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Method Article ***Open Access***

IDENTIFICATION OF EXON 12 MUTATIONS IN THE *CFTR* GENE USING A COST-EFFECTIVE CAPILLARY ELECTROPHORESIS (CE) ASSAY

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Abstract *Cystic Fibrosis (CF) is an autosomal recessive genetic disorder caused by mutations in the CFTR gene. The F508del mutation in exon 11 of the CFTR gene is prevalent worldwide, affecting approximately 70% of CF patients, but it is less common in the local Pakistani population. Exon 12 mutations, such as S549N and S549R, have been observed in CF patients with Pakistani ancestry. This research provides accessible and affordable mutation detection using reproducible and cost-effective capillary-electrophoresis (CE) methods. Therefore, this study investigated whether local Pakistani CF patients lacking exon 11 mutations, such as F508del, harbor any common or rare CF-causing mutations in exon 12 of the CFTR gene using a cost-effective Sanger sequencing assay. To achieve this, a new set of primers was meticulously designed and optimized for the amplification of exon 12 through PCR. Additionally, Sanger sequencing-based CE assay was fine-tuned for the sequencing of exon 12 amplicons. The Sanger sequencing results revealed no mutations in exon 12 among the 17 local CF patients who participated in the study. This absence of mutations in both exons 11 and 12 suggests that CF-causing mutations may be located in these patients' other regions of the CFTR gene. Furthermore, it indicates that exon 12 mutations are less prevalent among local Pakistani CF patients. The optimized Sanger sequencing-based CE assay is at least five times more cost-effective and can be employed for the identification of mutations in any sequence with a length of up to 500 bases.*

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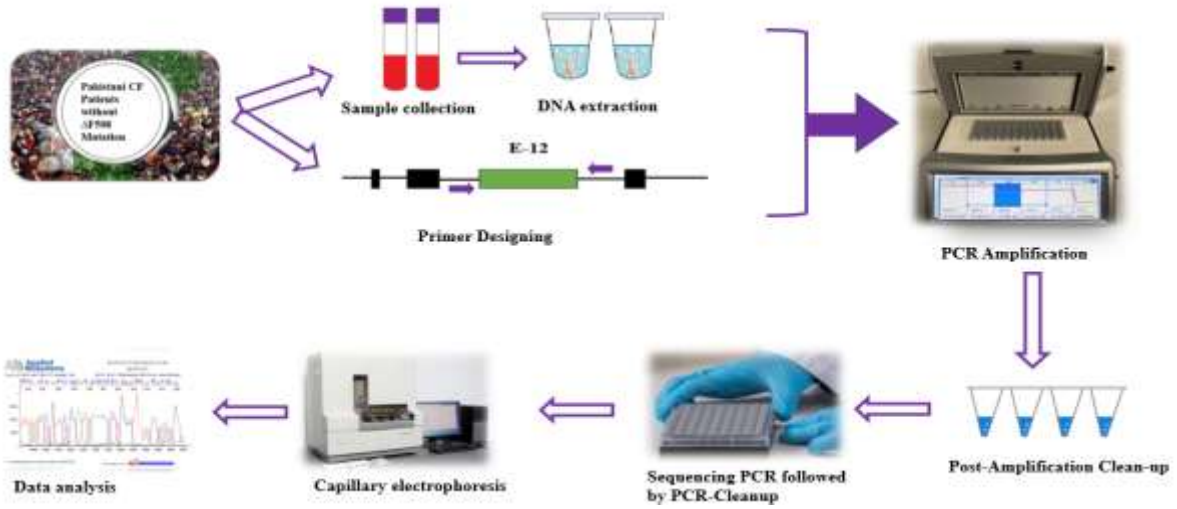
Keywords: *CFTR gene; Exon 12 mutations; Cystic Fibrosis; Sanger sequencing; Capillary Electrophoresis*

Graphical Abstract



Introduction

Cystic fibrosis (CF) is a genetic, life-limiting disease characterized by chronic endobronchial airway infections and pancreatic insufficiency. This condition is marked by the blockage of essential enzymes like bicarbonate, amylase, and pancreatic lipase, leading to issues such as protein and fat malabsorption, hypoalbuminemia, steatorrhea, and failure to thrive (Ashraf et al., 2022; Férec and Scotet, 2020). Chronic airway infections contribute to the development of bronchiectasis, which can ultimately lead to respiratory failure, making it the leading cause of death among patients with cystic fibrosis (pwCF) (Goetz and Ren, 2019). A major concern in pwCF is the production of thick and viscous secretions in the intestines, pancreas, and bronchi (Merjaneh et al., 2022) giving rise to various clinical complications. These include diabetes mellitus (Granados et al., 2019), gastrointestinal and hepatobiliary diseases (Gabel et al., 2019), rhino sinusitis (Safi et al., 2019), hyponatremic dehydration, distal and proximal intestinal obstruction syndrome (DIOS and PIOS) (Alattar et al., 2019; Carroll et al., 2021; Morales et al., 2020), and male infertility (AlMaghamsi et al., 2020; Bieniek et al., 2021). Additionally, it is crucial to raise awareness regarding female CF and the connection between *CFTR* mutations and pregnancy (Tournier et al., 2019). Some individuals with mild disease may remain asymptomatic until diagnosed in adulthood. Common symptoms of CF encompass dyspnea, wheezing, recurrent pneumonia, atypical asthma, productive cough, chronic airway disease, bronchial inflammation, and haemoptysis (Martínez-Jiméne et al., 2017; Rafique et al., 2024; Sabar et al., 2023). CF cases are diagnosed using various methods, including newborn screening to assess levels of pancreatic enzyme immunoreactive trypsinogen (Castellani et al., 2016); the nasal potential difference test, which measures voltage across the nasal epithelium (Aalbers et al., 2020); and the sweat chloride test (SCT), considered the gold standard for confirming CF. SCT is known as pilocarpine iontophoresis: detection of high levels of chloride in the sweat (Massie et al., 2000); Additionally, *CFTR* (cystic fibrosis transmembrane conductance regulator) gene mutation screening plays a crucial role (Khan et al., 2024; Pagin et al., 2020) The genetic confirmation of CF is based on the identification of one or more CF-related mutations in the *CFTR* gene (Ghani et al., 2022). However, in modern mutation detection, if only one DNA mutation is found or no mutations are detected, an individual may still have CF due to rare mutations that standard genetic testing might overlook. This can occur because of limited knowledge about *CFTR* genotyping in diverse ethnic populations. The *CFTR* gene is located on chromosome 7 at position 7q31.2 and comprises 27 exons (Akram et al., 2024; Duz and Ozyavuz Cubuk, 2021) which encodes the *CFTR* protein. The *CFTR* protein is a member of the ABC (ATP-binding cassette) transporter superfamily. In this context, ABC transporters function as chloride channels in the exocrine glands, regulating the movement of chloride ions in and out of epithelial cells. This mechanism helps maintain the balance of fluid in the passageways (Aqeel et al., 2025; Hanssens et al., 2021).Genetic mutations in the *CFTR* gene are the underlying causes of complications related to CF. CF has a 25% inheritance probability when both parents are carriers with heterozygous disease-causing *CFTR* gene mutations. The distribution and prevalence of common genetic mutations can vary among CF patients of different ethnic backgrounds. For a long time, CF was primarily associated with Caucasians, but recent studies have shown that its prevalence has been reported in various populations, including Asians (Akram et al., 2022; Ghani et al., 2019b; Mehdi et al., 2023; Sabar et al., 2020; Safdar et al., 2025; Safdar et al., 2023).

In the context of CF within the Pakistani population, there were three cases of native Pakistani children reported with a confirmed CF diagnosis in Birmingham in 1974, with two of these cases being siblings (Goodchild et al., 1974). A study conducted at the University of Karachi aimed to identify *CFTR* mutations in Pakistani CF individuals and found that the frequency of the F508del mutation is lower among Pakistani CF children compared to its reported frequency in the Caucasian population (Bhutta et al., 2000). Another study from Aga Khan University in Karachi reported that the F508del homozygous mutation was present in only 33% of their enrolled CF patients (Shah et al., 2006) which is notably lower than in other global populations. Shah et al. 2006 proposed that some symptomatic Pakistani CF individuals who were heterozygous for F508del may have compound mutations in the *CFTR* gene, further indicating that the prevalence of F508del is lower in the Pakistani population (Bhutta et al., 2000). These studies provide clear evidence that relying solely on the presence of a single mutation (F508del) for CF diagnosis could result in false-negative diagnoses. Consequently, in the absence of comprehensive genetic testing for CF, the SCT remains the gold standard for CF diagnosis (Shah and Moatter, 2006; Yousaf et al., 2024). It's worth noting that in some cases, even normal values in SCT may not rule out a CF diagnosis, highlighting the critical importance of genetic testing (Stewart et al., 1995). Regrettably, in some metropolitan cities, SCT is available but facilities are not quite in scope to render their services to every patient on time which may yield the under-diagnosis status of this disease in the country. Consequently, clinical conditions of this disease may often be confused with pneumonia or pancreatitis which direly affects the management of the disease. Clinical complications related to CF patients in Pakistan majorly include chronic cough (69.76%), steatorrhea 5 (11.62%), and meconium ileus (9.3%) with only 27.90% of patients having F508del

mutation (Aziz et al., 2017). The CF-causing mutation in the remaining 72.10% of patients was unknown.

After an extensive literature survey, this study formulated a hypothesis suggesting that the predominant mutation F508del located in exon 11 was not the only underlying cause of CF disease in a significant portion of the studied CF patients of Pakistani origin. Based on the latest cohort study conducted by Shah et al. on CF patients of Pakistan, it was observed that mutations from exon 12 i.e., S549 were identified as the additional mutation in this population following the F508del mutation (Shah et al., 2009). Thus, the primary aim of this study was to conduct a genetic analysis of exon 12 in the *CFTR* gene, specifically focusing on two mutations, S549R and S549N, which have been previously identified in CF patients from Pakistan (Curtis and Richardson, 1993; Safdar et al., 2024; Shah et al., 2009). The objective was to optimize the conventional sequencing method for exon 12 of the *CFTR* gene. To achieve this, a cost-effective CE electrophoresis assay (Sanger sequencing) was used for DNA sequencing of exon 12. However, the results revealed that no disease-causing mutations were detected in exon 12 of the *CFTR* gene among the CF patients who participated in the study.

Materials and methods

Samples

Seventeen paediatric pwCF , 7 males and 10 females, was enrolled for study from "University of Child Health Sciences, The Children's Hospital Lahore - Pakistan (54600)" with written informed consents from the guardians/parents of the patients who were tested positive for CF through SCT (>90 mmol) and negative for homozygous F508del (exon 11) mutation. Genomic DNA was extracted from peripheral blood using inorganic salting out method (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; 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; 5 M Sodium Chloride 100ml).

Amplification of exon 12 of CFTR gene

Primers for exon 12 of the *CFTR* gene were designed using Primer3 (https://primer3.ut.ee) followed by NCBI Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) analysis for auto dimerization and self-complementarity etc. Primers were designed approximately 125 bp upstream and downstream from the actual sequence of the exon 12. PCR amplification optimization of exon 12 from genomic DNA was done using designed (F: 5'-TTTCAGCAATGTTGTTTTTGACC-3 R: 5' CCACTAGCCATAAAACCCCAG-3) with 2X HotStart PCR Mix in 10 or 20µl PCR reaction. The 10ul reaction mixture comprised of 5ul 2X Master Mix, 1.6 pmol forward and reverse primer each while 1µlof template (25ng/µl). reaction was carried out by placing in gradient PCR at annealing temperature of 58-62 °C.

Product purification and Sanger Sequencing

After confirmation of amplified PCR products of the *CFTR* gene's exon 12 on 2% agarose gel electrophoresis, PCR amplicons were subjected to purification by ethanol precipitation method followed by confirmation of purified PCR products again on 2% agarose gel electrophoresis. After purification, in-house optimization was done for sequencing PCR reaction with Big Dye Terminator Ver. 3.1 Cycle Sequencing kit (chain termination method) (**Table *1***) using thermal cycler profile as given in **Table 2**. After the sequencing PCR, products were purified by the ethanol-EDTA precipitation method. The purified sequencing PCR products were dissolved in 13µl formamide and given heat-shock at 95°C for five minutes to denature the sequencing PCR products, and placed in ABI Genetic Analyzer 3130xl for capillary electrophoresis, subsequently, exon 12 of CF samples was sequenced.

Table 1. Optimization of Sequencing PCR Reaction Mixture

Reagent (Concentration)	Standard Reaction	Volume		
		Optimization-I	Optimization-II	Optimization-III*
PCR Product (416 bp)	3-10 ng	7-8 ng	7-8 ng	7-8 ng
Ready Reaction Premix (2.5X)	4 µl	2	1	0.7 µl
Primer (UCF-12-F)	3.2 pmol	1.6 pmol	1.6 pmol	1.6 pmol
BigDye Sequencing Buffer (5X)	2 µl	1	1.5	1.65 µl
Nuclease Free Water	To adjust volume up to 10 µl	To adjust volume up to 10 µl	To adjust volume up to 10 µl	To adjust volume up to 10 µl
Final Volume	20 µl	10 µl	10 µl	10 µl
* Suitable choice for sequencing of PCR products upto 500 bp				

Table 2. Thermal profile for sequencing PCR

Step	Temperature	Time	Cycles
Hot Start	96 °C	1 min	1

Denaturing	96 °C	30 s	32
Annealing	50 °C	15 s	
Elongation	60 °C	4 min	
Final elongation	60 °C	4 min	1
s- seconds, mint- minutes			

Analysis of Sequencing Data

The electropherogram of all samples generated after capillary electrophoresis was analysed by experts for validation of sequencing and capillary electrophoresis followed by NCBI-Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for similarity check. Successfully sequenced data was also analysed with 'Mutation Surveyor' software for detection of possible mutations.

Results

The descriptive analysis of the enrolled patients is given in **Table *3***.

Table 3. Descriptive Analysis of the Enrolled Patients

Variable	Value
Patients Enrolled	17
Mean Age (Years ± SD)	7.7 ± 6.8
Consanguinity in Parents	70.58 %
Male	41.17%
Female	58.92%
Symptoms	Coughing, Shortness of breath, Pulmonary Infection, Intestinal Manifestations

The optimized primers designed for *CFTR* gene's exon 12 successfully amplified the *CFTR* gene's exon 12 from the genomic DNA with optimized results at 5 µM concentration of primers at all annealing temperatures 58/59/60/61/62°C. However, the annealing temperature of 60°C was used for amplification PCR of all samples in 10 µl PCR reaction (Figure 1). The sequencing results were satisfactory (Figure 2). However, no *CFTR* mutation was found in the exon 12 of the *CFTR* gene in processed samples.

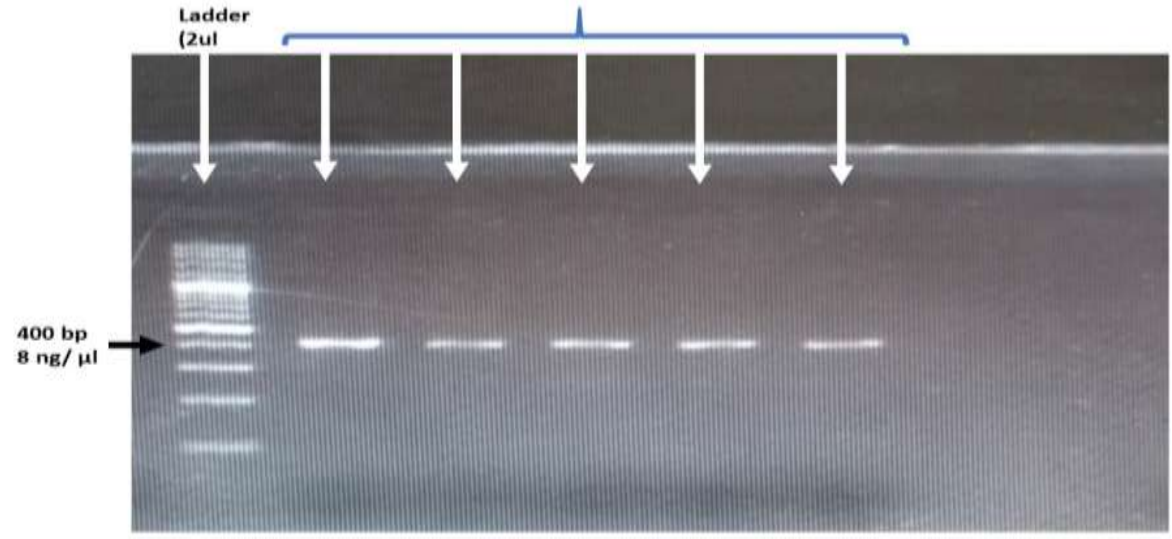


Figure 1. Amplified CFTR gene's exon 12 of CF samples. Size of exon 12 is 416 base pairs. Lane 1 contains DNA ladder in parallel to the amplified PCR products in lane 2, 3, 4, 5 and 6

Discussion

Admittedly, socio-economic factors hinder appropriate neonatal screening and CF diagnosis for every patient in developing countries like Pakistan. Most CF disease diagnosis is based on the physical appearance of CF-related complications at the later stage of the patient's life. It is reported that CF disease in Pakistan is usually diagnosed later in life due to the unavailability of rapid and fast CF diagnostic facilities (Shah et al., 2009). As the Cystic fibrosis (CF) is associated with a diverse spectrum of *CFTR* mutations, a comprehensive understanding of the mutation landscape in the *CFTR* gene among individuals with CF in Pakistan is currently lacking. In this study, seventeen CF patients from the local population were included for genetic analysis of exon

12 of the *CFTR* gene using Sanger sequencing. Among these patients, 41.17% were male, while the other 58.83% were female. The mean age of the participants was 7.7 ± 6.8 years. Upon conducting a descriptive analysis of the enrolled patients, it was observed that a significant 70.58% of the parents of CF patients had consanguineous relationships (as shown in Table 3). This notable prevalence of consanguinity may be a major contributing factor to the occurrence of rare autosomal genetic disorders like cystic fibrosis in Pakistan.

In-family marriages are the highlighted factor of inheritance of recessively inherited disorders and the outcome of consanguineous marriages is an increase in the number of homozygous diseased individuals (Bhinder et al., 2019; Ghani et al., 2019a; Naeem et al., 2020). In-family marriages are also predominant in the patriarchal society of Pakistan. It is reported that first-degree consanguinity in Pakistan is significantly associated with neonatal deaths and genetic disorders (Ghani et al., 2017; Sabar et al., 2018; Zakar et al., 2014). Therefore, promoting genetic counselling and raising awareness among the general public about the potential drawbacks of cousin marriages could be instrumental in reducing the incidence of genetic disorders in Pakistan (Ashfaq et al., 2023; Ghani et al., 2017). In the *CFTR*2 database (https://CFTR2.org), till April 7, 2023, worldwide prevalence of mutations of *CFTR* gene's exon 12 among 89,052 patients was found as following: S549N (0.14%), L558S (0.02%), S459R (0.6%), A559T (0.06%), G551D (2.10%), R560K (0.01%), G551S (0.01%) and R560T (0.24%). In the current study, none of the previously reported or novel mutations were detected in exon 12 of the *CFTR* gene among the seventeen CF patients. Furthermore, sixteen of these patients tested negative for mutations in exon 11. There was one CF patient who was found to be heterozygous for the F508del mutation in exon 11, and this individual had a single mutant allele of F508del, while the status of the other mutant allele remained unknown, necessitating further investigation. The lack of mutation in exons 11 and 12 may be indicative of the genetic diversity inherent in the CF patient population. It may also be possible that the specific characteristics of the study sample, such as geographic location or ethnic composition, could contribute to the absence of certain mutations. Thus, the absence of mutations in these exons for the present sample size may reflect the variability in the *CFTR* gene across patients.

It is essential to recognize the limitations of this study, which may have contributed to the absence of findings. The relatively small sample size, consisting of only seventeen CF patients, may not be sufficient to detect mutations in exon 12 of the *CFTR* gene among the local CF patients. The intriguing absence of mutations in both exon 11 and exon 12 of the *CFTR* gene in the studied CF patients underscores the wide-ranging mutation spectrum of the *CFTR* gene. Nevertheless, the findings from this study suggest the possibility of *CFTR* gene variants from other regions of the *CFTR* gene playing a role in the development of CF in the enrolled patients. The findings of this study should be interpreted within the context of the confined sample size, and caution should be exercised when attempting to generalise the results to larger and heterogeneous populations. The genetic diversity within different geographic regions, ethnic groups, and populations may influence the prevalence and variations of the *CFTR* gene. Therefore, the outcomes of this study may not be representative of the broader CF patient demographic, and any generalizations should be made with careful consideration of these limitations. Furthermore, this study has successfully refined an economical Sanger sequencing method that utilizes capillary electrophoresis (CE) to efficiently sequence exon 12 amplicons. Notably, the authors have previously published an optimized SNaPshot reaction for detecting asthmatic SNPs (Ghani et al., 2021). The optimized Sanger sequencing CE approach presented in this study offers a significant cost advantage, being at least five times more economical than the standard reaction. Additionally, it is versatile, capable of sequencing PCR products up to 500 base pairs in size, making it a suitable choice for a wide range of genetic analyses. A comparison between the standard and optimized methods of Sanger sequencing for DNA templates of 500 base pairs (Table-1).

The cost-effectiveness of the method can facilitate broader genetic studies, enabling researchers to analyze larger sample sizes within limited budgets. The economical nature of the optimized sequencing method also enhances its feasibility for clinical applications. Clinical laboratories, which often operate under budget constraints, can benefit from a cost-effective sequencing method for routine genetic testing, diagnosis, and monitoring of genetic disorders. This can help in identifying carriers of specific genetic mutations or assess genetic predispositions for certain conditions, contributing to preventive healthcare and early intervention strategies. In educational and resource-limited settings, where budget constraints may pose a significant barrier to genetic testing, the cost-effectiveness of the optimized method makes genetic analysis more accessible. This is particularly relevant for research and diagnostic purposes in regions with limited financial resources. The versatility of the optimized Sanger sequencing CE approach, capable of sequencing PCR products up to 500 base pairs, makes it suitable for a wide range of genetic analyses contributing to its versatility and applicability in diverse research and diagnostic scenarios.

Conclusion

Given the dearth of comprehensive research on *CFTR* genotyping in Pakistan, it is essential to embark on a nationwide study covering a larger and more diverse sample size. This broader approach is necessary to uncover the full spectrum of CF-causing genetic

mutations among CF patients in Pakistan. Such an undertaking not only has the potential to advance our understanding of the genetic foundations of CF but also to pave the way for improved genetic diagnosis of the disease within the Pakistani population. Furthermore, the present research sought to refine the methodology for traditional sequencing of exon 12 of the *CFTR* gene while reducing costs, particularly in light of the high expenses associated with whole exome sequencing. This optimization can prove invaluable in making genetic testing more accessible and cost-effective for CF patients in Pakistan.

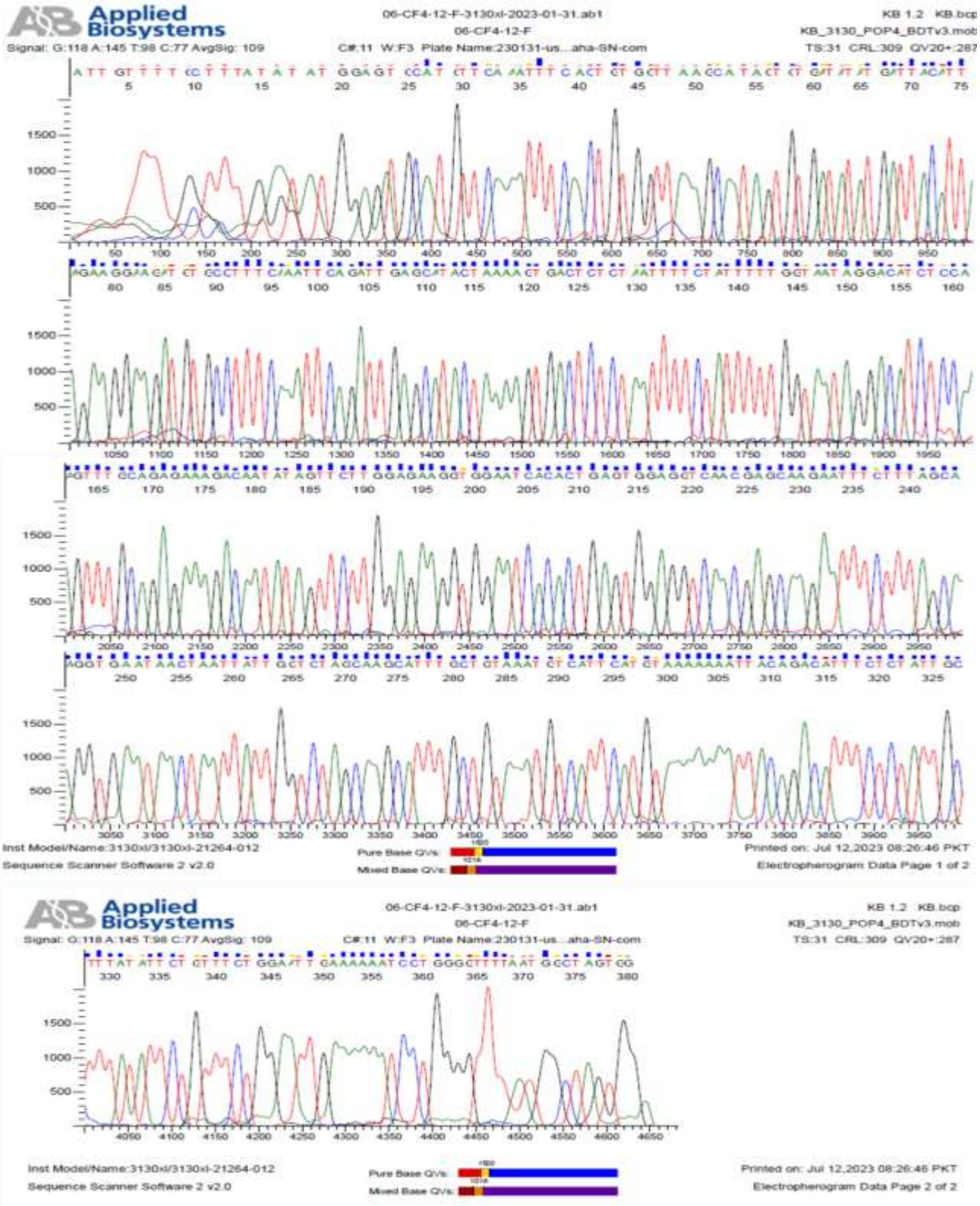


Figure- 2: Electropherogram of Sanger sequencing of CFTR gene's exon 12 from a CF patient with UCF-12 F

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Declaration

Ethical Statements

The study was ethically approved by the institutional ethical review board vide Diary No. as No.D/331/FIMS dated: 23-09-2022.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Author contribution

M.U.G surveyed the literature and conceptualized the study. H.R, A.S, I.B, and N.S did the sampling and collected patients' data. A.S and H.R did the genomic DNA extraction from peripheral blood, designed the primers and performed PCR. M.U.G, M.F.S and M.S optimized the sequencing protocol. H.R and R.A wrote the manuscript. T. M, M.U.K, A.A, and F.K did the proofreading and editing of the manuscript. All authors reviewed the final manuscript.

Consent for Publication

Not applicable