In-silico Characterization and Mutational Analysis of Genetic Variants in Exon-6 of *CFTR* gene among Cystic Fibrosis Pakistani Patients

SUPERIOR UNIVERSITY

Thesis Submitted to

The Superior University Lahore

In Partial Fulfillment of the

Requirement for the Degree of

MS Zoology

By

SHAZIA TANVEER

SU92-MSZGW-F22-039

Session: 2022-2024

Faculty of Sciences

Author's Declaration

I hereby state that my MS/M.Phil thesis titled **"*In-silico* Characterization and Mutational Analysis of Genetic Variants in Exon-6 of *CFTR* gene among Cystic Fibrosis Pakistani Patients"** is my work and has not been submitted previously by me for taking any degree from this University, **The Superior University, Lahore** or anywhere else in the country/world.

At any time if my statement is found to be incorrect even after my graduation, the university has the right to withdraw my MS/M.Phil degree.

Sha Tanveer

Name of Student: **Shazia Tanveer**

Date: 20-8-2026

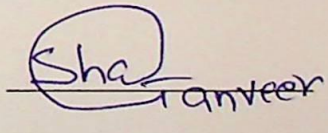
Plagiarism Undertaking

I solemnly declare that research work presented in the thesis titled **"*In-silico* Characterization and Mutational Analysis of Genetic Variants in Exon-6 of *CFTR* gene among Cystic Fibrosis Pakistani Patients"** is solely my research work with no significant contribution from any other person. Small contribution/help wherever taken has been duly acknowledged and that complete thesis has been written by me.

I understand the zero-tolerance policy of the HEC and University,

The Superior University, Lahore

towards plagiarism. Therefore, I as author of the above-titled thesis declare that no portion of my thesis has been plagiarized, and any material used as a reference is properly referred/cited. I undertake that if I am found guilty of any formal plagiarism in the above-titled thesis, even after awarding of MS/M.Phil. degree, the University reserves the rights to withdraw/revoke my MS/M.Phil. degree and that HEC and the University have the right to publish my name on the HEC/University website on which names of students are placed who submitted a plagiarized thesis.

Student/Author Signature: 

Name of student: **SHAZIA TANVEER**

Research Completion Certificate

This is to certify that the thesis entitled "***In-silico* Characterization and Mutational Analysis of Genetic Variants in Exon-6 of *CFTR* gene among Cystic Fibrosis Pakistani Patients"** submitted by **"Shazia Tanveer"** has been accepted towards the partial fulfillment of the requirement for MS/M.Phil.,**"MS Zoology"**. The quality of the work contained in this thesis is adequate for the award of degree.

Supervisor Name: **Dr. Muhammad Sarfraz Kiani**

Designation: **Assistant Professor**

Signature: _____

Co-supervisor: **Dr. Muhammad Usman Ghani**

Designation: **Assistant professor**

PGRL, CAMB Punjab University, Lahore

Signature: _____

CERTIFICATE OF APPROVAL

This is to certify that the research work presented in this thesis, titled **"*In silico* characterization and mutational analysis of genetic variants in exon -6 of the *CFTR* gene among cystic fibrosis patients"** was conducted by **"Shazia Tanveer"** under the supervision of **"Dr. Muhmmad Sarfraz kiani ".**

No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Faculty of Sciences, The Superior University, Lahore in partial fulfillment of the requirements for the degree of Master of Science/Master of Philosophy in the field of "**MS Zoology"** in Faculty of Sciences at The Superior University, Lahore.

Student Name: Shazia Tanveer Signature: Sha Tanveer

Examination Committee:

a) **Session Chair:** Dr. Hania Ramzan Signature:

b) **External Examiner:**
Name: Dr.Nazim Hussain Signature:
Designation: Assistant Professor
University Name: Center for Applied Molecular Biology (CAMB), Lahore

c) **Internal Examiner:**
Name: Dr. Usman Elahi Signature:
Designation: Assistant Professor
University Name: Superior University, Lahore

d) **Supervisor Name:** Dr. Muhammad Sarfraz Kiani Signature:

e) **Name of HOD:** Prof. Dr. Uqba Mehmood Signature:

f) **Name of Dean:** Prof. Dr. Muhammad Naveed Babur Signature:

g) **Controller Examination:** Dr. Muhammad Haris Signature:

Dedication

To My Parents and Siblings

Best Support That I Can Ever Have

Acknowledgements

Every praise and excellent glory belongs to THE ALMIGHTY **ALLAH** who is the ultimate source of knowledge. Indeed, only He commands and directs us to the straight path of life in this world, preparing us for the Final Day of Judgment. All worship to HIM, who bestowed HIS grace and mercy upon me and honored me with the blessing of ISLAM. All the reverence for HIS LAST MESSENGER **MUHAMMAD**ﷺ whose perfect life is the role model for the entire human race. Certainly, his guidance and teaching are the ultimate sources of salvation for the whole of mankind.

It is in my best interest to make known that ALMIGHTY **ALLAH** has always blessed me. May HE always keep me blessed with the unmatched love and support of my dearest parents who have always been at my side through every thick and thin of life and raised me doing their best. Not mentioned much here, but they have supported with every penny of their kindness and finance in the accomplishment of this thesis would be in suffice to say.

I would like to acknowledge with reverence, the help and support that I received from my Supervisor **Dr. Muhammad Sarfraz Kiani**, FOS, The Superior University Lahore. Being an honor for me, I would love to extend my gratitude to my research Co-supervisor **Dr. Muhammad Usman Ghani**, CAMB, University of the Punjab, Lahore, for his unparalleled guidance efforts in the entire course of completion of the thesis. I appreciate and acknowledge their support, devotion, patience, and intellectual supervision to disseminate practical lessons in the research field.

Finally, I am grateful to the Lab attendant **Usman Haider** and PhD senior at CAMB **Anum Safdar** for their friendly and supportive behavior during my whole research work.

Shazia Tanveer

TABLE OF CONTENTS

Title page i
Author's Declaration ii
Plagiarism Undertaking iii
Research Completion Certificate iv
Certificate of Approval v
Dedication vi
Acknowledgements vii
Table of contents viii
Table of contents ix
List of figures x
List of Tables xi
List of Abbreviations xii
Abstract xiv
CHAPTER 1 1
1. INTRODUCTION 3
Problem statement 3
Hypothesis 3
Aim and Objectives 3
CHAPTER 2 4
2. LITERATURE REVIEW 4
2.1. History 4
2.1. Cystic fibrosis, 4
2.2. Diagnosis of CF 5
2.3. CFTR gene and its product 6
2.4. Genetic variants of *CFTR* 7
2.5. Treatments for Variants of *CFTR* 7
2.6. The Study Gap 8
CHAPTER 3 10
3. MATERIALS AND METHODS 10
3.1. *in-silico* Analysis 10
3.1.1. Data Retrieval 10
3.1.2. Filtering harmful nsSNVs 10
3.1.3. Impact of mutation of protein stability 11
3.1.4. Prediction of the Effect of Amino Acid Substitution 11
3.1.5. Structural Analysis of Native and Mutant 11
3.2 . Genetic analysis 12
3.2.1. Sample collection 12
3.2.2. Genomic DNA Extraction 12
3.2.3. Qualitative and Quantitative Analysis of extracted DNA 13
3.3. Optimization of Tetra-ARMS PCR 13
3.3.1. Primer designing 13
3.3.2. Optimization of Tetra ARMS PCR 13
3.4. Sanger sequencing 14
3.4.1. Amplification of Outer products 14
3.4.3. Sequencing PCR 14
3.4.2. Post-Sequencing PCR clean-up 15
3.4.4. Purification of Sequencing PCR Products 15

3.4.5. Sequencing analysis 15
CHAPTER 4 16
3.2. RESULTS 16
3.3. *in-silico* Analysis 16
3.3.1. Retrieval of Protein sequence 16
3.3.2. Identification of Deleterious nsSNVs 19
3.3.3. Impact of mutation of protein stability 20
3.3.4. Prediction of effect of Amino Acid Substitution on protein 20
3.3.5. Protein Structure Analysis: Native and Mutant 22
3.4. Genetic Analysis 22
3.4.1. Qualitative and Quantitative Analysis of Extracted Genomic DNA 22
3.4.2. Optimization of Tetra ARMS PCR 23
4.2.1. Tetra ARMS Primers design. 23
4.2.2. Optimization of Tetra ARMS PCR 23
3.5. Sanger sequencing 25
3.5.1. Amplification of exon-6 25
3.5.2. Sequencing analysis 26
3.5.3. **CHAPTER 5** 30
4. DISCUSSION 30
4.1. *In-silico* Analysis 30
4.2. Optimization of tetra ARMS PCR 31
4.3. Sanger sequencing for Validation of Tetra-ARMS PCR Results 32
Descriptive analysis 34

CONCLUSION 34
REFERENCES 35

APPENDICES 39
Appendix- 1 39
Appendix- 2 40
Appendix- 3 41
Appendix- 4 42
Appendix- 5 44
Appendix-6 46
Appendix-7 50
Appendix-8 51
Appendix-9 54

List of Figures

Figure No.	Description	Page No.
Figure 1:	Screening method of CF	*06*
Figure 2:	Structure of the human *CFTR* protein in the ATP-free conformation	*06*
Figure 3:	Distribution of *CFTR* mutations:	*7*
Figure 4:	Thermocycling profile for sequencing reaction	*14*
Figure 5:	The three-dimensional (3D) structure of *CFTR* protein:	*22*
Figure 6:	Extracted DNA from the blood of patients	*23*
Figure 7:	Results of Tetra-ARMS PCR:	*25*
Figure 8:	Amplified outer products of exon-6 of *CFTR* gene	*26*
Figure 9:	Electropherograms of Sanger sequencing	*27*
Figure 10:	Electropherograms of *CFTR* gene exon 6 L218X	*28*
Figure 11:	Exon 6 of a CF-sample sequence alignment sequence *CFTR* gene's exon 6	*29*

List of Tables

Table No.	Description	Page No.
Table 1: Inclusion and exclusion criteria for sample collection		12
Table 2 : Optimized reaction for Sequencing PCR (10ul		14
Table 3: Nucleotide sequence and encoded sequence of *CFTR* gene' exon 06 of *Homo sapiens*		15
Table 4: List of mutations obtained from Cystic fibrosis patients database		18
Table 5 : Results of Insilico analysis of *CFTR* exon-6		20
Table 6: Predicted effects of amino acid substitution on protein function using Mutepred2		20
Table 7: Primer sequences selected for optimization of tetra ARMS PCR		23
Table 8: Optimized PCR reaction mixture		24
Table 9: optimized tetra ARMS amplification profile		24
Table 10: PCR reaction mixture optimised using a manual procedure		25

LIST OF ABBREVIATIONS

ABC	ATP-Binding Cassette
ARD	Autosomal Recessive Disorder
ATP	Adenosine Tri-Phosphate
CF	Cystic Fibrosis.
IRT	immunoreactive trypsinogen
CFI	Cystic Fibrosis Individuals.
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator.
COPD	chronic obstructive pulmonary disorders
EDTA	Ethylene-Diamine-Tetra-Acetic acid
FDA	Food and Drug Administration.
NBD	Nucleotide Binding Domain.
NCBI	National Centre for Biotechnology Information.
nsSNV	Non Synonymous Single Nucleotide Variant.
PCR	Polymerase Chain Reaction
PIOS	Proximal Intestinal Obstruction Syndrome
SCT	Sweat Chloride Test
SIFT	Sorting Intolerant From Tolerant.
SOPs	Standard Operating Procedures
T.E	Tris-EDTA
TBE	Tris/Borate/EDTA



IN THE NAME OF ALLAH, THE MOST COMPASSIONATE, THE MOST MERCIFUL

"READ! IN THE NAME OF YOUR LORD, WHO HAS CREATED!"

(AL-ALAQ)

In-silico Characterization and Mutational Analysis of Genetic Variants in Exon-6 of *CFTR* gene among Cystic Fibrosis Pakistani Patients

ABSTRACT

Cystic Fibrosis (CF) is an autosomal recessive disorder caused by mutations in the *CFTR* gene, producing a dysfunctional *CFTR* protein. The mutated gene produces *CFTR* protein that is unable to maintain ion balance across cell membranes. More than 2,000 mutations have been identified in the *CFTR* gene. However, Pakistan's mutational spectrum is still unknown. This study aimed to enlist all known variants in exon-6 of the *CFTR* gene, perform *in silico* analysis, and to optimize Tetra-ARMS PCR for the mutation L218X, already reported in Pakistani patients. Bioinformatics pipelines such as SIFT, PolyPhen-2, Meta SNP, PANTHER, iMutant, and Mutpred2 were used to shortlist the most harmful. Structural analysis was performed using Chimera software. Fifteen CF patients were enrolled in this study to optimize the Tetra-ARMS PCR of L218X mutation. The results of tetra ARMS PCR were validated using Sanger Sequencing. *in silico* analysis predicted that 60% of exon 6 reported variants are deleterious. The tetra-ARMS were successfully optimized to determine the L218X mutation in fifteen CF patients that were negative forΔF508 mutation. The mutation L218X was identified in two of fifteen (13%) CF patients. This study contributes to the understanding of *CFTR* gene mutations and their impact on protein function, facilitating the development of personalized diagnostic and therapeutic strategies for CF patients. Our results also demonstrate the effectiveness of combining *in silico* and molecular approaches for comprehensive mutational analysis.

Key words: Cystic Fibrosis, *CFTR* gene, Tetra-ARMS PCR, L218X mutation, ΔF508 mutation, Sanger sequencing, *in silico*

CHAPTER NO # 1

INTRODUCTION

Cystic fibrosis (CF) is a hereditary multisystem disorder caused by variations in the *CFTR* gene [1]. Patients with CF suffered from progressive lung damage [2] due to frequent infections caused by *Pseudomonas aeruginosa, Staphylococcus aureus* and opportunistic fungi [3] as well as inflammation. Inflammation leads to the development of bronchiectasis and other obstructive conditions [4, 5]. CF patients often experience gastrointestinal diseases like distal intestinal obstruction syndrome, chronic pancreatitis [6], and the production of greasy stools due to blockage of ducts. Approximately 15-20% patients suffered meconium ileus at the time of birth [7, 8]. Male infertility is also affected by CF as it leads to the blockage of duct in male reproductive system [9].

Similarly, blockage of ducts in sweat glands, caused by *CFTR* mutation, leads to the accumulation of chloride ions in the extracellular environment, resulting in the production of salty sweat in CF patients. The sweat chloride test remains the gold standard for discriminating CF from other ailments, such as primary ciliary dyskinesia (PCD), chronic obstructive pulmonary disorders (COPD), and other similar disorders. This elevated chloride ion concentration is then measured using ionophoresis, a specialized process to determine conductivity [10].

CF is an autosomal recessive genetic disorder while the frequency of individual variants varies in different ethnic groups. Although it was previously believed that CF mostly affected Caucasians, emerging research has revealed the presence of CF in other regions including people of South Asia [8]. The prevalence of CF in Pakistani population was also reported first time in 1974 when three cases of native Pakistani children were identified in Birmingham; two of those three were siblings. [11]. A study from Karachi region in Pakistan reports that the frequency of the

ΔF508 mutation in Pakistani CF children is lower than in the Caucasian population [12]. In subsequent research of Agha Khan University in Karachi found that only 33% of the local CF patients had the homozygous mutation ΔF508, which is a relatively low percentage compared to global prevalence [13]. It has been reported

that 67% of CF patients have *CFTR* gene variants other than ΔF508. These results clearly show that diagnosing of CF based on one mutation (ΔF508) would result in a false-negative. Although, sweat chloride measurement remains the most reliable method for diagnosing CF in the absence of appropriate genetic testing [14]. Priority is given to genetic testing when normal sweat chloride test results are not sufficient for CF diagnosis [15]. Current research on *CFTR* gene mutations is limited in Pakistan and South Asia due to diverse population [16]. There are not enough comprehensive research studies on Asian populations, particularly Indian subcontinent populations, to identify the mutation profile of the *CFTR* gene in cystic fibrosis patients. Rather, the gene sequencing panels for the *CFTR* gene alteration are least useful in detecting *CFTR* mutation(s) in other Indian subcontinent populations. It is more difficult to diagnose CF in Pakistani patients due to a lack of knowledge on genetic diversity in the *CFTR* gene in Southeast Asian populations, including Pakistan.

Moreover, many treatments for CF have been developed based on the functional impact of *CFTR* mutations. For example, Ivacafter (Kalydeco) is a potentiator that responds to 97 mutations in the *CFTR* gene and increases the likelihood of channel opening [17]. Additional combination medicines authorized by the FDA for use in CF patients include Orkambi (Ivacaftor-Lumacaftor), Symdeko (Tezacaftor + ivacaftor), and Trikefta (Elexacaftor + tezacaftor + ivacaftor). These alliance medications are effective against various types of *CFTR* mutations [17]. With a focus on the problem that CF patients may not benefit from Ivacaftor lumacaftor medication utilized against the ΔF508 mutation in the absence of accurate mutational screening. Therefore, it is strongly advised to conduct an extensive investigation to find the genetic mutation in the *CFTR* gene in the Pakistani population [16]. Without genetic study, modulation therapy such as Ivacaftor lumacaftor might not be a feasible choice. Additionally, it is important to conduct genetic research to find *CFTR* gene variants in the Pakistani population [18].

Therefore, this study aims to identify common or rare variants in exon-6 of *CFTR* gene. This study was divided into three sections. In the first section, Insilico analysis of exon-6 of *CFTR* gene was performed to understand structural and functional impact of mutations on protein. In the second, section tetra ARMS PCR for L218X mutation, previously reported in patients of South Asia was optimized. In the third section,

Sanger sequencing of exon-6 was performed to validate the results of tetra ARMS PCR.

Problem Statement:

Cystic fibrosis (CF) is a life-threatening genetic disorder primarily caused by mutations in the *CFTR* gene, leading to dysfunctional chloride channels. Although over 2,000 variants of the *CFTR* gene have been identified, not all mutations are well-characterized, particularly those in exon 6. Understanding the mutational spectrum in exon 6 is crucial for improving diagnostic accuracy and treatment strategies for CF patients. However, there is limited data on the prevalence and pathogenic potential of specific exon 6 variants among Pakistani patients. The lack of comprehensive *in-silico* and molecular studies on these mutations hampers the development of precise, population-specific therapeutic interventions. Therefore, this study aims to bridge the gap by performing an *in-silico* characterization and mutational analysis of exon 6 variants in *CFTR* among cystic fibrosis patients, with a focus on Pakistani population data.

Hypothesis:

Genetic variants in exon 6 of the *CFTR* gene, identified through *in-silico* analysis, exhibit significant pathogenic effects that contribute to the development of cystic fibrosis, with specific mutations showing variable impacts on the severity of the disease.

Aim and Objectives:

The objectives of this study are:

i. To identify the structural and functional impact of mutation in exon-6 of *CFTR* gene using bioinformatics pipeline.

ii. To optimize Tetra-Arms PCR for L218X mutation for cost-effective screening of this variant in large cohort.

iii. To perform Sanger sequencing analysis to validate results obtained from tetra ARMS PCR.

CHAPTER No # 2

LITERATURE REVIEW

2.1 History

During the middle Ages, children in Europe with cystic fibrosis were believed to be bound by a witch's curse and were certain to die young. A myth was that child whose forehead tasted salty after a kiss was thought to be cursed and would soon die. Before the autosomal recessive inheritance pattern of CF was assumed in the late 20th century, salty skin was considered a sign of a fatal disease with no known cause or cure [19]. High salt concentrations in CF indicate that defect the disability of exocrine epithelial cells to transfer electrolytes. In 1990, it was assumed that both secretory and absorptive functions are affected by a tissue-specific depletion in the different membranes' ability to the passage of chloride (Cl-) ions. According to Quinton net electrolyte absorption in the gut remains normal, while it decreases in airway epithelia and sweat ducts [20].

As medical knowledge advanced, the term 'saline skin' was replaced with 'cystic fibrosis (CF),' an inherited disorder cause by mutation in *CFTR* gene. The mutated gene produces *CFTR* protein that is unable to maintain ion balance across cell membranes. This review examines the existing literature on cystic fibrosis, including its diagnosis, the *CFTR* gene and protein, major mutation classes associated with the disease, therapeutic options, and *CFTR* gene genotyping techniques. Also address the gaps in adhoc diagnosis and management of CF in Pakistan, taking into account the current knowledge.

2.2 Cystic fibrosis

Cystic fibrosis (CF) is a rare, life-limiting hereditary disease. Patients with CF suffered from progressive lung damage due to frequent infections caused by Pseudomonas aeruginosa, Staphylococcus aureus and opportunistic fungi as well as inflammation. Inflammation leads to the development of bronchiectasis and other obstructive conditions. CF also causes gastrointestinal diseases [21] like distal intestinal obstruction syndrome, chronic pancreatitis due to blockage of ducts and meconium ileus which leads to formation of greasy stools in some patients at the birth time meconium ileus CF also impact male fertility [22, 23] effect on reproductive system cause infertility. Male reproductive system is ducts system due to CF these ducts are

blocked. The blockage of tubes does not transport sperms [20, 24, 25] . Impact of *CFTR* mutations on female pregnancy and male fertility [26].

CF symptoms vary from person to person. Some individuals with minor diseases can go undiagnosed until later in life. Wheezing, productive cough, recurrent pneumonia, atypical asthma, bronchial inflammation, chronic airway disease, hemoptysis (coughing up bloody mucus), and shortness of breath are common symptoms [27].

2.3 Diagnosis of CF

Different diagnostic techniques used to identify cystic fibrosis (CF) include nasal potential difference test and pancreatic enzyme immunoreactive trypsinogen levels evaluation (new-born screening) (Figure 1). The gold standard for CF confirmatory testing is the sweat chloride test (SCT). The two main components of SCT, or pilocarpine iontophoresis, are the detection of elevated chloride levels in sweat and *CFTR* gene mutation screening, which genetically validates the diagnosis of CF by identifying one or more CF-related mutations in the *CFTR* gene [10, 28-30]. Due to uncommon mutations that normal genetic testing is unable to identify because of incomplete understanding of *CFTR* genotyping in various ethnic communities, an individual may still have cystic fibrosis (CF) even if just one DNA mutation or no mutation is found.

2.4 Newborn screening:

Sample of blood is drawn from the baby's foot or vein in his arm. On a piece of filter paper, a tiny drop of this blood is applied, and it is allowed to air dry. Once the material has dried, it is submitted to lab for examination where immunoreactive trypsinogen (IRT) levels are checked.

2.5 Sweat chloride test

The sweat chloride test remains the gold standard for discriminating CF from other ailments, such as primary ciliary dyskinesia (PCD), chronic obstructive pulmonary disorders (COPD), and other similar disorders. This elevated chloride ion concentration is then measured using ionophoresis, a specialized process to determine the conductivity [10].

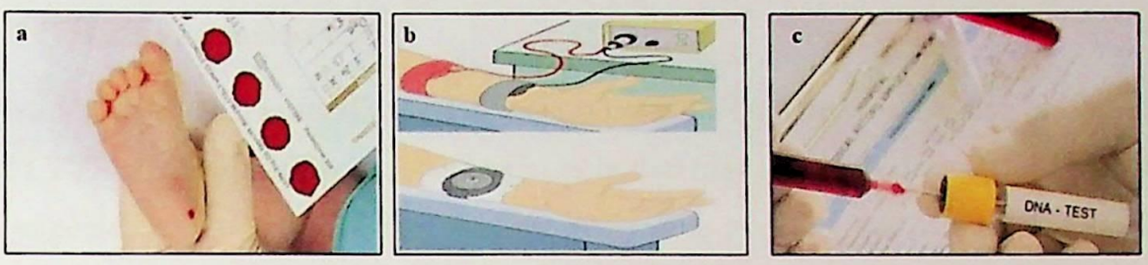


Figure 2.1: Screening method of CF: (a) Sampling for newborn screening, (b) Conductivity test for CF, also known as SCT, (c) Genetic testing for CF.

2.6 *CFTR* gene and its product

The location of the *CFTR* gene on chromosome 7 is 7q31.2 (Figure 3). Its 27 exons encode an associated protein of the ABC (ATP-binding cassette) transporter superfamily with 1480 amino acids [31, 32]. For example, chloride networks in exocrine glands, ABC transporters mainly control the passage of chloride ions via epithelium tissue to preserve liquid balance in numerous structures. NBDs (nucleotide-binding domains) bind ATP, phosphorylate regulatory domains (RDs), and cycle through these processes to activate channels. ATP binding and cAMP-dependent phosphorylation cause the channel to alter conformation, opening it and enabling chloride ions to pass from within the cell into the alveolar gaps [33]. Decreased chloride secretion affects several organ systems, including the liver, pancreas, lungs, colon, reproductive organs, and upper airways. It also causes an increase in sodium absorption across epithelial channels that carry sodium and the elimination of water from secretory fluids [5].

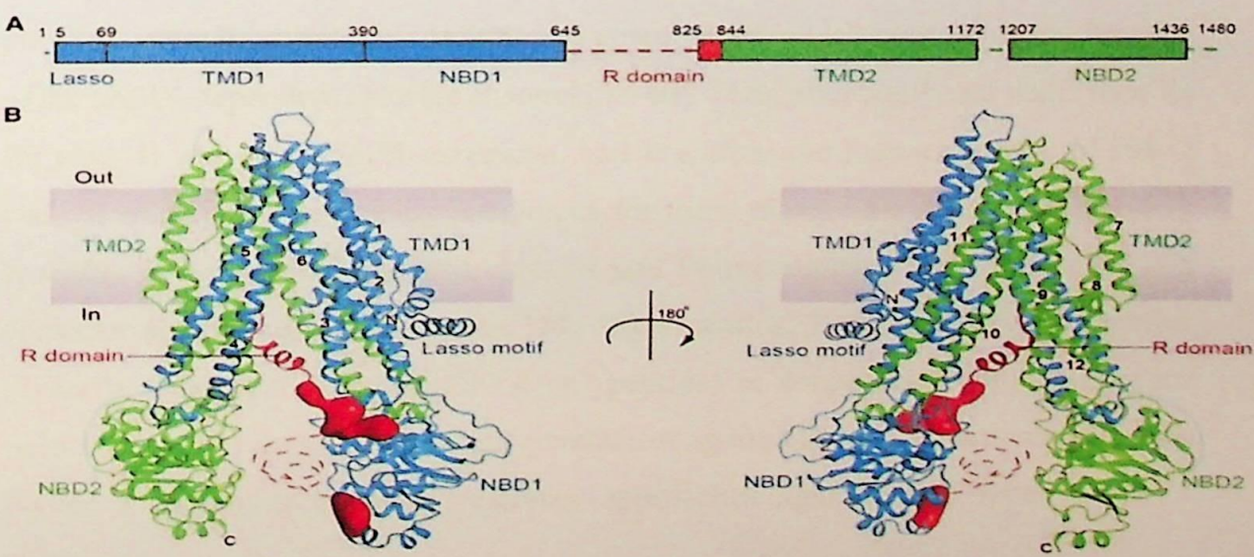


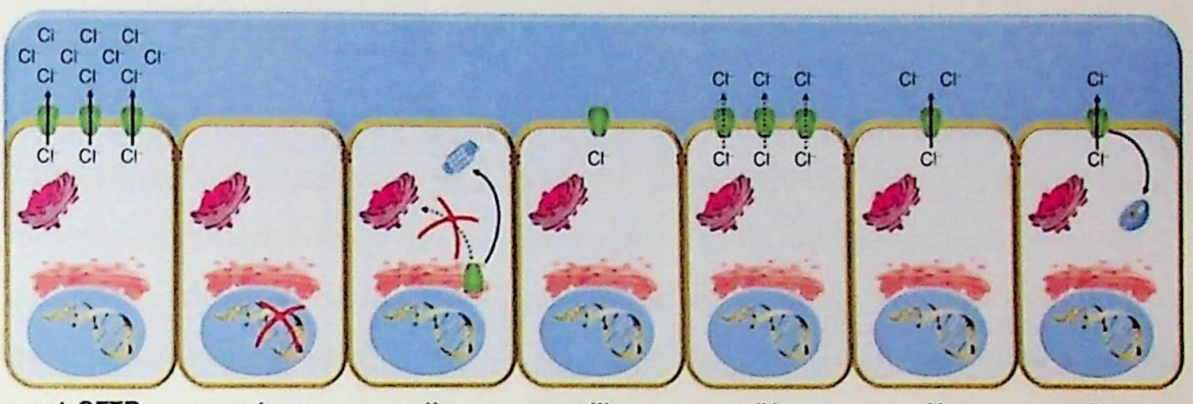
Figure 2.2: Structure of the human CFTR protein in the ATP-free conformation [34].

2.7 Genetic variants of CFTR

Cystic fibrosis (CF) is an inherited disease, a person with a mutation in one or both of the *CFTR* alleles will have an aberrant protein that may not fold correctly or function as expected, ultimately causing swelling (CF). The majority the mutations causing cystic fibrosis (CF), with the common ΔF508 mutation, cause the *CFTR* protein to fold incorrectly, averting it after achieving the situation optimal native three- dimensional shape. CF-related problems may also arise in carriers of CF (those with *CFTR* mutation(s) in only one allele)[35, 36].The *CFTR* gene may undergo exonic, intronic, *CFTR* changes, and mutations in non-coding areas, all of which can result in the development of CFTR variations. The six classes (class I through class VI) of *CFTR* variations are based on the effects of a mutation or mutations in the *CFTR* gene on the function of the *CFTR* protein. By way of the etiology of Cystic fibrosis in CFI varies from person to person, i.e., specific class of *CFTR* variation in CFI, different treatment techniques are being developed based on the *CFTR* variants, hence encouraging customized therapy for CFI. Research to detect various *CFTR* variations in the Pakistani inhabitants and build a indigenous group of *CFTR* alterations is crucial because truthful diagnosis and categorization of the *CFTR* abnormal class are necessary for optimal therapy and tailored medication [37].

2.8 Treatments for Variants of CFTR

Therapies for *CFTR* variations involve correctors, which try to stop the trafficking of the most prevalent mutation, ΔF508, and potentiators, which reestablish the function of the cAMP-dependent chloride channel [38-40]. Tezacaftor combined with Ivacaftor for class II and other *CFTR* variations, and Ivacaftor and Lumacaftor for ΔF508: a class II mutation is promising candidates for these therapeutic approaches [41-43]. Ivacaftor is a potentiator against class III and IV mutations, while Lumacaftor is a corrector against class II mutations [44, 45], Ivacaftor, Tezacaftor, and Elexacaftor (Trikafta: against the mutant *CFTR* polypeptide) in conjunction with Elexacaftor (which functions as a corrector and potentiator against class II mutations) [46, 47]. Figure 3 highlights possible treatment approaches against each type of *CFTR* mutations.



wt-CFTR (wt: wild-type)	I	II	III	IV	V	VI
Defect types	**No protein**	**No traffic**	**No function**	**Less function**	**Less protein**	**Less stable**
Mutation examples	p.Gly542* p.Arg553* p.Trp1282*	p.Gly85Glu p.Ile507del **p.Phe508del** p.Asn1303Lys	p.Val520Phe p.Ser549Arg p.Gly551Asp	p.Arg117His p.Arg334Trp p.Ser1235Arg	p.Arg455Glu c.1680-886A>G c.2657+5G>A	Residual p.Phe508del p.Gln1412*
Required approaches	Rescue protein synthesis	Correct protein folding	Restore channel conductance	Restore channel conductance	Maturation / Correct missplicing	Promote protein stability

Figure 2.3: Distribution of CFTR mutations

2.9 The Study Gap

First, the most prominent element in the inheritance of diseases with recessive inheritance is within-family marriages. Religious beliefs, cultural customs, family cast pride and family economic circumstances are some of the variables that may influence the decision to marry within the family [48]. Regarding Pakistan's socioeconomic standing, studies revealed that delay of the dowry couple's parents to force their daughters into consanguineous marriages. There is a substantial correlation between first-degree consanguinity and genetic diseases in Pakistan and infant fatalities [49, 50]. Given that consanguinity adds to an ARD and that there is a 25% probability of conceiving a kid with CF in Pakistan, consanguinity may have a disastrous effect on CF. As a result, appropriate genetic counseling before marriage the local population's ignorance of the *CFTR* gene's genotype. Secondly, in developing nations such as Pakistan, socioeconomic problems impede the proper diagnosis of CF and newborn screening for all patients. Physical manifestation of CF-related difficulties in the latter stages of a patient's life is mostly responsible for the diagnosis of CF illness. According to reports, lack of quick and efficient CF testing facilities in Pakistan causes CF illness to be identified later in life [51]. In addition, phenotypic manifestations of this illness might be mistaken for pancreatitis or pneumonia, which would seriously impair the illness's treatment. Though SCT is accessible in certain large cities, the institutions

may not be able to provide timely care to all patients, which might lead to an underdiagnosed of the condition in Pakistan.

Thirdly, vulnerable to variations in mutational patterns [52], in some populations, the diagnosis of CF is not consistent with the current global genetic panel. Modern mutation identification may rule out the diagnosis of CF if there are undiscovered CF-causing mutation(s) that traditional genetic testing is unable to identify because of incomplete understanding of the *CFTR* genotype spectrum in various populations. With improved knowledge of the *CFTR* genotypic and phenotypic spectrum of Pakistani people, it may be possible to do *CFTR* mutation screening in the country, which would aid in the appropriate management of cystic fibrosis.

In the end, Europeans suggested ability therapy for conventional *CFTR* mutation(s) in their population since the ad-hoc methods of CF therapies rely on information of the frequency of *CFTR* variation in cystic fibrosis individual. Unfortunately, there is a lack of research and adequate description of the facts regarding the widespread *CFTR* editions in Pakistan [16, 51, 53]. This information could help with both scenarios that is, a proper evaluation of cystic fibrosis using the regional facts of the universal *CFTR* versions and the enhancement of recent CF treatment strategies for Pakistani CF patients.

CHAPTER NO #3

MATERIALS AND METHODS

This study was divided into three stages *i.e., in silico* analysis of exon-6 of *CFTR* gene, optimization of tetra-ARMS assay for screening of relatively prevalent mutation of Exon-6, and the verification of Terta-ARMS result using Sanger sequencing.

3.1 *In-silico* Analysis

3.1.1 Data Retrieval

The sequence of exon-6 of the *CFTR* gene was retrieved using the NCBI web server. For the selection of non-synonymous single nucleotide polymorphism (nsSNP) cystic fibrosis mutation database (http://www.genet.sickkids.on.ca) was used. ClinVar and dbSNP tools from NCBI (https://www.ncbi.nlm.nih.gov) was used to retrieve clinical significance and rsID of variants. The amino acid sequence of the *CFTR* gene was retrieved from Uniprot (https://www.uniprot.org).

3.1.2 Filtering Harmful nsSNVs

Four bioinformatics tools were used to filter potentially harmful non-synonymous single nucleotide variants (nSNV).

SIFT (Sorting Intolerable from Tolerant) (https://sift.bii.a-star.edu.sg/) is a freely available online web server. It predicts pathogenicity of variants by performing multiple sequence alignment (MSA) and calculation conservation scores of each amino acid. It classifies variant as damaging if the probability score ≤ 0.05 otherwise the variant will be considered tolerated.

Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/) is another web-based server that can predict the impact of amino acid substitution rather than nucleotide changes. Similar to SIFT, it also performs multiple sequence alignment and predicts the evolutionary importance of amino acids. Its output is categorized as probably damaging (high confidence), possibly damaging (low confidence), and benign.

PANTHER (Protein Analysis through Evolutionary Relationships) (https://www.pantherdb.org/) another online web-based server that not only focuses

on conservation scores but also extracts data based on the functional annotation of protein.

Final filtering was performed using Meta-SNP (https://snps.biofold.org/meta-snp/). It uses a machine-learning model and combines the results of SIFT, PolyPhen-2, PANTHER, and PhD-SNP to predict the effects of substitution on protein. This step is used as the final line of screening to increase the accuracy and reliability of results obtained in previous steps.

3.1.3 Impact Of Mutation Of Protein Stability

The shortlisted nsSNVs were further analyzed to determine the effect of mutation on protein stability. iMutant (https://folding.biofold.org/i-mutant/i-mutant2.0.html) was used for this purpose. This tool predicts differences in free-energy of the normal with mutant amino acid.

3.1.4 Prediction Of The Effect Of Amino Acid Substitution

MutPred2 (http://mutpred.mutdb.org/), a web-based tool, was used to predict the functional consequences of mutations on catalytic domains, post-translational modifications, and DNA binding sites.

3.1.5 Structural Analysis of Native and Mutant

Protein databank (PDB) was used to obtain the experimental 3D structure of *CFTR* protein for the comparison of normal and mutant protein. There were two, 5UAK, and 6MSM, experimentally proven human *CFTR* structures present in PDB. Both structures were analysed keenly and 5UAK, as it was unmutated, and non-ATP bound, was selected for structural analysis. The *UCSF* chimera was used for visualization and analysis of the 3-D structure of *CFTR* protein. UCF chimera not only provides visualization, but also facilitates structural analysis, such as bond angles, bond length, and number of hydrogen bonds, clashes, contacts, and structural editing like mutagenesis.

3.2 Genetic Analysis

3.2.1 Sample collection

A total of fifteen patients, having elevated sweat chloride, were enrolled from The Children's Hospital & the Institute of Child Health, Lahore. Written consent was obtained from the parents or legal guardian of patients, and questionnaire was filled to obtain complete medical history as detailed in Annex-2. Blood samples were collected in EDTA via the Standard Operating Protocols (SOPs) detailed in Annex-4. The collected blood samples were then stored at -20°C in EDTA vials until further processing.

Table 3.1: Inclusion and exclusion criteria for sample collection

Inclusion criteria	**Exclusion criteria**
Individuals exhibiting one or more of the following clinical features: • Primary and secondary intestinal blockage syndrome; meconium ileus • The pancreas • The bronchiectasis • *Staphylococcus aureus / Pseudomonas aeruginosa* lung infection [54]	Patients who do not have any of the following symptoms: • Pancreatitis • Intestinal blockage syndrome • meconium ileus • bronchiectasis •*Staphylococcus aureus/Pseudomonas aeruginosa* lung infection.
Individuals with sweat chloride test results supported their CF being diagnosed.	Individuals with sweat chloride test results indicate a negative CF test.
Individuals that possess a heterozygous and negative Δf508 mutation.	Individuals carrying a homozygous Δf508 mutation.
Measurement of Sweat Cl- above 90 mmol/l	Less than 90 mmol/l of sweat Cl-

3.2.2 Genomic DNA Extraction

The genomic DNA was extracted from blood samples using the organic DNA extraction method. This involves RBC lysis using 20mMTris-HCl. The 0.2M sodium acetate was used for precipitation of DNA and maintained optimal pH. In WBC lysis, SDS was used as detergent and proteinase K for proteins digestion. The DNA was further purified using PCI to remove organic and inorganic impurities. After centrifugation, three layers such as aqueous, protein and organic, were observed. The DNA would be in aqueous layer that is why this layer was separated in new labeled eppendrof. DNA was further concentrated using absolute ethanol followed by washing with 70% ethanol. This step facilitates DNA purification and washing. DNA was allowed to dry by air and pellet was resuspended in 1XTE buffer. Appendix 5 has reagents recipes.

3.2.3 Assessment of DNA Quality and Quantity

The purified genomic DNA which had stored at low temperature in 1X TE buffer (pH 8), was examined using the electrophoretic separation using agarose gel. For gel electrophoresis, the 0.8% agarose was used see Appendix 2 for procedure.

3.3 Optimization of Tetra-ARMS PCR

3.3.1 Primer designing

Allele-specific oligonucleotide primers were designed for L218X by the Primer 1 tool (https://primer1.soton.ac.uk/primer1.html). The obtained primers were then validated using NCBI primer BLAST web tools. The outer primers were further refined to ensure the inclusion of entire exon and some flanking region. Final primers stood had visible size ranges.

3.3.2 Optimization of Tetra ARMS PCR

The 20 µl PCR reaction mixture was prepared for optimization. Gradient PCR, temperature ranging from 57-62°C, was performed to determine the optimized temperature of all primer pairs. The optimized primer concentration for better results was also determined by testing primer ratio (outer: inner) 1:1, 1:2, 1:3, and 1:4. The results were visualized using 2% agarose gel protocol given in Appendix 2. The optimized products were quantified using 100bp ladder.

3.4 Sanger sequencing

3.4.1 Amplification of Outer products

20ul reaction was planned to amplify the 441bp long outer product. These products were further purified using the ethanol precipitation method. See Appendix 5 for procedure.

3.4.2 Sequencing PCR

10ul sequencing reaction was prepared in 96 wells sequencing plate using forward primer and BigDye Terminator Ver. 3.1. The sequencing protocol is given in figure 4.

Figure 3.1: Thermocycling profile for sequencing reaction

Sanger sequencing was performed on purified PCR procedure. The optimized PCR reaction is given in Table 3.2.

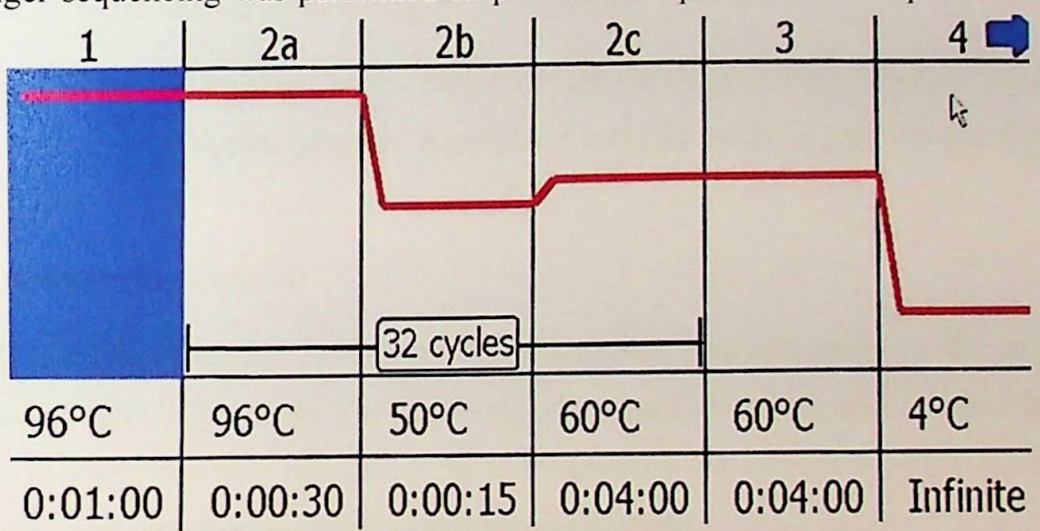


Table 3.2 : Optimized reaction for Sequencing PCR (10ul)

Concentration of the Reagent	Quantity
Product of PCR (441nucleotide)	7-8 ng
Prepared Reaction Mixture (2.5X)	0.7 µl
E6-F primer (5µM)	0.5 µl
Sequencing Buffer (5X)	1.65 µl
Nuclease free water	Adjustable

3.4.3 Post-Sequencing PCR clean-up

PCR products were purified to remove unused primers, ddNTPs, dNTPs etc. For purification, 3M sodium acetate (pH 5.2) was mixed with 125mM EDTA and this mixture was then mixed with 96-wells sequencing plate. This plate was incubated at 4°C for 20 minutes after adding absolute ethanol. After incubation, the plate was centrifuged at 3200 rpm at 4°C for 20 minutes. Ethanol was discarded by placing the plate upside down after brief spinning. Then, 70% ethanol was added and centrifuged again. After discarding ethanol, the plate was air-dried. The pellet was re-suspended in 13ul formamide. The plate was then placed in thermocycler for heat shock at 95°C and placed in ABI genetic v.3.1 analyzer for capillary electrophoresis.

3.4.4 Purification of Sequencing PCR Products

Sequencing PCR products of the exon 06 of the *CFTR* gene were then purified using the ethanol-EDTA precipitation. Appendix 4 contains the PCR products purification protocol.

3.5 Sequencing analysis

ABI Genetic Analyser 3130xl performed capillary electrophoresis on the purified sequencing PCR products. The purified product was dissolved in 13µl formamide and heat-shocked for five minutes at 95°C to denature the sequencing PCR products. Sequencing data was analyzed using chromas software.

CHAPTER No # 4

RESULTS

4.1 *In-silico* Analysis

4.1.1 Retrieval of Protein sequence

The FASTA sequence of the 1480 amino acid *CFTR* protein, which was retrieved from UniProt, is given below. Amino acid encoded by exon-6 is bolded.

>sp|P13569|CFTR_HUMAN Cystic fibrosis transmembrane conductance regulator OS=Homo sapiens OX=9606 GN=CFTR PE=1 SV=3

MQRSPLEKASVVSKLFFSWTRPILRKGYRQRLELSDIYQIPSVDSADNLSEKL
EREWDRELASKKNPKLINALRRCFFWRFMFYGIFLYLGEVTKAVQPLLLGRII
ASYDPDNKEERSIAIYLGIGLCLLFIVRTLLLHPAIFGLHHIGMQMRIAMFSLI
YKKTLKLSSRVLDKISIGQLVSLLSNNLNKFDE**GLALAHFVWIAPLQVALL**
MGLIWELLQASAFCGLGFLIVLALFQAGLGRMMMKYRDQRAGKISERL
VITSEMIENIQSVKAYCWEEAMEKMIENLRQTELKLTRKAAYVRYFNSSAFF
FSGFFVVFLSVLPYALIKGIILRKIFTTISFCIVLRMAVTRQFPWAVQTWYDSL
GAINKIQDFLQKQEYKTLEYNLTTTEVVMENVTAFWEEGFGELFEKAKQNN
NNRKTSNGDDSLFFSNFSLLGTPVLKDINFKIERGQLLAVAGSTGAGKTSLL
MVIMGELEPSEGKIKHSGRISFCSQFSWIMPGTIKENIIFGVSYDEYRYRSVIK
ACQLEEDISKFAEKDNIVLGEGGITLSGGQRARISLARAVYKDADLYLLDSPF
GYLDVLTEKEIFESCVCKLMANKTRILVTSKMEHLKKADKILILHEGSSYFY
GTFSELQNLQPDFSSKLMGCDSFDQFSAERRNSILTETLHRFSLEGDAPVSWT
ETKKQSFKQTGEFGEKRKNSILNPINSIRKFSIVQKTPLQMNGIEEDSDEPLER
RLSLVPDSEQGEAILPRISVISTGPTLQARRRQSVLNLMTHSVNQGQNIHRKT
TASTRKVSLAPQANLTELDIYSRRLSQETGLEISEEINEEDLKECFFDDMESIP
AVTTWNTYLRYITVHKSLIFVLIWCLVIFLAEVAASLVVLWLLGNTPLQDKG
NSTHSRNNSYAVIITSTSSYYVFYIYVGVADTLLAMGFFRGLPLVHTLITVSKI
LHHKMLHSVLQAPMSTLNTLKAGGILNRFSKDIAILDDLLPLTIFDFIQLLLIVI
GAIAVVAVLQPYIFVATVPVIVAFIMLRAYFLQTSQQLKQLESEGRSPIFTHL
VTSLKGLWTLRAFGRQPYFETLFHKALNLHTANWFLYLSTLRWFQMRIEMI
FVIFFIAVTFISILTTGEGEGRVGIILTLAMNIMSTLQWAVNSSIDVDSLMRSVS
RVFKFIDMPTEGKPTKSTKPYKNGQLSKVMIIENSHVKKDDIWPSGGQMTV

KDLTAKYTEGGNAILENISFSISPGQRVGLLGRTGSGKSTLLSAFLRLLNTEGE
IQIDGVSWDSITLQQWRKAFGVIPQKVFIFSGTFRKNLDPYEQWSDQEIWKV
ADEVGLRSVIEQFPGKLDFVLVDGGCVLSHGHKQLMCLARSVLSKAKILLL
DEPSAHLDPVTYQIIRRTLKQAFADCTVILCEHRIEAMLECQQFLVIEENKVR
QYDSIQKLLNERSLFRQAISPSDRVKLFPHRNSSKCKSKPQIAALKEETEEEV
QDTRL

Table 4.1: Nucleotide sequence and encoded sequence of *CFTR* gene' exon 06 of Homo sapiens

Characteristic	Exon-6
Nucleotide Sequence	GGACTTGCATTGGCACATTTCGTGTGGATCGCTCCTTTGCAA GTGGCACTCCTCATGGGGCTAATCTGGGAGTTGTTACAGGCG TCTGCCTTCTGTGGACTTGGTTTCCTGATAGTCCTTGCCCTTT TTCAGGCTGGGCTAGGGAGAATG ATGATGAAGTACAG
Amino Acid Sequence	GLALAHFVWIAPLQVALLMGLIWELLQASAFCGLGFLIVLALFQA GLGRMM MKY

Total twenty-six nsSNV were present in Cystic fibrosis patient's database; these all are enlisted in table 4.1.

Table 4.2: List of mutations obtained from Cystic fibrosis Patients database

Sr.#	Nucleotide Change	Mutation	rsID
1	G to A at 712	G194R	rs376008630
2	G to T at 713	G194V	rs397508763
3	G to A at 724	A198T	rs193922529
4	G to C at 724	A198P	rs193922529
5	Gly to Val at 194	G194V	rs397508763
6	A to G at 728	H199R	rs397508764
7	T to G at 729	H199Q	rs397508765
8	T to A at 730	F200I	rs397508766
9	G to A at 733	V201M	rs138338446
10	C to G at 741	I203M	rs1800081
11	C to T at 745	P205S	rs121908803
12	C to G at 746	P205R	rs397508769
13	T to G at 749	L206W	rs121908752
14	G to T at 750	L206F	rs397508770
15	G to T at 757	A209S	rs397508772
16	G to T at 780	W216C	rs397508776
17	A to G at 782	E217G	rs121909046
18	T to C at 805	C225R	rs397508780
19	T to G at 812	L227R	rs397508782
20	T to A at 827	V232D	rs397508783
21	829C>T	L233F	rs775713428
22	C to G at 841	Q237E	rs397508784
23	843G>C	Q237H	rs397508785
24	G to A at 847	G239R	rs397508786
25	G to A at 853	G241R	rs397508789
26	T to A at 863	M244K	rs397508790

4.1.2 Identification of Deleterious nsSNVs

The predicted results of SIFT, Polyphen-2, and Meta-SNP use for prediction of deleterious effects of twenty-six missense mutation in exon 6 are provided in Table 4.3.

Table 4.3: Results of *In Silico* analysis of *CFTR* exon-6

rsID	Mutation	SIFT		Polyphen-2		Meta-SNP	
		Score	Prediction	Score	Prediction	Score	Prediction
rs376008630	G194R	0.002	Del	0.1	Del	0.776	Dis
rs397508763	G194V	0.003	Del	0.37	Prob. Del	0.470	Neu
rs193922529	A198T	0	Del	0.2	Prob. Del	0.489	Neu
rs193922529	A198P	0	Del	0.06	Del	0.817	Dis
rs397508764	H199R	0	Del	0	Del	0.825	Dis
rs397508765	H199Q	0	Del	0	Del	0.629	Dis
rs397508766	F200I	0	Del	0.05	Prob. Del	0.558	Dis
rs138338446	V201M	0.001	Del	0	Del	0.497	Dis
rs1800081	I203M	0	Del	0.01	Del	0.464	Neu
rs121908803	P205S	0	Del	0	Del	0.580	Dis
rs397508769	P205R	0	Del	0	Del	0.833	Dis
rs121908752	L206W	0	Del	0	Del	0.812	Dis
rs397508770	L206F	0.002	Del	0	Del	0.486	Neu
rs397508772	A209S	0.008	Del	0	Del	0.455	Neu
rs397508776	W216C	0	Del	0	Del	0.788	Dis
rs121909046	E217G	0.006	Del	0.3	Prob. Del	0.464	Neu

rs397508780	C225R	0.001	Del	0	Del	0.823	Dis
rs397508782	L227R	0	Del	0	Del	0.824	Dis
rs397508783	V232D	0.001	Del	0	Del	0.805	Dis
rs775713428	L233F	0.1	Del	0.1	Prob. Del	0.475	Neu
rs397508784	Q237E	0	Del	0.1	Prob. Del	0.769	Dis
rs397508785	Q237H	0	Del	0	Del	0.002	Dis
rs397508786	G239R	0	Del	0.4	Del	0.563	Dis
rs397508789	G241R	0.002	Del	0	Del	0.788	Dis
Del – Deleterious, dis– Disease, .ProbDel. – Probably Damaging, Neu - Neutral							

4.1.3 Impact of mutation of protein stability

The seventeen nsSNVs that were found deleterious by all the tools were selected to study impact of mutation on stability of protein. Results are indicated in table 4.4.

4.1.4 Prediction of effect of Amino Acid Substitution on protein

Mutpred2 was applied to predict the effect of sixteen shortlisted mutations in both the structure and function of the protein. With iMutant and Mutpred2 score, P (P-Value), and molecular shift, the Mutpred2 result is displayed in Table 4.4.

Table 4.4: Predicted effects of amino acid substitution on protein function using Mutepred2

rsID	**iMutant analysis**	**Mutatio n**	**MutPre d2 score**	**P-value**	**Molecular Variation**
rs376008630	Stability decrease	G194R	0.720	1.2e-04	Altered Transmembrane protein
rs193922529	Stability decrease	A198P	0.885	0.03 4.1e-03	Altered Ordered interface Altered Transmembrane protein
rs397508765	Stability decrease	H199Q	0.821	4.2e-03	Altered Transmembrane protein
rs397508764	Stability decrease	H199R	0.882	4.8e-03	Altered Transmembrane protein

rs397508766	Stability decrease	F200I	0.844	4.4e-03	Altered Transmembrane protein
rs397508769	Stability decrease	P205R	0.909	4.2e-03	Gain of Helix
rs121908803	Stability decrease	P205S	0.842	0.02	Gain of Helix
rs121908752	Stability decrease	L206W	0.860	0.02	Altered Transmembrane protein
rs397508776	Stability decrease	W216C	0.907	3.9e-03	Altered Transmembrane protein
rs397508780	Stability decrease	C225R	0.789	0.02	Altered Transmembrane protein
rs397508782	Stability decrease	L227R	0.922	0.03	Altered Transmembrane protein
rs397508783	Stability decrease	V232D	0.758	7.0e-04	Altered Transmembrane protein
rs397508785	Stability decrease	Q237H	0.755	0.04	Altered Transmembrane protein
rs397508789	Stability decrease	G241R	0.524	3.8e-04 0.01 0.01	Altered Transmembrane protein Altered Disordered interface Loss of Acetylation at K246
rs397508790	Stability decrease	M244K	0.582	4.9e-03 1.1e-03 0.02 0.04	Loss of Acetylation at K246 Altered Transmembrane protein Altered Disordered interface Altered Stability
rs397508784	Stability decrease	Q237E	0.719	0.03	Altered Transmembrane protein

4.1.5 Protein Structure Analysis: Native and Mutant

The 5UAK, structure was visualized on UCF chimera. Figure 4.1 indicates experimentally proven structure of human *CFTR* protein.

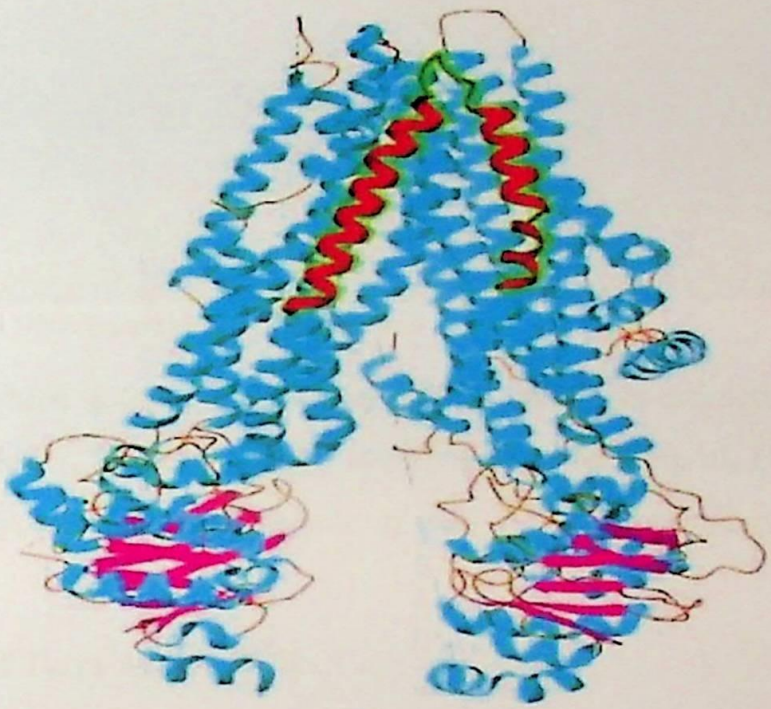


Figure 4.1: The three-dimensional (3D) structure of *CFTR* protein:

CFTR protein consists of helices (cyan), β-sheets (magenta), and coils and loops (orange). The exon-6 of *CFTR* gene encodes part of transmembrane domain (red). A comparison of mutant vs. normal proteins is represented in Anex-6.

4.2 Genetic Analysis

4.2.1 Qualitative and Quantitative Analysis of Extracted Genomic DNA

Genomic DNA was extracted from the blood cells of all patient blood samples by processing. Using an agarose gel and UV light, the isolated DNA was then measured and examined. The effective extraction of DNA was demonstrated by the bright bands on the gel, as seen in Figure 6.

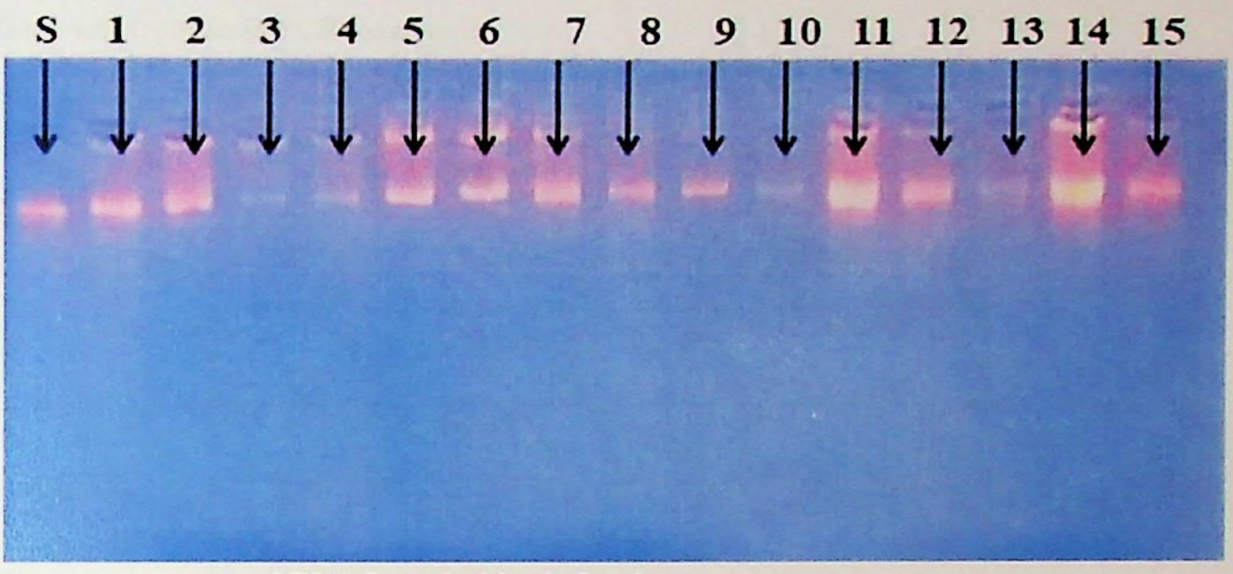


Figure 4.2: Extracted DNA from the blood of patients:

Here S=standard which is 25ng/ul. DNA in well 14 and 11 is estimated to be 35-40ng/ul, DNA in well 1, 2,5,6,7,9 and 15 is estimated to be 25ng/ul, DNA in well 4,10,13 are estimated to be 13ng/ul, while in well 3 DNA was approximately 7-10ng/ul.

4.3 Optimization of Tetra ARMS PCR

4.3.1 Tetra ARMS Primers design.

Primers given by primer 1 and primer 3 tools are given in Table 4.5.

Table 4.5: Primer sequences selected for optimization of tetra ARMS PCR.

Primers	Sequence	Oligomer length	Product length
E6-F	GGGAAAACCGATTCTATGTGTAGAA	25	441
E6-R	TCCTGGTTTTACTAAAGTGGGC	22	
N6-R	AAGGCAGACGCCTGTATCA	19	295
Mu6-F	CATGGGGCTAATCTGGGACTA	21	185

4.3.2 Optimization of Tetra ARMS PCR

Best results were obtained with a primer concentration of 1:3 at Tm 60°C. The protocol of optimized PCR reaction is represented in table 4.6.

Table 4.6: Optimized PCR reaction mixture

Components	Volume Per Reaction (µl)
Nuclease-free H_2O	9.4
PCR buffer	2
$MgCl_2$	1.8
dNTPs	1.5
Primer (OR=0.5) (5 µM) Primer (OF=0.5) (5 µM)	3
Primer (InnF=1.5 µM) Primer (innR=1.5 µM)	
Taq DNA polymerase	0.8
DNA (25 ng/µl)	1.5
Total volume	**20**

The optimized cycling conditions for tetra ARMS PCR is given in Table 4.7.

Table 4.7: optimized tetra ARMS amplification profile

Step	Temperature	Time	Cycles
Hot Start	95 °C	5 min.	1
Denaturing	95 °C	30 s	**30**
Annealing	60°C,	45 s	
Elongation	72 °C	90 s	
Final elongation	72 °C	10 mints	1

The results were visualized using 2% agarose gel protocol given in Appendix2.

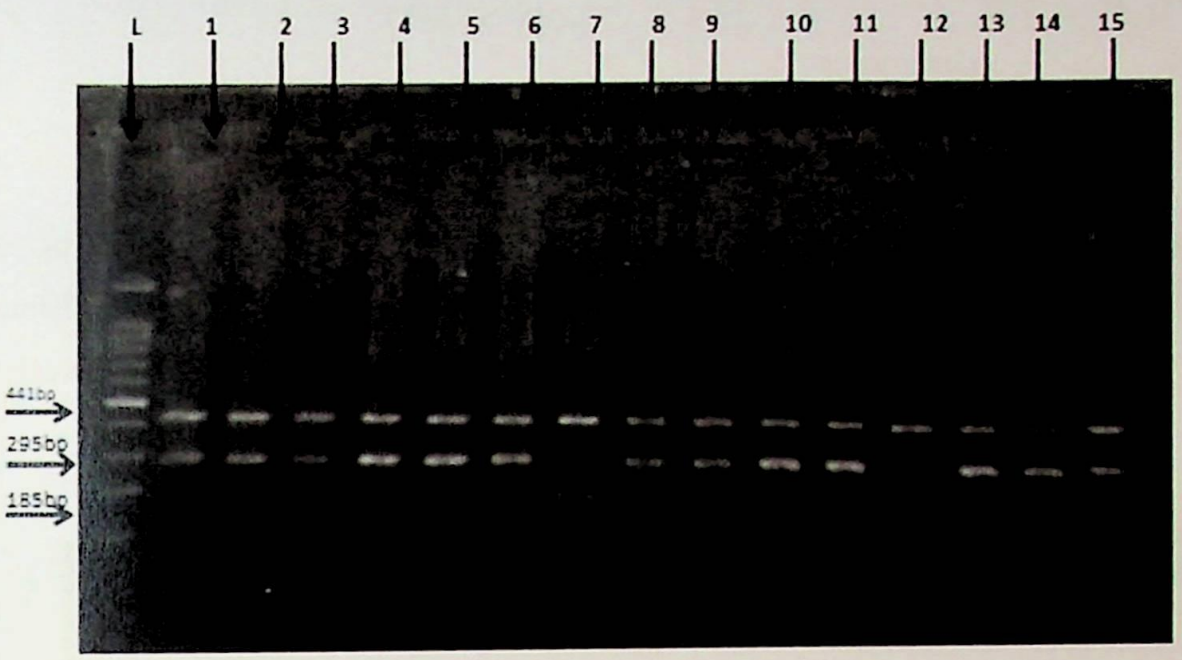


Figure 7: Results of Tetra-ARMS PCR:

A 100 bp DNA ladder was used for product size analysis. Sample numbers 7 and 12 were homozygous for L218X mutation (band at 185bp) and other patients were homozygous for normal allele (band at 295bp).

4.4 Sanger sequencing

4.4.1 Amplification of exon-6

The optimized PCR reaction of Exon-6 amplification is indicated in table 4.8.

Table 4.8: PCR reaction mixture optimised using a manual procedure.

Components	Volume Per Reaction (µl)
Nuclease-free H_2O	Variable depending upon DNA
PCR buffer	2
$MgCl_2$	1.5
dNTPs	1.5
Primer (OF=1) (5 µM)	1
Primer (OR=1) (5 µM)	1
Taq DNA polymerase	0.4

DNA (25 ng/µl)	Variable according to concentration
Total volume	**20**

The amplification of the *CFTR* gene's exon 06 on an agarose gel was analysed and quantified by visualizing and measuring the amplified PCR products under UV light, as shown in figure 8.

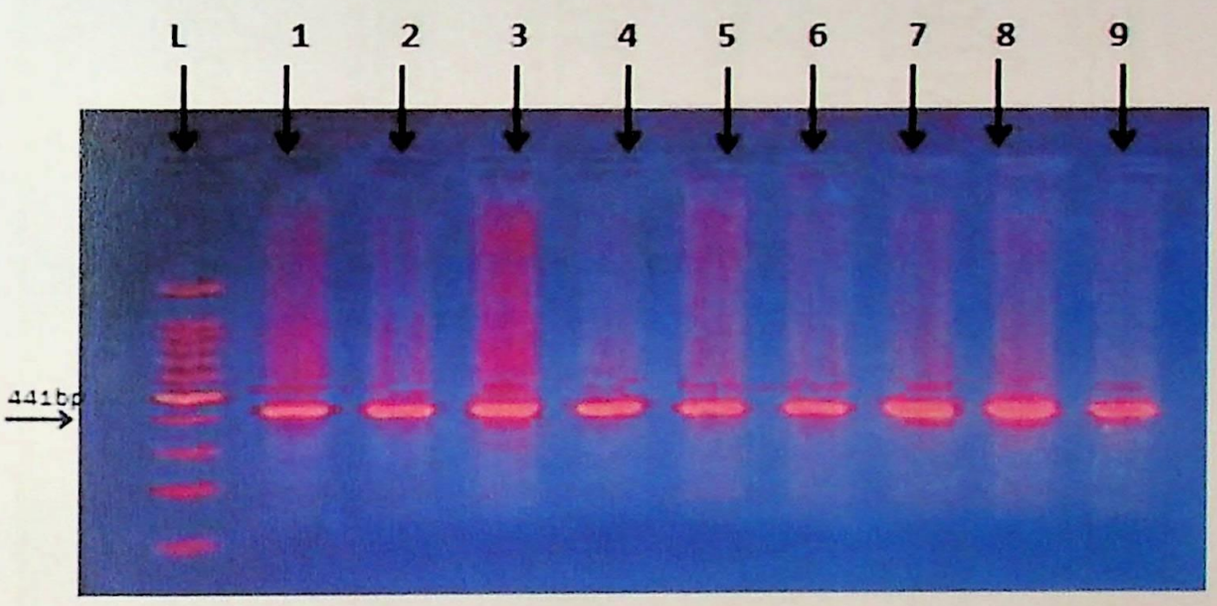


Figure 8: Amplified outer products of exon-6 of *CFTR* gene:

Exon 06 has a length of 441 base pairs. The amplified PCR products are aligned to a 100b DNA ladder in Row 1.

4.4.2 Sequencing analysis

ABI Genetic Analyser 3130xl performed capillary electrophoresis on the purified sequencing PCR products. The purified product was dissolved in 13µl formamide and heat-shocked for five minutes at 95°C to denature the sequencing PCR products [55]. Sequencing data was analyzed using chromas software. The illustrative sequencing electropherograms of one sample is given in Figures 9 & 10.

Figure 9: Electropherograms of Sanger sequencing of *CFTR* gene of exon 6 from a CF patient

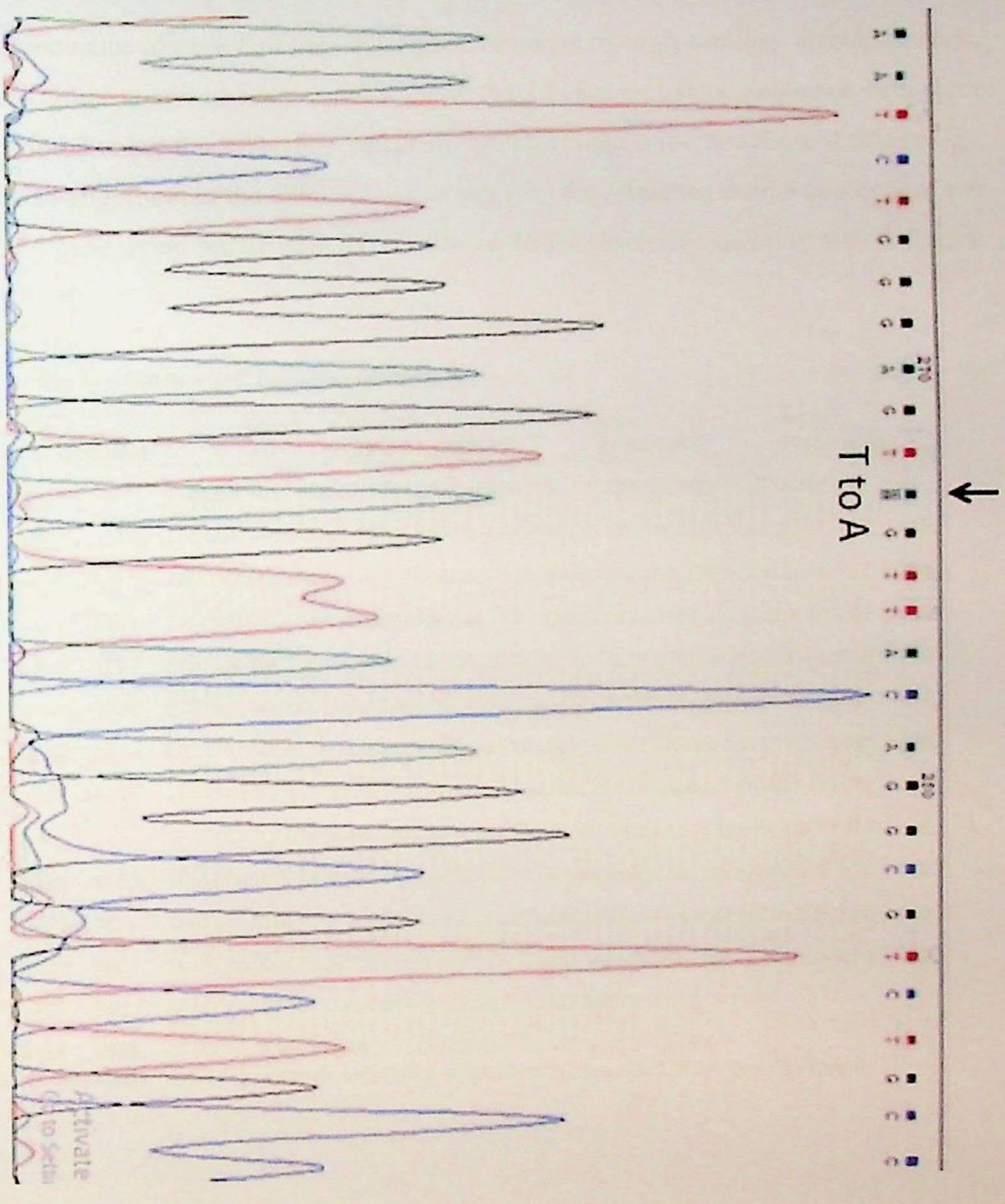


Figure 10: Electropherograms of *CFTR* gene exon 6 L218X in CF patient

4.4.3 Mutation Analysis of Sequenced Exon 6

The sequence of exon 6 of the *CFTR* gene, obtained through capillary electrophoresis, was further analyzed for the presence of the L218X mutation. Sequence data were examined using the 'Chromas' software, which revealed the mutation. Additionally, manual alignment of the obtained sequences with the reference exon 6 sequence of the *CFTR* gene using NCBI BLAST confirmed 100% similarity, as illustrated in Figure 11.

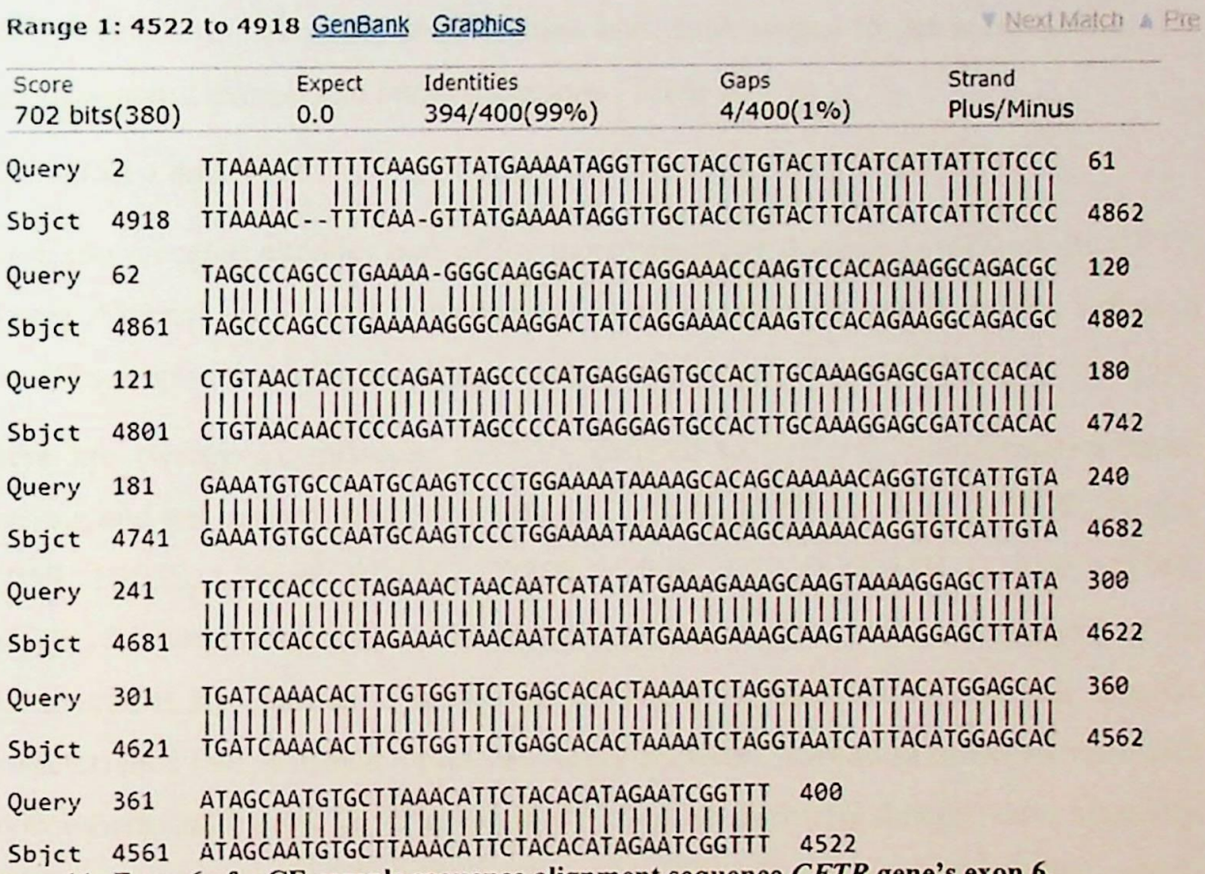


Figure 11: Exon 6 of a CF-sample sequence alignment sequence *CFTR* gene's exon 6.

CHAPTER No # 5

DISCUSSION

Cystic fibrosis (CF) is a genetic disorder inherited in an autosomal recessive manner (ARD) affecting multiple systems of human body. Its severity depends upon the class and type of mutation in *CFTR* gene. Limited research data on *CFTR* gene variants in Pakistani CF patients is available (Table 11). Therefore, the primary focus of current genetic research on local CF patients is to identify presence of L218X mutation in local population. This study was divided into three stages to get a comprehensive overview about exon-6 and lethal mutations (Table *5*).

5.1 *In-Silico* Analysis

The Exon 6 region encodes part of the transmembrane domain (TMD) of the CFTR protein. Normal amino acid sequence in Exon 6 is relatively stable, with a balanced mix of hydrophobic and hydrophilic residues, and a stable alpha-helical structure [56].

There are twenty-six missense nsSNVs enlisted to perform bioinformatics-based analysis and get sixteen mutations such as G194R, A198P, H199Q, H199R, F200I, P205R, P205S, L206W, W216C, C225R, L227R, V232D, Q237H, G241R, M244K and Q237E were determined to be harmful (Table 5). The structural analysis of all these variants by chimera is present in appendix 6. Each amino acid has specific characteristics that facilitate either secondary structure formation or interaction with environment and ligand. Glycine is a highly flexible molecule that provides elasticity to the molecule. Substitution of arginine at position 194 disrupts flexibility, which ultimately results in alteration of helical structure. Proline a rigid ring alters the structure and loses the flexibility. On the other hand, histidine is a charged aromatic basic amino acid. It supports gating activity of ions by maintaining pH substitution of glutamine or arginine might disrupt the gating activity of CFTR gene.

Leucine is non-polar, while tryptophan is aromatic. This change can alter protein hydrophobicity. Leucine has smaller aliphatic side chain while tryptophan has a larger aromatic ring that affects protein stability. Size differences also affect protein-

protein interaction. Tryptophan has aromatic ring that is replaced by thiol group altering the protein hydrophobicity. Tryptophan has a larger aromatic ring while cysteine has smaller spherical thiol. This changes the effect of protein packing and also participates in redox reaction introducing new properties. Cysteine has smaller spherical thiol group replaced by arginine positively charged guanidinium group altering the protein electrostatic substitution and loss redox property. Leucine has nonpolar side chain replaced by positive guanidinium group altering the protein electrostatic interactions. Leucine has a smaller positive charge while Arginine has larger positively charged group. This change affects protein packing and stability and also alters the bounding properties. Valine non-polar side chain is replaced by Aspartic acid's negatively charged carboxyl group, altering protein electrostatics and interactions. Valine has a smaller, aliphatic side chain, while Aspartic acid has a larger, negatively charged group. This change can affect protein conformation and packing. Change in charge and size can alter binding properties, potentially affecting protein-ligand or protein-protein interactions. Methionine non-polar side chain is replaced by Lysine positively charged amino group, altering protein electrostatics interactions. Methionine has a medium-sized, flexible side chain, while Lysine has a larger, positively charged group. The change in charge and size can alter binding properties, potentially affecting protein-ligand or protein-protein interactions.

5.2 Optimization of tetra ARMS PCR

Local residents with cystic fibrosis (CF) have been selected for tetra arm PCR. Tetra-ARMS PCR was used to detect the L218X mutation in exon 6 of the CFTR gene [57]. Tetra-ARMS PCR is a reliable, allele-specific method for detecting SNPs and mutations, distinguishing between mutant and wild-type alleles in a single reaction using four primers. After *in silico* analysis of exon 6 of the CFTR gene, specific primers were designed to target the L218X mutation, a stop-gain mutation that results in a truncated CFTR protein (Table 7). The primer design focused on high specificity for the mutant allele while avoiding non-specific amplification. Two primers were designed for the mutant allele and two for the wild-type allele. These primers were optimized for annealing temperature and for efficient amplification. The Tetra-ARMS PCR successfully identified the L218X mutation in two patients out of fifteen samples (Figures 9 & 10). Tetra ARMS PCR clearly distinguished the

mutant allele from the wild-type allele by the size of the PCR products (Figure7). The mutant allele produced a distinct band at the expected size, confirming the mutation, while the wild-type allele showed a different band pattern.

Tetra-ARMS PCR has several advantages for mutation detection in this study, being rapid, cost-effective, and eliminating the need for post-PCR sequencing [58]. It also allowed the simultaneous detection of mutant and wild-type alleles in a single reaction, making it efficient for diagnostics. However, some limitations were noted, such as potential cross-reactivity between primers and primer-dimer formation, though these issues were minimized through careful primer design. The technique proved highly effective for detecting specific mutations in a clinical setting.

5.3 Sanger sequencing for Validation of Tetra-ARMS PCR Results

Sanger sequencing was used to validate the results from Tetra-ARMS PCR for detecting the L218X mutation in exon 6 of the *CFTR* gene. As the gold standard for DNA sequencing, Sanger sequencing provides high accuracy in confirming specific mutations, making it crucial for verifying results from methods like Tetra-ARMS PCR. While Tetra-ARMS PCR is a fast and cost-effective method for detecting known mutations like L218X, it carries a risk of false positives or nonspecific amplification due to primer issues [59]. To ensure accuracy and eliminate false results, Sanger sequencing was used as a validation tool [57], providing precise identification of nucleotide changes by directly sequencing the amplified region of exon 6. The results from Sanger sequencing confirmed the L218X mutation in patient samples that tested positive with Tetra-ARMS PCR (Figures 9 & 10). The sequencing provided precise nucleotide confirmation of the mutation at the 218th codon, validating the Tetra-ARMS PCR results, and also confirmed the wild-type allele sequence, further supporting the findings. These findings were in consistency with [60].

The combination of Tetra-ARMS PCR and Sanger sequencing was a strong method for mutation analysis in this study. Tetra-ARMS PCR allowed for rapid screening of multiple patient samples, while Sanger sequencing offered precise confirmation of mutations at the nucleotide level. This two-step approach improved the reliability of genetic analysis; especially in clinical settings where accuracy is essential (figure 11 NCBI BLAST alignments).

5.4 Descriptive analysis:

The prevalence of consanguinity among parents of CF young patients was 75%, according to our descriptive study of the enrolled individuals in the current investigation. This was in harmony with the findings of [18]. Pakistan has a high proportion of first-degree consanguinity [61, 62], which is strongly linked to an increased risk of genetic diseases and newborn mortality. Therefore, it is suggested that genetic illnesses such as cystic fibrosis are probably caused due to this widespread consanguinity among Pakistani population. Genetic counseling may assist in lowering the prevalence of genetic illnesses [13, 51, 63].

Though, cousin marriages are prevalent in Pakistan, but mutations in exon 06 of *CFTR* gene among Pakistani population are poorly investigated. In the current study, 13 out of fifteen patients did not have L218X mutations in exon 06 of the *CFTR* gene. Therefore, it is pertinent to acknowledge the limited sample size of young patients as it is a rare disease and targeting a single mutation would make it difficult to identify mutations in exon 06 of the *CFTR* gene in nearby CF patients [63].

CONCLUSION

This study improves our understanding of *CFTR* gene mutation in young Pakistani patients. Children hospital Lahore has the only public facility for CF diagnosis in all over Pakistan. While the range of *CFTR* mutations in Pakistani population is still unknown, our analysis of Exon 6 has revealed the presence of L218X mutation in 2 out of 15 young patients (13%). The identification of the L218X mutation in current study reveals its potential significance within the Pakistani CF population. Our findings highlight the importance of genetic screening for personalized treatment and disease management in CF patients. Future research should expand the study to include a larger cohort which may provide a more comprehensive understanding of *CFTR* gene mutations among Pakistani population.

REFERENCES

1. Jia, S. and J.L. Taylor-Cousar, *Cystic fibrosis modulator therapies.* Annual review of medicine, 2023. **74**(1): p. 413-426.
2. Young, T.J., D.A. Li, and P.H. Eshaghian, *Cystic Fibrosis: an Update on Disease Pathophysiology, Management, and Novel Modalities of Therapy.* Current Treatment Options in Allergy, 2019. **6**: p. 226-237.
3. Antony, S., et al., *Incidence, prevalence, clinical features, and outcomes of COVID-19 in persons with cystic fibrosis: a systematic review protocol.* JBI evidence synthesis, 2022. **20**(11): p. 2721-2726.
4. Beswick, D.M. and R.J. Schlosser, *Chronic rhinosinusitis in people with Cystic Fibrosis: Expanding evidence and future directions*. 2022. p. 737-738.
5. Naehrig, S., C.-M. Chao, and L. Naehrlich, *Cystic fibrosis: diagnosis and treatment.* Deutsches Ärzteblatt International, 2017. **114**(33-34): p. 564.
6. Bianchimani, C., et al., *Impact of pancreatitis-associated protein on newborn screening outcomes and detection of CFTR-related metabolic syndrome (CRMS)/cystic fibrosis screen positive, inconclusive diagnosis (CFSPID): a monocentric prospective pilot experience.* International Journal of Neonatal Screening, 2022. **8**(3): p. 46.
7. Fajac, I. and I. Sermet-Gaudelus, *Emerging medicines to improve the basic defect in cystic fibrosis.* Expert Opinion on Emerging Drugs, 2022. **27**(3): p. 229-239.
8. Safdar, A., et al., *P027 Mutational analysis of CFTR gene in Pakistani cystic fibrosis patients.* Journal of Cystic Fibrosis, 2023. **22**: p. S71-S72.
9. Taussig, L.M., et al., *Fertility in males with cystic fibrosis.* New England Journal of Medicine, 1972. **287**(12): p. 586-589.
10. Massie, J., et al., *Sweat testing following newborn screening for cystic fibrosis.* Pediatric pulmonology, 2000. **29**(6): p. 452-456.
11. Goodchild, M., et al., *Cystic fibrosis in 3 Pakistani children.* Archives of Disease in Childhood, 1974. **49**(9): p. 739.
12. Bhutta, Z.A., T. Moattar, and U. Shah, *Genetic analysis of cystic fibrosis in Pakistan: a preliminary report.* Journal of Pakistan Medical Association, 2000. **50**(7): p. 217.
13. Shah, U., T. Moatter, and Z. Bhutta, *Profile and factors determining outcome in a cohort of cystic fibrosis patients seen at the Aga Khan University Hospital, Karachi, Pakistan.* Journal of tropical pediatrics, 2006. **52**(2): p. 132-135.
14. Shah, U. and T. Moatter, *Screening for cystic fibrosis: the importance of using the correct tools.* Journal of Ayub Medical College, 2006. **18**(1).
15. Stewart, B., et al., *Normal sweat chloride values do not exclude the diagnosis of cystic fibrosis.* American journal of respiratory and critical care medicine, 1995. **151**(3): p. 899-903.
16. Sabar, M.F. and F. Arshad, *Genetic Mutations in CFTR Protein Gene Cause Cystic Fibrosis-Its Symptoms, Treatment, and Incidence in Pakistan.* 2019.
17. Lopes-Pacheco, M., N. Pedemonte, and G. Veit, *Discovery of CFTR modulators for the treatment of cystic fibrosis.* Expert Opinion on Drug Discovery, 2021. **16**(8): p. 897-913.
18. Shah, U., P. Frossard, and T. Moatter, *Cystic fibrosis: defining a disease under-diagnosed in Pakistan.* Tropical Medicine & International Health, 2009. **14**(5): p. 542-545.

19. Rafique, H., et al., *Exploring the diversity of CFTR gene mutations in cystic fibrosis individuals of South Asia.* Journal of Asthma, 2024. **61**(6): p. 511-519.
20. Esposito, C., et al., *Advances in the cystic fibrosis drug development pipeline.* Life, 2023. **13**(9): p. 1835.
21. Quinton, P.M., *Cystic fibrosis: a disease in electrolyte transport.* The FASEB Journal, 1990. **4**(10): p. 2709-2710.
22. Gabel, M.E., G.J. Galante, and S.D. Freedman. *Gastrointestinal and hepatobiliary disease in cystic fibrosis*. in *Seminars in Respiratory and Critical Care Medicine*. 2019. Thieme Medical Publishers.
23. AlMaghamsi, T., et al., *Cystic fibrosis gene mutations and polymorphisms in Saudi men with infertility.* Annals of Saudi Medicine, 2020. **40**(4): p. 321-329.
24. Bieniek, J.M., C.D. Lapin, and K.A. Jarvi, *Genetics of CFTR and male infertility.* Translational andrology and urology, 2021. **10**(3): p. 1391.
25. Goetz, D. and C.L. Ren, *Review of cystic fibrosis.* Pediatric annals, 2019. **48**(4): p. e154-e161.
26. Merjaneh, L., et al., *The role of modulators in cystic fibrosis related diabetes.* Journal of clinical & translational endocrinology, 2022. **27**: p. 100286.
27. Tournier, A., et al., *Fertility of women with cystic fibrosis: a French survey.* Reproductive BioMedicine Online, 2019. **39**(3): p. 492-495.
28. Martínez-Jiménez, S., M.L. Rosado-de-Christenson, and B.W. Carter, *Specialty Imaging: HRCT of the Lung E-Book: Specialty Imaging: HRCT of the Lung E-Book*. 2017: Elsevier Health Sciences.
29. Castellani, C., et al., *Newborn screening for cystic fibrosis.* The Lancet Respiratory Medicine, 2016. **4**(8): p. 653-661.
30. Aalbers, B.L., et al., *Nasal potential difference in suspected cystic fibrosis patients with 5T polymorphism.* Journal of cystic fibrosis, 2020. **19**(4): p. 627-631.
31. Pagin, A., I. Sermet-Gaudelus, and P.-R. Burgel, *Genetic diagnosis in practice: From cystic fibrosis to CFTR-related disorders.* Archives de Pédiatrie, 2020. **27**: p. eS25-eS29.
32. Farrell, P.M., et al., *Diagnosis of cystic fibrosis: consensus guidelines from the cystic fibrosis foundation.* The Journal of pediatrics, 2017. **181**: p. S4-S15. e1.
33. Duz, M.B. and P. Ozyavuz Cubuk, *Analysis of rearrangements of the CFTR gene in patients from Turkey with CFTR-related disorders: frequent exon 2 deletion.* Journal of Human Genetics, 2021. **66**(3): p. 315-320.
34. Férec, C. and V. Scotet, *Genetics of cystic fibrosis: Basics.* Archives de Pédiatrie, 2020. **27**: p. eS4-eS7.
35. Mall, M.A. and D. Hartl, *CFTR: cystic fibrosis and beyond*. 2014, Eur Respiratory Soc.
36. Liu, F., et al., *Molecular structure of the human CFTR ion channel.* Cell, 2017. **169**(1): p. 85-95. e8.
37. Patrick, A.E. and P.J. Thomas, *Development of CFTR structure.* Frontiers in pharmacology, 2012. **3**: p. 162.
38. Miller, A.C., et al., *Cystic fibrosis carriers are at increased risk for a wide range of cystic fibrosis-related conditions.* Proceedings of the National Academy of Sciences, 2020. **117**(3): p. 1621-1627.
39. Bareil, C. and A. Bergougnoux, *CFTR gene variants, epidemiology and molecular pathology.* Archives de Pédiatrie, 2020. **27**: p. eS8-eS12.

40. Rowe, S.M. and A.S. Verkman, *Cystic fibrosis transmembrane regulator correctors and potentiators.* Cold Spring Harbor perspectives in medicine, 2013. **3**(7): p. a009761.
41. Southern, K.W., et al., *Correctors (specific therapies for class II CFTR mutations) for cystic fibrosis.* Cochrane Database of Systematic Reviews, 2018(8).
42. Hanrahan, J.W., H.M. Sampson, and D.Y. Thomas, *Novel pharmacological strategies to treat cystic fibrosis.* Trends in pharmacological sciences, 2013. **34**(2): p. 119-125.
43. Condren, M.E. and M.D. Bradshaw, *Ivacaftor: a novel gene-based therapeutic approach for cystic fibrosis.* The Journal of Pediatric Pharmacology and Therapeutics, 2013. **18**(1): p. 8-13.
44. Patel, S., et al., *Potentiators (specific therapies for class III and IV mutations) for cystic fibrosis.* Cochrane Database of Systematic Reviews, 2015(3).
45. Rogan, M.P., D.A. Stoltz, and D.B. Hornick, *Cystic fibrosis transmembrane conductance regulator intracellular processing, trafficking, and opportunities for mutation-specific treatment.* Chest, 2011. **139**(6): p. 1480-1490.
46. Deeks, E.D., *Lumacaftor/ivacaftor: a review in cystic fibrosis.* Drugs, 2016. **76**: p. 1191-1201.
47. Boyle, M.P., et al., *A CFTR corrector (lumacaftor) and a CFTR potentiator (ivacaftor) for treatment of patients with cystic fibrosis who have a phe508del CFTR mutation: a phase 2 randomised controlled trial.* The lancet Respiratory medicine, 2014. **2**(7): p. 527-538.
48. Sala, M.A. and M. Jain, *Tezacaftor for the treatment of cystic fibrosis.* Expert review of respiratory medicine, 2018. **12**(9): p. 725-732.
49. Laselva, O., et al., *Rescue of multiple class II CFTR mutations by elexacaftor+ tezacaftor+ ivacaftor mediated in part by the dual activities of elexacaftor as both corrector and potentiator.* European Respiratory Journal, 2021. **57**(6).
50. Saadat, M., *Consanguineous marriages in Iranian folktales.* Public Health Genomics, 2006. **10**(1): p. 38-40.
51. Ghani, M.U., et al., *A report on asthma genetics studies in Pakistani population.* Advancements in Life Sciences, 2017. **4**(2): p. 33-38.
52. Zakar, R., M.Z. Zakar, and N. Aqil, *Adverse birth outcomes associated with consanguineous marriage in Pakistan.* International Journal of Gynecology and Obstetrics, 2014. **127**(2): p. 211-212.
53. Kabra, S., et al., *Cystic fibrosis in India.* Pediatric pulmonology, 2007. **42**(12): p. 1087-1094.
54. Shah, U., et al., *P0277 CYSTIC FIBROSIS IN PAKISTANI PATIENTS: THE TIP OF THE ICEBERG?* Journal of Pediatric Gastroenterology and Nutrition, 2004. **39**: p. S163.
55. Ghani, M., M. Sabar, and F. Awan, *WS21. 03 Low-cost chain termination DNA sequencing PCR reaction to diagnose CFTR gene mutations.* Journal of Cystic Fibrosis, 2022. **21**: p. S41.
56. Amaral, M.D., et al., *Quantitative methods for the analysis of CFTR transcripts/splicing variants.* Journal of Cystic Fibrosis, 2004. **3**: p. 17-23.
57. Hwang, T.C. and D.N. Sheppard, *Gating of the CFTR Cl− channel by ATP-driven nucleotide-binding domain dimerisation.* The Journal of physiology, 2009. **587**(10): p. 2151-2161.

58. Zhou, Z., et al., *The two ATP binding sites of cystic fibrosis transmembrane conductance regulator (CFTR) play distinct roles in gating kinetics and energetics.* The Journal of general physiology, 2006. **128**(4): p. 413.
59. Shishido, H., et al., *CFTR trafficking mutations disrupt cotranslational protein folding by targeting biosynthetic intermediates.* Nature Communications, 2020. **11**(1): p. 4258.
60. Sigoillot, M., et al., *Domain-interface dynamics of CFTR revealed by stabilizing nanobodies.* Nature communications, 2019. **10**(1): p. 2636.

APPENDICES

Appendix- 1

Genomic DNA Extraction

Organic Method

Blood Collection: Collect blood samples using suitable anticoagulants (e.g., EDTA) to prevent clotting. Transfer 1-2 mL of blood into a microcentrifuge tube.

Cell Lysis:

Add an equal volume of TE buffer (pH 8.0) to the blood sample. Gently mix by inverting the tube several times to lyse the cells.

Protein Precipitation:

Add an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, pH 8.0). Vortex the mixture thoroughly for 15-30 seconds to ensure complete mixing.

Centrifugation:

Centrifuge the tube at 12,000 × g for 10 minutes at room temperature. This step separates the aqueous (upper) phase containing DNA from the organic (lower) phase.

DNA Precipitation:

Transfer the upper aqueous phase (containing DNA) to a fresh microcentrifuge tube. Add 0.7 volumes of isopropanol and mix by gently inverting. Incubate at room temperature for 5-10 minutes to precipitate the DNA.

DNA Collection:

Centrifuge at 12,000 × g for 10 minutes at 4°C to pellet the DNA. Carefully remove the supernatant without disturbing the DNA pellet. Wash the DNA pellet with 70% ethanol by gently adding ethanol, briefly centrifuging, and removing the ethanol.

DNA Resuspension:

Air-dry the DNA pellet briefly (~5-10 minutes). Resuspend the DNA pellet in TE buffer or sterile water by gentle pipetting or vortexing. Store the extracted DNA at -20°C or -80°C for long-term storage.

Appendix- 2

Agarose Gel Preparation

- Measure 50 ml of 0.5X TBE buffer in a measuring flask and add the measured buffer in a 200 ml flask.
- Weigh the agarose according to the required volume and percentage of the gel as per the following conditions:

Agarose %	Buffer Volume	Agarose Weight
0.8	50 ml	0.4 g
1	50 ml	0.5 g
1.5	50 ml	0.75 g
2	50 ml	1 g
3	50 ml	1.5 g

- Add weighted agarose powder to the flask containing the measured buffer.
- Put the flask in the Oven and heat it for 45 seconds (until the solution becomes clear).
- After heating the flask, remove it from the oven and wait until you can touch the flask with bare hands. (Meanwhile prepare the gel tray i.e., attach casting gates and place the comb in the gel tray)
- Then add 2-4 µl Ethidium bromide in the gel and slightly pour it into the gel caster and weigh until the gel is hard.
- When the gel gets hard, remove the comb(s) and place the gel tray in the gel caster with the same buffer and ensure that the gel is dipped in the buffer. The agarose gel is ready for sample loading.

Appendix- 3

Purification of Nucleic Acid-DNA (Ethanol Precipitation)

Use 96 well plate and label wells with corresponding sample names.

1. First add 1/10 volume of 3M Sodium Acetate (pH=5.2) i.e., 2µl of 3M Sodium Acetate in each well
2. Then add 20 µl sample's PCR product.
3. Add 2-2.5 volume of absolute ethanol i.e., 52 µl of absolute ethanol in each well-containing sample's PCR product followed by pipette mixing. Then gently vortex the plate.
4. Place the well plate at 4°C for > 20 minutes.
5. Spin the plate at maximum speed (3200 rpm) in the centrifuge at 4° C for 25 minutes.
6. Adjust the tissue paper on the upper surface of the well plate and place the well plate upside down in the centrifuge and short spin up to 250 rpm and immediately stop the centrifuge (This step is used to discard ethanol from wells).
7. Then add 50 µl of 70% ethanol in the wells containing the pellet of DNA and spin at maximum speed (3200 rpm) for 20 minutes at 4° C in the centrifuge.
8. Repeat step 6.
9. Air dry the wells/pellet or dry the well plate in a PCR machine at 52°C for 5 minutes.
10. When the wells/pellet are completely dried, re-suspend the pellet in an equal volume of T.E buffer (pH=8) as the initial sample's PCR product (If the initial sample's PCR product concentration is low, decrease the volume of
11. Add T.E. buffer as per need.

Appendix- 4

Purification of 'Sequencing PCR' Products

(Ethanol-EDTA Precipitation)

Use 96 well plate and label wells with corresponding sample names.

1. First, prepare SALT MIXTURE.

SALT MIXTURE

Component	Volume (For 10 µl volume of product)
125 mM EDTA	2 µl
3M Sodium Acetate	2 µl

2. Then distribute 4 µl of the salt mixture in each well with the same label.
3. Add 10 µl Sequencing PCR products of each sample in corresponding labeled wells of 96 well plate.
4. Then add 25 µl of Absolute Ethanol in each well containing Sequencing PCR product + SALT MIXTURE.
5. Slightly vortex the well plate and short spin the well plate (for 250 rpm for 1- 2 seconds).
6. Incubate the well plate at room temperature for more than 20 minutes.
7. After incubation, spin the well plate at maximum speed (3200 rpm) at 4°C for 25 minutes.
8. Adjust the tissue paper on the upper surface of the well plate and place the well plate upside down in the centrifuge and short spin up to 250 rpm and immediately stop the centrifuge. (This step is used to discard ethanol from wells).
9. Then add 30 µl of 70% ethanol in the wells containing the pellet, slightly vortex it, and give the well plate a short spin.

10. Then spin the well plate at maximum (3200 rpm) speed for 20 minutes at 4°C in the centrifuge.

11. Repeat step 8.

12. Air dry the wells/pellet or dry the well plate in a PCR machine at 52°C for 5 minutes.

13. When the wells/pellet is completely dried, then add 13µl formamide in each well with the sample. Slightly shake/vortex and spin.

14. Heat at 95°C for 5 minutes and immediately put the well plate on ice. (Heat-shock). The samples are now ready for sequencing.

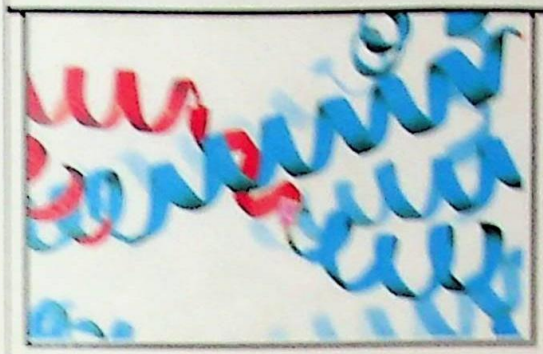
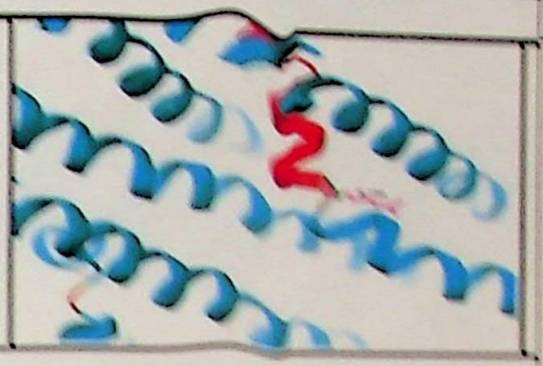
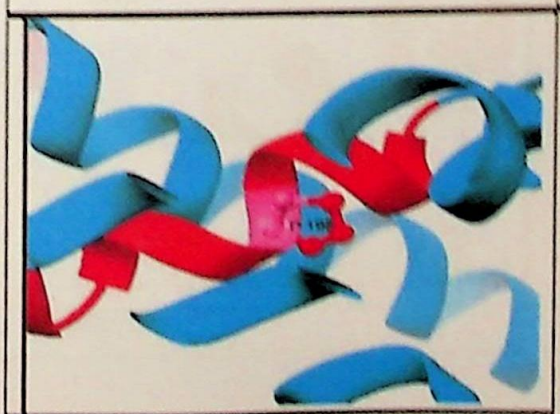
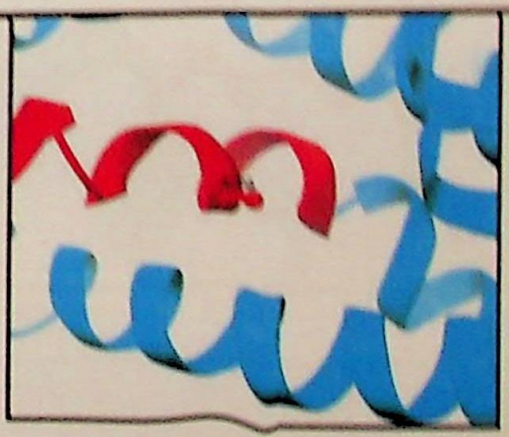
Appendix- 5

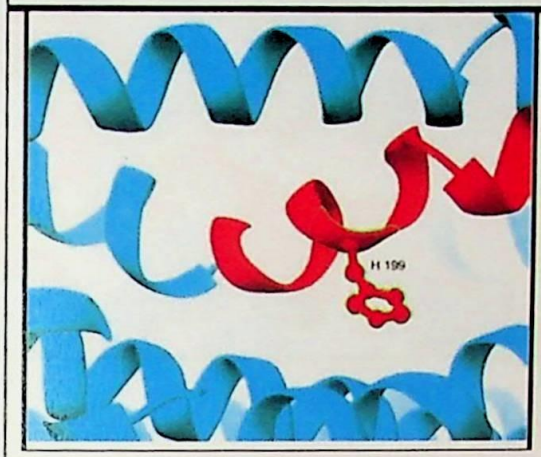
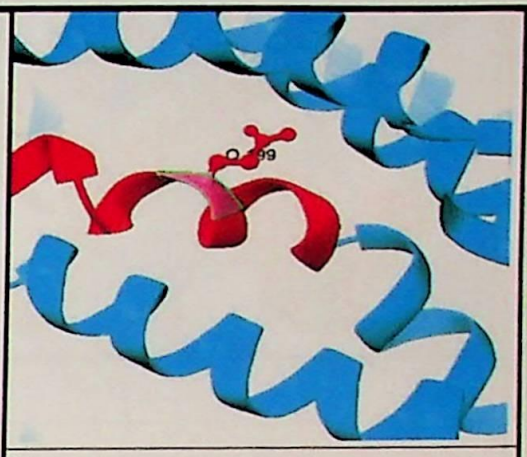
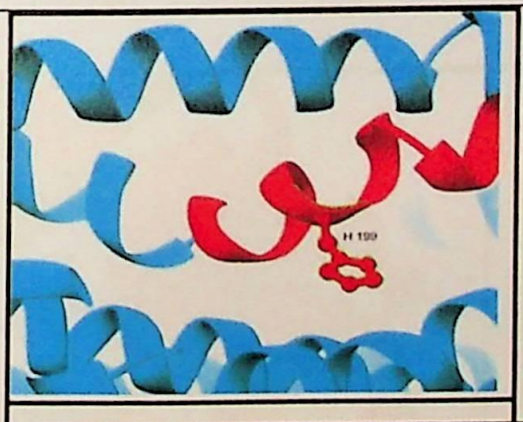
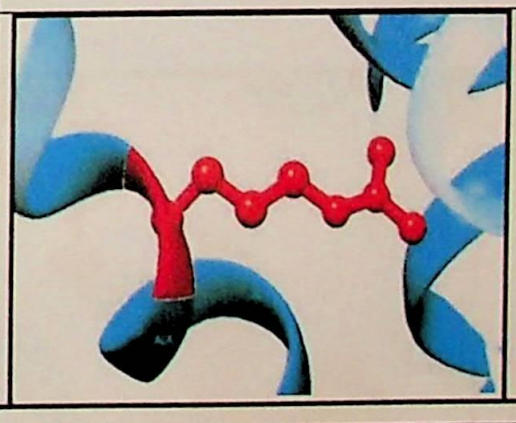
Reagent Preparation

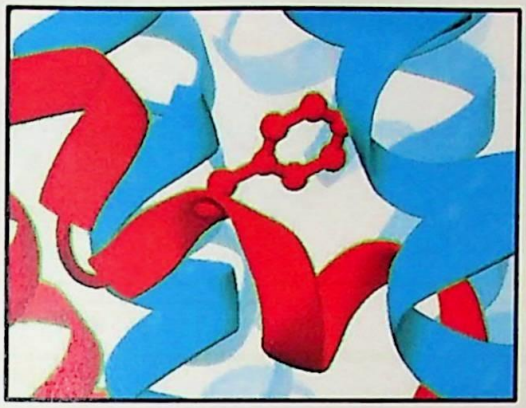
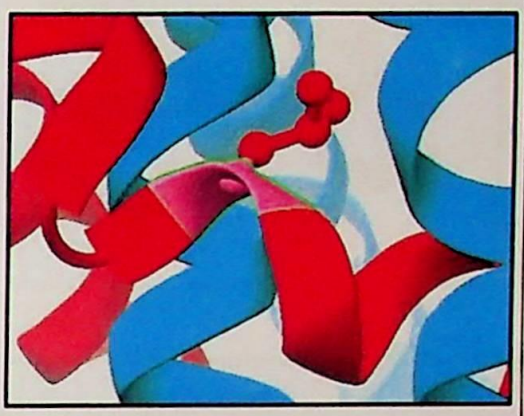
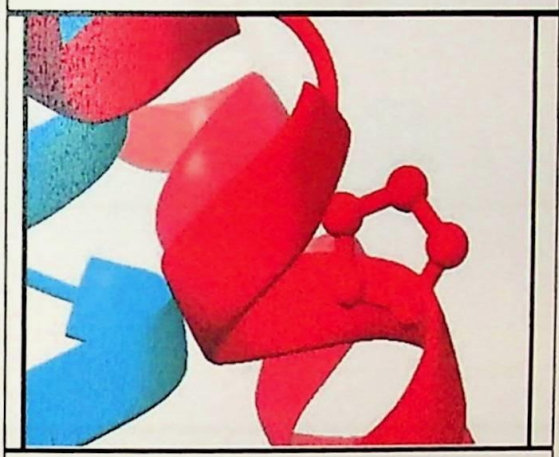
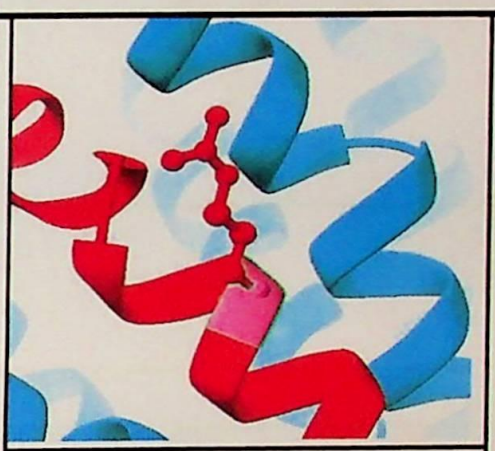
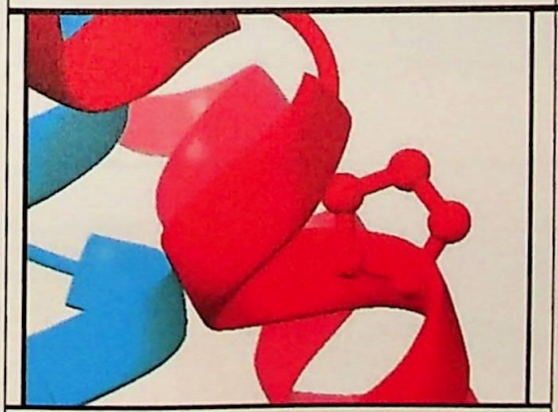
Components	Vol/Weight
0.5 M EDTA (pH=8.0)	
EDTA	186.1g
NaOH	2g
Distilled Water	1000ml
1M TrisHCl	
Tris-base	121.1g
Conc. HCl	10.20ml
Distilled Water	**1000ml**
1X Tris-EDTA (TE) Buffer	
1M TrisHCl	150µl
0.5M EDTA	30µl
Distilled Water	up to 15ml
5X TBE Buffer	
0.5M EDTA	20ml
Boric acid	27.5g
Tris-Base	54g
Distilled Water	1000ml
0.5X TBE Buffer	
5X TBE Buffer	100ml
Distilled water	900ml
3M Sodium Acetate (pH=5.2)	
Sodium Acetate	9.84 g

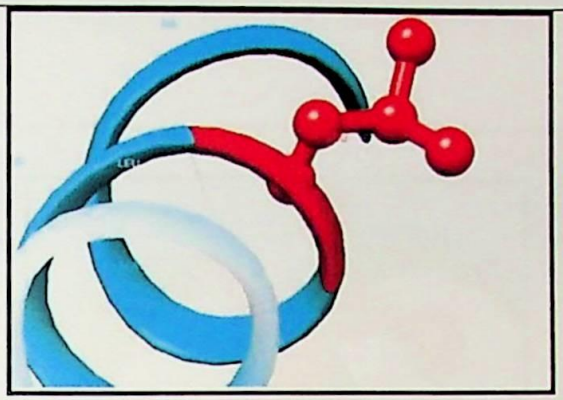
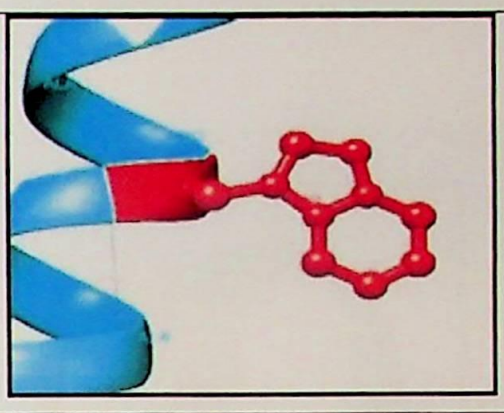
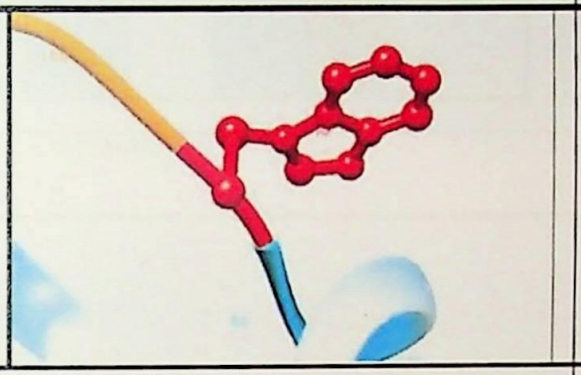
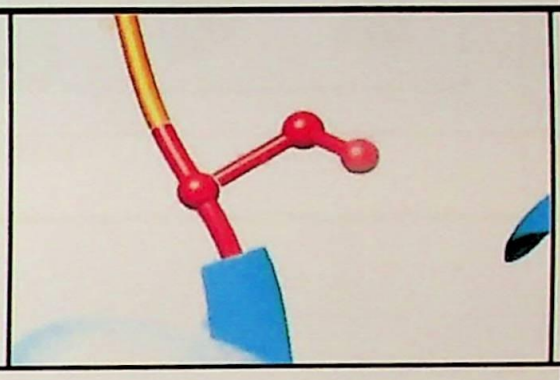
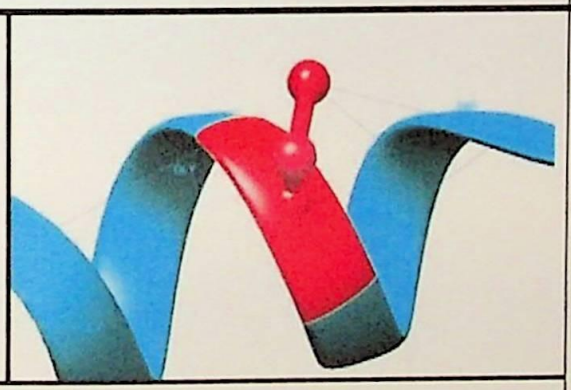
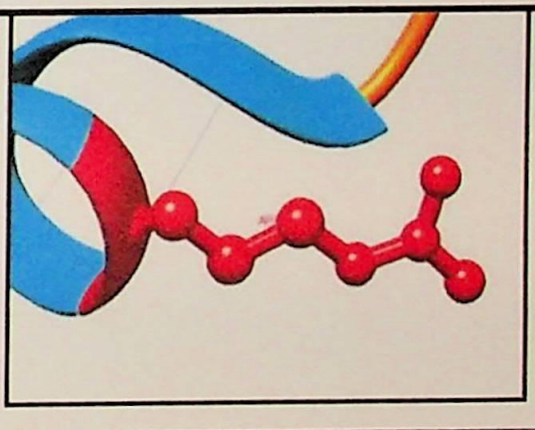
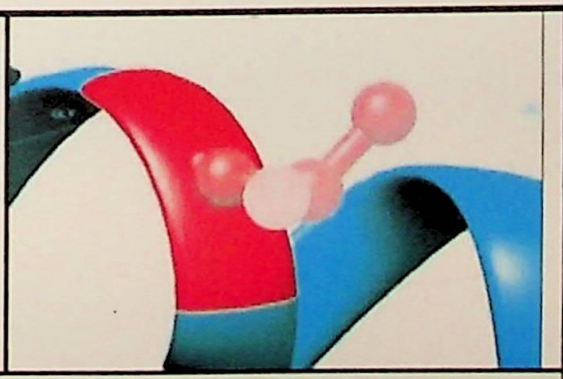
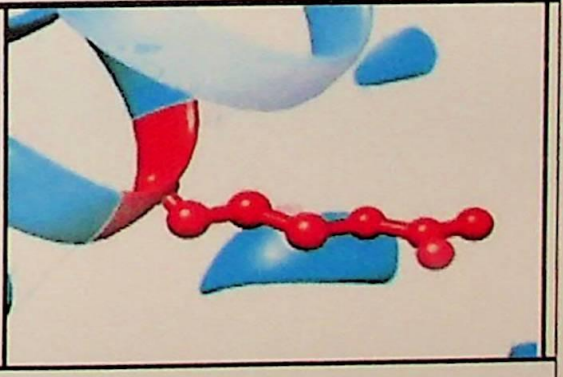
Distilled water	Up-to 40 ml (Adjust pH with glacial acetic acid
6X Gel Loading Dye	
Bromophenol Blue	0.25g
Glycerol	3ml
Distilled water	7ml
Ethidium Bromide	
Ethidium Bromide	0.01 g
Distilled Water	1 ml
Red Blood Cell (RBC) Lysis Buffer	
0.01 M Tris Base	300 µl
340 mM Sucrose	3.28 g
5 mM MgCl2	30.4 mg
1 & Triton X-100	0.3 ml
Distilled Water	up to 30 ml
White Blood Sample (WBC) Lysis Buffer	
0.01 Tris HCL	100 µl
11.4 mM Sodium Citrate	0.0335 g
10% SDS	0.1 mg
1mM EDTA	20 µl
Distilled Water	up to 10 ml

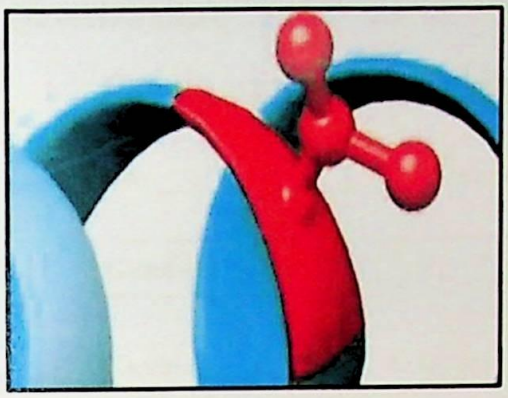
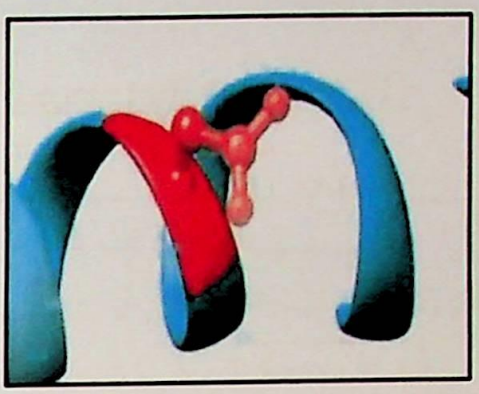
Appendix-6

rs376008630	G194R	
Wild type	Mutant	Interpretation
		Glycine is highly flex ible whil e argi nine has a bul ky resi due the flex ibili ty of heli x
rs193922529	A198P	
		Altered amino acid proline provide rigidity to the structure and there are high chance that helical structure if protein will be

		disturbed
rs397508765	**H199Q**	
		Altered amino acid reduce the hydrophobicity of protein because it is neutral and wild-type amino acid is good at making hydrogen bonding
rs397508764	**H199R**	
		Mutant residue can disturb the hydrogen bonding pattern of the protein.
		
rs397508766	**F200I**	

		Wild type has larger in size and mutant has smaller in size due to size differences effect on protein stability
rs397508769	**P205R**	
		Arginine, a charged polar molecule, disrupts helical structure of the protein. proline residue is buried at this position while the arginine side chain is exposed.
rs121908803	**P205S**	
		Serine is small molecule that reduce rigidity of helix hence disrupt protein structure
rs121908752	**L206W**	

		Wild type has non-polar. Mutant has aromatic this change can alter protein hydrophobi
rs397508776	**W216C**	
		Wild type larger Mutant has spherical which effect of packing participates reaction introducing properties
rs397508780	**C225R**	
		Wild type smaller thiol group. has charged group protein substitution redox
rs397508782	**L227R**	
		Wild type smaller charge. Mutant has positively group this effect packing and and also bounding properties

		Wild type has a smaller, aliphatic side chain. Mutant has a larger, negatively charged group. This
rs397508785	**Q237H**	

Appendix-7

Questionnaire

Questionnaire for Cystic Fibrosis Individuals (CFI)

Name: _____ **Father:**_____ **Age:**_____

Gender: ☐ ♂, ☐ ♀, ☐ Other

Height:_____

Weight: ____kg/____lb

Caste: _____ **Contact:** _____ *Sample Id:* **CF-**_____

Address:_____

Residential city:_____ **Province:**_____ **Nationality**_____

S-Economic Status: **Low** ☐, **Middle** ☐, **Upper** ☐ **Mother Tongue:**_____

Family Information

Father relates to Mother as: _____ **Consanguinity:** Yes | No

No of Siblings: _____ **Order of Birth:** _____ Remarks

- Any relative with Cystic fibrosis?/ Relation? _____
- Any relative died because of the same disease? _____

Medical History

Symptoms	CF related Complications	Any Medication
☐ Persistent Coughing	☐ Pulmonary infections	_____
☐ Shortness of Breath	☐ Intestinal manifestations	_____
☐ Male Infertility	☐ Pancreatitis	_____
☐ Greasy Stools	☐ Others _____	_____
		_____
		_____

Appendix-8

Consent Form

مریض کا کلینیکل تحقیق میں شمولیت کا اظہار نامہ

ادارہ: سینٹر فار ایلائیڈ مالیکیولر بیالوجی، یونیورسٹی آف پنجاب، لاہور

مطالعہ کا شمار:

سینیئر تحقیق کار: ڈاکٹر محمد عثمان غنی

مطالعہ کا عنوان : پاکستانی آبادی میں "سسٹک فائبروسس" کا جنیاتی مطالعہ

تحقیق کا تعارف

ہم آپ کو سنٹر فار اپلائڈ مالیکیولر بیالوجی، یونیورسٹی آف پنجاب، لاہور کی جانب سے تحقیق کے مطالعہ میں حصہ لینے کی دعوت دیتے ہیں. یہ بہت اہم ہے کہ آپ وہ تمام اصول پڑھیں اور سمجھیں جو

کہ ان تمام لوگوں پر لاگو ہوتے ہیں جو ہمارے مطالعے میں حصہ لیں گے.

1 . اس مطالعہ میں حصہ لینا مکمل طور پر رضاکارانہ ہوگا.

2 . اس مطالعے میں حصہ لینے سے ہو سکتا ہے آپ کو ذاتی طور پر کوئی فائدہ نہ ہو لیکن اس سے جو علم حاصل ہو سکے گا اس سے دوسروں کو فائدہ پہنچ سکتا ہے.

3 . آپ کسی بھی وقت بغیر جرمانے یا فائدے کے حصول میں رکاوٹ کی وجہ سے، جو کہ بصورت دیگر آپ کو حاصل ہے، اس مطالعے میں حصہ لینے سے انکار کر سکتے ہیں.

مطالعے کی نوعیت، خطرات، مشکلات اور دوسری اہم معلومات نیچے بیان کی گئی ہیں. آپ سٹاف کے رکن سے اس کے متعلق کوئی بھی سوال کرنے کی خواہش کا اظہار کر سکتے ہیں

جو کہ آپ کو اس کی وضاحت پیش کریں گے.

مطالعہ کس بارے میں ہے؟

تحقیقاتی ٹیم پاکستان میں اس جینز میں تبدیلیاں تلاش کرنے کی کوشش کر رہی ہے جو کہ سسٹک فائبروسس کا موجب بنتی ہیں. ایسے افراد جو اس موروثی بیماری میں مبتلا ہیں، ان میں تحقیق کرنے

والوں کو ان جینز کی تلاش ہے جو کہ پاکستان میں موجود سسٹک فائبروسس کا موجب بن رہی ہے.

آپ سے کیا توقع کی جائے گی؟

اس مطالعہ میں حصہ لینے کے لئے درج ذیل باتوں پر عمل کرنا ہوگا.

۱ .اپنے خاندان کے تمام افراد، جو اس بیماری سے متاثر ہیں، کے متعلق معلومات فراہم کرنا اور ممکنہ طور پر ڈاکٹر یا تحقیقاتی ٹیم ممبران کا آپ کے خاندان کے دوسرے افراد سے رابطے میں مدد کرنا.

۲ .جینٹک فائبروسس کا ٹیسٹ کروانا

۳ .اپنے خون کا، تقریباً 3 سے 5 سی سی، نمونہ دی

وہ تمام معلومات جو آپ اپنے خاندان کے ممبران سے متعلق فراہم کریں گے انہیں صیغہ راز رکھا جائے گا. تحقیق کار آپ کے خون کے نمونے سے کروموسوم (سیل کا وہ حصہ جو وراثت کو کنٹرول کرتا ہے) کے ٹکرے علیحدہ کئے جائیں گے. ان علیحدہ کئے ہوئے ٹکڑوں کو جینز میں موجود تبدیلیوں کی موجودگی اور ان کے اثرات جاننے میں مدد کرنے کے لئے استعمال کیا جائے گا. اس تحقیق کا واحد خطرہ خون نکالنا ہے. خوب آپ کے بازو کی دریڈ سے بذریعہ سرنج نکالا جائے گا. آپ کو سوئی کے داخلے کی جگہ عارضی درد ہو سکتا ہے، سرنج والی جگہ پر وقتی سوجن ہو سکتی ہے مگر اس سے

کمزوری اور مقامی انفیکشن کا خطرہ بہت کم ہے۔

اپنے نتائج کو مدنظر رکھتے ہوئے ہم چند مزید ٹیسٹ کروانے کی گزارش کر سکتے ہیں، آپ کی اور آپ

کے خاندان کی تصویر حاصل کرنے کی درخواست بھی کی جاسکتی ہے. تصویر کی درخواست کرنے پر

آپ سے تصویر اتارنے کے اجازت نامہ پر دستخط لئے جائیں گے.

کلینیکل تحقیق کے مطالعہ میں شمولیت
کی منظوری

دستخط بالغ مریض :

دستخط کم عمر مریض کے والدین:

مطالعہ نمبر

Appendix-9

SOPs for Blood Sampling

Introduction

This standard operating procedure defines the collection of blood samples for genetic analysis of the *CFTR* gene in Cystic Fibrosis patients in Pakistan. The study will be conducted at the Centre for Applied Molecular Biology (CAMB), Lahore. Practically, blood is an easily accessible research material for DNA studies.

Constraints

Blood will be collected from patients who fall under the inclusion criteria of this study. The informed consent form is necessary for collection of blood sample. Only a designated person will collect the blood sample.

Materials and Equipment Required

- Disposable gloves.
- Vein Infusion set.
- Sharps disposal container.
- Alcohol swabs.
- Tourniquet.
- Vacutainer specimen tubes.
- Vacutainer holders.
- Appropriate size sterile disposable syringes.
- 10% household bleach.
- Permanent labelling markers.
- Refrigerator.

Procedure

- Patients must have completed the consent form.
- Required materials for the procedure should be assembled at one place before performing the procedure.

- The local phlebotomy procedure will be followed.
- New vacutainer tubes (EDTA Tubes) and sterile syringes and are to be used for each blood draw.
- After completion, needles must be properly disposed of in a puncture- resistant container.
- Label the vacutainer tube with the sample ID as filled in the questionnaire.
- The preferred site for phlebotomy is the median antecubital veins of the upper extremity.
- Possible alternative sites are the veins on the dorsum of the hand and other forearm veins.
- A tourniquet may be used to transiently distend veins before drawing blood.
- Using the tip of the index finger, feel the vein, and decide exactly where to place the puncture.
- Collect up to 5 ml of blood in one attempt. If difficult 3ml is sufficient. Avoid second attempt.
- Transfer the blood from syringe to the vacutainer and mix by inverting it 8- 10 times to ensure that anticoagulant is distributed evenly.
- Release the tourniquet after the blood draw, cover the puncture site with a cotton swab.

Sample Transport

The sample from the hospital will be transported to the CAMB, by the designated person under sterile and appropriate conditions in a damage-free container or box.

Protocol For Sample Storage

The samples will be stored at -20°C until further processing

tayyab ali

For Plagiarism-Shazia Tanveer-SU92-MSZGW-F22-039

M.PHIL

Document Details

Submission ID
trn:oid:::3618:129034566

Submission Date
Feb 24, 2026, 11:35 AM GMT+5

Download Date
Feb 24, 2026, 11:40 AM GMT+5

File Name
For Plagiarism-Shazia Tanveer-SU92-MSZGW-F22-039.docx

File Size
57.7 KB

25 Pages

6,235 Words

36,781 Characters

8% Overall Similarity

The combined total of all matches, including overlapping sources, for each database.

Filtered from the Report

- Bibliography
- Quoted Text
- Small Matches (less than 8 words)

Match Groups

48 Not Cited or Quoted 8%
Matches with neither in-text citation nor quotation marks

1 Missing Quotations 0%
Matches that are still very similar to source material

0 Missing Citation 0%
Matches that have quotation marks, but no in-text citation

0 Cited and Quoted 0%
Matches with in-text citation present, but no quotation marks

Top Sources

7% Internet sources

4% Publications

0% Submitted works (Student Papers)

Integrity Flags

0 Integrity Flags for Review

Our system's algorithms look deeply at a document for any inconsistencies that would set it apart from a normal submission. If we notice something strange, we flag it for you to review.

A Flag is not necessarily an indicator of a problem. However, we'd recommend you focus your attention there for further review.

February 24, 2026

PLAGIARISM CERTIFICATE

For MPhil and PhD

This is certified that the thesis entitled:**In-Silico Characterization And Mutational Analysis Of Genetic Variants In Exon-6 Of CFTR Gene Among Cystic Fibrosis Pakistani Patients.**

Student Name:Shazia Tanveer

Reg /Roll No:SU92-MSZGW-F22-039

Submitted on date:24.02.2026

We have checked this document and found 8 % plagiarism/similarity,1% single source, and 0% AI in the document. It is certified that this document meets the HEC criteria.

SIRC
SUPERIOR UNIVERSITY

Supervisor Signature

Research Project / Thesis

Librarian Hafsan Khalil
24-02-2026

Superior Information Resource Center