



Original Article

Mitochondrial DNA mutations in rRNA and tRNA loci associated with maternally inherited deafness

Shafee Ur Rehman, PhD¹, Khushi Muhammad, PhD²

¹Department of Medicine, Ala-Too International University, Bishkek, Kyrgyzstan, ²Department of Biotechnology and Genetic Engineering, Hazara University, Mansehra, Pakistan.

*Corresponding author:

Shafee Ur Rehman,
Department of Medicine, Ala-
Too International University,
Bishkek, Kyrgyzstan.

shafeeur.rehman@alatoou.edu.kg

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ABSTRACT

Objectives: Mutations in mitochondrial deoxyribonucleic acid (mtDNA) are a known cause of maternally inherited deafness and other mitochondrial disorders. These mutations often disrupt mitochondrial translation and oxidative phosphorylation, particularly affecting high-energy tissues such as the auditory system. This study aimed to identify mtDNA mutations in the 12S ribosomal RNA (rRNA), 16S rRNA, and mitochondrial transfer RNA for valine gene (*MT-TV*) loci and assess their pathogenicity using updated bioinformatic tools.

Methods: A cross-sectional genetic study was performed from 2015 to 2016 involving 71 students (31 females and 40 males) aged 12–16 years, all exhibiting bilateral sensorineural hearing loss with maternal inheritance. DNA was isolated from saliva samples utilizing the phenol–chloroform technique. A 795 base pair fragment encompassing the 3' terminus of 12S rRNA, the entire *MT-TV*, and the 5' terminus of 16S rRNA was amplified and sequenced. Variants were aligned with the revised Cambridge reference sequence and evaluated utilizing the Mitochondrial transfer RNA informatics predictor (MitoTIP) pathogenicity scoring system.

Results: Fifty samples produced high-quality sequences, uncovering 78 mtDNA variants. Significant mutations comprised m.1438A>G and m.1544A>T in 12S rRNA, along with m.1603A>C, m.1607T>G, and m.1623G>C in *MT-TV*, many of which exhibited MitoTIP scores surpassing 60%.

Conclusion: Recurrent variants found in rRNA and transfer RNA loci may signify mutational hotspots responsible for maternally inherited deafness. These findings advocate for regional mtDNA screening and functional validation to enhance the diagnosis and management of mitochondrial hearing loss.

Keywords: Hearing loss, Mitochondrial transfer RNA informatics predictor analysis, Mitochondrial deoxyribonucleic acid, Ribosomal RNA mutations, transfer RNA pathogenicity

Key Messages

The research confirms that mtDNA mutations in 12S rRNA, 16S rRNA, and tRNA regions disrupt mitochondrial translation and energy production, which results in maternally inherited deafness. The study shows that pathogenic mtDNA variants such as 1438A>G and 1603A>C need complete mtDNA screening for improved diagnosis and therapy development.

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INTRODUCTION

The energy-producing components of the oxidative phosphorylation (OXPHOS) system and the mitochondrial translation machinery, which mitochondrial deoxyribonucleic acid (mtDNA) encodes, function in high-demand tissues, including the brain, muscles, and auditory system.^[1,2] The scientific community has proven that mtDNA mutations lead to maternally inherited deafness, which represents one of the primary mitochondrial dysfunction-related phenotypes.^[3] The 12S and 16S ribosomal RNA (*rRNA*) genes serve as the structural foundation of the mitochondrial ribosome, which translates mitochondrial messenger RNAs into OXPHOS proteins.^[4,5] The 12S rRNA region mutations create structural problems in ribosomes, which result in translation errors and defective protein production. Mutations in the mitochondrial transfer RNA for valine gene (*MT-TV tRNA-Val*) create problems with transfer RNA (tRNA) folding and aminoacylation, which prevent valine from being properly incorporated during translation. The resulting defects in mitochondrial protein synthesis and adenosine triphosphate (ATP) production primarily affect tissues with high energy demands, such as the cochlea.^[6,7] The maternal inheritance pattern of progressive sensorineural hearing loss occurs because mtDNA mutations affect the cochlea and other high-energy tissues.^[8]

Evaluating the functional impact of mtDNA mutations is challenging because these regions contain numerous variants, making it difficult to distinguish pathogenic mutations from benign polymorphisms. The evaluation of pathogenic mtDNA mutations requires predictive bioinformatic tools to differentiate them from harmless polymorphisms. The mitochondrial tRNA informatics predictor (MitoTIP) system combines structural modeling with evolutionary conservation analysis and clinical data to evaluate mitochondrial transfer RNA (mt-tRNA) variants for pathogenic potential.^[9] The MitoTIP system provides specific pathogenicity scores for mitochondrial tRNA variants through its evaluation of tRNA architectural structures.^[10]

The current study examines genetic alterations in the 12S rRNA and 16S rRNA and *MT-TV* (tRNA-Val) regions from Pakistani adolescents who inherited their hearing loss from their mothers. The research uses Sanger sequencing together with MitoTIP pathogenicity scoring to detect known and potential new mtDNA genetic alterations. The research uses modern bioinformatics methods to analyze historical Pakistani genetic data, which generates fresh regional findings about mitochondrial deafness and extends the worldwide collection of mtDNA-related hearing loss mutations.

MATERIALS & METHODS

Saliva samples were obtained from deaf students who attended a special education school in Peshawar within

Khyber Pakhtunkhwa (KP) Province during 2015 and 2016. The participants used 3 mL of sterile sugar solution to rinse their mouths for 2–3 min before giving their sample. The researchers stored the obtained samples at –20°C until they performed DNA extraction.

Study design and participants

A cross-sectional genetic association study was designed throughout the period from January 2015 to December 2016. The research took place at a special education school for deaf students in Peshawar, KP Province, Pakistan, while working with the Department of Biotechnology and Genetic Engineering, Hazara University, Mansehra, Pakistan. The Human Genetics Laboratory at the Department of Biotechnology and Genetic Engineering, Hazara University, conducted all laboratory tests, including DNA extraction and polymerase chain reaction (PCR) amplification, and electrophoresis.

The research involved 71 students who were 12–16 years old (31 female and 40 Male) from a special education school for the deaf in Peshawar, Pakistan [Table 1]. The participants had bilateral symmetric sensorineural hearing loss, which was confirmed through school medical records and audiometric evaluations. The family histories obtained from parents/guardians were used to determine the maternal inheritance pattern, which was defined as multiple affected siblings or maternal relatives with hearing loss and no paternal transmission. Participants who had hearing loss due to environmental factors such as birth trauma, meningitis, or ototoxic drug exposure, or those with syndromic features, were excluded. This ensured a cohort enriched for maternally inherited deafness. The study included participants who met three conditions: (i) They had congenital or early-onset hearing loss confirmed by medical evaluation, (ii) they showed a clear pattern of deafness inheritance from their mothers, and (iii) they provided consent to participate, along with their guardians' consent for minor participants. The study excluded participants who had deafness from environmental factors such as trauma, infection, or ototoxic drug use, those with unclear family history, and those who refused to consent. The saliva samples were obtained at the school location

Table 1: Baseline demographic and clinical characteristics of participants.

Characteristic	Value (n=71)
Age range (years)	12–16
Mean age (years)	~14
Sex ratio (Male: female)	40:31
Bilateral sensorineural hearing loss	100%
Maternal inheritance pattern	Documented in all cases
Excluded cases (environmental causes)	0

before freezing them at -20°C for transportation to the Human Genetics Laboratory, where phenol–chloroform extraction methods were used for DNA extraction from the saliva.

Sample collection and sequencing

In this study, 71 saliva samples from deaf student participants were obtained. Good-quality DNA from all samples was collected before successfully performing PCR amplification and Sanger sequencing. The sequencing process targeted a 795 base pair area that included the 3' end of the 12S *rRNA* gene together with the complete mitochondrial transfer RNA for valine gene (*MT-TV*) and the 5' end of the 16S *rRNA* gene. This specific DNA region was chosen because it has been linked to deafness that passes through maternal lines and mitochondrial disorders.

Reference alignment

The sequencing data were aligned to the revised Cambridge reference sequence (rCRS; GenBank Accession: NC_012920) using CLUSTALW. MitoTIP allowed the evaluation and annotation of tRNA variants after mutation detection.

DNA isolation and quality assessment

In the current study, we employed a modified phenol–chloroform extraction technique to extract DNA from their samples. The mixture containing 1 mL of saliva and 100 μL of lysis solution with 0.5% sodium dodecyl sulfate and 250 mM ethylenediaminetetraacetic acid (EDTA) and 500 mM Tris(hydroxymethyl)aminomethane hydrochloride (pH 8.0) and 3 μL β -mercaptoethanol, and 10 μg proteinase K underwent 60°C incubation for 90 min with a second proteinase K treatment at the 30-min mark. The aqueous solution underwent phenol extraction followed by centrifugation before isopropanol precipitation at cold temperatures. The solution was then washed with 70% ethanol before air drying and dissolving in 40 μL of double-distilled water at 56°C .

DNA quality assessment

The spectrophotometer NanoDrop 2000 from Thermo Scientific measured DNA concentration and purity through absorbance readings at 260 nm and 280 nm. The A260/A280 ratio between 1.7 and 1.9 in DNA samples indicated suitable quality for PCR amplification. The DNA integrity assessment included 1% agarose gel electrophoresis with Tris-acetate-EDTA (TAE) buffer and ethidium bromide staining. The DNA bands became visible under Ultraviolet (UV) light after performing electrophoresis at 80–100 V.

Agarose gel electrophoresis of extracted DNA

The extracted DNA quality was evaluated using 1% agarose gel electrophoresis. The agarose solution needed TAE buffer to dissolve, while ethidium bromide served for detection purposes. The DNA samples underwent electrophoresis at 80–100 volts after being mixed with loading dye before being visualized under UV light.

PCR amplification

The 3' end of the 12S *rRNA* and the complete tRNA-Val (*MT-TV*) gene and the 5' end of the 16S *rRNA* region in mitochondrial DNA were amplified using a specific primer pair. The forward primer sequence was 5'-CGATCAACCTCACCACCTCTT-3' and the reverse primer sequence was 5'-CTTGGACAACCAGCTATACCA-3'. National Center for Biotechnology Information (NCBI) reference mitochondrial genome (rCRS, NC_012920) was used to design new primers, which were tested for specificity through NCBI Primer-Basic Local Alignment Search Tool (BLAST) *in silico* analysis to prevent non-specific amplification and pseudogene (nuclear mitochondrial pseudogene) binding. The preliminary agarose gel electrophoresis test confirmed the presence of a single distinct amplification band that matched the expected 795 base pair (bp) fragment, which validated primer efficiency and specificity. The PCR mixture consisted of 25 μL with 2 μL deoxynucleotide triphosphates (dNTPs) and 2.5 μL MgCl_2 and 2.5 μL of $\times 10$ Taq buffer and 2 μL of each primer and 0.5 μL of Taq DNA polymerase and 1.5 μL of template DNA, and 12 μL of ddH₂O. The thermal cycling process started with 5 minutes of denaturation at 94°C , followed by 35 cycles of denaturation at 94°C for 30 s and annealing at 53°C for 1 min, and extension at 72°C for 1 min before a final extension at 72°C for 5 min.

Agarose gel electrophoresis of the PCR product

The analysis of PCR products used 1% agarose gel following the procedures previously described. The samples received loading dye before undergoing 100-volt electrophoresis for 30 min. The UV light exposure revealed bands, which were then photographed.

Extraction of the desired PCR product from the gel

The PCR bands obtained from agarose gels were placed into microtubes with labels for storage at -20°C . The GeneAll Gel Extraction Kit from GeneAll Biotechnology Korea was used to purify DNA samples according to the manufacturer's instructions. The NanoDrop spectrophotometer from Thermo Scientific verified DNA purity and concentration, while a 1% agarose gel electrophoresis confirmed DNA integrity by showing a single band without signs of degradation before sequencing.

Sequence analysis of the PCR product

The PCR products underwent Sanger sequencing at Microgen (Seoul, South Korea) through forward and reverse (bidirectional) sequencing to achieve accurate base-calling results. The sequence chromatograms received visual inspection to verify peak quality and the absence of background noise. The analysis included only reads that achieved a Phred quality score of 30 or higher (99.9% base-calling accuracy). The sequencing data reached an average coverage of $\times 2$ per locus through forward and reverse reads. The obtained sequences underwent alignment against the rCRS with GenBank accession number NC_012920 using CLUSTALW software. The researchers used Mitochondrial DNA Mutation Database (MITOMAP) to annotate mutations and MitoTIP to evaluate their pathogenic potential.

Sequence alignment and variant annotation

Sequencing reads were aligned to the rCRS (rCRS; NC_012920) using CLUSTALW v2.1. Using the MitoTIP v3.0, which was available via the MITOMAP platform (MITOMAP; accessed March 2025), mitochondrial tRNA variants were annotated and scored. During the same access period, data on pathogenicity and variant frequency were compared to MITOMAP and related databases of mitochondrial variants.

Software and computational tools

Chromas Lite v2.6.6 and BioEdit version 7.2.5 were used to manually examine the sequence chromatograms. Microsoft Excel 2021 and GraphPad Prism v9.0 (GraphPad Software, USA) were used for statistical and data visualization analyses. The current study presented mutation data through counts and percentages, and proportions that spanned across three genomic areas, including 12S rRNA and 16S rRNA, and tRNA loci. The study did not perform formal statistical tests for subgroup differences between male and female participants or between transition and transversion mutations because it had only 50 successfully sequenced samples and an exploratory research design. The study did not execute any

statistically significant subgroup analysis. The researchers determined mutation frequency through sequencing results of targeted mitochondrial loci without adjusting for complete mtDNA genome coverage because they analyzed a 795 bp segment instead of the full mtDNA genome. The method used for this study generated proportional mutation distribution data within the analyzed segment, which served as an appropriate method for detecting recurring mtDNA variants linked to maternal deafness.

RESULTS

Saliva samples were collected from deaf students at Peshawar's special education school. Participants received information about the study procedure, and their basic demographic information was recorded. The DNA extraction results indicated high-quality DNA samples [Figure 1] as shown below.

Optimization of PCR conditions

In current research, we evaluated different primer sets to optimize the desired PCR product amplification. We optimized PCR amplification through modifications in annealing temperature and extension time to increase reaction efficiency. The results of PCR amplification for the mtDNA m.1555A>G primers, including specific bands observed during optimization and the final optimized PCR reaction, are presented in Figures 2 and 3.

Sequence analysis of nucleotide

In this study, we used the amplified PCR fragment from 71 participants to proceed with nucleotide sequencing. Fifty nucleotide sequences were retrieved after sequencing. The sequence peaks were analyzed and trimmed for further analysis.

Sequencing outcomes

All 71 saliva samples yielded high-quality DNA for analysis. The sequencing process yielded successful results for 50 out of 71 samples (70.4%). The remaining 21 samples failed

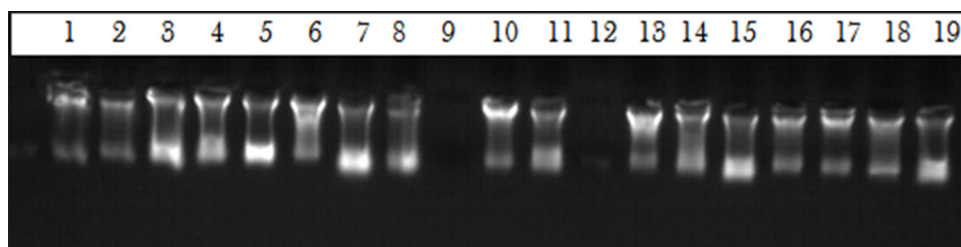


Figure 1: The 1% agarose gel electrophoresis shows genomic DNA extracted from saliva samples. The DNA samples from ten random patients appear in Lanes 1–10, while the 1 kb marker DNA ladder runs through Lane M. The high-molecular-weight DNA bands show excellent extraction quality and PCR amplification readiness of mtDNA.

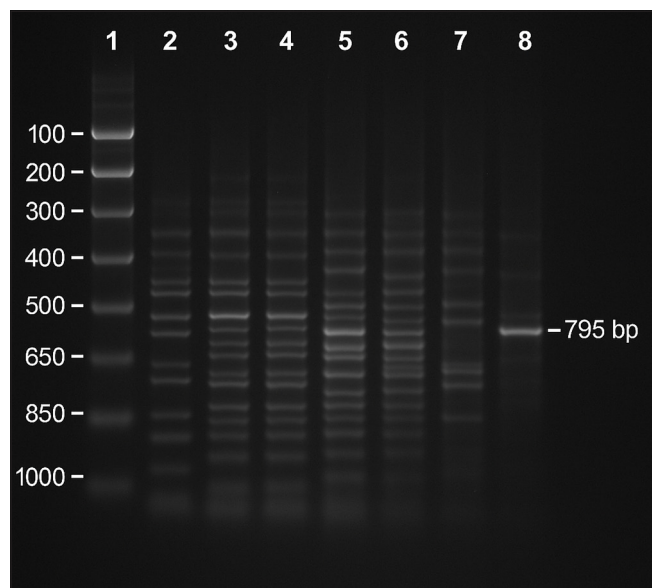


Figure 2: Agarose gel electrophoresis for PCR optimization of a 795 bp mtDNA fragment spanning the 12S rRNA, MT-TV (tRNA-Val), and 16S rRNA loci. Lane M: 100 bp DNA ladder; Lanes 1–8: PCR amplification under varying conditions; arrow indicates the expected 795 bp product.

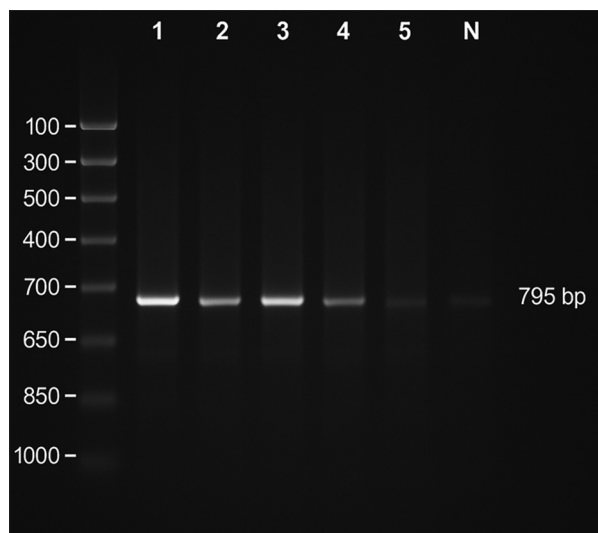


Figure 3: Final PCR amplification of the 795 bp mtDNA fragment targeting the 12S rRNA, MT-TV, and 16S rRNA regions. Lane M: DNA ladder; Lanes 1–5: successful amplification; Lane N: negative control; arrow indicates the 795 bp product.

sequencing because their DNA quality was insufficient, or their DNA had degraded during storage or PCR amplification produced weak or non-specific agarose gel bands. The extraction of DNA from field saliva samples faces typical difficulties because storage and transportation conditions differ between samples.

Mutation spectrum and novelty

The 50 successfully sequenced samples contained 78 mtDNA variants, which spread across the 12S rRNA, 16S rRNA, and tRNA-Val (*MT-TV*) regions. The MITOMAP database (accessed 2025) shows that m.1438A>G and m.1544A>T mutations in the 12S rRNA gene match known pathogenic variants that cause maternally inherited deafness. The *MT-TV* (tRNA-Val) gene variants m.1603A>C, m.1607T>G, and m.1623G>C appear to be discoveries because they do not exist in MITOMAP or the rCRS variant catalogs. The study-specific variants need additional population-based and functional analysis because they have not been documented in previous research [Table 2].

Across the 12S rRNA, 16S rRNA, and *MT-TV* (tRNA-Val) regions, 78 different forms of mtDNA were found. Because they were not included in MITOMAP (accessed March 2025) or other publicly accessible databases of mitochondrial variants, five of these were determined to be novel. These new variations included m.1492A>C* and m.1546A>T* in the 12S rRNA gene, as well as m.1603A>C*, m.1607T>G*, and m.1623G>C* in the *MT-TV* region. In addition, previously identified variants linked to deafness were found, such as m.1438A>G and m.1544A>T, which are known mutational hotspots in mitochondrial rRNA genes. These variations have been regularly documented in cases of ototoxicity caused by aminoglycosides and maternally inherited deafness. A number of novel mutations exhibited moderate-to-high predicted pathogenicity, according to MitoTIP scoring of the tRNA variants (e.g., m.1603A>C* = 61.4%; m.1623G>C* = 77.6%), suggesting possible functional relevance and requiring additional validation. Variants that are deemed novel in this study are indicated by asterisks (*) in Table 2.

Mitochondrial DNA variants identified

Multiple samples underwent mtDNA sequence analysis through comparison with the rCRS [Supplementary file Sample sequences]. We discovered different types of mutations throughout the 12S rRNA, 16S rRNA, and tRNA regions of the mitochondrial genome [Figure 4]. In the current study, we identified 78 mtDNA variants, which included m.1438A>G and m.1544A>T mutations in the 12S rRNA gene that have been linked to maternally inherited deafness. The *MT-TV* (tRNA-Val) region contains three newly discovered variants m.1603A>C, m.1607T>G, and m.1623G>C, which do not appear in MITOMAP or the rCRS variant databases. The newly discovered mutations received MitoTIP pathogenicity scores of 50% or higher, which indicates their potential functional impact and requires additional research. In this study, we grouped the genetic changes into two categories according to their nucleotide change type: Transitions and transversions. The rRNA and tRNA loci show mutations

Table 2: Summary of mitochondrial DNA variants detected in study participants.

Gene/Locus	Variant (HGVS)	Mutation Type	Frequency (n/%)	MitoTIP Score (%)	MITOMAP Status
<i>MT-RNR1</i> (12S rRNA)	m. 1438A>G	Transition	40/80.0	—	Reported
<i>MT-RNR1</i> (12S rRNA)	m. 1443T>G	Transversion	12/24.0	—	Reported
<i>MT-RNR1</i> (12S rRNA)	m. 1492A>C*	Transversion	3/6.0	—	Novel
<i>MT-RNR1</i> (12S rRNA)	m. 1544A>T	Transversion	28/56.0	—	Reported
<i>MT-RNR1</i> (12S rRNA)	m. 1546A>T*	Transversion	2/4.0	—	Novel
<i>MT-RNR2</i> (16S rRNA)	m. 1709G>A	Transition	8/16.0	—	Reported
<i>MT-RNR2</i> (16S rRNA)	m. 1711C>A	Transversion	4/8.0	—	Reported
<i>MT-RNR2</i> (16S rRNA)	m. 1722A>C	Transversion	3/6.0	—	Reported
<i>MT-RNR2</i> (16S rRNA)	m. 1752T>A	Transversion	2/4.0	—	Reported
<i>MT-TV</i> (tRNA-Val)	m. 1603A>C*	Transversion	5/10.0	61.4	Novel
<i>MT-TV</i> (tRNA-Val)	m. 1607T>G*	Transversion	4/8.0	56.3	Novel
<i>MT-TV</i> (tRNA-Val)	m. 1623G>C*	Transversion	3/6.0	77.6	Novel
<i>MT-TV</i> (tRNA-Val)	m. 1629A>C	Transversion	3/6.0	51.3	Reported
Other tRNA loci	m. 1618A>C	Transversion	2/4.0	9.9	Reported
Other tRNA loci	m. 1634A>T	Transversion	2/4.0	30.5	Reported
Other tRNA loci	m. 1636A>C	Transversion	1/2.0	12.8	Reported

Variants marked with an asterisk (*) were not listed in MITOMAP (accessed March 2025) or other mitochondrial variant databases and are considered novel findings in this cohort. Gene names follow HGVS guidelines

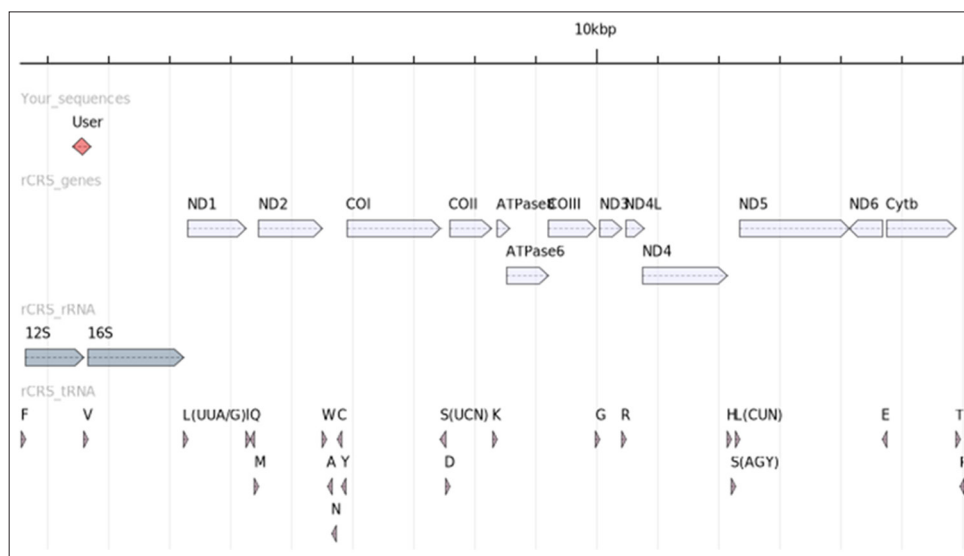


Figure 4: mtDNA gene map showing the amplified region (12S rRNA-tRNA-Val-16S rRNA) relative to the rCRS. Red marker indicates sequenced region.

where 12S rRNA region: A large number of mutations were detected that include the frequent mutations at positions m.1438A>G, m.1544A>T, and m.1443T>G. The mutations occur in conserved domains of the 12S rRNA locus, which is necessary for both ribosome assembly and protein translation. tRNA region: The tRNA regions contained mutations at m.1603A>C, m.1607T>G, and m.1629A>C positions, where MitoTIP pathogenicity scores were between 9.9% and 77.6%.

The tRNA-disrupting potential of m.1623G>C (77.6%) variants is higher than other variants. The results of mutation analysis were as follows: Transitions (e.g., A↔G, T↔C): The mutations occurred in large numbers as expected for mtDNA, which tends to produce such changes. Transversions (e.g., T↔G, A↔C): These mutations are less common, and they can have a larger impact on structure, especially when they occur in conserved regions.

The mutations detected at m.1438A>G and m.1544A>T positions in multiple samples indicate these regions function as mutation hotspots or hold critical functional positions under evolutionary selection. We used MitoTIP pathogenicity scores to evaluate the likelihood of tRNA mutations affecting mitochondrial function. The positions 1603 (61.4%), 1607 (56.3%), and 1629 (51.3%) had MitoTIP pathogenicity scores ranging from moderate to high, which suggests functional and clinical relevance [Table 2 and Figure 5].

The research findings indicate that mtDNA mutations disrupt mitochondrial function when they target the rRNA regions. The mutations at positions m.1443T>G and m.1544A>T located in the 12S rRNA locus may destabilize ribosomes, which impairs mitochondrial protein synthesis. The disruptions impair the pathway of oxidative phosphorylation because the pathway supports tissues that require high energy levels, particularly the auditory system. Medical significance exists in tRNA locus mutations because their high MitoTIP scores can lead to mitochondrial diseases. The m.1603A>C mutation (61.4%) has previously been associated with deafness, together with other mitochondrial syndromes. The study results match previous research that shows tRNA mutations act as primary factors in mitochondrial dysfunction, which leads to neurological and auditory disorders. The m.1438A>G mutations found in several samples point toward these sites being evolutionary hotspots or under selective pressure. The widespread occurrence of these mutations in various samples suggests their potential involvement in mitochondrial adaptation or increased vulnerability to dysfunction. The hearing loss occurs because of mutations in the 12S rRNA locus, which contains vital ribosomal elements that also affect patients with aminoglycoside-induced ototoxicity or

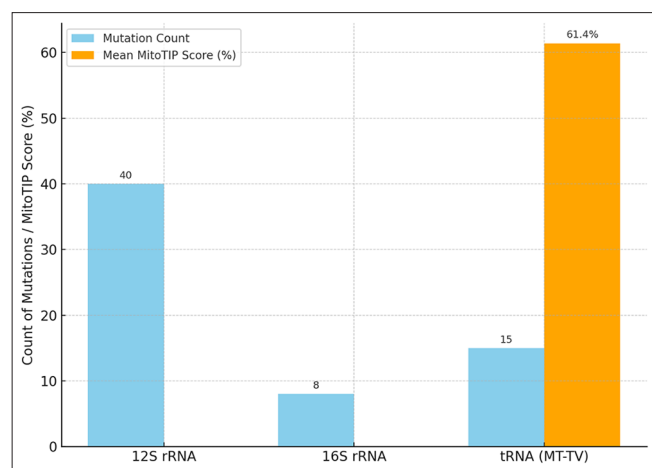


Figure 5: Distribution of mtDNA mutations across genomic regions. Bars represent mutation counts, and orange bars denote the average MitoTIP pathogenicity score for tRNA variants.

maternally inherited deafness. These detected mutations in the study could potentially contribute to the observed clinical manifestations. Collectively, the findings demonstrate a clear concentration of recurrent and novel mtDNA variants in critical rRNA and tRNA loci, underscoring their potential contribution to mitochondrial dysfunction and maternally inherited hearing loss.

DISCUSSION

The research examined mtDNA variants located in the 12S rRNA, 16S rRNA, and tRNA-Val (*MT-TV*) positions to determine their possible link with deafness that passes through maternal inheritance. The study identified multiple recurring genetic variants, which included known mutations and new variants specific to this research group. The research findings expand knowledge about mtDNA variations that affect hearing by disrupting mitochondrial translation and OXPHOS processes. The m.1438A>G and m.1544A>T mutations in the 12S rRNA gene have been linked to hearing loss caused by aminoglycoside treatment and maternal inheritance across different population groups.^[10,11,12] The repeated occurrence of these mutations in this study population confirms their probable involvement in mitochondrial dysfunction, which leads to hearing loss. The tRNA-Val G>C shows moderate to high MitoTIP pathogenicity scores, which indicate they could disrupt t (*MT-TV*) variants m.1603A>C, m.1607T>G, and 1623RNA folding and aminoacylation processes leading to impaired mitochondrial translation and decreased ATP production in cochlear tissues.^[12,13] The study discovered three new variants that have not been documented in the MITOMAP or rCRS databases.

Multiple *MT-TV* variants show high MitoTIP scores, which indicate these genetic locations play a significant part in the development of mitochondrial diseases.^[14] The predicted structural models show that tRNA stem and loop substitutions in conserved regions lead to unstable secondary structures, which decrease translation efficiency and generate the energy deficits that cause auditory neuron degeneration. The research indicates that rRNA and tRNA mutations work together to block mitochondrial protein synthesis, which results in maternally inherited deafness. The research used data from insights that were inaccessible before.^[15-17] The re-evaluation of these data through contemporary pathogenicity prediction tools 2015–2016, but the application of modern MitoTIP algorithms enabled researchers to discover new enhancements, both the understanding of variant effects and their clinical value for 2025 medical practice.^[18-21] The combination of past research data with present-day bioinformatic tools demonstrates their enduring scientific worth.

The research faces three main limitations because it works with a small number of participants and does not include

control subjects, and lacks experimental evidence to validate the results. Future research needs to sequence the complete mitochondrial genome through next-generation sequencing while conducting functional tests to validate pathogenic variants and studying how mutation accumulation affects disease progression.^[22,23] The research confirms that mtDNA variants located in 12S rRNA and *MT-TV* genes lead to maternally inherited deafness by disrupting mitochondrial translation and energy production.^[24,25] The research findings establish a basis for enhancing genetic testing and counseling services that focus on mitochondrial hearing loss within South Asian communities.

Strengths and limitations

The research offers a detailed examination of 12S rRNA and 16S rRNA and *MT-TV* (tRNA-Val) mutations in Pakistani patients who inherited deafness from their mothers. The study used Sanger sequencing to achieve precise mutation detection and MitoTIP as a specialized tool to evaluate mt-tRNA variant pathogenicity through integration of structural and clinical data. The research method provides strong variant interpretation results while identifying specific areas where hearing loss mutations tend to occur.

The research contains multiple restrictions that affect its results. The research included only 71 participants but only 50 of them received successful sequencing results which made it difficult to detect rare genetic variations and reduced statistical power. The study lacks healthy control samples and family segregation analysis which prevents researchers from confirming the pathogenic nature of identified variants. The results should be interpreted with caution because this study was exploratory in nature and relied on descriptive analyses without formal statistical power calculations. Even though recurrent variations point to biologically significant patterns, these findings need to be confirmed by larger cohort studies and inferential statistical techniques. The study focused on a 795 bp mtDNA region but failed to detect mutations that could exist in other important genetic locations. The study did not perform biological tests to prove how high-scoring variants from MitoTIP affect cellular functions. Hence, further research requires additional studies with bigger participant groups across different populations and complete mitochondrial genome sequencing and biological tests to validate these results.

CONCLUSION

The current research found specific mtDNA genetic alterations in 12S rRNA and tRNA-Val regions which could cause deafness that passes through maternal inheritance. The MitoTIP analysis showed that both known and potential new mutations had strong pathogenic potential. The study

demonstrates that complete mtDNA testing provides essential information for diagnosing and counseling families with mitochondrial hearing loss. Additional research must be conducted to validate the disease-causing effects of these genetic variants and develop better methods for early diagnosis and specific treatment approaches.

Author's contributions: Shafee Ur Rehman performed the experiment, wrote the manuscript, and did the analysis. Khushi Muhammad supervised and reviewed the final manuscript.

Ethical approval: This study was approved by the Ethics Committee of Hazara University, Mansehra, Pakistan, under Memo No. HU/ERC/2015/127 (2015). The committee reviewed and authorized the collection of saliva samples and subsequent DNA analysis for research purposes. All research procedures complied with institutional, national, and international ethical standards, including the principles of the Declaration of Helsinki (2013 revision).

Declaration of patient consent: Written informed consent was obtained from all adult participants before sample collection. For participants under 18 years of age, written consent was obtained from parents or legal guardians. All data were anonymized to ensure confidentiality.

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Conflicts of interest: There are no conflicts of interest.

Availability of data and material: The data and material are available in the manuscript and supplementary files.

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