin chains, which are significant protein constituents of hemoglobin. Hemoglobin is a tetrameric protein that is composed of two alpha ( $\alpha$ ) and two beta ( $\beta$ ) chains and is in charge of transporting oxygen to the tissues and the exhalation of carbon dioxide. The relative imbalance in the production of alpha and beta chains causes the structural and functional defects in hemoglobin, which cause a variety of clinical disorders known as thalassemia. Of them, one of the most clinically relevant forms is the  $\beta$ -thalassemia, which is characterized by a high prevalence rate and inconsistent disease severity. The mutations, which cause the disorder, are in the HBB gene, found in the short arm of chromosome 11, the gene that codes for the 11-globin chain. These mutations disrupt the synthesis of  $\beta$ -globin, which causes the imbalance between the production of alpha and beta chains and, as a result, intracellular precipitation of unpaired alpha chains and poor erythropoiesis (Wang *et al.*, 2025).

In patients with thalassemia minor, clinical manifestation is usually confined to mild anemia; therefore, therapeutic interventions are not usually needed. Thalassemia intermedia, in its turn, is characterized by mild or moderate anemia, and the cohort is clinically and genetically heterogeneous. Although routine blood transfusion is not compulsory complication of affected patients can be observed in the form of splenomegaly or skeletal deformity. Thalassemia major is the most severe phenotype, and this occurs due to the homozygous mutation of the  $\beta$ -globin

gene. The signs of the disease include severe anemia in early childhood, which requires life-long blood transfusion and blood iron chelation to remain under iron overload. Thalassemia major is untreated and may trigger growth retardation, skeletal defects, and organ dysfunction. Specific genetic mutations and other genotypic and environmental modifiers regulate the phenotypic severity of  $\beta$ -thalassemia. Diagnosis is based on molecular and hematologic studies, and treatment is personalized based on the type and severity of the disease, with intensive treatments applied in the significant cases, and the surveillance of the minor cases (Galanello *et al.*, 2009).

Approximately 1.5% of the global population carries the  $\beta$ -thalassemia gene, with the highest prevalence in South and Central Asia, the Middle East, the Mediterranean region, and parts of Africa and South America, including high carrier rates in Cyprus and Sardinia (Masih *et al.*, 2023). Access to long-term care remains limited in developing countries compared with Europe and North America (Sufi *et al.*, 2022). However, the limited availability of advanced gene and cell therapies in low-income regions underscores the need for affordable preventive and therapeutic strategies (Fazal *et al.*, 2021; Chiew *et al.*, 2024).

$\beta$ -thalassemia major is a significant public health issue in Pakistan, causing psychosocial problems such as anxiety and depression among patients and families (Ansari *et al.*, 2024). Limited healthcare access, lack of national policies, and inadequate surveillance exacerbate the disease burden (Waheed *et al.*, 2021). The country reports over 100,000 active cases with 5,000–9,000 new cases annually and a high carrier rate (Ehsan *et al.*, 2020). Recent studies indicate a potential protective role of higher serum calcium against ocular complications, warranting further investigation (Siddiqui *et al.*, 2024).

$\beta$ -thalassemia affects 5–7% of Pakistan's population, with a similar carrier frequency, creating a major public health burden (Ansari *et al.*, 2022). The most common HBB gene mutations are IVS-I-5 (G>C), codon 8/9 +G, codon 41/42 -TTCT, IVS-I-1 (G>T), and the 619-bp deletion. IVS-I-5 (G>C) is the most frequent across ethnicities, indicating a strong founder effect. IVS-I-5 and codon 8/9 are common in Punjab and Sindh, while the 619-bp deletion is more prevalent in Khyber Pakhtunkhwa and Baluchistan. These five mutations together account for about 90% of  $\beta$ -thalassemia alleles in Pakistan (Akbar *et al.*, 2024).

Thalassemia syndrome screening is clinically important in three main scenarios: correct diagnosis and treatment of anemia, prenatal clinics of parents with high risks of having an affected child, and postnatal screening to allow detection in infancy. Carriers of thalassemia are often mistaken for having iron deficiency anemia and end up with long-term iron supplementation, which is parenteral at times. The relevant screening procedures would also exclude the unwarranted use of iron on carriers, and the resulting sequelae and chronic diseases related to thalassemia intermedia would be minimized through careful follow-ups. The use of prenatal screening is aimed at early planning and counseling through identifying adult carriers (Kamil *et al.*, 2021). The Punjab Thalassemia and Genetic Disorders Institute (PTGD) offers free pre-marital and family screening using CBC, hemoglobin electrophoresis, and molecular tests, with cascade screening proving effective (PTGD, 2021). Community outreach with local languages and visual aids improves awareness and reduces stigma in consanguineous populations (Raja *et al.*, 2022).

Prenatal diagnosis of  $\beta$ -thalassemia is a crucial measure of prevention of the birth of the sick child(s) in populations at high risk. It is normally given to couples who carry both  $\beta$ -thalassemia phenotypes. Molecular analysis of chorionic villus samples (CVS) or amniotic fluid, which can be collected during weeks 10-18 with the help of amniocentesis, is the initial step in a workflow of diagnosis (Amin *et al.*, 2022).

The research aims to identify and analyze prevalent HBB gene mutations in the Pakistani population. The research will also evaluate the efficacy of prenatal diagnostic techniques, including chorionic villus sampling (CVS) and molecular methods like ARMS PCR. Finally, the research will promote genetic counseling and public awareness about  $\beta$ -thalassemia, emphasizing the importance of carrier screening to reduce the incidence of the disorder in high-risk populations.

The research on  $\beta$ -thalassemia in Pakistan is significant as it addresses a major public health challenge with high prevalence due to genetic and sociocultural factors, including consanguineous marriages. Understanding the distribution of HBB mutations and the effectiveness of carrier screening and prenatal diagnosis can inform targeted prevention strategies.

## MATERIALS AND METHODS

Before the beginning of the study, formal approval was obtained from the Institutional Review Board (IRB) of the National Institute for Biotechnology and Genetic Engineering (NIBGE). Faisalabad, Pakistan.

### Research Framework

This study investigated  $\beta$ -thalassemia in eight consanguineous families from Southern Punjab. The research followed a systematic framework: first, families were recruited with ethical approval and informed consent, and

detailed pedigree analyses were conducted to confirm autosomal recessive inheritance. Clinical assessments and sample collection (blood from parents/children and CVS from fetuses) were performed using standard protocols. Genomic DNA was extracted from blood and CVS samples were screened for common HBB mutations (IVS-1-5 and FSC 8/9) using ARMS-PCR. DNA quality was verified through agarose gel electrophoresis. This framework integrates clinical, molecular, and statistical approaches, providing a comprehensive strategy to study inheritance patterns, carrier frequency, and prenatal diagnosis of  $\beta$ -thalassemia (Fig.1).

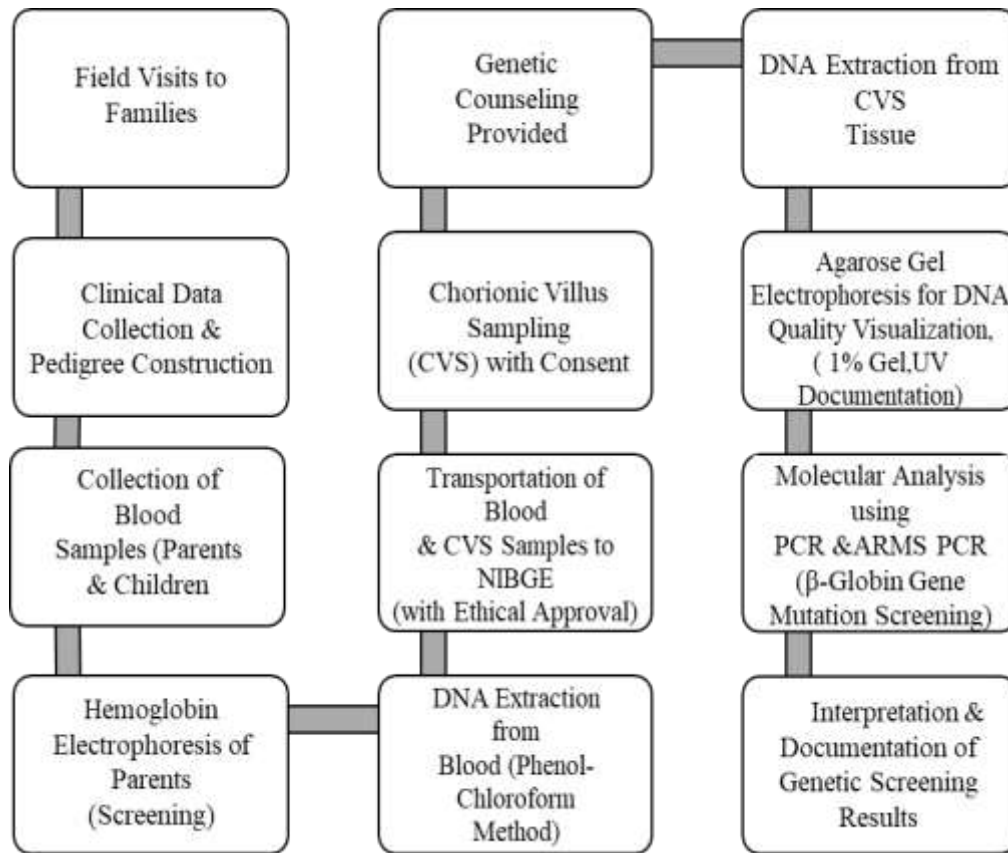


Fig. 1. Research Framework.

### Data Collection Family Profiles

A total of eight families with  $\beta$ -thalassemia were investigated in the present study. These families were identified by the collaborators in the Multan Institute of Nuclear Medicine and Radiotherapy (MINAR), Multan. A team was generated by our research supervisor. These team members belonged to Southern Punjab. These team members planned visits to their areas and gathered information from the local population and identified families with symptoms of anemia and blood transfusions. After taking proper tests, medical history was recorded before the molecular investigation. These families were told about the purpose and expected outcomes of this research and then informed approvals were obtained from these families. The individuals of these families were observed physically with care. The families were asked about any suspected environmental cause (trauma, infection etc.) that might lead to disease but the absence of associated environmental factors and the presence of other affected individuals in the family confirmed the expected genetic disorder in the family. On the basis of these findings and the group discussions, we selected the eight families for the most common mutations in the Pakistani population. All the eight families showed strong evidence of the autosomal recessive mode of inheritance and consanguinity.

### Pedigree Analysis

After approval for the study by the local ethical committee and a written consent from all families, pedigrees were constructed from all available information using standard methods introduced by Bennett (Bennett *et al.*, 1995). Cyrillic (Cherwell Scientific Publishing Ltd, Oxford, UK) software version 2.1.3 was used to draw all

extensive pedigrees. Different symbols are utilized in this software to explain the architecture of families. Males and females are represented by different symbols i.e., males by squares and females by circles. Numbers enclosed in these symbols represent the number of individuals/siblings and at least 1-3 affected member(s) of each family were subjected to genotypic examination. Parents of both families have consanguinity and were found as clinically healthy. Genetic screening of thalassemic families was performed for known mutations prior to prenatal diagnosis. CVS samples were collected at the 12th week of the pregnancy at the MINAR (Multan Institute of Nuclear Medicine and Radiotherapy) under the supervision of a medical team which all were experts with trained staff.

## Tools and Technology

### Collection of Blood Whole Blood in EDTA Vials) and CVS by (Giuseppe Simoni Method)

Field visits were carried out to collect blood samples from affected individuals and their family members using EDTA vacutainers. Parents were screened by hemoglobin electrophoresis and provided genetic counseling, followed by CVS collection at 12–16 weeks of gestation with informed consent. All samples were ethically approved and transferred to NIBGE Faisalabad for molecular analysis.

### Genomic DNA Extraction from Blood (Phenol-Chloroform Method)

Genomic DNA was extracted from blood using the phenol–chloroform method with proteinase K digestion, followed by precipitation and ethanol washing. DNA from chorionic villus samples was isolated using the salting-out method, involving tissue lysis, protein removal, and alcohol precipitation. Purified DNA was then dissolved in nuclease-free water or buffer for further molecular analysis.

### Genomic DNA Extraction from CVS (Salting-Out Method)

Genomic DNA from chorionic villus samples was extracted using the salting-out method with SDS and proteinase K for tissue lysis, followed by purification and centrifugation. DNA was precipitated with sodium acetate and chilled isopropanol, washed with 70% ethanol, and dried. The purified DNA was then dissolved in nuclease-free water or buffer for further analysis (Grimberg *et al.*, 1989).

### Agarose Gel Electrophoresis and DNA Visualization (Standard Molecular Biology Method)

DNA quality was checked using a 1% agarose gel prepared with 1.2 g agarose in 60 mL TAE buffer, containing ethidium bromide and loading dye. Electrophoresis was run at 100–120 V for 20–30 minutes. DNA bands were then visualized under UV light.

### Gel Preparation

A 1% agarose gel was prepared by dissolving 1.2 g agarose in 120 mL of 0.5X TBE buffer and heating until fully melted. After cooling to approximately 50°C, 6 µL of ethidium bromide was added, and the solution was poured into a gel tray with combs. The gel was left at room temperature for 20 minutes to solidify.

### Loading

The gel was placed in a 0.5X TBE buffer, and DNA samples mixed with loading dye were loaded into the wells. Electrophoresis was performed at 100 V for 20–30 minutes.

### Visualization

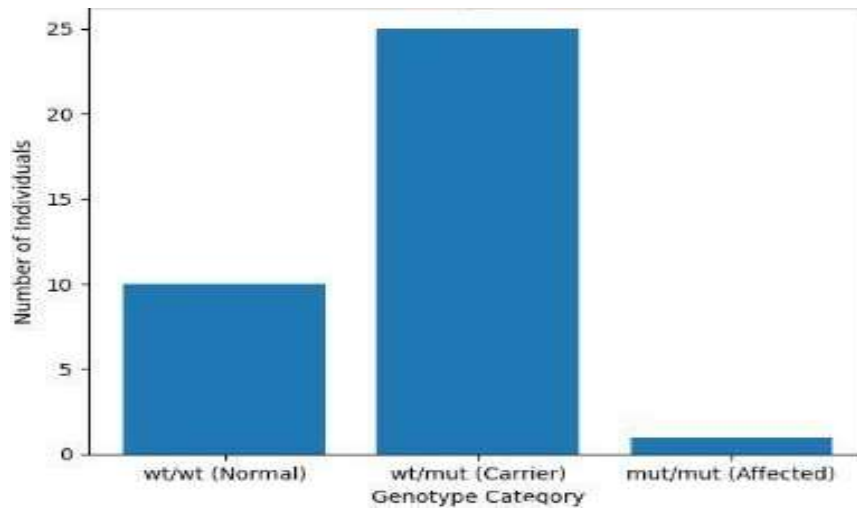
Gel was then put under UV light (310 nm) in a gel documentation system (BIORAD Gel Doc Imager) and visualized (Jeanpierre, 1987).

### Amplification Refractory Mutation System (ARMS) PCR

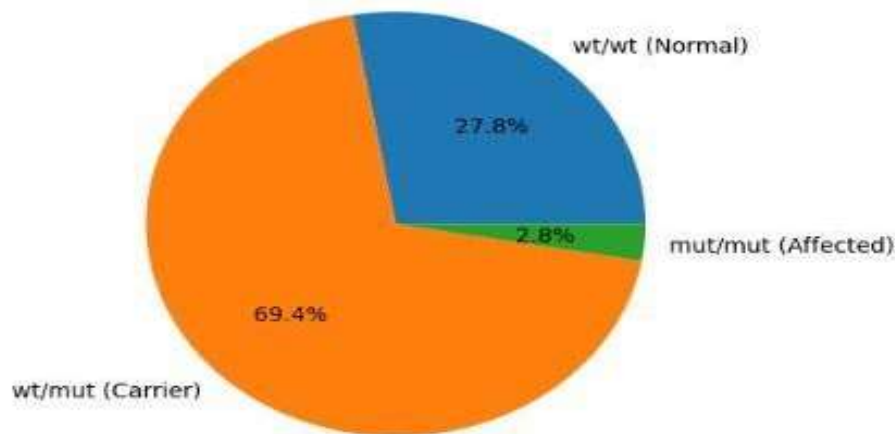
ARMS-PCR is a rapid and specific technique for detecting known  $\beta$ -globin gene mutations, using mutation-specific and internal control primers to genotype a bi-allelic system (Baysal and Huisman, 1994). It does not require amplicons or restriction enzymes for mutation analysis. The PCR mixture in a 0.2 mL tube contained DNA,  $MgCl_2 = 1.25mM$ ,  $DNTPs = 0.2mM$ , Taq buffer, primers (reverse and backward)  $= 0.5\mu M$ , Taq polymerase  $= 1/25\mu L$ , and PCR-grade water was used in variable amounts. The profile for ARMS-PCR was used during research i.e. Lid temperature was 105°C, initial denaturation of DNA temperature was at 95°C for 5 min repeat these steps for 30 cycles, annealing of primers and DNA amplification done at 94°C, for 45 sec, final extension was at 68°C for 5 min (Hassan *et al.*, 2013).

### Statistical Analysis

Genotype analysis of 36 individuals showed 10 normal (wt/wt), 25 carriers (wt/mut), and 1 affected (mut/mut), with allele frequencies  $p = 0.625$  and  $q = 0.375$ . Expected Hardy–Weinberg frequencies were 0.390 (wt/wt), 0.468 (wt/mut), and 0.140 (mut/mut) (Fig.2,3).



**Fig. 2.** Distribution of Genotypes in Studied Families.



**Fig. 3.** Genotype Proportion among Study Participant.

Table 1. Observed and Expected Genotype Frequencies (Hardy–Weinberg).

Genotype	Observed Count (O)	Expected Frequency	Expected Count (E)
wt/wt (Normal Homozygous)	10	0.390	14.04
wt/mut (Heterozygous Carrier)	25	0.468	16.85
mut/mut (Affected Homozygous)	1	0.140	5.04
Total	36	1.000	36.00

## RESULTS

### Molecular Analysis

This section presents the molecular, prenatal, and statistical evidence obtained by studying 8 high-risk Southern Punjabi families that had 8 cases of  $\beta$ -thalassemia. By using ARMS-PCR in addition to standard PCR, the

researchers aimed at determining the two most common HBB gene mutations in the Pakistani population- IVS 1- 5 (G C) and FSC 8/9 +G. The genotyping of parents, offspring, and chorionic villus sampling (CVS) samples and the pedigree evaluation led to the determination of the carrier, unaffected and affected statuses of the successive generations. Such findings provide a holistic knowledge on mutation inheritance pattern, burden of carrier frequency and effectiveness of prenatal diagnostic strategies. Combining the family and statistical studies provides more information on genetic segregation, population structure, and the role of consanguinity in the distribution of  $\beta$ -thalassemia in the examined communities (Bennett *et al.*, 1995).

### $\beta$ -Thalassemia Analysis

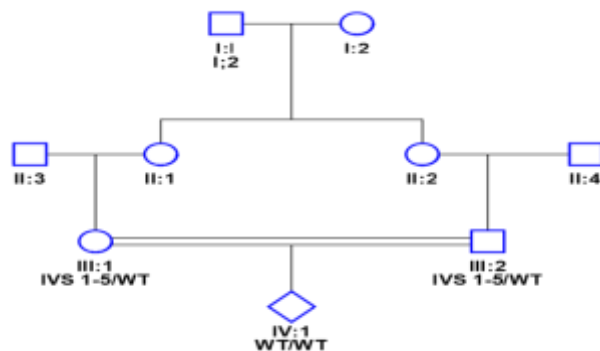
There is a facility for prenatal screening of  $\beta$ -Thalassemia in the NIBGE(National Institute for Biotechnology and Genetic Engineering), Faisalabad. A cascade of ARMS and routine PCR is performed to screen the most prevalent mutations in HBB gene in the Pakistani population.

### $\beta$ -thalassemia Families

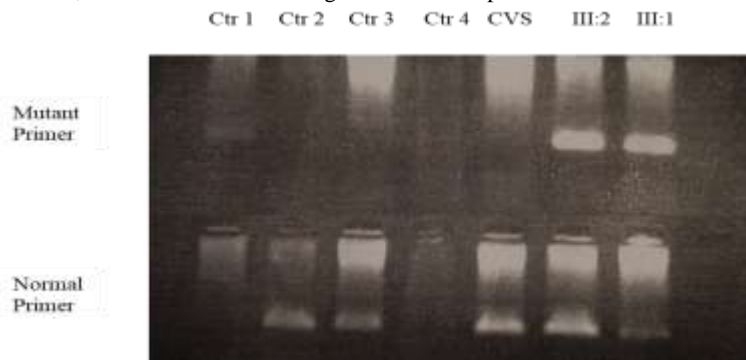
Eight  $\beta$ -thalassemia families (A–H) from Southern Punjab were sampled via MINAR Multan, Pakistan. Blood samples from parents and children, along with the ~12-week CVS, were collected and sent to HMG Laboratory, NIBGE Faisalabad. Pedigree analysis confirmed autosomal recessive inheritance in all families (Mackenzie and Boycott, 2012).

#### Family A

Family A consisted of parents and their fetus. ARMS-PCR for the IVS 1-5 (G>C) mutation showed both parents were heterozygous carriers. The fetal CVS sample was homozygous normal (wt/wt). These results demonstrate the value of family-based molecular screening and the importance of genetic counseling ( Fig. 4, 5).



**Fig. 4.** Genotype of family A segregating with  $\beta$ -thalassemia in autosomal recessive mode of inheritance; Carrier parents are mentioned in the third generation; the normal CVS is having both normal copies of alleles.

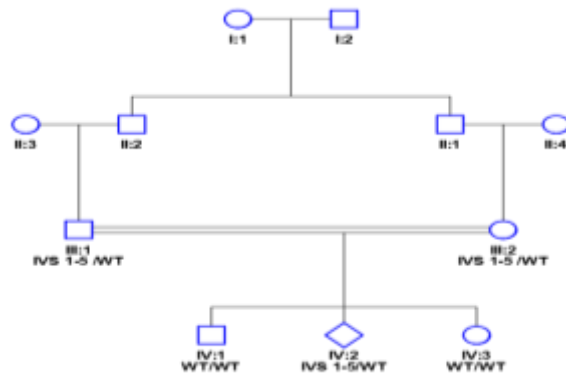


**Fig. 5.** Electropherogram of 1.8% gel shows the results of Family A for IVS- 1-5 mutation. Lane 1 mutant control, lane 2 and 3 normal control and lane 4 NTC were used as control samples for comparison. The order of screening family members consisted of the CVS, III:1 the mother and III:2 the father, respectively.

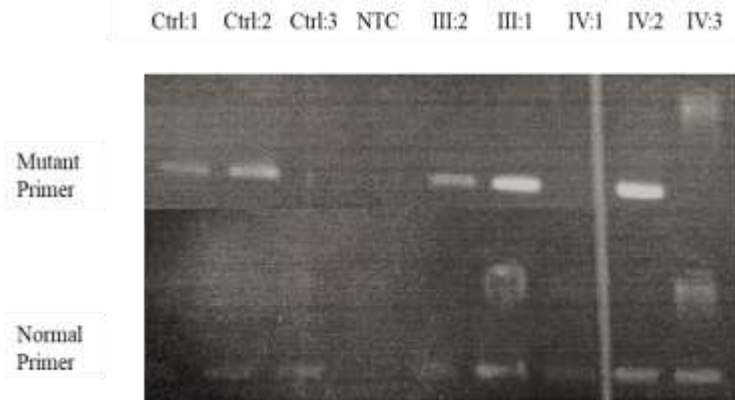
#### Family B

Family B consisted of parents, two healthy children, and one CVS sample. ARMS-PCR for the IVS-1-5 mutation showed the parents and CVS were heterozygous carriers, while the children were homozygous normal.

This highlights the effectiveness of molecular screening within a single family. Low awareness and limited use of genetic counseling hinder early detection and prevention (Fig. 6, 7).



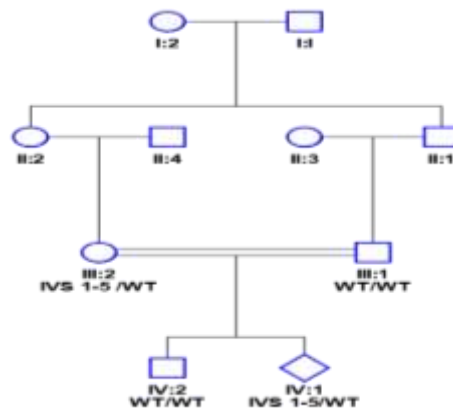
**Fig. 6.** Genotype of family B segregating  $\beta$ -thalassemia in autosomal recessive mode of inheritance. Carrier parents are mentioned in the third generation. Heterozygous CVS and the normal children are mentioned in the fourth generation.



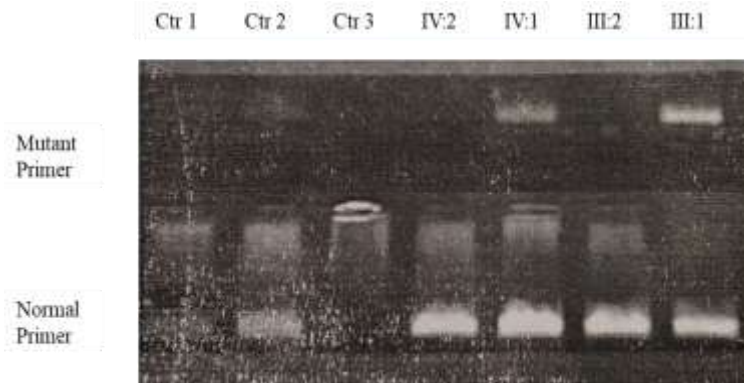
**Fig. 7.** Electropherogram of 1.8 gel shows the results of Family B for IVS-1-5 mutation. NTC was used as a control sample for comparison. The order of screening family members consisted of IV:1 a normal child, IV:2, IV:3 normal, III-2, the mother, and III:1 father, respectively.

**Family C**

Family C included parents and two children. ARMS-PCR for the IVS-1-5 mutation showed the mother and CVS were heterozygous carriers, while the father was negative and one child was homozygous normal. These findings emphasize the importance of molecular testing for reproductive planning. They also highlight the persistent lack of awareness about prenatal screening and genetic counseling (Fig. 8, 9).



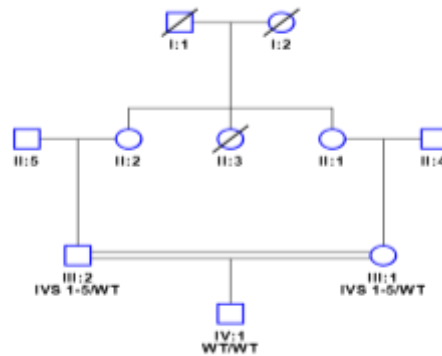
**Fig. 8.** Genotype of family C segregating  $\beta$ -thalassemia in autosomal recessive mode of inheritance. Carrier mothers and normal fathers are mentioned in the third generation. The Normal child was homozygous. CVS was a carrier for IVS 1-5 mutation.



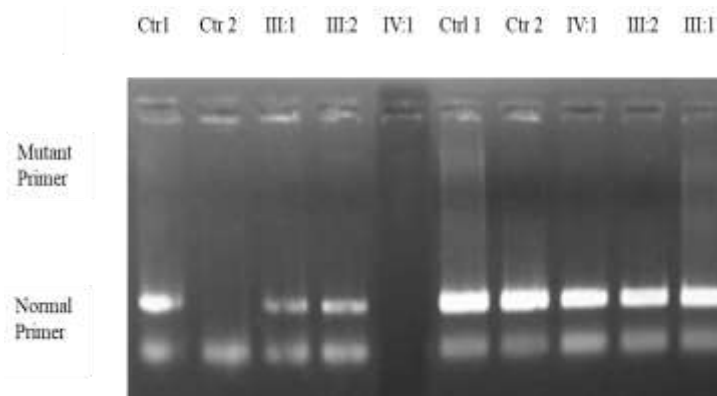
**Fig. 9.** Electropherogram of 1.8% gel shows the results of Family C for the IVS-1-5 mutation. Two homozygous and one NTC were used. The order of family members consisted of IV:2, healthy child, IV:1, CVS III: father, and III:2, mother, respectively.

#### Family D

Family D included two parents and one healthy child. ARMS-PCR for the IVS-1-5 mutation showed that both parents were heterozygous carriers, while the child was homozygous normal. This case illustrates a successful outcome of carrier parents having a healthy child. It underscores the importance of prenatal screening to reduce risks in high-risk pregnancies (Fig.10, 11).



**Fig. 10.** Genotype of family D segregating  $\beta$ -thalassemia in an autosomal recessive mode of inheritance. Carrier parents are mentioned in the third generation. The normal child has both normal (wt) copies of alleles.

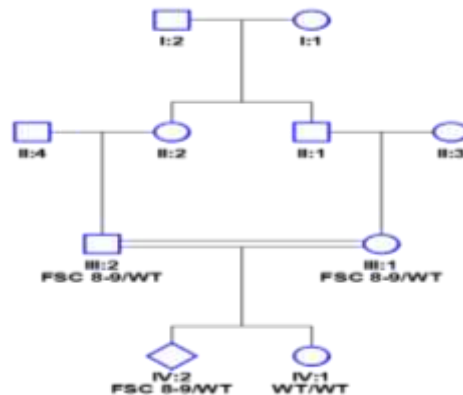


**Fig. 11.** Electropherogram of 1.8% gel shows the results of Family D. One heterozygous and one NTC were used as control samples. The order of screening family members consisted of III:1 mother, III-2 ,father, IV-1, a healthy child, respectively.

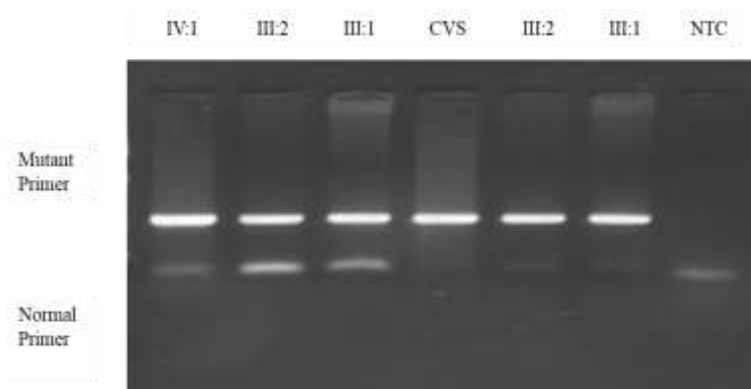
#### Family E

Family E comprised three members: two parental samples (mother and father) and one CVS. First of all, ARMS PCR was performed for the second most common mutation in Pakistani families, FSC 8/9. All the samples were screened along with a control sample. NTC control was used for the comparison of results. ARMS PCR results

showed that both parents were carriers (heterozygous) for the FSC 8/9 mutation. Their CVS was heterozygous; it carried one variant and one normal gene from parents (Fig. 12, 13).



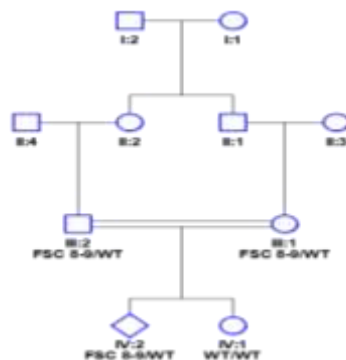
**Fig. 12.** Genotype of family E segregating with  $\beta$ -thalassemia in autosomal recessive mode of inheritance. Carrier parents are mentioned in third generation. The CVS is carrier for FSC 8-9.



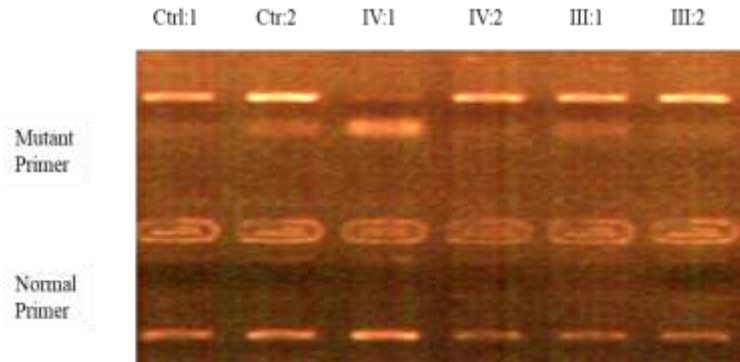
**Fig. 13.** Electropherogram of 1.8% gel shows the results of family E. NTC was used as a control sample. The order of family members consisted of IV:1 sick child; the CVS, heterozygous (carrier), III:2 mother and III:1 father, respectively.

#### Family F

Family F consisted of parents, one CVS, and one affected child. ARMS-PCR for the IVS 1-5 mutation showed the parents were heterozygous carriers, the CVS was homozygous normal, and the affected child had homozygous normal genes. This highlights the reliability of conventional ARMS-PCR for accurate family diagnosis. It also emphasizes the potential of non-invasive prenatal testing as a safer alternative (Fig. 14, 15).



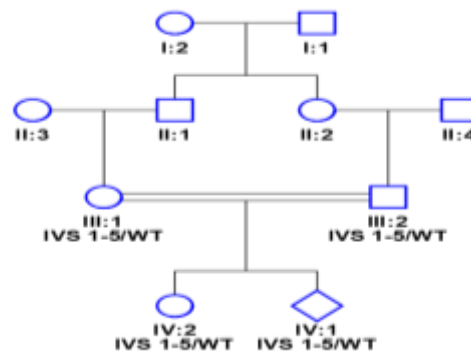
**Fig. 14.** Genotype of family F segregating with  $\beta$ -thalassemia in autosomal recessive mode of inheritance. Carrier parents are mentioned in the third generation. CVS was carrier for FSC 8-9 mutation and the normal child shown in fourth generation.



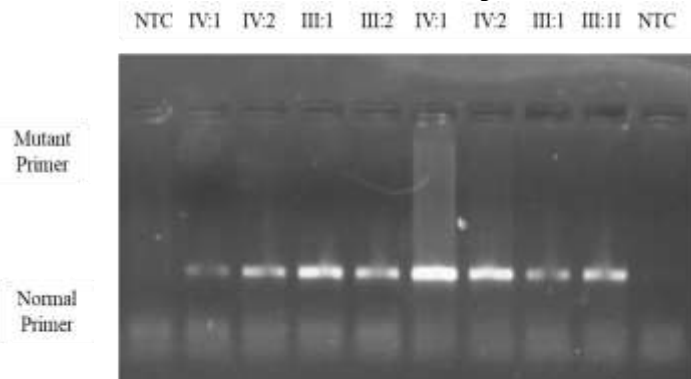
**Fig. 15.** Electropherogram of 1.8% gel shows the genotype of Family F. One (heterozygous) for FSC 8-9 were used as control. The upper row shows mutant primers and the lower row of bands shows normal primers. CVS IV:1, III: father and III:2 mother respectively.

### Family G

Family G consisted of parents, one child, and CVS. ARMS-PCR showed all samples, including parents, child, and CVS, were heterozygous carriers for the IVS 1-5 mutation. AS-PCR confirmed the inheritance of the carrier state. This highlights the importance of molecular testing and the role of community awareness in managing  $\beta$ -thalassemia (Fig. 16, 17).



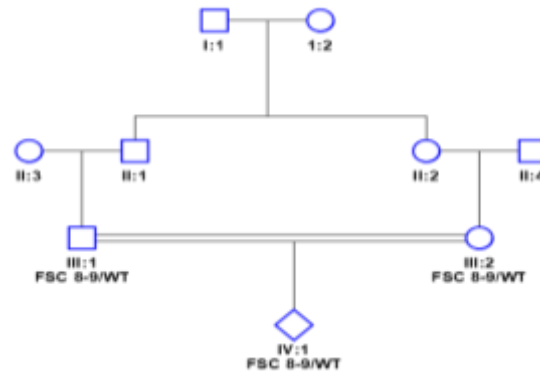
**Fig. 16.** Genotype of family G segregating with  $\beta$ -thalassemia in autosomal recessive mode of inheritance; Carrier parents are mentioned in third generation; Carrier child and CVS are mentioned in fourth generation.



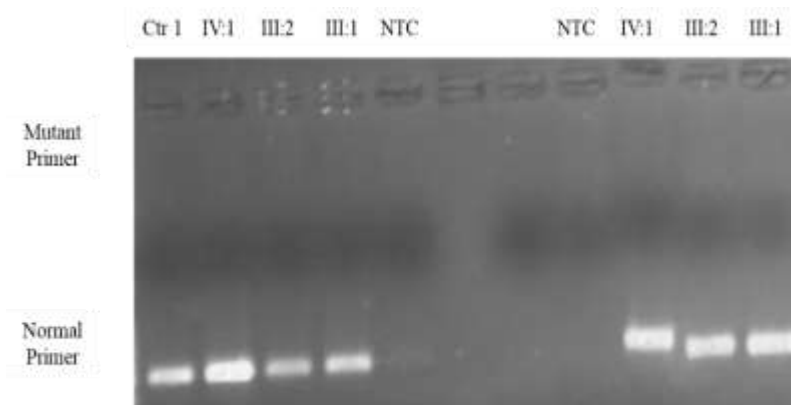
**Fig. 17.** Electropherogram of 1.8% shows the genotyping of family G for IVS 1-5 mutation. One NTC was used as control. The order of family screening consisted of IV:1 CVS, IV:2 carrier child, III:1 father, III:2 mother respectively.

### Family H

Family H included parents and one CVS sample. ARMS-PCR for the FSC 8-9 mutation showed that both parents and the CVS were heterozygous carriers. This demonstrates the effectiveness of mutation screening at the family level. It also emphasizes the importance of multiplex ARMS-PCR for detecting multiple mutations and assessing regional differences (Fig. 18, 19).



**Fig. 18.** Genotype of family H segregating with  $\beta$ -thalassemia in autosomal recessive mode of inheritance. Carrier parents are mentioned in the third generation, the CVS is shown as a carrier for the FSC 8-9 mutation.



**Fig. 19.** Electropherogram of 1.8% gel shows the genotyping of family H for FSC 8-9 mutation. One heterozygous and one NTC were used as control. The order of the family members consisted of IV:1 (CVS), III:2 mother, III:1 father respectively.

**Table 2.** Combined Genotyping and Electrophoresis Findings.

Family (Mutation Tested)	Electrophoresis Findings
<b>Family A (IVS-1-5 G&gt;C)</b>	Pedigree analysis showed both parents as heterozygous carriers. The ARMS-PCR electrophoresis confirmed the carrier status in parents, while the CVS sample was homozygous normal (wt/wt), consistent with autosomal recessive inheritance.
<b>Family B (IVS-1-5 G&gt;C)</b>	Genotyping revealed the carrier parents and a heterozygous CVS, whereas both children were homozygous normal. Gel electrophoresis supported these findings, confirming accurate segregation within the family.
<b>Family C (IVS-1-5 G&gt;C)</b>	Pedigree analysis showed a heterozygous mother and a normal father. The ARMS-PCR results confirmed a carrier CVS and a homozygous normal child, highlighting the value of prenatal molecular diagnosis.
<b>Family D (IVS-1-5 G&gt;C)</b>	Both parents were identified as heterozygous carriers, while the child was homozygous normal. Electrophoresis results corroborated the genotyping data, demonstrating a favorable reproductive outcome.
<b>Family E (FSC 8/9)</b>	Pedigree genotyping indicated both parents as carriers for the FSC 8/9 mutation. The ARMS-PCR electrophoresis confirmed the CVS as heterozygous, inheriting one mutant and one normal allele.
<b>Family F (IVS-1-5 / FSC 8-9)</b>	Genotyping showed both parents as carriers. ARMS-PCR analysis demonstrated a normal CVS and confirmed segregation of alleles within the family, supporting the reliability of the conventional ARMS-PCR for prenatal diagnosis.

**Family G (IVS-1-5 G>C)**

Pedigree analysis revealed that parents, child, and CVS were all heterozygous carriers. Electrophoresis patterns confirmed consistent inheritance of the carrier state across generations.

**Family H (FSC 8/9)**

Genotyping showed both parents and the CVS as heterozygous carriers. ARMS-PCR electrophoresis validated these results, emphasizing the effectiveness of the family-based mutation screening.

**DISCUSSION**

The statistical study of the findings was carried out to test the distribution of  $\beta$ -thalassemia genotypes and gauge their compliance with the Hardy-Weinberg equilibrium. The observed genotype frequencies showed a larger number of heterozygous carriers (wt/mut) as opposed to normal homozygous (wt/wt) and affected homozygous (mut/mut) society. Hardy-Weinberg law was used to compute the expected genotype frequencies, which were compared with the observed frequencies. Molecular screening also proved the presence of IVS-I-5 (G>C) and FSC 8-9 mutations as the most common variants in families under analysis. Across, the statistical results indicated high prevalence of carriers, effectiveness of molecular diagnostic methodology, and the necessity of carrier-selective screening, genetic counseling, and prenatal diagnostic methods to minimize carrier-to-offspring transmissions in high-risk groups with  $\beta$ -thalassemia.

Genetically acquired congenital conditions represent one of the major determinants of childhood deaths and the prevalence of chronic diseases in the world. According to the World Health Organization, in 2006, an estimated eight million live births were affected every year, and about half of the infants die before they attain the age of five years. The most common hemoglobinopathy in the global context is  $\beta$ -thalassemia. The level of affected births in Pakistan, on an annual basis, is more than five thousand cases, whereas the total number of carriers in the population is estimated to be more than ten million individuals. This high prevalence rate comes with a heavy burden to the affected families and the health care system of the country. The decrease in the frequency of such inherited conditions is possible based on preventive interventions, which are based on an in-depth knowledge of their underlying genetic etiology. The inbred populations have higher hereditary disorders, especially those with an autosomal recessive pattern of inheritance. Consanguinity unions have the effect of upholding the tendency of recessive variants, thus increasing the risk of disease. Consanguinity is a cultural practice that is very strong in Pakistan, and it is estimated that up to 70 % of marriages are consanguineous in some areas (Ghazanfar et al., 2022).

The study will help to reduce the incidence of thalassemia in the Pakistani population. The most significant challenge of the future is to gain more insight into the mechanisms of diseases and clarify the role of genes in various pathologies and the way inborn errors can be prevented. It is expected that rapid progress in genomics will allow an elaborate examination of individual genomic DNA with a view to discovering the connection between diseases and the inherent genetic variability; hence, it is likely that the number of affected births will drop. Towards achieving this goal, the study should be extended to population-based research in Pakistan (Alter, 1984).

This study provides valuable insights into molecular diagnosis and genetic screening of  $\beta$ -thalassemia in Pakistani families but has several limitations. The small cohort of eight families may not represent the broader population, and the focus on common mutations (IVS-I-5 and FSC 8-9) excluded rarer or region-specific variants. Recruitment of families with a known history may have introduced selection bias, and limited access to advanced molecular facilities prevented the use of complementary diagnostics like sequencing or multiplex assays. Socioeconomic and behavioral data, long-term outcomes of prenatal diagnosis, and follow-up of screened cohorts were not assessed. Being laboratory-based, the study does not reflect operational challenges of community-level screening, especially in rural areas with low health literacy. Future research should include larger, more diverse populations and advanced non-invasive diagnostics to guide comprehensive thalassemia prevention strategies in Pakistan.

The current study found that  $\beta$ -thalassemia is one of the most common hereditary erythrocytic disorders in Pakistan, which is mainly due to high rate of occurrence of consanguineous marriages and lack of public awareness about the autosomal inheritance. Molecular investigation was performed in eight families with known cases of  $\beta$ -thalassemia history using the Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) assay which was shown to be effective in detection of common  $\beta$ -globin gene mutations. The results showed that the most common mutation of the investigated families was IVS-I-5 (G>A) followed by a frameshift mutation FSC 8-9 (+G); other mutations, such as IVS-I-1 (G>T), codon 41/42 (-TTCT) and a 619-bp deletion were also identified. These variants are in line with the mutation spectrum of the Pakistani population previously described (see also the recently reported mutation study by Rehman *et al.* (2025). Chorionic villus sampling (CVS) performed for prenatal diagnosis at about the twelfth week of gestation correctly identified the affected fetuses, carriers, and normal was identified in the families. The homozygous mutations were found in the congenital materials and definitely

confirmed the diagnosis of  $\beta$ -hematological disease major, while the heterozygous allelic patterns were associated with dispensers. The results suggest that ARMS-PCR is a technique that is reliable, inexpensive and quick for the detection of these mutations in both peripheral blood and chorionic villus specimens. Moreover, the study found that none of the participants had any previous knowledge of thalassemia before planning a child with the disease; similarly, only a small minority of respondents had any idea about the existence of premarital or prenatal screening options. Taken together, the results confirm the high rate of the IVS - 1 - 5 and FSC 8 - 9 pathogenic variants in the local cohort and thus elucidate the value of carrier screening that is being supplemented by prenatal diagnostic strategies to direct the identification of at-risk couples to reduce the passage of pathology over to offspring. These findings indicate the need for extensive public awareness programs, extensive genetic counseling infrastructure and systematic implementation of thalassemia prevention programs across the length and breadth of Pakistan.

### Conclusion

$\beta$ -thalassemia is a major genetic health problem in Pakistan, largely due to high rates of consanguineous marriages, limited awareness, and inadequate genetic counseling. Using ARMS-PCR, this study identified the most common  $\beta$ -globin gene mutations, including IVS-I-5 (G>C), FSC 8-9 (+G). IVS-I-5 and FSC 8-9 being predominant are consistent with earlier studies. Chorionic villus sampling (CVS) enables early prenatal detection of affected fetuses and carrier status in high-risk families, allowing informed reproductive decisions. ARMS-PCR is shown to be an accurate, efficient, and cost-effective tool for mutation detection, applicable in diagnostic and preventive programs. Strengthening national public health interventions, including carrier screening, premarital testing, and genetic counseling, can significantly reduce at-risk births. Modern techniques such as non-invasive prenatal testing (NIPT) provide safer alternatives to traditional invasive methods. Increasing public education, particularly in rural and high-risk populations, is critical for awareness and informed decision-making. Integrating molecular diagnostics into national programs ensures equitable access to preventive measures. These interventions collectively offer a pathway to mitigate the burden of  $\beta$ -thalassemia in Pakistan.

### Recommendations

Drawing on the empirical evidence given through this investigation, it is suggested that universal carrier screening and prenatal diagnosis, diagnosis, and genetic counseling to prevent  $\beta$ -thalassemia in Pakistan. Integrating premarital screening into health policies, strengthening programs like PTGD, expanding public awareness, investing in advanced diagnostics, and training healthcare professionals are essential for sustainable disease prevention and control.

### Future Directions

Public-private partnerships can strengthen thalassemia prevention in Pakistan. Future research should focus on identifying rare  $\beta$ -thalassemia mutations and developing affordable diagnostic tools. Systematic data sharing, strengthened public education, capacity building, and effective policy enforcement are essential to evaluate prevention strategies and achieve long-term disease control and elimination.

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