SNaPshot Assay for the Detection of the Most Common CFTR Mutations in Infertile Men

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Abstract

Congenital bilateral absence of vas deferens (CBAVD) is the most common CFTR-related disorder (CFTR-RD) that explains about 1–2% of the male infertility cases. Controversial data have been published regarding the involvement of CFTR mutations in infertile men with non-obstructive azoospermia and oligozoospermia. Here, we describe single base extension (SNaPshot) assay for detection of 11 common CFTR mutations: F508del, G542X, N1303K, 621+1G-->T, G551D, R553X, R1162X, W1282X, R117H, 2184insA and 1717-1G-->A and IVS8polyT variants. The assay was validated on 50 previously genotyped samples and was used to screen a total of 369 infertile men with different impairment of spermatogenesis and 136 fertile controls. Our results show that double heterozygosity of cystic fibrosis (CF) and CFTR-related disorder (CFTR-RD) mutations are found in a high percentage (22.7%) of infertile men with obstructive azoospermia, but not in other studied groups of infertile men. The SNaPshot assay described here is an inexpensive, fast and robust method for primary screening of the most common CFTR mutations both in patients with classical CF and CFTR-RD. It can contribute to better understanding of the role of CFTR mutations in impaired spermatogenesis, ultimately leading to improved management of infertile men.

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Introduction

Infertility which is defined as an inability to conceive in a period of one year has prevalence of about 15% among couples of reproductive age worldwide and approximately in 50% of these cases the male factor is responsible for the failure of conception [1,2]. The causes for male infertility can be classified as genetic and non-genetic. Chromosomal aberrations and microdeletions of the Y chromosome represent the major genetic factors of male infertility. Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (OMIM 602421) are responsible for one of the best known autosomal genetic disorders contributing to reproductive failure [3,4].

Mutations in CFTR gene are causing cystic fibrosis (CF) or related phenotypes, named CFTR-related disorders (CFTR-RD). Altered CFTR protein leads to malfunction of chloride channels and subsequent defective chloride and bicarbonate anions transport in the epithelial cells of intestine, respiratory system, pancreas, gall bladder and sweat glands. CF is a multi-system disorder that may affect normal functioning of pulmonary, pancreatic, gastro-intestinal and reproductive systems [5]. Infertility is one of the clinical manifestations of CF affecting equally both sexes with/without clinically apparent disease.

CFTR-RD are milder clinical entities associated with CFTR dysfunction, such as congenital bilateral absence of the vas deferens (CBAVD), disseminated bronchiectasis, chronic pancreatitis or chronic rhinosinusitis. CBAVD is the most common CFTR-RD and accounts for 1–2% of the population of infertile, but otherwise healthy, males and up to 25% of those with obstructive azoospermia [6]. Impaired CFTR protein function during the embryonal stage affects the development of male reproductive system leading to improper differentiation of Wolffian ducts causing CBAVD [7].

There are more than 1900 CFTR mutations described to date [http://www.genet.sickkids.on.ca/StatisticsPage.html]. The severity and presentation of the disease (CF or CFTR-RD) may depend on the type of mutation and many studies have investigated the association between the genotype and the phenotype [8,9].

The three CFTR mutations most commonly associated with CBAVD are: c.1521_1523delCTT (F508del), c.1210-12T(5) (5T

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involved are F508del with 5T or R117H [6,13,14]. The 5T allele is known to modify the expression of the R117H mutation when it is present on the same chromosome (cis). R117H mutation can be found in cis with IVS8-5T or -7T underlying a mild CF-causing complex allele when in cis with IVS8-5T, or as a CFTR-RD mutation when in cis with IVS8-7T [15].

Recent studies present an evidence for involvement of CFTR protein in several pathways in the process of spermatogenesis [16,17,18,19,20,21]. In addition, several studies have shown increased frequencies of CFTR mutations in infertile patients with non-obstructive azoospermia, impaired spermatogenesis and low sperm quality [22,23,24,25]. However, other studies have shown no association of CFTR mutations with idiopathic male infertility [26,27,28]. Therefore, larger studies of CFTR mutation screening among unselected infertile males with different sperm counts may contribute to the elucidation of the role of CFTR gene in the impaired spermatogenesis and male infertility.

Here, we present a rapid, reliable, cost-effective and simple assay for testing of 11 CFTR (NM_000492.3) mutations: F508del (c.1525_1526delCTT), G542X (c.1624G>T), N1303K (c.3909C>G), 621+1G>T (c.489G>A), T508A (c.1567G>A), R1162X (c.3484C>T), W1282X (c.3846C>G), A1526G>A, R117H (c.350G>A), 2184insA (c.2052_2053insA) and 1717-1G>A (c.1585-1G>A). These are the most common CFTR mutations overall with the highest frequencies found in Southern Europe [29] and Republic of Macedonia populations [30,31]. Moreover the assay tests for polyT variants (5T, 7T and 9T) in intron 8 (c.1210-12T[5_9]).

Materials and Methods

Reference samples

To optimize and validate the assay genomic DNA from 25 CF patients and carriers, and from 25 normal individuals, previously genotyped using commercial genotyping kits Inno-LiPA CFTR19 and Inno-LiPA CFTR17+Tn Update Kits (Innogenetics, Ghent, Belgium) were used as the reference material. These samples were used to determine the reproducibility and performance (specificity and sensitivity) of the single-nucleotide primer extension (SNaPshot) method for detection of CFTR mutations.

Patients and controls

A total of 505 DNA samples were screened for CFTR mutation using the newly developed CFTR SNaPshot assay. Briefly, DNA from peripheral blood was extracted from 260 infertile men with azoospermia (n=79), oligozoospermia (n=108) and normospermatozoospermia (n=73) as well as 136 fertile controls. The assay was also used to detect CFTR mutations in 109DNA samples extracted from testicular biopsies (Formalin Fixed Paraffin Embedded-FPPE samples) of patients with azoospermia (n=99) and severe oligozoospermia (n=10).

DNA from peripheral blood was isolated using standard phenol-chloroform protocol while from FPPE samples was isolated using AllPrep DNA/RNA FFPE Kit (Qagen, Hilden, Germany) following manufacturer’s protocol. The quality and quantity of the extracted DNA was assessed using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA).

Clinical information and the results of spermograms including the sperm counts were available for all patients in the study. The histopathology results were available for all testicular biopsies. Based on these results 22 azoospermic patients were diagnosed with obstructive azoospermia (normal spermatogenesis on histopathology), and 87 patients with nonobstructive azoospermia or severe oligozoospermia (34 with hypospermatogenesis, 18 with Sertoli Cell Only Syndrome (SCOS), 12 with maturational arrest (MA) and 23 with atrophy/fibrosis/hyalinization of the testis).

The study was approved by the Ethical Committee of the Macedonian Academy of Sciences and Arts, and all subjects have given written informed consent for participation in the study according to Declaration of Helsinki.

Primer design and optimization of the CFTR SNaPshot assay

The SNaPshot method is based on hybridization of oligonucleotide primer adjacent immediately to the variant nucleotide analyzed and extension with complementary fluorescently labeled dideoxynucleotide (ddNTP). The template for annealing and extension of a primer is previously amplified PCR fragment that contains the sequence of interest. We have amplified in one reaction, multiplex of eight different DNA fragments with length from 194 bp to 480 bp that were used for the detection of 12 CFTR mutations (Table 1).

The design and optimization of the multiplex PCR (Table 1) and extension primers mixes (Table 2) were performed using Primer3 software [36], while the specificity was confirmed using BLAST software (http://blast.ncbi.nlm.nih.gov). Primers were designed not to include regions with known SNPs as polymorphisms and mutations within primer-annealing regions of the template, especially those close to the 3’ end of a primer, could create imperfections in base paring and decrease primer extension efficiency.

Electrophoretic mobility and separation of extended oligonucleotides depends not only on their size but also on their sequence and type of label (extended ddNTP). For oligonucleotides that had same electrophoretic mobility and/or size overlap, we either redesigned the sequence or added a polyC tail on the 5’ end on one of the ambiguous primers (Table 2). Also, we further optimized the SNaPshot multiplex by testing each of the extension primers for CFTR gene variants individually and in multiplex on samples with known CFTR genotypes. For this purpose we used 50 samples with 14 different CFTR genotypes (Table 3).

PCR conditions and capillary electrophoreses

PCR reaction mix contained 1X reaction buffer B2, 2.5 mM MgCl2, 200 μM each dNTP, 100 nM each primer, 1X Solution S, 1 U HOT FIREPol DNA polymerase (Solis BioDyne, Tartu, Estonia) and 100 ng genomic DNA. Cycling conditions were: initial ‘hot-start’ denaturation at 95°C for 10 min followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min 30 s, and a final extension at 72°C for 10 min. To remove unincorporated nucleotides and excess primers 1 ul of PCR product was cleaned up with 1 ul of ExoSAP-IT (Affymetrix, Inc., Santa Clara, California, USA) for 60 min on 37°C followed by enzyme heat inactivation for 20 min on 86°C. Subsequently, 2 ul of purified PCR multiplex mix was combined with 1 ul extension primer mix and 1 ul of SNaPshot Multiplex Ready Reaction Mix (Life Technologies, Carlsbad, California, USA).

Primer extension was performed on a thermal cycler for 25 cycles with the following cycling conditions: 96°C for 10 sec, 50°C for 5 sec and 60°C for 30 sec. The unincorporated ddNTPs were removed from the reaction mix by the use of 1 U shrimp alkaline phosphatase (Affymetrix, Inc., Santa Clara, California, USA) for 60 min on 37°C, followed by enzyme heat inactivation for 15 min.
Table 1. Primers used for PCR amplification of seven CFTR exons and intron 8 fragment.

| Mutation analyzed | Name | Sequence 5’→3’ | Exon/intron amplified (bp) | Length of PCR fragment amplified in bp |
|-------------------|------|----------------|---------------------------|----------------------------------------|
| 621+1G→T, R117H   | CFTR ex4/F | TCTTGTTGTAATCTCTAGGTA | exon4 (216)               | 374                                    |
|                   | CFTR ex4/R | CCAGCTCAGTCAATCAAGTCA |                          |                                        |
| delF508           | CFTR ex10/F | TGAATCTGAGTGATTTT | exon10 (192)              | 302                                    |
|                   | CFTR ex10/R | TGGATGTTGAAAGGTTATCT |                      |                                        |
| G542X, G551D, R553X | CFTR ex11/F | GCCTTTCAATTCAGATTGAC  | exon11 (95)               | 288                                    |
|                   | CFTR ex11/R | CTAGCCATAAACCCCAAGGA |                        |                                        |
| 2184insA          | CFTR ex13/F | TCAATTATTTTTATGTGACG | exon13 (216)             | 480                                    |
|                   | CFTR ex13/R | GGGGTCCTTTGGACACATGG |                     |                                        |
| R1162X            | CFTR ex19/F | TGTGAAATGGTCTGGCATCTT | exon19 (249)             | 369                                    |
|                   | CFTR ex19/R | TGCTTGCTGGGATT     |                           |                                        |
| W1282X            | CFTR ex20/F | CTGATTAAATTTTTTGACG  | exon20 (156)             | 249                                    |
|                   | CFTR ex20/R | TTTCTTGCTGGATCTTTTG |                        |                                        |
| N1303K            | CFTR ex11/F | GCCTTTCAATTCAGATTGAC  | exon11 (95)               | 288                                    |
|                   | CFTR ex11/R | CTAGCCATAAACCCCAAGGA |                        |                                        |
| IVS8-5T/7T/9T     | CFTR intron 8/F | GGCCATGTGCTTTTCAAACT | intron8 (194)           | 194                                    |
|                   | CFTR intron 8/R | AAGAAGAGGCTGTCATCCCA |                        |                                        |

*Legacy name.

The exon/intron numbering is based on legacy exon intron nomenclature (http://www.genet.sickkids.on.ca/).

Table 2. CFTR multiplex SNaPshot primer extension mix with primer orientation, size and concentrations.

| CFTR mutation | cDNA name according to HGVS (ref. seq. NM_000492.3) | Sequence (5’→3’) | Orientation | SNAPshot Result (normal/mutant allele) | Size of extended fragment in base pairs (normal allele/mutant allele) | Concentration in mix (µM) |
|----------------|---------------------------------------------------|-------------------|-------------|----------------------------------------|-----------------------------------------------------------------|--------------------------|
| G542X          | c.1624G→T                                         | CAGTGTGATCCACCTTCTC | Reverse     | C/A                                    | (24.9/25.9)                                                     | 3                        |
| N1303K         | c.3909C→G                                         | CCCACTTTCTAGGGATCAA | Reverse     | G/C                                    | (26.3/26.9)                                                     | 5                        |
| F508del        | c.1521_1523delCTT                                  | CCGGTGACCATTTAAAG- | Forward     | C/T                                    | (29.6/31.0)                                                     | 1                        |
| R117H          | c.350G→A                                          | 15(C)GATAGCAAGGAGGAC | Forward     | G/A                                    | (33.6/35.3)                                                     | 7                        |
| IVS8-5T/7T/9T  | c.1210-12T[5_9]                                   | TGTGGTGGGTTGGGTTT | Forward     | A/T                                    | (31.0 - 32.3, 7T, 9T - 33.4)                                    | 1                        |
| 621+1G→T       | c.489+1G→T                                        | CCCTAGTATTTTTGTGTTTT | Forward     | G/T                                    | (37.2/38.2)                                                     | 5                        |
| IVS8-7T/9T     | c.1210-12T[2_9]                                   | 14(C)GTGTTGGGTTGGGTTTTTTTT | Forward | A/T                                    | (31.0 - 44.0, 9T - 44.9)                                       | 2                        |
| 2184insA       | c.2052_2053insA                                    | 13(C)GTCTTCTGACAGAAAC- | Forward     | C/A                                    | (38.7/39.7)                                                     | 8                        |
| 1717-1 G→A     | c.1585-1G→A                                       | 9(C)GACTCTTCACTTTTC- | Forward     | G/A                                    | (41.3/41.7)                                                     | 2                        |
| G551D          | c.1652G→A                                         | 21(C)GATATCCACATGGAG | Forward     | G/A                                    | (43.4/43.9)                                                     | 4                        |
| R553X          | c.1657C→T                                         | 24(C)AATACACTGAGG- | Forward     | C/T                                    | (46.2/47.2)                                                     | 2                        |
| W1282X         | c.3846G→A                                         | 28(C)GATAGCAATACCTTGAGCATG | Forward | G/A                                    | (51.6/52.6)                                                     | 1                        |
| R1162X         | c.3484C→T                                         | 29(C)ATTTTCAATGGCTTTTGA | Forward | C/T                                    | (51.0/52.0)                                                     | 4                        |

Data generated on ABI PRISM 3130 Genetic Analyzer with POP-4 polymer, 36-cm capillary array and sized against GeneScan-120 LIZ size standard.

*Concentration in the SNaPshot extension primer mix adjusted to produce relatively equal peak heights.

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on 65°C. A 1 ul of cleaned products was combined with 12 ul HiDi formamide (Life Technologies) and 0.5 ul GeneScan-120LIZ size standard (Life Technologies), heat denatured at 95°C for 5 min then transferred on ice for 2 minutes and loaded onto an ABI PRISM 3130 Genetic Analyzer (Life Technologies). Capillary electrophoresis was conducted following manufacturer instructions on a 36 cm length capillary and POP-4 polymer. Data were analyzed using GeneMapper analysis software version 4.0. A representative electrophoreogram is given in Figure 1a.

### Determination of Intron 8 polyT strand (5T/7T/9T) with single base extension approach

To determine the length of intron 8 polyT strand we designed a set of PCR primers (forward and reverse) amplifying the region containing the T-alleles and two extension primers for subsequent single base extension analysis. One of the extension primers, designated as ‘5T/7T/9T extension primer’ can detect any of the polyT alleles (5T, 7T and 9T) while the other one, designated as ‘7T/9T extension primer’ is further discriminating the 7T and 9T alleles. This primer has 7 thymidine residues at the 3' end and in the presence of 7T or 9T alleles it is extended with dideoxythymidine (red peak). Electrophoreograms of samples with the 5T allele from 7T/9T alleles. This primer has 5 thymidine residues at the 3' end and in the presence of 5T allele it is extended with dideoxyadenosine (green peak) while in the presence of 9T allele it is extended with dideoxythymidine (red peak). Electrophoreograms of samples with three different IVS8polyT genotypes (5T/7T, 5T/9T and 7T/9T) are given in Figure 1b, 1c and 1d.

### Determination of the number of TG repeats adjacent to the IVS8polyT alleles

The TG number immediately upstream of the IVS8polyT allele was determined by multiplex allele-specific fluorescent PCR in 25 men who carried IVS8-5T allele (5 compound heterozygotes for a CF mutation and IVS8-5T and 20 IVS8-5T heterozygotes with no other CF mutation) following the procedure described previously [37].

### Statistical analysis

Statistical non-parametric tests for categorical variables (Pearson chi-square or Fischer exact test) were used when appropriate. The level of significance was set at p<0.05 and data analysis was conducted using Statistical Package for Social Sciences Version 19 (SPSS, Chicago, IL, USA).

### Results

The validation of the CFTR SNaPshot assay carried out on 50 previously genotyped DNA samples demonstrated that it could accurately detect the 11 tested CFTR mutations and IVS8polyT alleles. Feasibility and reproducibility of the assay was assessed first by testing the normal samples and the specificity and sensitivity was further tested by analyzing the CF patients and CF carriers. All extension primers multiplexed in one SNaPshot mix, had electrophoretic mobility and fragment sizes in agreement with those obtained when each was tested individually without any nonspecific peaks or ambiguous results. Our results showed 100% concordance with the independently determined genotypes attesting that the new assay was highly accurate (Table 3).

The CFTR SNaPshot assay was used for detection of 12 different CFTR mutations in a total of 505 men, of whom 136 were fertile controls with proven paternity by DNA analysis. The infertile men consisted of 260 men who carried IVS8-5T allele (5 compound heterozygotes for a CF mutation and IVS8-5T and 20 IVS8-5T heterozygotes with no other CF mutation) following the procedure described previously [37].

### Table 3. CFTR genotypes in 50 DNA samples used for validation of the SNaPshot method.

| Genotype with legacy name from Inno Lipa kits | cDNA name according to HGVS (reference sequence NM_000492.3) | Number of patients tested | Concordance with CFTR SNaPshot assay |
|-----------------------------------------------|---------------------------------------------------------------|---------------------------|------------------------------------|
| [–]/[–] [–]/[–]                               | [–]/[–]                                                       | 25                        | 100%                               |
| F508del/F508del                              | c.[1521_1523delCTT]                                          | 9                         | 100%                               |
| F508del/[–] [–]/[–]                          | c.[1521_1523delCTT][–]/[–]                                    | 3                         | 100%                               |
| G542X/[–] [–]/[–]                            | c.[1624G>T][–]/[–]                                            | 2                         | 100%                               |
| 621+1G>T/[–] [–]/[–]                         | c.[489+1G>T][–]/[–]                                           | 2                         | 100%                               |
| G542X/F508del                                | c.[1624G>T][1521_1523delCTT]                                  | 1                         | 100%                               |
| R117H/F508del                                | c.[350G>A][1521_1523delCTT]                                   | 1                         | 100%                               |
| 2184insA/F508del                             | c.[2052_2053insA][1521_1523delCTT]                            | 1                         | 100%                               |
| W1282X/[–] [–]/[–]                           | c.[3846G>A][–]/[–]                                            | 1                         | 100%                               |
| R1162X/[–] [–]/[–]                           | c.[3484C>T][–]/[–]                                            | 1                         | 100%                               |
| R553X/R553X                                 | c.[1657C>T][1657C>T]                                          | 1                         | 100%                               |
| N1303K/[–] [–]/[–]                           | c.[3999G>C][–]/[–]                                            | 1                         | 100%                               |
| 1717-1G>A/[–] [–]/[–]                        | c.[1585-1G>A][–]/[–]                                          | 1                         | 100%                               |
| G551D                                        | c.[1652G>A][–]/[–]                                            | 1                         | 100%                               |

Note: [–] means no CF mutation detected on single chromosome.

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CBAVD diagnosis, and two patients were heterozygotes for F508del mutation. In the group of 87 patients with histopathology consistent with nonobstructive azoospermia/severe oligozoospermia only one patient with F508del/IVS8-5T genotype was detected who presented with azoospermia and histopathological result of testicular atrophy. Two patients were F508del heterozygotes and two patients were heterozygotes for 5T allele variant (Table 4).

The distribution of the CFTR and IVS8polyT genotypes in the group of 260 infertile patients categorized in accordance to the sperm counts is shown in Table 5. Nine azoospermic patients were heterozygous for 5T allele variant. Among the oligozoospermic men five patients carried CF mutation (3 with F508del and 2 with G542X) but one of them also carried the CFTR-RD mutation, i.e. 5T allele. This patient manifested with mild oligozoospermia with sperm concentration of 11 × 10^6/ml additionally, four patients were heterozygous for 5T allele variant. Three normoasthenoteratozoospermic men were carriers of F508del mutation and additional 6 were carriers of 5T allele. Among the fertile controls, two men carried F508del mutation and additional 9 were carriers of 5T allele.

Table 6 summarizes the results of the CFTR genotypes among the five groups of infertile men in comparison to the fertile controls. Compound heterozygosity for CF mutation (F508del or G542X) and CFTR-RD mutation (IVS8-5T) was present in higher frequency among patients with obstructive azoospermia (22.7%), when compared to the fertile controls (0%) p = 3.42 × 10^{-2} and all other groups of infertile men: non-obstructive azoospermia/severe oligozoospermia (1.14%, p = 1.16 × 10^{-2}), azoospermia (0%, p = 3.32 × 10^{-4}), oligozoospermia (0.92%, p = 4.89 × 10^{-4}) and normoasthenoteratozoospermia (0%, p = 4.54 × 10^{-4}). Although the heterozygosity for CF mutation was present with higher frequency among patients with obstructive azoospermia than among fertile controls and all other groups of infertile men, these differences were not statistically significant. There was also no statistically significant difference in the distribution of the IVS8-5T carriers among the studied groups.

Figure 1. Representative electrophoreograms of CFTR SNaPshot multiplex assay. a) Electrophoreogram of CFTR SNaPshot multiplex assay for detection of 12 common CFTR gene mutations showing a compound heterozygote for F508del/IVS8-5T. The fluorescence intensity is represented on the Y axis of the electrophoreogram and the fragments‘ size on the X axis. N = normal allele (wild type) M = mutant allele; b-d) Electrophoreograms of samples with different IVS8polyT genotypes: 5T/7T (b), 5T/9T (c) and 7T/9T (d). doi:10.1371/journal.pone.0112498.g001
Figure 2. Strategy for detection of IVS8polyT alleles with single base extension approach. a) ‘5T/7T/9T extension primer’ in the presence of 5T allele is extended with dideoxyadenine while in the presence of 7T/9T alleles is extended with dideoxythymidine; b) ‘7T/9T extension primer’ in the presence of 7T allele is extended with dideoxyadenine while in the presence of 9T allele is extended with dideoxythymidine. Results of the two primers extension reactions give the final genotype. Extension primer sequences are given in 5’–3’ orientation, while the alleles represent the complementary (minus) strand.

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Table 4. Distribution of CFTR and IVS8polyT genotypes in the two groups of patients divided according to the histopathological results: obstructive azoospermia and nonobstructive azoospermia/oligozoospermia.

| Histopathological diagnosis                       | CFTR genotype | Intron8 polyT genotype | N  | %    |
|---------------------------------------------------|---------------|------------------------|----|------|
| Obstructive azoospermia                           |               |                        |    |      |
| delF508/[−−]                                      | 5T/9T         | 5                      | 22.7% |
| delF508/[−−]                                      | 7T/9T         | 2                      | 9.1% |
| [−]/[−]                                           | 7T/7T         | 13                     | 59.1% |
| [−]/[−]                                           | 7T/9T         | 2                      | 9.1% |
| Total                                             |               | 22                     | 100.0% |
| Nonobstructive azoospermia + severe oligozoospermia|               |                        |    |      |
| delF508/[−−]                                      | 5T/9T         | 1                      | 1.1% |
| delF508/[−−]                                      | 7T/9T         | 3                      | 3.4% |
| [−]/[−]                                           | 5T/7T         | 2                      | 2.3% |
| [−]/[−]                                           | 7T/7T         | 60                     | 69.0% |
| [−]/[−]                                           | 7T/9T         | 21                     | 24.1% |
| Total                                             |               | 87                     | 100.0% |

[−] means no CF mutation detected on single chromosome.

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Our study has proven that the new SNaPshot assay developed to detect 12 different CFTR mutations (11 mutations and IVS8-5T allele) can be accurately used for screening and diagnosis of CFTR mutations among different groups of affected patients including infertile men. Cystic fibrosis has different degrees of severity and clinical manifestations depending on the type of

Table 5. Distribution of CFTR and IVS8polyT genotypes in infertile men with different sperm counts and fertile controls.

| Patients                        | CFTR genotype | Intron8 polyT genotype | N  | %      |
|---------------------------------|---------------|------------------------|----|--------|
| Azoospermia                     | [-]/[-]       | 5T/7T                  | 8  | 10.1%  |
|                                 | [-]/[-]       | 5T/9T                  | 1  | 1.3%   |
|                                 | [-]/[-]       | 7T/7T                  | 56 | 70.9%  |
|                                 | [-]/[-]       | 7T/9T                  | 14 | 17.7%  |
| Total                           |               |                        | 79 | 100.0% |
| Oligozoospermia                 | delF508/[-]   | 7T/9T                  | 2  | 1.9%   |
|                                 | delF508/[-]   | 9T/9T                  | 1  | 0.9%   |
|                                 | G542X/[-]     | 5T/9T                  | 1  | 0.9%   |
|                                 | G542X/[-]     | 7T/9T                  | 1  | 0.9%   |
|                                 | [-]/[-]       | 5T/7T                  | 4  | 3.7%   |
|                                 | [-]/[-]       | 7T/7T                  | 79 | 73.1%  |
|                                 | [-]/[-]       | 7T/9T                  | 20 | 17.8%  |
| Total                           |               |                        | 108| 100.0% |
| Normoasthenoteratozoospermia    | delF508/[-]   | 7T/9T                  | 1  | 1.4%   |
|                                 | delF508/[-]   | 9T/9T                  | 2  | 2.7%   |
|                                 | [-]/[-]       | 5T/7T                  | 6  | 8.2%   |
|                                 | [-]/[-]       | 7T/7T                  | 49 | 67.1%  |
|                                 | [-]/[-]       | 7T/9T                  | 14 | 19.2%  |
|                                 | [-]/[-]       | 9T/9T                  | 1  | 1.4%   |
| Total                           |               |                        | 73 | 100.0% |
| Fertile Controls                | delF508/[-]   | 7T/9T                  | 2  | 1.5%   |
|                                 | [-]/[-]       | 5T/7T                  | 8  | 5.9%   |
|                                 | [-]/[-]       | 5T/9T                  | 1  | 0.7%   |
|                                 | [-]/[-]       | 7T/7T                  | 96 | 70.6%  |
|                                 | [-]/[-]       | 7T/9T                  | 27 | 19.9%  |
|                                 | [-]/[-]       | 9T/9T                  | 2  | 1.5%   |
| Total                           |               |                        | 136| 100.0% |

Note: [-] means no CF mutation detected on single chromosome.
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Discussion

Our study has proven that the new SNaPshot assay developed to detect 12 different CFTR mutations (11 mutations and IVS8-5T allele) can be accurately used for screening and diagnosis of CFTR mutations among different groups of affected patients including infertile men. Cystic fibrosis has different degrees of severity and clinical manifestations depending on the type of

Table 6. Distribution of genotypes (compound heterozygous, heterozygous or normal) in patients with and without histopathological data.

| Genotypes                        | Histopathological data | No histopathological data |
|-----------------------------------|-------------------------|---------------------------|
|                                  | Obstructive azoospermia (n = 22) | Non-obstructive azoospermia + severe oligozoospermia (n = 87) | Azoospermia (n = 79) | Oligozoospermia (n = 108) | Normoasthenoteratozoospermia (n = 73) | Fertile controls (n = 136) |
| CF mutation/5T                   |                         |                          |                   |                         |                          |                         |
|                                  | 5 (22.7%)               | 1 (1.4%)                 | 0                  | 1 (0.92%)              | 0                          | 0                        |
| CF mutation/[–|–]                 | 2 (9.09%)               | 3 (3.44%)                | 0                  | 4 (3.70%)              | 3 (4.10%)                  | 2 (1.47%)                |
| ST/[–|–]                           | 0                       | 2 (2.29%)                | 9 (11.39%)         | 4 (3.70%)              | 6 (8.21%)                  | 9 (6.61%)                |
| Total                            | 7 (31.8%)               | 6 (6.89%)                | 9 (11.39%)         | 9 (8.33%)              | 9 (12.33%)                 | 11 (8.08%)               |
| [–|–|–]                            | 15 (68.20%)             | 81 (93.11%)              | 70 (88.61%)        | 93 (86.11%)            | 64 (87.67%)                | 125 (91.92%)             |

Note: [-] means no CF mutation detected on single chromosome.
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mutations (severe or mild) and their combination (cis or trans positions on chromosomes) which could result in ‘classical’ or ‘atypical’ form of the disease. The ‘atypical CF’ or CFTR-RD is mainly organ oriented. CBAVD is the most common CFTR-RD, which is usually associated with the presence of one severe mutation (most commonly F508del) and one mild mutation (most commonly R117H or IVS8-5T) or two mild mutations.

The intron 8 polyT tract, adjacent to the CFTR exon 9 splice acceptor site contains 5, 7 or 9 thymidine bases. This variation affects the efficiency of the splice site, with the 5T variant being associated with the least efficient splicing of exon 9 and subsequently the highest levels of mRNA lacking exon 9, leading to reduced production of functional CFTR protein. It is estimated that the 5T allele is found in approximately 5% of alleles in the general population. It has been reported that exon 9 skipping on IVS8-5T alleles can be further modulated by the number of adjacent TG repeats [38]. It was shown that most of the patients with CBAVD carry 12 or 13 TG repeats, while most of the normal individuals have 11 TG repeats immediately upstream of the IVS8-5T allele [39]. Apart from these three mutations there are reports for other mutations found in men with CBVAD, most frequently in combination with the three previously mentioned [13].

Several studies have reported an increased frequency of CFTR gene mutations in men with non-obstructive azoospermia and impaired spermatogenesis [22,23,24,25]. An evidence for involvement of CFTR protein in several pathways in the process of spermatogenesis has also been suggested [16,17,18,19,20,21].

With an aim to contribute to the elucidation of the role of CFTR gene in the impaired spermatogenesis and male infertility, we designed a screening assay for 11 most common CFTR gene mutations and IVS8polyT variant, based on primer extension with fluorescent deoxyribonucleotides and subsequent detection on capillary electrophoresis. The assay was validated with samples previously genotyped with commercial kits.

This method was used to screen for the presence of the most common CF and CFTR-RD mutations among 369 infertile males divided in two groups (109 patients with and 260 patients without histopathological data) and in 136 fertile controls. Our results show that CFTR mutations are found in a high percentage of infertile men with obstructive azoospermia (5/22 or 22.7%), which is consistent with the data from other studies [40]. All 5 patients were double heterozygotes for a CF F508del mutation and a CFTR-RD IVS8-5T variant. Although the double heterozygosity in the patients with F508del/IVS8-5T genotype was not confirmed by studies of the patient’s parents, 5T was inferred to be in trans with F508del, since this mutation has never been found in linkage disequilibrium with 5T [39].

We could not demonstrate an increased frequency of double heterozygosity of CFTR mutations among other studied groups of infertile men. We have genotyped one patient to have F508del/IVS8-5T genotype, with histopathological diagnosis of testicular atrophy and one oligozoospermic patient with G542X/IVS8-5T genotype. The analysis of the TG repeats adjacent to the 5T showed 11TG repeats in the patient with testicular atrophy and 12TG repeats in the oligozoospermic patient. These results imply that F508del/IVS8-11TG-5T genotype might not be a cause of infertility in the first patient, while G542X/IVS8-12TG-5T genotype is most probably a cause of infertility in the second patient. Similar to F508del, G542X has never been found in linkage disequilibrium with IVS8-5T [39].

The analysis for determination of TG numbers were also performed in 3 of the 5 obstructive azoospermic patients with F508del/IVS8-5T genotypes and in 20 men with 5T allele and no other CFTR mutation. Two men with F508del/IVS8-5T genotype had 12, while one had 13TG repeats. Fourteen of the 20 men with 5T allele had 11TG repeats, while 6 had 12TG repeats. All fertile controls analyzed (n = 4) had 11TG repeats. Three of the 6 men with 12TG-5T were azoospermic, one was oligozoospermic, one was oligozoospermic and two were normoasthenoterozoospermic. It might be interesting to search for other CF mutations in the azoospermic patients with 12TG-5T allele, especially if they have CBAVD. Unfortunately, we do not have any other clinical or laboratory data on these patients apart from the sperm counts to confirm that they have obstructive azoospermia and CBAVD.

The heterozygosity for CF mutation was highest among patients with obstructive azoospermia (9.09%). With the exception of the patients with azoospermia where no CF mutation was detected in all other groups of infertile men the CF heterozygosity was higher (ranging from 3.44 to 4.10%) when compared to the fertile controls (1.47%). Although these differences were not statistically significant, the higher percentage of CF mutations among infertile patients, especially those with obstructive azoospermia might imply a presence of some other mild CFTR-RD variant in these patients that was not investigated by our screening assay.

Assisted reproduction techniques that are rapidly evolving are increasing the chances for reproduction among infertile men with CFTR mutations along with the increased risk of passing the mutation to the offspring. Our assay may serve as primary screening tool for the most common CFTR mutations. In case a single CF or CFTR-RD mutation is detected the search for second mutation should be extended with sequencing of all exons and splicing sites or appropriate molecular diagnostic techniques for detecting larger genomic rearrangements. This is giving the importance of this test in the view of proper risk calculations for prospective parents and adequate genetic counseling.

Previously, one study used the SNaPshot method, but only for the detection of paternally-inherited fetal mutations in maternal plasma in three families at risk for a child with CF [41]. Here, we describe the first fully optimized and validated method for screening of several common CFTR mutations, based on multiplex single nucleotide base extension using fluorescently labeled deoxyribonucleotides. This method utilizes the use of Genetic Analyzers which are becoming standard equipment in most molecular diagnostic laboratories. The multiplex SNaPshot assay for CFTR mutation detection is a robust, inexpensive and fast method with a minimum hands-on time suited for screening of a large number of samples. It can be reliably used as a diagnostic and screening test both in patients with classical CF and atypical CF or CFTR related disorders.

Author Contributions
Conceived and designed the experiments: DPK. Performed the experiments: PN SM MM. Analyzed the data: PN DPK. Contributed reagents/materials/analysis tools: DPK TP VF. Contributed to the writing of the manuscript: PN SM DPK.

References
1. Poongotlai J, Gopenath TS, Manonayagi S (2009) Genetics of human male infertility. Singapore Med J 50: 336–347.

2. Kolettis PN (2003) Evaluation of the subfertile man. Am Fam Physician 67: 2165–2172.
3. Chan HC, Ruan YC, He Q, Chen MH, Chen H, et al. (2009) The cystic fibrosis transmembrane conductance regulator in reproductive health and disease. J Physiol 587: 2187–2195.

4. Visser L, Repping S (2010) Unravelling the genetics of spermaticogenic failure. Reproduction 139: 303–307.

5. Rowntree RK, Harris A (2003) The phenotypic consequences of CFTR mutations. Ann Hum Genet 67: 471–485.

6. Yu J, Chen Z, Ni Y, Li Z (2012) CFTR mutations in men with congenital bilateral absence of the vas deferens (CBVAD): a systemic review and meta-analysis. Hum Reprod 27: 25–35.

7. Wong PY (1998) CFTR gene and male fertility. Mol Hum Reprod 4: 107–110.

8. Zielenkis J, Tsui LC (1995) Cystic fibrosis: genotypic and phenotypic variations. Annu Rev Genet 29: 777–807.

9. Welsh MJ, Smith AE (1993) Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. Cell 73: 1251–1254.

10. Dork T, Dworniczak B, Aulenh-Behm CL, Wiegertz E, Bohm I, et al. (1997) Distinct spectrum of CFTR gene mutations in congenital absence of vas deferens. Hum Genet 100: 365–377.

11. Casals T, Bassas L, Egozcue S, Ramos MD, Gimenez J, et al. (2000) CFTR-related disorders–updated European recommendations. Eur J Hum Genet 7: 51–63.

12. Xu WM, Shi QX, Chen WY, Zhou CX, Ni Y, et al. (2007) Cystic fibrosis: genotypic and phenotypic variations. Mol Human Reprod 13: 703–713.

13. De Braekeleer M, Ferec C (1996) Mutations in the cystic fibrosis gene in men with congenital absence of the vas deferens. Hum Reprod 11: 513–517.

14. Casals T, Bassas L, Egozcue S, Ramos MD, Gimenez J, et al. (2000) Heterogeneity for mutations in the CFTR gene and clinical correlations in patients with congenital absence of the vas deferens. Hum Reprod 15: 1476–1483.

15. De Brackelee M, Ferec C (1996) Mutations in the cystic fibrosis gene in men with congenital bilateral absence of the vas deferens. Mol Hum Reprod 2: 669–677.

16. De Decker E, Stuhrmann M, Morris MA, Casals T, Castellani C, et al. (2009) Spectrum of CFTR mutations in cystic fibrosis and in congenital absence of the vas deferens in France. Hum Mutat 16: 143–156.

17. De Waeme E, Stuhrmann M, Morris MA, Casals T, Castellani C, et al. (2009) Best practice guidelines for molecular genetic diagnosis of cystic fibrosis and CFTR-related disorders–updated European recommendations. Eur J Hum Genet 17: 51–63.

18. Xu WM, Chen J, Chen H, Diao RY, Fok KL, et al. (2011) Defective CFTR-dependent CREB activation results in impaired spermatogenesis andazoospermia. PLoS One 6: e19120.

19. Ruan YC, Shum WW, Belleau C, Da Silva N, Beeton S (2012) ATP secretion in the male reproductive tract: essential role of CFTR. J Physiol 590: 4209–4222.

20. Chen H, Ruan YC, Xu WM, Chen J, Chan HC (2012) Regulation of male fertility by CFTR and implications in male infertility. Hum Reprod Update 18: 703–713.

21. Li CY, Jiang LY, Chen WY, Li K, Sheng HQ, et al. (2010) CFTR is essential for sperm fertilizing capacity and is correlated with sperm quality in humans. Hum Reprod 25: 317–327.

22. Chen J, Fok KL, Chen H, Zhang XH, Xu WM, et al. (2012) Cryptorchidism-induced CFTR down-regulation results in disruption of testicular tight junctions through up-regulation of NF-kappaB/COX-2/PGH2. Hum Reprod 27: 2385–2397.

23. Xu WM, Shi QX, Chen WY, Zhou CX, Ni Y, et al. (2007) Cystic fibrosis transmembrane conductance regulator is vital to sperm fertilizing capacity and male fertility. Proc Natl Acad Sci U S A 104: 9016–9021.

24. van der Ven K, Messer L, van der Ven H, Jeyendran RS, Ober C (1996) Cystic fibrosis mutation screening in healthy men with reduced sperm quality. Hum Reprod 11: 513–517.

25. Schulz S, Jakubczik S, Kropf S, Nickel I, Muschik P, et al. (2006) Increased frequency of cystic fibrosis transmembrane conductance regulator gene mutations in fertile males. Fertil Steril 85: 135–138.

26. Jakubczik S, Bettechen T, Stumm M, Nickel I, Mesebeck J, et al. (1999) Frequency of CFTR gene mutations in males participating in an ICSI programme. Hum Reprod 14: 1833–1834.

27. Yu J, Chen Z, Zhang T, Li Z, Ni Y (2011) Association of genetic variants in CFTR gene, IVS8 c.1210-12T>TA and c.1210-53_1210-12GT[8_12], with spermaticogenic failure: case-control study and meta-analysis. Mol Hum Reprod 17: 594–603.

28. Tuorling JH, Mol B, Kremer JA, Loorman M, Muleman EJ, et al. (1996) Mutation frequency of cystic fibrosis transmembrane regulator is not increased in oligozoospermic male candidates for intracytoplasmic sperm injection. Fertil Steril 69: 899–903.

29. Fallaha-Ruiz N, Carles S, Des Georges M, Guittard C, Arnaul F, et al. (1999) Complete mutational screening of the cystic fibrosis transmembrane conductance regulator gene: cystic fibrosis mutations are not involved in healthy men with reduced sperm quality. Hum Reprod 14: 3035–3040.

30. Mak V, Zilenkis J, Tsui LC, Dure F, Zini A, et al. (2000) Cystic fibrosis gene mutations and infertile men with primary testicular failure. Hum Reprod 15: 436–439.

31. CFPCG (1994) Population variation of common cystic fibrosis mutations. The Cystic Fibrosis Genetic Analysis Consortium. Hum Mutat 4: 167–177.

32. Petreska L, Koeveva S, Plaseska D, Mickovski M, Gjorgova-Muratovska A, et al. (1998) Molecular basis of cystic fibrosis in the Republic of Macedonia. Clin Genet 54: 203–209.

33. Koeveva S, Plaseka/Karanfiliska D, Fistoe-Naceva S, Caparreka M, Efremov G (2001) Cystic Fibrosis In Macedonia: An Update. Balkan Journal of Medical Genetics 4: 47–51.

34. Noveski P, Trivodolave S, Efremov G, Plaseka-Karanfiliska D (2009) Y Chromosome single nucleotide polymorphisms typing by SNAPSHOT minisequencing. Balkan Journal of Medical Genetics 12 (2).

35. Plaseki T, Noveski P, Popeska Z, Efremov GD, Plaseka-Karanfiliska D (2012) Association study of single-nucleotide polymorphisms in FASLG, JMJDDIA, LOC203413, TEX15, BRDT, OR2W3, INS1, and TAS2R38 genes with male infertility. J Androl 33: 673–683.

36. Madjunkova S, Volk M, Peterlin B, Plaseka-Karanfiliska D (2012) Detection of thrombophilic mutations related to spontaneous abortions by a multiplex SNAPSHOT method. Genet Test Mol Biomarkers 16: 259–264.

37. Atanasovska B, Bozhinovski G, Plaseka-Karanfiliska D, Chakalova L (2012) Efficient detection of Mediterranean beta-thalassemia mutations by multiplex single-nucleotide primer extension. PLoS One 7: e18116.

38. Untergrasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, et al. (2012) Primer3–new capabilities and interfaces. Nucleic Acids Res 40: e115.

39. Costa C, Costa JM, Martin J, Bousier B, Goossens M, et al. (2008) Multiplex allele-specific fluorescent PCR for haplotyping the IVS8 (TG)m/n locus in the CFTR gene. Clin Chim Acta 391: 156–160.

40. Patrizio P, Asch RH, Handelin B, Silber SJ (1993) Aetiology of congenital absence of the vas deferens: genetic study of three generations. Hum Reprod 8: 215–220.

41. Bustamante-Aragones A, Gallego-Merlo J, Trujillo-Tiebas MJ, de Alba MR, Gonzalez-Gonzalez C, et al. (2008) New strategy for the prenatal detection/exclusion of paternal cystic fibrosis mutations in maternal plasma. J Cyst Fibros 7: 305–310.