Screening for Common CFTR Mutations in Palestinian Cystic Fibrosis Patients

Ayat N. Azarah

Supervised by:
Prof. Dr. Fadel A. Sharif
Molecular Biology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology

Aug. /2017
Screening for Common CFTR Mutations in Palestinian Cystic Fibrosis Patients

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

Screening for Common CFTR Mutations in Palestinian Cystic Fibrosis Patients

أقر بأن ما اشتملت عليه هذه الرسالة إنما هو نتاج جهدي الخاص، باستثناء ما تم الإشارة إليه حينما ورد، وأن هذه الرسالة ككل أو أي جزء منها لم يقدم من قبل الآخرين لنقل درجة أو لقب علمي أو بحثي لدى أي مؤسسة تعليمية أو بحثية أخرى.

Declaration

I understand the nature of plagiarism, and I am aware of the University’s policy on this.

The work provided in this thesis, unless otherwise referenced, is the researcher’s own work, and has not been submitted by others elsewhere for any other degree or qualification.

<table>
<thead>
<tr>
<th>Student's name:</th>
<th>Ayat N. Azarah</th>
<th>Signature:</th>
<th>التوقيع:</th>
</tr>
</thead>
<tbody>
<tr>
<td>آيات نعيم عزارة</td>
<td>آيات عزارة</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Date:</td>
<td>2017/8/26</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
نتيجة الحكم على أطروحة ماجستير

بناءً على موافقة شئون البحث العلمي والدراسات العليا بالجامعة الإسلامية بغزة على تشكيل لجنة الحكم على أطروحة الباحثة/ ايات نعم اسمااعيل عزاره لنيل درجة الماجستير في كلية العلوم قسم التكنولوجيا الحيوية. وموضوعها:

الكشف عن الطفرات الشائعة لمرض التليف الكيسي في فلسطين

Screening for Common CFTR Mutations in Palestinian Cystic Fibrosis Patients

وبعد المناقشة التي تمت اليوم الاثنين 16 ذو العقدة 1438 هـ الموافق 2017/8/7، الموافق

الساعة العاشرة صباحاً، اجتمعت لجنة الحكم على أطروحة المكونة من:

أ.د. فضيل أكرم الشريف

مرشراً ورئيساً

د. صائب حسين العويني

مناقشاً داخلياً

د. نبيل عبد الله العيللة

مناقشاً خارجياً

وبعد المداولة أوصت اللجنة بمنح الباحثة درجة الماجستير في كلية العلوم/ قسم - التكنولوجيا الحيوية.

واللجنة إذ تمنحها هذه الدرجة فإنها توصي بها بتقوى الله وزروماً طاعته وأن يسخر علمها في خدمة دينها ووطنها.

والله والترفوف،

نائب الرئيس لشؤون البحث العلمي والدراسات العليا

أ.د. عبد الرؤوف علي المناعية
Abstract

Problem:
Cystic Fibrosis (CF) is a severe life threatening inherited disease characterized by dysfunction of the exocrine glands. The disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene the product of which is responsible for the transport of chloride ions across the membranes of epithelial cells. In Palestine, mutations in CFTR gene are not fully identified.

Objectives:
The present study aims to identify the common CFTR mutations in Gaza Strip.

Methodology:
This study was performed on blood genomic DNA samples from 30 patients (18 males and 12 females) residing in Gaza who were clinically diagnosed with cystic fibrosis. Several polymerase chain reaction (PCR) techniques such as the amplification refractory mutation system (ARMS), tetra-primer ARMS-PCR and restriction fragment length polymorphism (RFLP-PCR) were applied to examine the following mutations: 508delF, W1282X, 3120+1kb, S549R, 1548delG, I1234V, 711+1G, G115X, 4010del4 and H139L.

Results:
Of the 60 studied alleles, four mutations were detected. The most common mutations were: 508delF and G115X (6.67% each), followed by W1282X (5%) and 3120+1kb (1.67%).

Conclusions & Recommendations:
Identification of CFTR mutations in the Palestinian CF population is necessary to set up feasible molecular diagnostics, genetic counseling and patient management.

Key words: Cystic fibrosis; CFTR; mutations; PCR; Gaza Strip-Palestine.
المملص:
مشكلة البحث:
التمييز الكيسي هو مرض وراثي حاد يمتاز بخلق في وظائف الغدد الإفرازية ويهدد حياة المصاب. المرض ناجم عن حدوث طفرات في جين التمييز الكيسي (CFTR) الذي يشفر البروتين المسؤول عن نقل أيونات الكلور عبر غشاء الخلايا الطلائية. في فلسطين، طفرات جين التمييز الكيسي غير محددة كلياً.

هدف البحث:
تهدف الدراسة الحالية إلى تحديد طفرات جين التمييز الكيسي الشائعة في قطاع غزة.

منهجية البحث:
أجريت هذه الدراسة على عينات الحمض النووي الريبوزي منقوص الأكسجين، حيث تم استخلاص هذا الحمض من عينات دم لثلاثين مريضاً (18 ذكرًا، 11 أنثى) متواجدين في غزة وتشخيصهم سريرياً بإصابة التمكين الكيسي. تم استخدام العديد من تقنيات تفاعل البوليميراز المتسلسل (PCR) مثل: نظام تضخيم الطفرة الحراري (ARMS) وتمارين البريمير (tetra-primer) ونظام تضخيم الطفرة الحراري (RFLP) في الكشف عن العشر طفرات الأليفة: W1282X, 3120+1kb, S549R, 1548delG, I1234V, 711+1G, G115X, H139L, 508delF 4010del4

نتائج البحث:
تم تحديد أربع طفرات في ال60 أليل الذين تم دراستهم، والطفرات هي: 508delF بنسبة 6.67% لكل منهما، و G115X بنسبة 5% ثم W1282X بنسبة 3120+1kb بنسبة 1.67%.

خلاصة البحث:
تحديد طفرات جين التمييز الكيسي لدى المرضى الفلسطينيين يعد ضرورياً لإجراء التشخيص الجزيئي، تقديم الاستشارات الوراثية وبحث سبل العلاج.

كلمات مفتاحية:
التمييز الكيسي، جين التمييز الكيسي، الطفرات، تفاعل البوليميراز المتسلسل، قطاع غزة، فلسطين.
Dedication

To all of them I dedicate this work, fulfillment and recognition:
The soul of my dear father (may Allah have mercy on him) and my dear mother (may Allah protect her), who enlightened my way all the time.
My husband who has been a great source of motivation and inspiration.
All my wonderful brothers and sisters, for their endless love and support.
    My teachers, friends and great family.
    The souls of the martyrs of Palestine.
    All Muslims all over the world.
Acknowledgments

First and above all, I praise and thank Allah, the almighty, for granting me the strength, patience, faith, wisdom, and physical ability to proceed throughout my research work and complete it successfully. I wouldn’t reach to where I am now without his kindness, mercy, and showers of blessings throughout my life.

I’d like to thank Prof. Fadel A. sharif, the supervisor of the study, who did not spare any effort to overcome all the difficulties aroused during the theoretical and practical parts and for his constructive scientific advice.

I’d like also to thank all members of genetics lab in the medical technology department in particular Mr. Mohammmed Ashour and Mr. Shadi El-Ashi.

I’d like also to thank Cystic Fibrosis Friend Center in Gaza for their generosity in providing us with the samples and valuable information.

I am grateful and thankful to all the CF patients and their families for their generosity in providing us with the precious samples without which this thesis could not be accomplished.

Finally, profuse thanks, love and appreciations to my lovely parents, my husband, my sisters, my brothers, my family and my friends for all unlimited support and encouragement they provided me.
Table of Contents

Declaration……………………………………………………………………………… I
Abstract………………………………………………………………………………… II
Abstract in Arabic………………………………………………………………… III
Dedication……………………………………………………………………………… IV
Acknowledgment…………………………………………………………………… V
Table of Contents…………………………………………………………………… VI
List of Tables……………………………………………………………………… VIII
List of Figures……………………………………………………………………… IX
List of Abbreviations……………………………………………………………… X

Chapter 1 Introduction……………………………………………………………… 1
  1.1 Background and Context………………………………………………………… 2
  1.2 Scope and Objectives…………………………………………………………… 4
  1.3 Significance……………………………………………………………………… 4
  1.4 Limitations……………………………………………………………………… 5
  1.5 Overview of Thesis……………………………………………………………... 5

Chapter 2 Literature Review………………………………………………………… 6
  2.1. Cystic Fibrosis (CF)…………………………………………………………… 7
  2.2. CFTR gene……………………………………………………………………… 8
  2.3. CFTR protein………………………………………………………………….. 8
  2.4. CFTR mutations……………………………………………………………… 11
    2.4.1. Previous studies…………………………………………………………… 12
    2.4.2. Classes of CFTR mutations…………………………………………… 13
  2.5. Diagnosis……………………………………………………………………… 16
    2.5.1. Sweat test…………………………………………………………………. 17
    2.5.2. Genetic testing…………………………………………………………… 17
    2.5.3. Preimplantation Genetic Diagnosis (PGD)…………………………… 18
    2.5.4. Antenatal testing………………………………………………………… 18
    2.5.5. Newborn screening……………………………………………………… 19
  2.6. Cystic fibrosis mutation specific treatment………………………………… 20

Chapter 3 Materials and Methods………………………………………………… 23
  3.1. Materials……………………………………………………………………… 24
    3.1.1. Equipment………………………………………………………………. 24
    3.1.2. Chemicals, Kits, and Disposables……………………………………… 25
  3.2. Study sample…………………………………………………………………… 26
    3.2.1. Study design……………………………………………………………… 26
    3.2.2. Study location…………………………………………………………… 26
    3.2.3. Study subjects…………………………………………………………… 26
    3.2.4. Ethical considerations………………………………………………… 26
  3.3. Genotyping…………………………………………………………………… 26
    3.3.1. DNA extraction………………………………………………………… 26
    3.3.2. PCR primers reconstitution…………………………………………… 26
    3.3.3. Detection of CFTR mutations………………………………………… 27
      3.3.3.1. Allele refractory mutation system (ARMS)-PCR method……… 27
      3.3.3.2. Tetra-ARMS-PCR method……………………………………… 29
      3.3.3.3. PCR-RFLP method……………………………………………… 31
## List of Tables

**Table (2.1):** Classes of the selected *CFTR* mutations ................................. 15

**Table (3.1):** The major equipment used in this study. ................................. 24

**Table (3.2):** Chemicals, kits and disposables used in this study ....................... 25

**Table (3.3):** ARMS-PCR components for amplification of the *CFTR* gene fragments ................................................................................................................. 28

**Table (3.4):** PCR primers, annealing temperatures and lengths of PCR products for genotyping *CFTR* mutations by ARMS-PCR method ............................. 28

**Table (3.5):** Thermal cycler program for ARMS-PCR amplification of the *CFTR* gene target fragments ......................................................................................... 29

**Table (3.6):** Tetra-ARMS-PCR components for amplification of the *CFTR* gene ................................................................. 30

**Table (3.7):** PCR primers, annealing temperatures and lengths of PCR products for genotyping *CFTR* mutations by Tetra-ARMS-PCR method .......................... 30

**Table (3.8):** Thermocycler program for Tetra-ARMS-PCR amplification of the *CFTR* gene ................................................................................................................. 31

**Table (3.9):** PCR-RFLP components for amplification of the *CFTR* gene .......... 32

**Table (3.10):** PCR primers, annealing temperatures and restriction enzymes for genotyping *CFTR* mutations by PCR-RFLP method .............................................. 31

**Table (3.11):** Thermocycler program for PCR-RFLP amplification of the *CFTR* gene ...................................................................................................................... 32

**Table (3.12):** The enzymatic digestion components of amplified *CFTR* gene .......... 32

**Table (3.14):** PCR components for amplification of G115X mutation .................. 33

**Table (3.13):** PCR primers and lengths of PCR products for G115X mutation ...... 33

**Table (3.15):** Thermocycler program for PCR amplification of G115X mutation ... 34

**Table (4.1):** Frequency of the observed genotypes in the cystic fibrosis patients ... 34

**Table (4.2):** Frequency of the detected *CFTR* mutant alleles ............................ 41

**Table (5.1):** Frequency of common Palestinian *CFTR* mutations compared to other countries. ................................. 48
List of Figures

Figure (2.1): Schematic diagram of CFTR showing transmembrane topology and domain organization ........................................10

Figure (2.2): Classes of CFTR mutations ........................................15

Figure (4.1): A photograph of ethidium bromide stained 3% agarose gel showing the ARMS-PCR products of F508del........................................... 36

Figure (4.2): A photograph of ethidium bromide stained 3% agarose gel showing the ARMS-PCR products of 3120+1kb.............. 36

Figure (4.3): A photograph of ethidium bromide stained 3% agarose gel showing the ARMS-PCR products of W1282X............................ 37

Figure (4.4): A photograph of ethidium bromide stained 3% agarose gel showing the ARMS-PCR products of 1548delG................................. 37

Figure (4.5): A photograph of ethidium bromide stained 3% agarose gel showing the Tetra-ARMS-PCR products of 711+1G................................. 38

Figure (4.6): A photograph of ethidium bromide stained 3% agarose gel showing the Tetra-ARMS-PCR products of H139L................................. 38

Figure (4.7): A photograph of ethidium bromide stained 3% agarose gel showing the PCR-RFLP products of I1234V................................. 39

Figure (4.8): A photograph of ethidium bromide stained 3% agarose gel showing the PCR-RFLP products of 4010del4................................. 39

Figure (4.9): A photograph of ethidium bromide stained 3% agarose gel showing the PCR-RFLP products of S549R................................. 40

Figure (4.10): A photograph of ethidium bromide stained 3% agarose gel showing the allele specific mutation analysis products of G115X mutation…..40

Figure (4.11): An illustration of the percentages of the detected CFTR mutations.................................................................42
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCC7</td>
<td>ATP-Binding Cassette sub-family C 7.</td>
</tr>
<tr>
<td>ARMS</td>
<td>Amplification Refractory Mutation System.</td>
</tr>
<tr>
<td>AS-PCR</td>
<td>Allele-Specific PCR</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate.</td>
</tr>
<tr>
<td>bp</td>
<td>base pair.</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate.</td>
</tr>
<tr>
<td>CBAVD</td>
<td>Congenital Bilateral Absence of Vas Deferens.</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic Fibrosis.</td>
</tr>
<tr>
<td>CF-PI</td>
<td>Pancreatic Insufficiency.</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic Fibrosis Transmembrane Conductance Regulator.</td>
</tr>
<tr>
<td>CFTR-RD</td>
<td>CFTR-Related Disorders.</td>
</tr>
<tr>
<td>DGGE</td>
<td>Denaturing Gradient Gel Electrophoresis.</td>
</tr>
<tr>
<td>dHPLC</td>
<td>Denaturing High Pressure Liquid Chromatography.</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid.</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine Tetraacetic Acid.</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial Sodium Channel.</td>
</tr>
<tr>
<td>IRT</td>
<td>Immunoreactive Trypsinogen.</td>
</tr>
<tr>
<td>IVF</td>
<td><em>In Vitro</em> Fertilization.</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic acid.</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucleotide Binding Domains.</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction.</td>
</tr>
<tr>
<td>PGD</td>
<td>Preimplantation Genetic Diagnosis.</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism.</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism.</td>
</tr>
<tr>
<td>SSCP</td>
<td>Single-Strand Conformation Polymorphism.</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane.</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane Domain.</td>
</tr>
<tr>
<td>UAE</td>
<td>United Arab Emirates.</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom.</td>
</tr>
<tr>
<td>US</td>
<td>United States.</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet.</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
Chapter 1
Introduction

1.1 Background and Context

Cystic fibrosis (CF) is one of the most lethal, autosomal recessive, monogenic disorders affecting over 70,000 individuals worldwide (Cystic Fibrosis Foundation, 2014). CF leads to significant morbidity and mortality with a median predicted age of survival of about 40 in 2014 (Collaco and Cutting, 2008). The disease is more common in the Caucasians with a prevalence of approximately 1 in 2500 live births and a carrier frequency of around 1 in 25. CF is the second most common life-shortening, inherited disorder occurring in childhood in the United States (US), after sickle cell anemia (American Lung Association, 2010). The incidence rate of CF among the Arab population is ranged from very rare to as prevalent as among Caucasians (Kakish, 2001).

In CF patients, the lungs, digestive system and other organs are clogged with viscous mucus. Through time, organs are deteriorated more and more, and lung transplantation may be the only option to prolong the patient's life (American Lung Association, 2010).

The disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which is the responsible gene for CF, encoding a transmembrane protein that functions as a chloride channel and a regulator of other channels across the epithelial cell membrane. The defective protein impairs water movement across epithelia leading to formation of viscous mucus that obstructs the airways of the lungs and ducts of the pancreas. CF is characterized by progressive lung disease, pancreatic dysfunction, elevated sweat electrolytes, and male infertility (Essawi et al., 2015).

Early diagnosis for CF means treatment can begin immediately. There are various tests available for CF diagnosis. These include: sweat chloride test, immunoreactive trypsinogen (IRT), and genetic testing (Wang and Freedman, 2002).
Analysis of sweat chloride is a laboratory “Gold Standard” for the diagnosis of CF. The cut-off chloride ions value of 60 mmol/L is considered diagnostic of CF, however in some patients chloride concentration might be less than 60 mmol/L but other clinical signs would also be present (Siryani et al., 2015).

There are about 2,000 CFTR mutations have been identified, with variable frequencies depending on the geographic and ethnic background. The most common mutation, called p.F508del, is a deletion of one amino acid at position 508 in the CFTR protein. The resulting abnormal channel breaks down shortly after it is made, so it never reaches the cell membrane to transport chloride ions. The p.F508del mutation, accounts for around two-thirds of all cystic fibrosis alleles worldwide (Cystic Fibrosis Mutation Database, 2011; Siryani et al., 2015; Dell’Edera et al., 2016).

CF is genetically diagnosed by applying a number of molecular genetic methods, namely, restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP), denaturing high pressure liquid chromatography (dHPLC) etc., but they are relatively slow and technically demanding. Several independent groups described a PCR-based approach for analyzing known point mutations in DNA and distinguishing between the normal, heterozygous and homozygous mutant genotypes. This method is commonly referred to as amplification refractory mutation system (PCR-ARMS) or (ARMS) (Soloviov et al., 2010).

CF prevalence in the Middle East ranges from 1 in 2,500 to 1 in 16,000 with different mutation frequencies depending on the population ethnic descent. However, reliable information about the frequency of CF among the Palestinians is still lacking and the spectrum and nature of mutations have not been documented yet, hampering molecular diagnostics. A good insight into the type and rate of CFTR mutations in a certain population is essential to set up adequate and cost-effective molecular diagnostics (Essawi et al., 2015).

Few studies from Palestine and other different countries (where Palestinian patients are living or treated) reported the Palestinian CFTR mutations, including
p.F508del, p.W1282X, p.N1303K, p.G85E and 3120+1Kbdel8.6Kb were the most common (Siryani et al., 2015).

In this study, 30 clinically diagnosed CF Palestinian patients residing in Gaza were tested in order to detect the common CFTR mutations and their prevalence in this population. We selected ten mutations: 508delF, W1282X, 3120+1kb, S549R, 1548delG, I1234V, 711+1G, G115X, 4010del4 and H139L. Selection of those mutations was based on their prevalence in various Arab countries.

1.2 Scope and Objectives

- Testing CF patients for ten documented CFTR mutations in Gaza Strip.
- Performing rapid detection of the investigated CFTR mutations in Gaza Strip by applying PCR techniques.
- Determining the frequency of the investigated CFTR mutations in Gaza Strip population.

1.3 Significance

- Optimizing techniques for rapid detection of investigated CFTR mutations in Gaza Strip.
- Decreasing the birth of CF children by recommending CF families to undergo preimplantation genetic diagnosis (PGD) and prenatal diagnosis.
- Minimizing the CF complications through the early diagnosis of the disease. Additionally, disease prognosis is associated with the type of CFTR mutation.
- Identifying CF patients who can benefit from mutation-specific treatment.
1.4 Limitations

Due to lack of adequate budget we could not make any DNA sequencing work for the CFTR gene.

1.5 Overview of Thesis

The thesis contents are covered within six chapters. Chapter one which presents the disease, definition, severity, prevalence and the objectives, significance and the limitations of this study.

In the second chapter, we describe in detail the disease, CFTR gene, CFTR protein, CFTR mutations, major diagnostic laboratory tests for CF and we review relevant studies that identified CFTR mutations in different populations.

The third chapter describes our approach to detect the common CFTR mutations in the recruited samples. This includes the materials we used and the methods we followed.

In Chapter four, we present the results of our work.

In Chapters five and six, we discuss our results in the light of previous studies. Finally, we outline the results in a brief conclusion and present our proposed recommendations.
Chapter 2

Literature Review
Chapter 2
Literature Review

2.1. Cystic Fibrosis (CF)

CF is a chronic, progressive, multiorgan genetic disease related to the buildup of thick, sticky mucus. The cystic fibrosis name is derived from the fibrous scar tissue which developed in the pancreas. CF can damage the pulmonary, pancreatic, gastrointestinal, and the reproductive systems (Schwarz et al., 2009).

The disease causes the lungs to be clogged with thick mucus which leads to a vicious cycle of infection, inflammation and lung tissue destruction, ultimately leading to life-threatening lung infections. The secreted mucus also clogs the pancreatic ducts, thereby obstructing the flow of bile digestive enzymes (which help in digestion and absorption of the food, especially of fats) to the small intestine. This leads to multitudes of disease conditions such as pancreatic insufficiency, gall stones, cysts, and chronic digestive problems (maldigestion and malabsorption) (Banjar and Angyalosi, 2015).

Moreover, there are also other complications may be associated with the disease: most males suffer from infertility; older patients may evolve CF-related diabetes that require multiple insulin injections daily; liver disease and hypertension may develop; nasal polyps and sinusitis are common. Psychological and behavioral problems may also appear due to severe long-treatment medical condition (NHS standard contract for cystic fibrosis, 2013).

CF is an autosomal recessive disorder, which means both parents must carry an abnormal copy of the gene for the problem to occur in their child. A person who has one copy of the abnormal gene for a recessive disorder is a carrier for that disorder, although he or she may not show any signs of it. If both parents are carriers, each of their children has a 25% chance of having the disorder (Cystic Fibrosis: Prenatal Screening and Diagnosis, 2016).
CF results from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, located on the long arm of human chromosome 7 at the q31.2 locus, with 27 exons. When both alleles fail to produce the CFTR protein in its functional form, an individual is likely to develop CF. On both CFTR alleles, a CF patient can carry, either the same or two different CFTR mutant alleles (Al-Kindy et al., 2014).

CF is most prevalent in European and European-derived populations. The spread of CF is highest in Caucasians and Ashkenazi Jews; where one child in every 2,500 and 2,300 births is affected, respectively. However, CF is present in other populations such as Native Americans (1 in 10,900), African Americans (1 in 15,000), and Asians (1 in 35,000). Although it was rarely considered in those of non-Caucasian origin, CF has now been found in all ethnic groups, with an evaluated prevalence of 1 in 10,000 births among Arabs and with affected individuals from India, Iran, South East Asia and Turkey (Siryani et al., 2015).

2.2. CFTR gene

The CFTR gene is the seventh member of the ATP-binding cassette sub-family C (ABCC7). The gene, discovered in 1989, spans 188,702 bp of genomic DNA at chromosomal location 7q31.2, with its coding sequence distributed over 27 exons that is transcribed into 6129 bp mRNA which in turn codes for the 1480 amino acids CFTR protein (Alibakhshi and Zamani, 2006; The CFTR mutations database, 2013).

2.3. CFTR protein

The cystic fibrosis transmembrane conductance regulator (CFTR) protein is a cAMP-regulated chloride channel located primarily at the apical or luminal surfaces of epithelial cells in the airway, intestine, pancreas, kidney, sweat gland, as well as male reproductive tract (Li and Naren, 2010).

The CFTR protein is made up of two repeated parts (Figure 2.1); each contains a transmembrane domain (TMD1 or TMD2); each part includes six transmembrane helices (M1–M6 and M7–M12) and two nucleotide binding domains
"NBDs" (NBD1 and NBD2). Both parts are linked by a cytoplasmic hydrophilic regulatory (R) domain, which is comprised of many charged amino acids and required for protein kinase A sensitivity of channel gating. The R-domain is a unique feature of CFTR within the ABC superfamily (Vankeerberghen et al., 2002).

The ordinary function of epithelial cells in the lungs, pancreas, intestine, gallbladder, and sweat glands depends mainly on the CFTR channel (Akhavan-Niaki et al., 2011). The charged chloride ions are negatively transported into and out of the cells across the channel. This transport of chloride ions enhances the control of water movement in tissues, which is requisite to produce thin, freely flowing mucus. Mucus is a slippery substance that lubricates and protects the lining of the airways, digestive system, reproductive system and other organs and tissues (Dell’Edera et al., 2016).

The CFTR dysfunction can be harmful and may lead to life-threatening disorders. CFTR hypofunctioning due to genetic defects results in cystic fibrosis, whereas CFTR hyperfunctioning due to various infections results in secretory diarrhea, the main cause of death in early childhood. Therefore, keeping a dynamic balance between CFTR up-regulating processes and CFTR down-regulating processes is critical for maintaining fluid and body homeostasis (Li and Naren, 2010).
Figure (2.1): The CFTR Schematic diagram shows the topology of the membrane and the organization domain. TRL: Threonine, Arginine, Leucine. (Adopted from: How does VX809 help F508del Part 2, 2013).

When c-AMP levels inside a secretory epithelial cell increased, the protein kinase A is activated which binds the phosphorylation site on the (regulatory) R-domain of the CFTR protein thus opening the channel. The CFTR chloride channel essentially works as an electrostatic attractant by drawing intracellular and extracellular anions toward positively charged transmembrane domains inside the channel. The CFTR protein has 12 transmembrane (TM) domains. Two of these (TM1 and TM6) attract and bind chloride (and/or bicarbonate) ions. As the chloride ions bind to these sites in the pore, the mutual repulsion accelerates expulsion of the ions from the cell. When normally functioning CFTR is activated, chloride ions are secreted out of the cell. However, in addition to chloride ion secretion, the epithelial sodium channel (ENaC) is also inhibited by CFTR and less sodium is absorbed into the cell, leaving a greater combined ionic gradient to allow water to leave the cell by osmosis, providing fluid for epithelial tissue secretions. In CF these mucus secretions become hyperviscous and it is this which accounts for the principal features of cystic fibrosis (Schwarz et al., 2009).
2.4. *CFTR* mutations

About 2,000 CFTR-causing mutations were reported. These mutations have a diverse effects on the protein product (http://www.genet.sickkids.on.ca/Home.html, 2011). *CFTR* mutations include insertions, deletions, splice site mutations, nonsense mutations as well as more than 650 missense mutations (Schwarz et al., 2009).

Although most mutations are rare, the three-base-pair deletion p.Phe508del is the most prevalent in the Caucasian population affecting about 70% of the patients whereas in the Jewish population the W1282X is the most common with a frequency of 60%. Which clearly shows that the occurrence of mutations is highly population specific. For many ethnic or geographic populations, the mutation spectrum has been determined (Essawi et al., 2015).

In the Middle East, the incidence rate of cystic fibrosis differs according to ethnic origin and the degree of consanguinity. In the Arab world, consanguinity is estimated to be around 65%, with estimates ranging between 1 in 2,560 and 1 in 15,876. Some of the mutations in the Middle East are also found in a number of other regions in the world. These include F508del, N1303K, W1282X and 3120+1G>A, although this last is more often found in people of African descent and may have spread from African to Arabic populations through mixed marriage (Kambouris et al., 2000)

There are mutations that appear to be more widely spread throughout the Middle East but are rarely observed elsewhere. In some cases, these more frequent mutations may be specific for a subset of the people in the Middle East defined by a common ethnic or religious background, e.g., the 1548delG mutation in Saudi Arabia, Bedouin tribes in the case of I1234V, the S549R (T > G) mutation in Bedouins from the United Arab Emirates and Oman, Muslim Arabs in case of CFTRdele2 (ins186), Christian Arabs in case of 4010delTATT and the 548A > T mutation in Bahrain (El-Falaki et al., 2014).
Therefore, CF also explains that, in addition to its indigenous founder mutations, the geographic location and ethnic admixture has made the Middle East a “melting pot” of different genetic influences from outside and over time (The molecular genetic epidemiology of cystic fibrosis, 2002).

2.4.1. Previous studies

EL-Shanti et al. (2014) identified nine CFTR mutations in Gaza Strip. These were: F508del, N1303K, 3120+1G>A, 1717+1G>A, G85E, G542X, 1209G>A, W1282X and 3120+1kbdel8.6kb (El-Shanti et al., 2014).

Essawi et al. (2014) identified 17 different mutations in 40 Palestinian CF patients which have previously been described as CF-causing mutations, including R75X, G85E (8%), R347P, 1525-1G>A, 1341+1G>A, ΔF508 (34%), 1717-1G>A, G542X, 2183AA>G (8%), 2221insA, 3120+1Kbde18.6Kb (13%), G1265R, D1270N, W1282X (31%), N1303K (5%) and 4382delA (Essawi et al., 2015).

Laufer-Cahana et al. (1999) conducted the CFTR mutation analysis on 42 unrelated Israeli Arab CF patients and revealed 12 different mutations. These were: N1303K, F508del, 3120+1Kbde18.6Kb, G85E, W1282X, R75X, 2183AA>G, 4010delTATT, S549R(T>G), del(exon2), G542X and S549R(A>C) (Laufer-Cahana et al., 1999).

Kambouris et al. (2000) revealed eight novel mutations of CFTR mutations among Arab populations. These were: 425del42, 475G>T, 548A>T, 711+1G>A, 1548delG, 1729T>C, 1811+2 and 3361A>T. The comparative frequencies of the most common mutations were: 1548delG> I123V=DF508=3120+1G>A > H139L. Screening for these five mutations identifies 60% of the CF alleles in Arab populations. The authors showed that the novel mutation1548delG is the most frequent (17%) among Arabs (Kambouris et al., 2000).

Al-Kindy et al. (2014) studied the clinical and the genetic aspects of CF in Oman. The mutation prevalence of CF in Oman is very similar to the one in neighboring countries with two major mutations, the p.S549R, which is the most common, ΔF508, 3120+1G>A, L578delTA, p.A357T, and 3849+10kbC>T. The
invention of this study was to identify two new CF-causing mutations, L578delTA and p.A357T (Al-Kindy et al., 2014).

El-Harith et al. (1997) identified six different CFTR mutations in CF Saudi Arab children. Among these mutations there were two novel mutations, 406-2A>G and 1548delG and the other mutations were 2043delG, 3120+1 G>A, I1234V and N1303K (El-Harith et al., 1997).

Siryani et al. (2015) identified nine different CFTR mutations in a group of patients living in Palestine. These mutations were 1525-1G>A which is the most frequent, W1282X, F508del, G85E, N1303K, 444delA, deletion exons 17a-17b-18, deletion exons 17a-17b and Q1100P. (Siryani et al., 2015).

Shahin et al. (2016) identified eleven different CFTR mutations in the Egyptian CF population. These were F508 del, N1303K, 2183AA/G, W1282X, I148T, G155D, 3199del6, CFTRdel2-3 (21 KB) ,R1162X, R347P and A544E (Shahin et al., 2016).

The manifestation and severity of the disease depends on the type of mutation and several studies have examined the relationship between the phenotype and the genotype (Schwarz et al., 2009).

Despite the total loss of the CFTR protein in exocrine tissues significantly affects the viscosity and thus the transfer of secretions, other CFTR mutations are not always well associate with the observed CF phenotype. This absence of association may be due to environmental factors and/or other compound genetic factors (Schwarz et al., 2009).

**2.4.2. Classes of CFTR mutations**

CFTR mutations are clustered in six different classes, demonstrated in figure (2.2), depending on their molecular mechanisms and consequences for different aspects of CFTR biogenesis, metabolism and function (The CFTR mutations database, 2013).
**Class I:** mutations that interfere with protein synthesis. They trigger the introduction of a premature signal to terminate the translation (stop codon) in the mRNA: nonsense, frameshift or severe splicing mutations. The truncated proteins are unstable, rapidly degraded, so, the net effect is no protein at the apical membrane (p.Gly542X, 621+1G>T, 711+1G>T). 50% of all CFTR mutations are belonged to this class (The CFTR mutations database, 2013; Schwarz et al., 2009).

**Class II:** Mutations that affect the maturation of CFTR protein. They result in the production of a protein that cannot be properly folded and trafficked to the site of its function on the apical membrane (p.Phe508del, p.Asn1303Lys, p.Ile507del) (The CFTR mutations database, 2013).

**Class III:** Mutations that change the regulation of the channel. The resulting mutated protein is correctly trafficked and localized to the plasma membrane but cannot be activated or function as a chloride channel (missenses located within the NBD), (p.Gly551Asp) (The CFTR mutations database, 2013).

**Class IV:** Mutations that affect the conductance of chloride. The CFTR protein is properly trafficked to the plasma membrane but lead to low chloride flow (most are missenses located within the membrane-spanning domain), (p.Arg117His and p.Arg334Trp) (The CFTR mutations database, 2013).

**Class V:** Mutations that reduce the normal performance level of CFTR at the apical membrane (partially aberrant splicing mutation or inefficient trafficking missenses ((5T allele, 3272-26A>G and 3849+10kbC>T) (The CFTR mutations database, 2013).

**Class VI:** Mutations that reduce the stability of CFTR present or that affect the regulation of other channels. Although these mutations have been observed in a few CF patients, most class VI mutants should be considered as severe (The CFTR mutations database, 2013).
EL-Shanti et al. (2014) classified the nine CFTR mutations which were identified in Gaza into three classes and reported that 51.2% of them belonged to class II, 19.5% to class V, 4.9% to class IV and 22% could not be assigned to any class (El-Shanti et al., 2014).

Table (2.1): Classes of the selected CFTR mutations

<table>
<thead>
<tr>
<th>Class I</th>
<th>Class II</th>
<th>Class III</th>
<th>Class IV</th>
<th>Class V</th>
</tr>
</thead>
<tbody>
<tr>
<td>W1282X 1548delG</td>
<td>S549R</td>
<td>H139L</td>
<td>I1234V</td>
<td>3120+1kb</td>
</tr>
<tr>
<td>G115X 711+1G</td>
<td>F508del</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4010del4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mutations fall within classes I-III and VI are considered as "severe" as they give little or no functional CFTR protein at the apical membrane and usually leading to a classic CF phenotype with pancreatic insufficiency (CF-PI) and lung disease with different severity. On the other hand, mutations fall within classes IV and V retain some activity of residual CFTR and give a more moderate phenotype.
For patients they have at least one “mild” CF allele, the function of CFTR is usually sufficient for digestion and the lung disease is less severe. Individuals with CFTR mutants that lead to about 10% of the normal level of CFTR mRNA may present with only one symptom, such as congenital bilateral absence of vas deferens (CBAVD) in males. Some patients who are heterozygous for a CFTR mutation may be more susceptible to "CFTR-related disorders" (CFTR-RD) such as sinusitis and pancreatitis. In these cases, genetic predisposition and environmental or genetic modifiers (eg. mannose-binding lectin, glutathione-S-transferase, transforming growth factor-beta1 and tumor necrosis factor-alpha (Merlo and Boyle, 2003)) can add other effects in addition to CFTR mutations (The CFTR mutations database, 2013).

Grouping mutations into different classes is useful to understand the mechanism of dysfunction, however many sequence variations are rare/unique and available data do not allow to evaluate their pathogenicity. Moreover, a single mutation can cause more than one type of abnormality or may confer variable phenotype (The CFTR mutations database, 2013).

2.5. Diagnosis

Accurate and early diagnosis of CF is necessary to manage the disease. Several criteria must be met to diagnose the CF, these include having one or more typical clinical manifestations, a family history of CF, or a positive newborn screening test, as well as laboratory evidence of CFTR dysfunction (Wang and Freedman, 2002).

In the past, the laboratory test of abnormal CFTR function was based largely on an elevated sweat chloride test result. The recent development of a genotypic CFTR mutation screen has greatly improved diagnostic accuracy and increased the likelihood of early diagnosis (Wang and Freedman, 2002). It should be useful as early diagnosis and treatment reduce symptoms, improve health and reduce costs associated with disease complications (Breathing in America: Diseases, Progress, and Hope, 2010).
**Major Diagnostic Laboratory Tests**

The major tests used to detect CFTR abnormality include:

### 2.5.1. Sweat test

Sweat test is the most common diagnostic test for cystic fibrosis. The test, which is described for the first time in 1959, measures the amount of salt in the sweat as the CF patients have a high amount of salt in their sweat. The test can be performed at any time from 1 week of age, but sufficient amounts of sweat may not be obtained before 6 weeks of age.

The reference values for the concentration of chloride in sweat are as follows:

- More than 60 mmol/L: consistent with CF (mean value around 100 mmol/L)
- A range between 40 and 60 mmol/L: borderline
- Less than 40 mmol/L: negative (mean value around 30 mmol/L) (Wang and Freedman, 2002).

Although the test can give an initial indication for the presence of the disease, there are some drawbacks that require further tests to confirm its result. One of these drawbacks is that the newborns may not produce enough amount of sweat, so the test may not work well (National Institutes of Health, 1995). On the other hand, a small proportion of patients with cystic fibrosis have normal chloride levels in their sweat and therefore molecular tests can be used (National Institutes of Health, 1995).

### 2.5.2. Genetic testing

Genetic testing is a more valuable and accurate test for CF diagnosis. The test checks the CFTR mutations in DNA samples of people they are suspected to have the disease. If two CFTR mutations are identified, this confirms the diagnosis of CF. In all patients who have been diagnosed with CF by a sweat test, the identification of the CFTR mutations provides more information about genotype/phenotype correlations and can be used to test a series of extended family (Smyth, 2005).

DNA can be obtained from white blood cells, mouthwash samples or buccal scrapes. After DNA extraction, samples are tested using one of the commercially available kits. Sufficient gene mutations should be tested to account for a high
proportion of known gene mutations in the region, or a specific ethnic group of the patient. Further testing can be done for other CFTR mutations if only one, or none, of the gene mutations are identified. When a diagnosis of CF is confirmed in a patient, it is good practice to investigate all other siblings, even those who do not have clinical features of the condition (Smyth, 2005).

2.5.3. Preimplantation Genetic Diagnosis (PGD)

PGD is a technique used to detect genetic defects in embryos who are created through in vitro fertilization (IVF) before pregnancy. The test is used when one or both parents has a known genetic abnormality and testing is performed on an embryo to determine if it also carries a genetic abnormality. If both parents are determined to be carriers, they may opt for IVF, where the resulting embryos can be tested for the disease and only healthy ones are implanted into the mother's uterus (Dayal, 2015).

PGD was developed in the UK in the mid-1980s as an alternative to current prenatal diagnoses, where it is presently applied for more than 300 different inherited disorders. The most frequent PGD indication is CF (Dayal, 2015; Rechitsky et al., 2013).

In a PGD for CF patients review, Rechitsky et al. (2013) showed very high accuracy of PGD for CF. In addition to the ability to test for one, two or three CFTR mutations simultaneously, the test can also test another genetic disorder (Rechitsky et al., 2013).

2.5.4. Antenatal testing

Antenatal testing is a test can be performed during pregnancy on the placenta (chorionic villus sampling) or the fluid around the fetus (amniocentesis). Testing. This can be carried out from 10 weeks of pregnancy. The test may be dangerous to the fetus as the chorionic villus sampling has a fetal death risk of 1 in 100 and amniocentesis of 1 in 200 (Fetal Screening, 2017).
2.5.5. Newborn screening

Newborn or neonatal screening is a test performed after birth on newborns and it became possible with the development, in 1979, of a radioimmunoassay for immunoreactive trypsin (IRT) (Smyth, 2005; Newborn screening, 2007).

IRT test is used as part of some newborn screening programs to screen for cystic fibrosis (CF). It may be used in conjunction with a sweat chloride test and/or a cystic fibrosis gene mutation panel to help identify CF (lab tests on line, 2017). IRT concentrations are increased in the first few weeks of life in babies with CF. Then, IRT level decreases after 1-2 months of age and therefore it becomes unreliable (Wang and Freedman, 2002). The test can be done on dried blood spots from a heel-prick test during 48 to 72 hours after birth (Newborn screening, 2007).

Ferrie et al. (1992) applied ARMS technique to detect 11 CFTR mutations found in the northwest of England using single ARMS tests. The researchers also develop a test that can detect the presence of the most common mutations (∆F508, G551D, G542X, and 621 + 1G>T) in a DNA sample (Ferrie et al., 1992).

Soloviov et al. (2010) used ARMS/PCR in the screening for ∆F508, R117H, W1282X, 2143delT, 621+1G>T mutations. designed the primers and optimized the conditions of ARMS-PCR. It was found that the ARMS technique is a reliable, fast and inexpensive method. (Soloviov et al., 2010).


Ashavaid et al. (2012) determined the frequency of six of the most common mutations of the CF world population including ∆F508, G551D, G542X, N1303K, R553X and 621+1G>T mutations in 23 suspected cases of Indian CF patients, using multiplex ARMS-PCR technique (Ashavaid et al., 2012).
2.6. Cystic fibrosis mutation specific treatment

The majority of cystic fibrosis therapies have been focused on treating the disease symptoms, such as clearing mucus from the lungs, treating infections and pancreatic enzyme replacement therapy (PERT). These treatments aim to lessen cystic fibrosis symptoms, and do not act on the fundamental cause of this disease. However, several companies took a different approach and focused on improving defective CFTR function as a novel way of treating cystic fibrosis (New Mutation-Specific Targeted Therapy for Cystic Fibrosis, 2016).

The recognition of the various molecular mechanisms of CFTR dysfunction, and thus the classification of CFTR mutations according to this, provides the scientific basis to develop drugs targeting a specific CF mutation (Kerem, 2006).

Mutation-specific therapies refer to potential treatments targeting the underlying genetic cause of a person's cystic fibrosis as there are various types of mutations in the CFTR gene that cause CF (Duchenne Connect., 2017).

These therapies include the use of compounds that have an effect on the cell function, involving the synthesis, trafficking and functioning of CFTR (Wilschanski, 2010).

Three major mutation-specific treatment approaches have been adopted to improve the function of CFTR: First, potentiators restore the function of CFTR at the apical surface of epithelial cells that is disrupted in class III and IV mutations. Second, correctors improve CFTR intracellular processing, increasing surface expression, in class II mutations. Last, production correctors or read-through agents promote transcription of CFTR in class I mutations (Brodlie et al., 2015).

Class I mutations, including mostly nonsense, frameshift, or missense mutations, (The most common are G542X and W1282X) are due to a premature termination codon (PTC), which terminates CFTR protein translation too early. The truncated protein is non-functioning and does not reach the cell surface. Mutation specific treatment approach is applied here through the use of Aminoglycoside antibiotics (Gentamicin and Ataluren) that in addition to their antimicrobial activity
can also suppress PTCs by inserting a random amino acid that allow the ribosome to read through and leads to the production of full-length protein ((Thursfield and Davies, 2013; Kerem, 2006).

Class II Mutations, including Phe508del, mostly lead to CFTR misfolding and eventually premature degradation. Even though it was classified within class II, F508del can also belong to class III mutations when it is rescued and inserted in the plasma membrane, resulting in defective regulation which is a characteristic of class III mutations (Rogan et al., 2011). Lumacaftor partially restored Phe508del-CFTR expression, resulting in up to 15% of wild-type channel activity in vitro, but failed to show significant benefit in the clinical trial. However, the combination of Lumacaftor (corrector), which is designed to increase CFTR level on the cell surface and Ivacaftor (potentiator), which is designed to enhance channel function once CFTR reaches the cell surface, showed significant clinical benefit (New Mutation-Specific Targeted Therapy for Cystic Fibrosis, 2016).

Class III mutations, including S549R and G551D, result in defective channel gating at the cell membrane and therefore the chloride transport function is impaired. Ivacaftor, the first mutation-specific drug, targets directly the gating defect of the class III mutations by increasing the opening time of the activated CFTR channels at the cell surface (a “potentiator”) (Brodlie et al., 2015; Ramsey et al., 2011).

Class IV mutations, including R117H, are mostly missense mutations that affect CFTR channel conductance. While no treatments are currently being sought for this mutation class, CFTR potentiators may be useful. (Thursfield and Davies, 2013).

Class V mutations result in the reduction of normal CFTR performance level at the apical membrane, this is mainly due to defective splicing and generation of both normal and aberrant transcripts. Potentiators could be of use in patients with these mutations. Another option would be to target these mutations with the use of anti-sense oligonucleotides (AONs). Antisense oligonucleotides correct splicing defects through exon skipping (CFTR-modulating therapies, 2017).
Class VI mutations result in the reduction of the stability of CFTR at the cell surface. Agents targeting these mutations should enhance retention and anchoring of the CFTR protein on the surface of the cell. This may include targeting cell signaling processes, such as activating Rac-1 signaling to encourage anchoring to the actin cytoskeleton (CFTR-modulating therapies, 2017).

The further progress in this therapeutic strategy confers a great hope for a better cystic fibrosis treatment in the future (Brodlie et al., 2015).
Chapter 3
Materials and Methods
Chapter 3
Materials and Methods

3.1. Materials

3.1.1. Equipment
The present work was carried out in the Genetics lab at the Islamic University of Gaza. The major equipment used in the study are listed in Table 3-1.

Table (3.1): The major equipment used in this study.

<table>
<thead>
<tr>
<th>Item</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Thermal Cycler</td>
</tr>
<tr>
<td>2</td>
<td>Horizontal electrophoresis chambers/tanks</td>
</tr>
<tr>
<td>3</td>
<td>Electrophoresis power supply</td>
</tr>
<tr>
<td>4</td>
<td>Digital balance</td>
</tr>
<tr>
<td>5</td>
<td>Vortex mixer</td>
</tr>
<tr>
<td>6</td>
<td>Gel documentation system</td>
</tr>
<tr>
<td>7</td>
<td>Safety cabinet</td>
</tr>
<tr>
<td>8</td>
<td>Microcentrifuge</td>
</tr>
<tr>
<td>9</td>
<td>Freezer, refrigerator</td>
</tr>
<tr>
<td>10</td>
<td>Micropipettes</td>
</tr>
<tr>
<td></td>
<td>0.1-2.5 μl</td>
</tr>
<tr>
<td></td>
<td>0.5-10 μl</td>
</tr>
<tr>
<td></td>
<td>5-50 μl</td>
</tr>
<tr>
<td></td>
<td>20-200 μl</td>
</tr>
<tr>
<td></td>
<td>100-1000 μl</td>
</tr>
<tr>
<td>11</td>
<td>Microwave Oven</td>
</tr>
</tbody>
</table>
3.1.2. Chemicals, Kits, and Disposables
Chemicals, kits and disposables used in this study are listed in **Table 3-2**.

**Table (3.2):** Chemicals, kits and disposables used in this study.

<table>
<thead>
<tr>
<th>Item</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Wizard ® Genomic DNA Purification Kit</td>
<td>Promega (Madison, USA)</td>
</tr>
<tr>
<td>2 PCR Go Taq® Green Master Mix</td>
<td>Promega (Madison, USA)</td>
</tr>
<tr>
<td>3 Agarose</td>
<td>Promega (Madison, USA)</td>
</tr>
<tr>
<td>4 PCR primers</td>
<td>Promega (Madison, USA)</td>
</tr>
<tr>
<td>5 Nuclease free water</td>
<td>Promega (Madison, USA)</td>
</tr>
<tr>
<td>6 Ethidium Bromide (EtBr) 10mg/ml</td>
<td>Promega (Madison, USA)</td>
</tr>
<tr>
<td>7 DNA molecular size marker (Ladder).</td>
<td>BioLab, New England (UK)</td>
</tr>
<tr>
<td>8 Restriction enzymes</td>
<td>BioLab, New England (UK)</td>
</tr>
<tr>
<td>9 Absolute Isopropanol and Ethanol 70%</td>
<td>(Sigma USA)</td>
</tr>
<tr>
<td>11 Microfuge tubes for PCR - thin wall 0.2 mL</td>
<td>Labcon, USA</td>
</tr>
<tr>
<td>12 Microfuge tubes - 1.5 mL capacity</td>
<td>Labcon, USA</td>
</tr>
<tr>
<td>14 Disposable tips</td>
<td>Labcon, USA</td>
</tr>
<tr>
<td>15 Disposables Gloves Free Powder</td>
<td>Weihai Sun Genius, China</td>
</tr>
</tbody>
</table>
3.2. Study sample

3.2.1. Study design

The current study is a descriptive analytical study.

3.2.2. Study location

Genetics lab-Islamic University, Gaza strip.

3.2.3. Study subjects

A total of 30 unrelated cystic fibrosis patients have participated in this study. There were 60% (18/30) male patients and 40% (12/30) females. The patients were from the different regions of Gaza strip, Palestine. The criteria for including participants in this study were based on previous clinical diagnosis of patients.

All study subjects were recruited from the Cystic Fibrosis Friend Center in Gaza.

3.2.4. Ethical considerations

Informed consent was taken from all the subjects who participated in the study (Appendix 1). The objective of the study was fully explained to all participants and their consent was taken.

3.3. Genotyping:

3.3.1. DNA extraction

About 2.0 ml of venous blood were drawn into sterile EDTA tubes. Samples were stored at 4–8°C pending DNA extraction. Genomic DNA was isolated from blood using Wizard Genomic DNA Purification Kit (Promega, USA) following the manufacturer instructions. The isolated DNA was stored at -20 °C until analysis of CFTR gene mutations.

3.3.2. PCR primers reconstitution

Primers were received in a lyophilized state. Primer containers were first centrifuged at 13,000 rpm for 3 minutes, and then reconstituted with ultrapure water (nuclease free water) to create a stock solution of each primer with a final concentration of 100 pmol/μl. The stock primer solution was then vortex mixed. Then, 10 μl aliquot was taken from the stock primer and diluted with 90 μl nuclease-
free water to make 10 pmol/μl working solution.

3.3.3. Detection of CFTR mutations

3.3.3.1. Allele refractory mutation system (ARMS)-PCR method

ARMS is a PCR-based method, which uses allele-specific priming for the detection of any mutation involving single base changes or small deletions (Little, 2001). This approach implies two PCR reactions. Amplification of the normal allele is accomplished using a primer complementary to the normal allele. Conversely, only the mutant allele will be amplified if the 3’ residue is complementary to the mutant sequence. Thus, as a result a normal individual generates PCR product only in the normal reaction; a heterozygote gives products in both reactions, and a homozygous mutant individual gives amplification only in the mutant reaction (Soloviov et al., 2010).

This method has many advantages over the traditional methods of mutation detection: it is rapid (the analysis is done in 2–3 hours), inexpensive, reliable when a positive control sample is used. However, the ARMS method depends on several factors: proper primers design and optimization of PCR conditions to avoid nonspecific amplification of the normal/mutant allele that could bring false positive or negative results (Soloviov et al., 2010).

The mutations F508del, 3120+1kb, WI282X and 1548delG were analyzed by ARMS-PCR technique.

Polymerase chain reaction (PCR) was carried out in a total volume of 20 μl. The primers and lengths of PCR products are shown in Table 3.4 and the reaction components were as described in Table 3.3.

Microfuge tubes were then placed in a thermal cycler and for all mutations PCR amplification was carried out by the program provided in Table 3.5 except the difference in the annealing temperatures as indicated in Table 3.4.
Table (3.4): PCR primers, annealing temperatures and lengths of PCR products for genotyping CFTR mutations by ARMS-PCR method.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer sequence (5' - 3')</th>
<th>Annealing Temp. (°C)</th>
<th>PCR product size (bp)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1548delG</td>
<td>C: TGGAGGCAAGTGAATCCCTGA N: CTGAAGGCTCCAGTTCTCCC M: CTGAAGGCTCCAGTTCTCCA</td>
<td>60</td>
<td>106</td>
<td>Primer 3 software</td>
</tr>
<tr>
<td>F508del</td>
<td>C: ACC ATT AAA GAA AAT ATC ATC TT N: ACC ATT AAA GAA AAT ATC ATT GG M: TGC AAG CTT CTT AAA GCA TA</td>
<td>60</td>
<td>262</td>
<td>Perone et al., 2010</td>
</tr>
<tr>
<td>3120+1kb</td>
<td>C: CTT ACC ATA TTT GAC TTC ATC CAG G N: CTT ACC ATA TTT GAC TTC ATC CAG A M: TTA CTA AAC TTA TGT CTA TTT TTA AGG C</td>
<td>62</td>
<td>235</td>
<td>Perone et al., 2010</td>
</tr>
<tr>
<td>W1282X</td>
<td>C: GGG ATT CAA TAA CTT TGC AAC AGT GG N: GGG ATT CAA TAA CTT TGC AAC AGT GA M: TCT GCC TAT GAG AAA ACT GCA CTG GAG</td>
<td>67</td>
<td>203</td>
<td>Perone et al., 2010</td>
</tr>
</tbody>
</table>

Legend: C: Common primer; N: Normal allele-specific primer; M: Mutant allele-specific primer.

Table (3.3): ARMS-PCR components for amplification of the CFTR gene fragments.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common primer</td>
<td>2</td>
</tr>
<tr>
<td>Mutant primer / Normal primer</td>
<td>2</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>4</td>
</tr>
<tr>
<td>PCR master mix (2X)</td>
<td>10</td>
</tr>
<tr>
<td>DNA</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>
Table (3.5): Thermal cycler program for ARMS-PCR amplification of the CFTR gene target fragments.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp.</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>94 °C</td>
<td>5 min.</td>
</tr>
<tr>
<td>Annealing and extension</td>
<td>94 °C</td>
<td>1 min.</td>
</tr>
<tr>
<td>(35 Cycles)</td>
<td>annealing temp. 72°C</td>
<td>30 sec.</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>10 min.</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td></td>
</tr>
</tbody>
</table>

3.3.3.2. Tetra-ARMS-PCR method

The Tetra-ARMS is a PCR-based method, which uses four primers in a single PCR tube to determine the genotype. In the beginning of the reaction, two non-allele-specific primers amplify the region that comprises the SNP. They are named outer primers, then as the outer primers fragment is produced, it serves as a template to the two allele-specific primers (inner primers) which will produce the allele-specific fragments. By placing the outer primers at different distances from the polymorphic nucleotide, the two allele specific fragments can be distinguished by their different sizes in an agarose gel (Medrano and de Oliveira, 2014).

The use of tetra-primer ARMS–PCR attends the expectations of modern genomic research and allows the study of SNPs in a fast, reliable, and low-cost way (Medrano and de Oliveira, 2014).

Tetra-ARMS-PCR was applied in searching for 711+1G and H139L mutations. The primers were designed using a freely available "Primer1" software (http://primer1.soton.ac.uk/primer1.html) using the CFTR reference sequence flanking the mutations. PCR was carried out in a total volume of 20 μl. The primers and lengths of PCR products are shown in Table 3.7 and the reaction components were as described in Table 3.6. Microfuge tubes were then placed in a thermal cycler and for all mutations PCR amplification was carried out by the program provided in Table 3.8 except the difference in the annealing temperature as indicated in Table 3.7.
Table (3.7): PCR primers, annealing temperatures and lengths of PCR products for genotyping CFTR mutations by Tetra-ARMS-PCR method.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer sequence (5' - 3')</th>
<th>Annealing Temp. (°C)</th>
<th>PCR product size (bp)</th>
<th>Ref.</th>
</tr>
</thead>
</table>
| H139L    | Forward inner primer (T allele): TCTCTTTATTGTGAGGACACTGCTCTGCT  
           | Reverse inner primer (A allele): GTGATGAAGGCCAAAAATGGCTGAGT  
           | Forward outer primer (5' - 3'): GGGAAGAATCATAGCTTCTATGACCCG  
           | Reverse outer primer (5' - 3'): ACGATACAGAATATATGTGCCATGGGC | 63   | 231/151 | Primer 3 software |
| 711+1G>A | Forward inner primer (A allele): CAACAACCTGAACAAATTTGATGCAA  
           | Reverse inner primer (G allele): GCCTAAAAGATTAAATCAATAGGTACA  
           | Forward outer primer (5' - 3'): TTCTTTTAGACTTTAAGCTGCTCAAGCC  
           | Reverse outer primer (5' - 3'): TGCTCTGCTATACAATTTGACCTTTCTTA | 60   | 190/263 | Primer 3 software |

Table (3.6): Tetra-ARMS-PCR components for amplification of the CFTR gene.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward inner primer</td>
<td>1</td>
</tr>
<tr>
<td>Reverse inner primer</td>
<td>1</td>
</tr>
<tr>
<td>Forward outer primer</td>
<td>1</td>
</tr>
<tr>
<td>Reverse outer primer</td>
<td>1</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>4</td>
</tr>
<tr>
<td>PCR master mix (2X)</td>
<td>10</td>
</tr>
<tr>
<td>DNA</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>
Table (3.8): Thermocycler program for Tetra-ARMS-PCR amplification of the CFTR gene.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp.</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95 °C</td>
<td>3 min.</td>
</tr>
<tr>
<td>Annealing and extension (35 Cycles)</td>
<td>95 °C, annealing temp. 72°C</td>
<td>30 sec., 30 sec., 45 sec.</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>5 min.</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td></td>
</tr>
</tbody>
</table>

3.3.3.3. PCR-RFLP method

The mutations S549R, I1234V and 4010del4 were detected using standard polymerase chain reaction (PCR)/restriction fragment length polymorphism (RFLP) protocol (PCR followed by digestion with restriction enzyme). PCR was carried out in a total volume of 20 μl, the reaction components were as described in Table 3.9. The primers, lengths of PCR products, related restriction enzymes, as well as digested bands are shown in Table 3.10.

Table (3.10): PCR primers, annealing temperatures and restriction enzymes for genotyping CFTR mutations by PCR-RFLP method.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer sequence (5’-3’)</th>
<th>Annealing Temp. (°C)</th>
<th>Restriction enzyme</th>
<th>Digested bands</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>S549R</td>
<td>Forward primer: GGAAGATGTGCTTTTCAAATTCA Reverse primer: ACTAGCCATAAAAACCCCAGGA</td>
<td>59</td>
<td>DdeI</td>
<td>Normal 130+170 Mutant 300</td>
<td>Primer 3 software</td>
</tr>
<tr>
<td>I1234V</td>
<td>Forward primer: GGGGCCAAATGACTGTCAAA Reverse primer: TCTGCTAACACATTTGCTTCAGG</td>
<td>58</td>
<td>DdeI</td>
<td>Normal 178 Mutant 77 +101</td>
<td>Primer 3 software</td>
</tr>
<tr>
<td>4010del4</td>
<td>Forward primer: CTTGATGGTAACTGATGGGTG Reverse primer: GTTTTTCTAAATGTTCCAGaaaGA A Underlined G is mutated to generate XmnI site</td>
<td>57</td>
<td>XmnI</td>
<td>Normal 176 Mutant 139 +37</td>
<td>Primer 3 software</td>
</tr>
</tbody>
</table>
Table (3.9): PCR-RFLP components for amplification of the *CFTR* gene.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>2</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>2</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>4</td>
</tr>
<tr>
<td>PCR master mix</td>
<td>10</td>
</tr>
<tr>
<td>DNA</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>

Microfuge tubes were then placed in a thermocycler and PCR amplification was performed according to the program provided in Table 3.11. PCR products were digested with restriction enzymes following the instructions of the manufacturer. RFLP was carried out in a reaction mixture in a final volume of 20 μl by mixing PCR product with 10X Buffer, nuclease free water and the restriction endonuclease. The quantities and volumes were as shown in Table 3.12.

Table (3.11): Thermocycler program for PCR-RFLP amplification of the *CFTR* gene.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp.</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>94°C</td>
<td>5 min.</td>
</tr>
<tr>
<td>Annealing and extension (35 Cycles)</td>
<td>94 °C annealing temp. 72°C</td>
<td>1 min. 45 sec. 45 sec.</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>10 min.</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td></td>
</tr>
</tbody>
</table>

Table (3.12): The enzymatic digestion components of amplified *CFTR* gene.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product</td>
<td>7.5</td>
</tr>
<tr>
<td>Restriction endonuclease</td>
<td>0.5</td>
</tr>
<tr>
<td>10X Buffer</td>
<td>1</td>
</tr>
<tr>
<td>BSA</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10</strong></td>
</tr>
</tbody>
</table>

Microfuge tubes were then placed in a thermocycler at 37°C overnight to allow the restriction endonuclease to digest the PCR product. Digested PCR products
was then electrophoresed on 3.0% agarose gel and was visualized by ethidium bromide staining.

### 3.3.3.4. Allele Specific Mutation Analysis

Screening for G115X mutation was performed by allele-specific PCR (AS-PCR) The PCR was carried out in a total volume of 20 μl. The primers and lengths of PCR products are shown in **Table 3.13** and the reaction components were as described in **Table 3.14**.

Microfuge tubes were then placed in a thermal cycler and PCR amplification was carried out in a hybrid touch-down PCR according to the program provided in **Table 3.15**.

**Table (3.13):** PCR primers and lengths of PCR products for G115X mutation.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer sequence (5’ - 3’)</th>
<th>PCR product size (bp)</th>
<th>Ref.</th>
</tr>
</thead>
</table>
| G115X    | Forward primer (G allele): CTATGACCGGGATAACAAGG  
Reverse primer (G allele): AATGGCTGGGTGTAGGAGC  
Forward primer (T allele): TCCTATGACCGGGATAACAAGt  
Reverse primer (T allele): CTGGGTGTAGGACGAGTGC | Normal 103  
Mutant 100 | Primer 3 software       |

**Table (3.14):** PCR components for amplification of G115X mutation.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward inner primer</td>
<td>1</td>
</tr>
<tr>
<td>Reverse inner primer</td>
<td>1</td>
</tr>
<tr>
<td>Forward outer primer</td>
<td>1</td>
</tr>
<tr>
<td>Reverse outer primer</td>
<td>1</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>4</td>
</tr>
<tr>
<td>PCR master mix (2X)</td>
<td>10</td>
</tr>
<tr>
<td>DNA</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>
Table (3.15): Thermocycler program for PCR amplification of G115X mutation.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp.</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95°C</td>
<td>10 min.</td>
</tr>
<tr>
<td>Annealing and extension</td>
<td>94 °C</td>
<td>30 sec.</td>
</tr>
<tr>
<td>(35 Cycles)</td>
<td>67 °C</td>
<td>30 sec.</td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>1 min.</td>
</tr>
<tr>
<td></td>
<td>94 °C</td>
<td>30 sec.</td>
</tr>
<tr>
<td></td>
<td>65 °C</td>
<td>30 sec.</td>
</tr>
<tr>
<td></td>
<td>72 °C</td>
<td>1 min.</td>
</tr>
<tr>
<td>Final Extension</td>
<td>63°C</td>
<td>7 min.</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td></td>
</tr>
</tbody>
</table>

3.3.4. Agarose gel electrophoresis (3.0%)

1. Dried agarose gel (2.4 gm) was dissolved in 80 ml 1x Tris-Acetate-EDTA buffer (2M Tris base 1M Glacial Acetic Acid, 0.05 M EDTA) by heating.
2. Then 4.0 μl Ethidium Bromide (10mg/ml) was added and mixed, the gel was casted into a mold which was fitted with wells-forming comb.
3. The agarose gel was submerged in electrophoresis buffer within a horizontal electrophoresis apparatus.
4. After amplification, the PCR products and a DNA ladder size marker (Promega, Madison, WI, USA) were loaded into the sample wells to aid in fragment size determination.
5. PCR fragments were detected by size in the agarose gel.
6. Electrophoresis was performed by using a power supply (BioRad, USA) at 70 volts for 40 min at room temperature, and the DNA bands were visualized and documented using a UV trans-illuminator documentation system.
Chapter 4

Results
Chapter 4

Results

4.1. ARMS-PCR Genotyping Results

The following figures (4.1 through 4.4) represent in a respective manner, genotyping examples of the F508del, 3120+1kb, WI282X, and 1548delG CFTR mutations detected in this study. The first track in each pair is the product of the mutant (m) ARMS reaction, and the second is the product of the normal (n) ARMS reaction.

**Figure (4.1):** A photograph of ethidium bromide stained 3% agarose gel showing the ARMS-PCR products of F508del. M: 100 bp DNA ladder. Sample 1 indicates homozygous for mutant product (262bp) and sample 2 indicates the negative control (m=mutant; n=normal).

**Figure (4.2):** A photograph of ethidium bromide stained 3% agarose gel showing the ARMS-PCR products of 3120+1kb. M: 100 bp DNA ladder. Sample 1 indicates heterozygous for mutant product (350+235 bp) and sample 2 indicates the negative control.
Figure (4.3): A photograph of ethidium bromide stained 3% agarose gel showing the ARMS-PCR products of W1282X. M: 100 bp DNA ladder. Sample 1 indicates heterozygous for mutant product (203 bp) and sample 2 indicates the negative control.

Figure (4.4): A photograph of ethidium bromide stained 3% agarose gel showing the ARMS-PCR products of 1548delG. M: 100 bp DNA ladder. Sample 1 and 2 indicates homozygous for normal product (107 bp) and sample 3 indicates the negative control.
4.2. Tetra-ARMS-PCR Genotyping Results

The following figures (4.5 and 4.6) represent in a respective manner, genotyping examples of the 711+1G and H139L CFTR mutations detected in this study.

**Figure (4.5):** A photograph of ethidium bromide stained 3% agarose gel showing the Tetra-ARMS-PCR products of 711+1G. M: 100 bp DNA ladder. Samples 1 to 4 indicate heterozygous for normal product (129 + 263 bp) and sample 5 indicates the negative control.

**Figure (4.6):** A photograph of ethidium bromide stained 3% agarose gel showing the Tetra-ARMS-PCR products of H139L. M: 100 bp DNA ladder. Samples 1 and 2 indicate heterozygous for normal product (231+136 bp) and sample 3 indicates the negative control.
4.3. PCR-RFLP Genotyping Results

The following figures (4.7 through 4.9) represent in a respective manner, genotyping examples of the S549R, I1234V and 4010del4 CFTR mutations investigated in this study.

**Figure (4.7):** A photograph of ethidium bromide stained 3% agarose gel showing the PCR-RFLP products of I1234V. M: 100 bp DNA ladder. Samples 1 and 2 indicate homozygous normal (undigested) product (178 bp) and sample 3 indicates the negative control.

**Figure (4.8):** A photograph of ethidium bromide stained 3% agarose gel showing the PCR-RFLP products of 4010del4. M: 100 bp DNA ladder. Samples 1, 2 and 3 indicate homozygous normal (undigested) product (176 bp) and sample 4 indicates the negative control.
Figure (4.9): A photograph of ethidium bromide stained 3% agarose gel showing the PCR-RFLP products of S549R. M: 100 bp DNA ladder. Samples 1 and 2 indicate homozygous normal (digested: 130 + 170 bp) product and sample 3 indicates the negative control.

4.4. Allele Specific Mutation Analysis Results

The following figure (4.10) represents genotyping examples of the G115X mutation investigated in this study.

Figure (4.10): A photograph of ethidium bromide stained 3% agarose gel showing the allele specific mutation analysis products of G115X mutation. M: 100 bp DNA ladder. Samples 1 and 2 indicate heterozygotes for T\G genotype (100 + 103 bp) and sample 3 indicates the negative control.
4.5. Cystic Fibrosis Mutation Spectrum

In the present work 30 CF patients were screened for \textit{CFTR} gene mutations: (F508del, 3120+1kb, WI282X, S549R, 1548delG, I1234V, 711+1G, G115X, 4010del4 and H139L). The frequency of the encountered genotypes are presented in Table 4.1. The genotyping analysis of patients DNA samples showed the following results: 2 patients were heterozygous for (Δ F508/G115X ) and 1 patient was heterozygous for (W1282X/ G115X). Whereas in 6 patients only one mutation could be identified.

\textbf{Table (4.1):} Frequency of the observed genotypes in the cystic fibrosis patients.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ F508/G115X</td>
<td>2</td>
</tr>
<tr>
<td>W1282X/G115X</td>
<td>1</td>
</tr>
<tr>
<td>WI282X/???</td>
<td>2</td>
</tr>
<tr>
<td>Δ F508/???</td>
<td>2</td>
</tr>
<tr>
<td>3120+1kb/???</td>
<td>1</td>
</tr>
<tr>
<td>G115X/???</td>
<td>1</td>
</tr>
<tr>
<td>unknown</td>
<td>21</td>
</tr>
<tr>
<td>\textbf{Total}</td>
<td>\textbf{30}</td>
</tr>
</tbody>
</table>

The frequency of the mutant alleles among the investigated 60 chromosomes or alleles (of the 30 patients) is illustrated in Table 4.2.

\textbf{Table (4.2):} Frequency of the detected \textit{CFTR} mutant alleles.

<table>
<thead>
<tr>
<th>Mutant allele</th>
<th>Frequency</th>
<th>Percentage %</th>
<th>% of known mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ F508</td>
<td>4</td>
<td>6.67</td>
<td>33.33</td>
</tr>
<tr>
<td>3120+1kb</td>
<td>1</td>
<td>1.67</td>
<td>8.33</td>
</tr>
<tr>
<td>G115X</td>
<td>4</td>
<td>6.67</td>
<td>33.33</td>
</tr>
<tr>
<td>W1282X</td>
<td>3</td>
<td>5.00</td>
<td>25.00</td>
</tr>
<tr>
<td>Unknown</td>
<td>48</td>
<td>80.00</td>
<td>-</td>
</tr>
<tr>
<td>\textbf{Total}</td>
<td>\textbf{60}</td>
<td>\textbf{100}</td>
<td>\textbf{100}</td>
</tr>
</tbody>
</table>
As illustrated in the pie chart below (Figure 4.11) the mutations F508del and G115X represented the most common (33.33% each) mutations among the observed CFTR mutations. The second common mutation is the W1282X (25.00%) followed by 3120+1kb (8.33%). S549R, 1548delG, I1234V, 711+1G, 4010del4 and H139L were not detected in any of our CF patients.

**Figure (4.11):** An illustration of the percentages of the detected CFTR mutations.
Chapter 5
Discussion
Chapter 5
Discussion

Cystic fibrosis (CF) is the most common autosomal recessive disease in white Caucasians; it is however, considered to be rare in the Arab populations (Farra et al., 2010). The disease prevalence in Gaza strip is around 4 cases per 100,000 (El-Shanti et al., 2014) i.e., about 10 times less than its prevalence in white Caucasians. There are only few reports that describe the distribution and frequency of CFTR gene mutations in Palestine as illustrated in El-Shanti A. et al (2014), Essawi O. et al (2014) studies and Siryani I. et al (2015) studies. The aim of this study was to detect the common CFTR mutations and their prevalence in Palestinian CF population which enables the development of a better molecular diagnosis of CF.

The identification of the CF mutations commonly seen in any given population is valuable when mutation analysis is used as a ‘diagnostic test’ for CF (Ashavaid et al., 2012). Where the recognition of the type and prevalence of CF mutations in a specific population is a prerequisite to set up adequate and cost-effective molecular diagnostics and marks a new era for genetic counseling and prevention (Essawi et al., 2015). Therefore, knowing the most common CFTR mutations in our population and developing a specific mutation screening test will enable earlier and rapid diagnosis for our patients which have a great effect on raising the median survival age of the CF patients who usually die in infancy or early childhood in the 1950's to 40 years in 2014. (Siryani et al., 2015; Cystic Fibrosis Foundation, 2014).

Identifying common CFTR mutations in patients clinically presenting with CF symptoms is important not only to the patients and their relatives, but it also enables more reliable carrier detection in population-screening programs (Siryani et al., 2015). Carrier screening for cystic fibrosis involves analysis for common mutations in the CFTR gene from people with no personal history, or family history, of the disease. This analysis shows whether a person is a carrier, at risk (one in four) of having a baby with cystic fibrosis if their partner is also a carrier (Dell’Edera et
Such a program will be significant for the Palestinian population who has a very high risk to develop autosomal recessive diseases including CF, due to the high consanguinity rates.

Most cystic fibrosis (CF) cases, particularly in European whites, are caused by the F508del mutation in which one phenylalanine residue at position 508 in the CFTR gene is deleted (Koff, 2016). The mutant protein fails to traffic out of the endoplasmic reticulum and is subsequently degraded by the proteasome (Patrick et al., 2010). F508del mutation nearly accounts 70% of the CFTR mutations in CF patients all over the world (CFTR.Info, 2017). This could be due to the occurrence of this mutation in the remote past that made it a founder mutation in many populations.

The number of known CFTR mutations approaches 2000 but each population seems to have its peculiar panel of mutations that accounts for most of its CF patients. For example, a panel of 11 mutations has been suggested for Arabs. This panel consists of the following mutations: F508del, 3120 + 1G> A, N1303K, W1282X, G115X, 711 + 1 G >A, S549R, I1234V, 1548delG, H139L and 4010del4, and it is claimed that this panel covers around 70% of Arabs CF mutations (Wei et al., 2006). In this study we screened our 30 CF patients for ten mutations (i.e., F508del, W1282X, G115X, 711+1G, S549R, I1234V, 1548delG, H139L, 4010del4, and 3120+1kb). Apart from N1303K and 3120+1G>A, all the mutations in the suggested panel were thus examined. Despite screening our samples for almost all the possible common Arab mutations, only 20% of the mutant chromosomes could be identified. Interestingly, only 3 mutations (F508del, G115X, and W1282X) out of the 11 stated in the panel could be detected (Table 4.2). This finding indicates that Palestinians, as compared to Arabs, should have a different spectrum of CFTR mutations e.g., the mutation G115X, which is not present in any Arab countries, constituted the most common mutation (6.67% of the mutant CF alleles) in our population (Table 4.2). On the other hand the mutation 1548delG is commonly encountered in Arabs, sometimes as the most common CF mutation (Banjar and Angyalosi, 2015). This mutation, however, was not encountered in any of our patients. Similarly, one of the most frequent CF mutations in Oman S549R (65.2%)
was not found in our CF patients (Al-Kindy et al., 2014). Likewise, S549R was not encountered in many Arab countries such as Jordan, Lebanon, Egypt and Syria. Once again, these reports indicate that Palestinian Arabs may have their own and peculiar common CF mutations that need to be fully characterized.

The nature and distribution of the CFTR gene mutations among the Palestinian population is slightly different from mutations reported in neighboring and regional countries such as Jordan, Lebanon, Egypt, Syria, Turkey and Israel as seen in Table 5.1. As reported from several Arab countries, F508del also appeared to be the most common mutation in our patients despite its overall low frequency (6.67%) as compared to other countries such as Israel, Jordan, Lebanon, areas of Arab Gulf and Europe which have a high incidence of this mutation. For example, in a cohort of 22 unrelated Lebanese CF patients, F508del (34%) was reported as the most common mutation followed by N1303K (27%), W1282X (7%), and S4X (7%).

A study conducted in America by Wei S. et al (2006) on Arabic CF patients living in America, reported that 1548delG mutation was the most common (13%) after F508del (15.1%). This is similar to Banjar H. and Angyalosi G. (2015) study conducted in Saudi Arabia which displayed 1548delG mutation as the most common (20%) in Saudi Arabian CF patients. However, this mutation was absent from our patients investigated in this study. Furthermore, most mutations reported from Arab Americans and Saudi Arabian CF populations such as H139L, 711+1G, S549R and I1234V were not found in our population.

While W1282X mutation, a nonsense mutation which is caused by a substitution of an G to A at nucleotide position 3846 (Cystic Fibrosis Mutation Database, 2011), was the second most common mutation in our CF patients with a frequency of 5%, in Israel W1282X has been reported as the most common mutation in Jewish patients (43%). Whereas in Europe it was the least common mutation (1%) and has been reported in other Arab countries at close rates.

Interestingly, one mutation, G115X, not previously reported in the Palestinian CF patients was identified in the present study. This nonsense mutation results from a G to T transversion at the coding nucleotide 475 (c. 475 G>T) in exon 4 and
replaces the glutamic acid codon by a stop codon (Glu_{115}→ Stop). The mutation leads to premature termination of translation and its pathogenicity is well-documented (Kambouris et al., 2000). The *CFTR* mutation G115X was found at the same frequency of F508del mutation (6.67%) and seems to be a quite common mutation in our CF patients. Although G115X mutation did not appear to be present in any of the CF neighboring Arab populations, it was found among Arab-Americans (1.6%).

Our results showed one patient (1.67%) with the 3120+1kb mutation. This mutation deletes 8.6Kb in the *CFTR* gene spanning the exons 17a, 17b and 18 (Lerer et al., 1999). As mentioned earlier, this mutation has been considered a founder mutation in Palestinian Arabs and it is rarely encountered in many other populations.

By comparing our results with previous studies conducted on Palestinian CF patients, we observed some similarities with those of El-Shanti A. et al (2014) and Essawi O. et al (2014) studies which all showed comparable rates (around 5%) for WI282X mutation. Siryani I. et al (2015) study, however, reported a higher frequency (14.3%) of W1282X mutation. Another similarity with El-Shanti study concerns the 3120+1kb (4.9%) mutation. This was also observed in one (1.67%) of our recruited patients. In the contrary, G115X mutation which was identified in the present study was not reported in any of the previous studies in Palestine. This piece of information indicates that our population has a diverse spectrum of *CFTR* mutations and that the mutation detection rate is still far from complete.

**Patients and mutation-specific treatment:**

According to our results,

- Patients who have G115X and WI282X mutations (class I) can benefit from production correctors in their treatment program.
- Patients who have F508del mutation (class II) can benefit from potentiators and correctors in their treatment program.
- Patients who have 3120+1kb mutation (class V) can benefit from potentiators in their treatment program.
Table (5.1): Frequency of common Palestinian CFTR mutations compared to other countries.

<table>
<thead>
<tr>
<th>Region or Country</th>
<th>F508del</th>
<th>WI282X</th>
<th>3120+1kb</th>
<th>G115X</th>
<th>H139L</th>
<th>4010del4</th>
<th>711+1G</th>
<th>S549R</th>
<th>I1234V</th>
<th>1548delG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palestine</td>
<td>6.67%</td>
<td>5%</td>
<td>1.67%</td>
<td>6.67%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lebanon</td>
<td>34%</td>
<td>7%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syria</td>
<td>18%</td>
<td>12%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egypt</td>
<td>58%</td>
<td>4%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jordan</td>
<td>23.7%</td>
<td>15%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4%</td>
<td></td>
</tr>
<tr>
<td>Saudi Arabia</td>
<td>12%</td>
<td>3%</td>
<td>9%</td>
<td>8%</td>
<td>3%</td>
<td>11%</td>
<td>20%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Turkey</td>
<td>27%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oman</td>
<td>13%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>65.2%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israeli Arab</td>
<td>23.5%</td>
<td>10.6%</td>
<td>13%</td>
<td></td>
<td>2.3%</td>
<td>1.2%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israel Jewish</td>
<td>33.5%</td>
<td>43%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Europe</td>
<td>66.8%</td>
<td>1%</td>
<td></td>
<td>6.9%</td>
<td>2.6%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UAE</td>
<td>30.4%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>19%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>African continent</td>
<td>48.3%</td>
<td>4.3%</td>
<td></td>
<td>5%</td>
<td>11.8%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arab-Americans</td>
<td>15.1%</td>
<td>4.3%</td>
<td>1.6%</td>
<td>3.8%</td>
<td>2.2%</td>
<td>1.6%</td>
<td>1.6%</td>
<td>10.2%</td>
<td>13%</td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td>Present study</td>
<td>Farra et al., 2010</td>
<td>Jarjour et al., 2015</td>
<td>Shahin et al., 2016</td>
<td>Al Sheyab et al., 2007</td>
<td>Banjar &amp;Angyalosi, 2015</td>
<td>Al Sheyab et al., 2007</td>
<td>Al-Kindy et al., 2014</td>
<td>Cahana et al., 1999</td>
<td>Quint et al., 2005</td>
</tr>
</tbody>
</table>

Legend: UAE–United Arab Emirates
Chapter 6

Conclusion and Recommendations
Chapter 6
Conclusion and Recommendations

Conclusion:

- The commonest mutations detected in our CF Palestinians were 508delF and G115X (6.67% each), followed by W1282X (5%) and 3120+1kb (1.67%).
- The CFTR mutations: 711 + 1 G > A, S549R, I1234V, 1548delG, H139L and 4010del4 were not detected in the recruited patients.
- Determining the mutations of CFTR gene in the Palestinian CF population is an important for molecular screening, genetic counseling and treatment.
Recommendations:

1. Extensive molecular techniques e.g., CFTR gene sequencing, must be carried out in order to identify all possible mutations circulating in the Palestinian CF patients.

2. Establishing a database for the identified CFTR gene mutations in Palestine.

3. Studies of CF samples from different geographical locations of Palestine should be undertaken to better understand the prevalence and type of CFTR gene mutations among Palestinians.

4. Constructing a CF mutation panel with a mutation detection rate of >90%.

5. CFTR gene mutations and disease pathophysiology should be evaluated to develop better counseling, management and treatment for the CF patients and their families.
References
The Reference List


Appendix 1

بسم الله الرحمن الرحيم

إقرار موافقة

أنا الموقع أدناه

أوافق على سحب عينة دم بانبوية 2 مل EDTA وذلك لإجراء بحث يخص الكشف عن الطفرات الشائعة لمرض التليف الكيسي في فلسطين.

توقيع