Osmangazi Journal of Med, 2025;47(1): 110-127
DOI: 10.20515/otd.1537723

Research Article
Araştırma Makalesi

Osmangazi Journal of Medicine
e-ISSN: 2587-1579

An Original Genetic Protocol for the Investigation of Y Microdeletions in Male Infertile Patients

Özgün Bir Genetik Protokol ile Erkek İnfertil Hastalarda Y Mikrodelesyonlarının İncelenmesi

[1]Aysen Akhundova, [2]Sezin Canbek, [3]Burcu Turkgenç

[1] Uskudar University, Institute of Natural and Applied Sciences, Department of Molecular Biology, Istanbul, Türkiye
[2] Health Sciences University, Umraniye Training and Research Hospital, Department of Medical Genetics, Istanbul, Tükiye
[3]Uskudar University, Faculty of Medicine, Department of Basic Medical Sciences, Department of Medical Biology, Istanbul, Türkiye

ORCID ID of the authors
AA. 0009-0007-8003-4049
SC. 0000-0001-9516-0047
BT. 0000-0001-7306-5866

Correspondence / Sorumlu yazar:
Burcu TÜRKGENÇ

Uskudar University, Faculty of Medicine, Department of Basic Medical Sciences, Department of Medical Biology, Istanbul, Türkiye

e-mail: burcu.turkgenc@uskudar.edu.tr

Abstract: The inability to conceive during 12 months or more of consistent, unprotected vaginal sex is known as infertility. In 30% of patients diagnosed with male infertility due to oligozoospermia and azoospermia, infertility is related to genetic causes. Y chromosome microdeletions (YMCs) are seen in idiopathic azoospermia (15-20%) and idiopathic oligozoospermia (7-10%). Complete or partial deletion status affects the patient's production of viable sperm. In this study we aimed to investigate partial and complete Y microdeletions in male infertile patients with a unique genetic protocol, taking into account the recommendations of the EAA/EMQN 2023 guidelines. In this study, DNA materials from 45 patients, most of whom had azoospermia, 1 healthy female, and 1 healthy male control DNA were analyzed for Y chromosome AZF complete and partial microdeletions using the gold standard two-step multiplex PCR methodology, and data from 45 patients were compared with results from another commercial kit. In our study, the Y microdeletion rate was 11.1% in 45 male patients diagnosed with male infertility. Of a total of 5 patients with deletions, 2 (Patients 21 and 31) had partial AZFb+c (40%) with loss of the Y chromosome terminal heterochromatin region (Sy160), 1 (Patient 23) had complete AZFc (20%) with preservation of the Y chromosome terminal heterochromatin region (Sy160), and 2 (Patients 25 and 35) had complete AZFa+b+c (40%) microdeletions. ZFY/ZFX and SRY (sY14) were preserved in Patients 25 and Patient 35 with complete AZFa+b+c deletions. The karyotype analysis result of Patient 35 was 46, XX male. A unique genetic protocol, based on the current 2023 EAA/EMQN guidelines, has been developed to examine Y microdeletions in male infertile patients with high sensitivity and accuracy and provides advantages over many previous protocols.
Keywords: Azoospermia, AZF, male infertility, Y chromosome microdeletion, multiplex PCR

Özet: Düzenli, korunmasız vajinal cinsel ilişkiden 12 ay veya daha uzun süre sonra gebe kalamama, infertilite olarak bilinmektedir. Oligozospermi ve azospermiye bağlı erkek infertilitesi tanılı hastaların %30'unda infertilite, genetik nedenlerle ilişkilidir. İdiyopatik azospermide (%15-20) ve idiyopatik oligozoospermide (%7-10), Y kromozom mikrodelesyonları görülmektedir. Komple veya parsiyel delesyon durumu, hastanın canlı sperm üretimini etkilemektedir. Çalışmamızda, EAA/EMQN 2023 klavuzu önerileri dikkate alınarak, özgün bir genetik protokol ile erkek infertil hastalarda parsiyel ve komple Y mikrodelesyonlarının incelenmesi amaçlandı. Çalışmada çoğu azospermili, 45 hastaya ait DNA materyali, 1 sağlıklı kadın ve 1 sağlıklı erkek kontrol DNA'sı, Y kromozomu AZF komple ve parsiyel mikrodelesyon tayinini gerçekleştirmek üzere, altın standart olarak görülen iki aşamalı multipleks PZR metodolojisini kullanıldı ve 45 hastanın verileri, diğer bir ticari kitle elde edilen sonuçlarla karşılaştırıldı. Çalışmamızda erkek infertilitesi tanısı almış 45 erkek hastada Y mikrodelesyon oranı %11,1 olarak belirlenmiştir. Delesyon belirlenen toplam 5 hastanın 2'sinde (Hasta 21 ve 31), Y kromozomu terminal heterokromatin bölgenin (Sy160) de kaybıyla eşlik eden parsiyel AZFb+c (%40), 1'inde (Hasta 23) Y kromozomu terminal heterokromatin bölgenin (Sy160) korunduğu komple AZFc (%20), 2 hastada (Hasta 25 ve 35) ise komple AZFa+b+c (%40) mikrodelesyonu saptanmıştır. Komple AZFa+b+c delesyonları saptanan Hasta 25 ve Hasta 35'de ZFY/ZFX ve SRY (sY14) pozitif olarak değerlendirilmiştir. Hasta 35'in karyotip analiz sonucu 46,XX erkek olarak tespit edilmiştir. Güncel 2023 EAA/EMQN kılavuzları dikkate alınarak geliştirdiğimiz benzersiz genetik protokol ile, erkek infertil hastalarda, Y mikrodelesyonlarının yüksek hassasiyet ve doğrulukla incelenmesi sağlanmıştır ve önceki çoğu protokolelere göre avantajlar sağlamaktadır. AZF komple ve parsiyel delesyonların tanısı prognostik öneme sahiptir ve hastanın tedavi seçeneklerini büyük oranda etkiler.
Anahtar Kelimeler: Azospermi, AZF, erkek infertilitesi, Y kromozom mikrodelesyon, multipleks PZR.

Ethics Committee Approval: This study was approved by the Ethics Committee of the Uskudar University (Decision no: 20244/96-1, Date: 29.01.2024).

Informed Consent: The authors declared that informed consent form was signed by the patient.

Authorship Contributions: All authors contributed equally to the study.

Copyright Transfer Form: Copyright Transfer Formwas signed by all authors.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

Received 27.08.2024

Accepted : 03.01.2025

Published : 04.01.2025

How to cite/ Atıf için: Akhundova A, Canbek S, Turkgenc B, An Original Genetic Protocol for the Investigation of Y Microdeletions in Male Infertile Patients, Osmangazi Journal of Medicine, 2025;47(1):110-127

1. Introduction

Infertility has always been the main problem of reproductive medicine. According to the World Health Organization (WHO), infertility is a specific disease state and should be considered a public disease due to its prevalence (1,2). The absence of conception after regular, unprotected sexual activity for a minimum of a year is known as infertility (3). The causes of infertility are wide-ranging and still not fully understood; in approximately 30% of infertile couples, no cause can be identified (called idiopathic infertility). Infertility is influenced by many biological factors, making it highly complex and heterogeneous. Therefore, it is controversial whether infertility is a disease, a condition, or a symptom (4).

The male factor accounts for 40-60% of infertility. Male infertility can present with a variety of semen phenotypes including qualitative defects associated with sperm cells, including azoospermia (complete absence of spermatozoa in the ejaculate), oligozoospermia (reduced number of spermatozoa), necrozoospermia (presence of dead sperm) and reduced sperm. These anomalies can be caused by various factors including other health complications (e.g. varicocele, cystic fibrosis, and obesity), infections, lifestyle choices, and environmental and genetic factors (4).

Y chromosome microdeletions (YCM) represent the absence of DNA segment(s) or gene(s) from the functionally active portion of the Y chromosome (5). Y chromosomal microdeletions are considered to be the most common structural chromosomal abnormality associated with failure of sperm production, with an overall prevalence of 1% to 58%, specifically 15-20% in idiopathic azoospermic men and 7-10% in idiopathic oligozoospermic men, and 2-3% of ICSI candidates are microdeletion carriers (6). The frequency of Y microdeletion was reported to be 9.6% in azoospermic and oligozoospermic infertile Turkish men (7).

In addition to the factors controlling testicular differentiation and maturation, a third genetic factor or gene cluster located on the Y chromosome in the distal part of the long arm (q) of the Y chromosome (Yq11) and controlling spermatogenesis is called azoospermia factor (5). Detailed molecular analyses have divided the azoospermia factor into three subregions AZFa, AZFb, and AZFc (8). Although the AZFd region has been proposed as the fourth region (9), it is known that the AZFd region does not exist in the current literature (10). Partial or complete deletion of AZF regions disrupts spermatogenesis. Since the Y chromosome sequence was mapped in 2003, microdeletions have been categorized based on the palindromic structure of euchromatin, which is made up of amplicons, which are repeat units (11). Several clinically relevant microdeletion patterns have been defined at the AZF locus including AZFa, P5-proximal P1 (AZFb), P5-distalP1 (AZFbc), P4-distal P1 (AZFbc), and b2/b4 (AZFc) (12). Deletions are caused by intrachromosomal recombinations between homologous sequences (11). The most common complete deletion type in the literature is AZFc deletion (70-80%), followed by AZFa (0.5-9%), AZFb (1-7%) and AZFbc (1-20%) (10). It has been reported that deletions detected as AZFabc are most probably associated with an abnormal karyotype such as 46,XX male, or iso(Y) (13). The frequency of Y microdeletions in infertile male patients with mosaic karyotype was reported as high as 71.43% (14). It was documented that AZFa and AZFb microdeletions were found in four (28.6%) of 14 patients with Klinefelter syndrome, and screening for Y microdeletions should be part of the diagnostic workup for those patients, especially in those under consideration for assisted reproductive techniques (15). In a recent study, it was emphasized that it is very important to perform a routine chromosomal karyotype analysis in patients presenting with male infertility along with an examination for YMCs (16).

The diagnosis of AZF deletions has prognostic importance and greatly affects the treatment options of the patient. Depending on whether the deletion is complete or partial, the sperm production of the patient may be variable and the success of benefiting from assisted reproductive techniques may be affected. Testicular sperm extraction (TESE) or (ICSI) applications are not recommended in patients with complete AZFa or AZFb deletion because there is no sperm in the ejaculate (17,18). In contrast, live sperm can be found in case of partial deletion (18–20). In case of partial AZFa and AZFb and complete AZFc deletion, a successful TESE or ISCI procedure can be performed (10). The importance of partial AZFc deletions in terms of assisted reproductive techniques is controversial (21). The cost of treatment options for infertile male patients is high. Therefore, chromosome analysis and YMC tests should be performed in infertile male patients before assisted reproductive techniques are implemented (22).

Although various methodologies have been implemented in the literature to determine AZF deletion, the gold standard protocol currently used in the genetic diagnosis of the disease is Multiplex Polymerase Chain Reaction (PCR). Even though there are many diagnostic tests and genetic protocols in the current literature, technical limitations and analysis restrictions of the methodologies have been explained in detail in the EAA/EMQN 2023 guidelines (10). Therefore, it is crucial to differentiate complete and partial AZFa, b, and c deletions with a low-cost genetic protocol with high sensitivity and specificity. In accordance with the new recommendations of the EAA/EMQN 2023 guideline, Sequence-Tagged-Site (STS) primers recommended to be used in Y microdeletion studies for accurate and sensitive discrimination of AZFabc complete and partial deletions have attracted attention.

Therefore, based on the new recommendations in the EAA/EMQN 2023 guidelines and a comprehensive literature review, we have established a new protocol for the diagnosis of Y microdeletion that will be superior to the current protocols used in Y microdeletion diagnosis and cost-effective compared to the competitors of real time PCR or fragment analysis in the market. The study aims to create a unique genetic protocol that can be determined by a simple PCR device and agarose gel electrophoresis protocol available in every genetic laboratory, can be easily applied, will be preferred for use in Turkey and the international market, and is superior to its local competitors in terms of the number of regions and scope compared to its competitors. At the same time, the study aims to ensure that patients benefit from assisted reproductive techniques in the most effective way by combining genetic data with clinical data and to create a road map for future studies.

2. Materials and Methods

Collection of Patient Materials

DNA material belonging to 45 patients diagnosed with male infertility who previously applied to the Genetic Diagnosis Centre of Ümraniye Training and Research Hospital, 1 healthy female and 1 healthy male control DNA were used. Ethics committee approval of our study was obtained by the Üsküdar University Non-Interventional Research Ethics Committee No: 61351342/ OCAK 2024-96). YMC analysis of the DNA material previously obtained from the patients included in the study was performed at the Medical Genetics Laboratory of Üsküdar University Faculty of Medicine. Patients between 20-60 years of age, male individuals with at least 1 sperm parameter abnormality, and patients whose karyotype chromosome analysis resulted in normal (46,XY) or sex chromosome abnormality (47,XXY, 47,XYY... etc.) were included in the study within the scope of inclusion criteria. Individuals under 20 years of age and over 60 years of age, patients with semen infection, and patients diagnosed with infertility as a result of another hereditary disease (congenital adrenal hyperplasia, cystic fibrosis, primary ciliary dyskinesia, Kalmann syndrome, Prader Willi syndrome, sickle cell anemia, etc.) other than sex chromosome anomalies were excluded from the study. 1 healthy male control (20-60 years of age, no infertility diagnosis, no suspicion of a sex-related disease, normal karyotype analysis) and 1 healthy female control (20-60 years of age, no suspicion of a sex-related disease, normal karyotype analysis) were used in the study. Study subjects underwent Y microdeletion testing with one of the routinely used and validated commercial kits for subsequent protocol comparison.

Optimization of the Y Microdeletion Analysis Genetic Protocol

A genetic protocol for complete and partial AZF a, b, and c deletions on the Y chromosome was established from DNA samples. A 2-step YMC genetic protocol was optimized by using 45 infertile male infertile patient DNA, 1 healthy female control DNA, and 1 healthy male control DNA. All DNA materials were sent from Umraniye Training and Research Hospital, and the accuracy, sensitivity, and specificity of the protocol were ensured. In the genetic protocol we developed, we identified and orderedSTS primer pairs located at specific intervals on the Y chromosome, taking into account a comprehensive literature review and the recommendations of the EAA/EMQN 2023 Y microdeletion guideline. We accessed the STS primer sequences with the help of MSY Breakpoint Mapper (http://breakpointmapper.wi.mit.edu/mapper.html) and EAA/EMQN 2023 guideline (10). We checked the accuracy of the forward and reverse primer sequences via the UCSC In-Silico PCR website (https://genome.ucsc.edu/cgi-bin/hgPcr). A comprehensive list of all primer pairs (30 pairs) planned to be used in the first and second-step PCR steps, along with primer sequences, chromosome

locations, and PCR product sizes is presented in Table 1.

Table 1. STSprimer pairs used in the Y microdeletion study and their locations on the Y chromosome (Human GRCh38/hg38)

Primer Name	PCR Step	Region	Location	Size (bp)	Forward sequence	Reverse sequence
ZFY /ZFX	**First Step**	**p arm**	**Y:2978942- 2979436**	**495**	**ACCRCTGTACTGACTGTGATTACAC**	**GCACYTCTTTGGTATCYGAGAAAGT**
SRY /sY14	**First Step**	**p arm**	**2787066- 2787535**	**472**	**GAATATTCCCGCTCTCCGGA**	**GCTGGTGCTCCATTCTTGAG**
sY81	**First Step**	**AZFa**	**11975704- 11975912**	**209**	**AGGCACTGGTCAGAATGAAG**	**AATGGAAAATACAGCTCCCC**
sY82	Second Step	AZFa	12207374- 12207637	264	ATCCTGCCCTTCTGAATCTC	CAGTGTCCACTGATGGATGA
sY1064	Second Step	AZFa	12321376- 12321485	110	GGGTCGGTGCACCTAAATAA	TGCACTAAAGAGTGATAATAAATTCTG
sY86	**First Step**	**AZFa**	**12495697- 12496014**	**320**	**GTGACACACAGACTATGCTTC**	**ACACACAGA GGG ACAACCCT**
sY84	**First Step**	**AZFa**	**12678105- 12678432**	**328**	**AGAAGGGTCCTGAAAGCAGGT**	**GCCTACTACCTGGAGGCTTC**
sY1065	Second Step	AZFa	13110497- 13110735	239	TCAGGTACTGTGATGCCGTT	TGAAGAGGACACAAAGGGAAA
sY88	Second Step	AZFa	13492084- 13492206	123	TTGTAATCCAAATACATGGGC	TGCACTAAAGAGTGATAATAAATTCTG
sY182	**First Step**	**AZFa**	**13868910- 13869034**	**125**	**TCAGAAGTGAAACCCTGTATG**	**GCATGTGACTCAAAGTATAAGC**
sY105	Second Step	AZFb	17245408- 17245708	301	AAGGGCTTCTTCTCTTGCTT	AGGGAGCTTAAACTCACCGT
sY108	Second Step	AZFb	17510991- 17511350 18394662- 18395021	360	TTGTGGATTGTTGTTTTTGTTG	AAGACAATGTTGTACCGGCA
sY1224	Second Step	AZFb	18449739- 18450378	640	GGCTTAAACTTGGGAGGGTG	CAAAGAGCCTCCCAGACCA
sY117	**First Step**	**AZFb**	**18549981- 18550242 18770164- 18770425**	**262**	**GTTGGTTCCATGCTCCATAC**	**CAGGGAGAGAGCCTTTTACC**
sY121	Second Step	AZFb	18890192-18890381	190	AGTTCACAGAATGGAGCCTG	CCTGTGACTCCAGTTTGGTC
sY124	Second Step	AZFb	19974696- 19974804	109	CAGGCAGGACAGCTTAAAAG	ACTGTGGCAAAGTTGCTTTC
sY127	**First Step**	**AZFb**	**20408532- 20408804**	**273**	**GCTCACAA ACG AAAAGAAA**	**CTGCAGGCAGTA ATAAGGGA**
sY130	**First Step**	**AZFb**	**21082005- 21082177**	**173**	**AGAGAGTTTTCTAACAGGGCG**	**TGGGAATCACTTTTGCAACT**
sY134	**First Step**	**AZFb**	**21394175- 21394477**	**301**	**GTCTGCCTCACCATAAAACG**	**ACCACTGCCAAAACTTTCAA**
sY1258	Second Step	AZFc	21924767- 21925734	968	AACCCCATCTCTAGCAAAAATATG	TAGGTGACAGGGCAGGATTC
sY1161	Second Step	AZFc	22092892- 22093221 22493184- 22493513	330	CGACACTTTTGGGAAGTTTCA	TTGTGTCCAGTGGTGGCTTA
sY1192	Second Step	AZFc	22726631- 22726885	255	ACTACCATTTCTGGAAGCCG	CTCCCTTGGTTCATGCCATT
sY1191	Second Step	AZFc	22729473- 22729857	385	CCAGACGTTCTACCCTTTCG	GAGCCGAGATCCAGTTACCA
sY153	**First Step**	**AZFb**	**22866498- 22866636**	**139**	**GCATCCTCATTTTATGTCCA**	**CAACCCAAAAGCACTGAGTA**
sY255	**First Step**	**AZFb**	**23168670- 23168793 23179510- 23179633 23190358- 23190481 23228061- 23228184**	**124**	**GTTACAGGATTCGGCGTGAT**	**CTCGTCATGTGCAGCCAC**
sY254	**First Step**	**AZFc**	**23170046- 23170425 23180886-23181265 23191734- 23192113 23226429- 23226808**	**380**	**GGGTGTTACCAGAAGGCAAA**	**GAACCGTATCTACCAAAGCAGC**
sY1291	Second Step	AZFc	23358923- 23359449	527	TAAAAGGCAGAACTGCCAGG	GGGAGAAAAGTTCTGCAACG
sY1206	**First Step**	**AZFc**	**25289447- 25289840**	**394**	**ATTGATCTCCTTGGTTCCCC**	**GACATGTGTGGCCAATTTGA**
sY1201	Second Step	AZFc	26311169- 26311845	677	CCGACTTCCACAATGGCT	GGGAGAAAAGTTCTGCAACG
sY160	First Step	Terminal	50000000– 50000235	236	TACGGGTCTCGAATGGAATA	TCATTGCATTCCTTTCCATT

First-Step of PCR

The STS primers used in the first-step of the genetic protocol design were determined by considering the recommendations of the EAA/EMQN 2023 guideline (10). In the multiplex PCR method, ZFX/ZFY and SRY primers were used as internal controls. Because the ZFX/ZFY primer pair shows homology in the short arm of both the X and Y chromosomes. Therefore, this primer pair is expected to amplify in DNA material from both males and females. The SRY primer is another sex-determining marker located on the short (p arm) of the chromosome and is expected to amplify in all male individuals, whether infertile or not. The other primers ordered in our protocol were male-specific and amplified only when male DNA material was used. Three sets of multiplex PCR (sets A, B, and C) were pooled, each containing four to six primer pairs, to identify deletions in the AZFa, b, and c regions. The primers to be used in mix sets A, B, and C in the multiplex PCR method are shown in Table 2. Stock primers (100 pmol/µl) to be used in each of the A, B, and C mix primer sets were pooled using the formula M1 x V1= M2 x V2 with a final concentration of 5 pmol/µl.

Table 2. Primers of Mix Set A, B, and C in the first-step of PCR

A Mix	B Mix	C Mix
SY1206-394 bp	ZFX/ZFY-495 bp	SRY-472 bp
SY86-320 bp	SY254-380 bp	SY134-301 bp
SY117-262 bp	SY84-328 bp	SY160-236 bp
SY130-173 bp	SY127-274 bp	SY255-124 bp
SY182-125 bp	SY81-209 bp	
	SY153-139 bp	

For the fmultiplex PCR reaction, 3 separate A, B, and C Master mixes for primer sets A, B, and C were prepared as shown in Table 3. The table shows the PCR components, the amounts to be collected from each PCR component, and the expected final concentrations. For each master mix, 1 DNA sample from a fertile male, 1 DNA sample from a fertile female, and a DNA-free water control sample were used in the PCR reactions along with the infertile patient samples whose deletion status was questioned. For each A, B, and C master mix, 1 more reaction mix was prepared than the total number of patient and control samples and distributed into PCR tubes at 22.5 µl per reaction. 2.5 µl DNA was added to the reaction mixes .

Table 3. Master mix preparation for primer sets A, B, and C

PCR Components	Amount	Final Concentration
10X Taq Buffer	2,5 µL	1x
dNTP Mix (2.5 mM)	3 µL	0,3 mM
Primer Mix A, B or C (5 pmol/µL)	2 µL	0,4 µM
25 mM MgCl2	2 µL	2 mM
Taq DNA Polymerase (5 U/µL)	0,5 µL	2,5 Unit
Nuclease-free water	12,5 µL	-
Subtotal	22,5 µL	-
Template DNA (~30-50 ng/µL)	2,5 µL	75-125 ng
Total volume	25 µL	

The prepared samples were amplified by a Thermal Cycle Device (Applied Biosystem, Thermo Fisher Scientific, USA). After pre-denaturation at 95°C for 3 minutes, 40 cycles of amplification were performed at 95°C for 30 seconds, 57°C for 40 seconds, 72°C for 45 seconds, and 72°C for 7 minutes for the last elongation step. After PCR, the samples were loaded into the wells of a 3% 100 ml

agarose gel prepared in 1 x TBE buffer containing ethidium bromide at a final concentration of 0.3 µg/mL. After the cap and power cables were replaced, the current was applied from the power supply. PCR products were run for 45 minutes at 100 volts (400 mA) using a horizontal gel electrophoresis system (Bio-Rad, USA). The band positions and presence of PCR products were visualized using a UV Gel Imaging system (Gel Doc XR+ Gel Documentation System, Bio-Rad, USA).

Second Step of PCR

DNA samples in which no deletion was detected in the first-step PCR study were excluded from the second-step PCR analysis and were considered to have no deletion. However, if a deletion was detected in 1 or more of the primers used in the first-step multiplex PCR study, additional primers were used behind and in front of the deletion, region to determine the start and end of the deletion (breakpoints) and to determine whether the deletion was complete or partial.

Following PCR, the samples were visualized by horizontal agarose gel electrophoresis and UV imaging as described in the previous step. Complete or partial deletions in AZFa, b, and c regions were determined by analyzing the results of the second multiplex PCR.

Data Analysis

All patient data studied with the Y microdeletion protocol were evaluated for partial and complete deletions using the current EAA/EMQN guideline (10). Clinical and genetic data were summarized in a table, and the results found in 45 patients were compared and interpreted. The demographic and clinical characteristics of the patients and Y microdeletion genotype results were presented as frequency distributions (n and %), Mean + SD, and Median (min-max) for numerical data.

3. Results

In the study, as a result of Y microdeletion analysis, we tested and compared the results of 1 healthy male and 1 healthy female control individual and the DNA material of a total of 45 individuals who were previously admitted to the Genetic Diagnosis Centre of Umraniye Training and Research Hospital and diagnosed with male infertility. No deletion was detected in 40 patients (88.9%) and 5 (11.1%) of the 45 patients studied. Of the 45 patients, 43 (95.6%) were diagnosed with azoospermia, 1 (2.2%) with oligoasthenoteratospermia, and 1 (2.2%) with asthenoteratospermia (Figure 1). As a result of Physical Examination/Ultrasonography (USG) findings, left-sided stage 1 varicocele was detected in 9 patients (20%). Follicle-stimulating hormone (FSH) was found to be elevated in 5 (Patients 4, 20, 28, 31, 31, and 35), luteinizing hormone (LH) in 5 (Patients 4, 28, 30, 31, and 35) and testosterone (TT) in 1 (Patient 5) of 11 patients. Karyotype examination revealed 47,XXY karyotype (Klinefelter syndrome) in 1 of 40 patients (Patient 28) (2.5%) and 46,XX male syndrome in 1 of 5 patients (Patient 35) (20%). The mean age of the study patients was 34.6±6.8 years. We also compared the Y microdeletion results of our genetic protocol with the results of other commercial kit in Table 4.

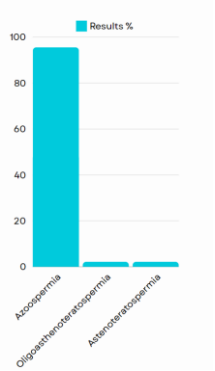

Figure 1. Spermiogram results of 45 patients used in the study. 43 patients (95,6%) were diagnosed with azoospermia, 1 patient (2,2%) with oligoastenoteratospermia, and 1 patient (2,2%) with astenoteratospermia.

Table 4. Examination, laboratory, cytogenetic, and molecular genetic results

Patient No	Age	Spermiogram	Physical Examination /Hormone USG findings	Values	Pathological Diagnosis	Chromosome Analysis	YMD Analysis (Other Commercial Kit*)	YMD* Analysis
1	47	Azoospermia	-	-	Primary infertility	46,XY	Deletion (-)	Deletion (-)

2	60	Azoospermia	-	-	Primary infertility	46,XY	Deletion (-)	Deletion (-)
3	39	Azoospermia	-	-	Primary infertility	46,XY	Deletion (-)	Deletion (-)
4	34	Azoospermia	-	FSH:40.3 LH:33.3 TT:5.11	Primary infertility	46,XY	Deletion (-)	Deletion (-)
5	36	Azoospermia	-	FSH:5.7 LH:3.5 TT:12.88	Primary infertility	46,XY	Deletion (-)	Deletion (-)
6	36	Azoospermia	-	-	Primary infertility	46,XY	Deletion (-)	Deletion (-)
7	35	Oligoasthenoteratospermia	Grade 1 varicocele on the left side	-	-	46,XY	Deletion (-)	Deletion (-)
8	38	Azoospermia	-	-	Primary infertility	46,XY	Deletion (-)	Deletion (-)
9	33	Azoospermia	-	-	-	46,XY	Deletion (-)	Deletion (-)
10	44	Azoospermia	-	-	Primer infertilite	46,XY	Deletion (-)	Deletion (-)
11	30	Azoospermia	-	-	-	46,XY	Deletion (-)	Deletion (-)
12	26	Azoospermia	-	-	Primary infertility	46, XY	Deletion (-)	Deletion (-)
13	38	Azoospermia	-	-	-	46,XY	Deletion (-)	Deletion (-)
14	37	Azoospermia	Grade 1 varicocele on the left side	-	-	46,XY	Deletion (-)	Deletion (-)
15	35	Azoospermia	-	-	Primary infertility	46, XY	Deletion (-)	Deletion (-)
16	31	Azoospermia	-	-	-	46,XY	Deletion (-)	Deletion (-)
17	39	Azoospermia	Grade 1 varicocele on the left side	-	Primary infertility	46,XY	Deletion (-)	Deletion (-)
18	30	Astenoteratospermia	Grade 1 varicocele on the left side	-	-	46,XY	Deletion (-)	Deletion (-)
19	39	Azoospermia	-	FSH:4.8 LH:3.8 TT:4.39	Primary infertility	46,XY	Deletion (-)	Deletion (-)

Table 4. Examination, laboratory, cytogenetic, and molecular genetic results (Continued)

Patient No	**Age**	**Spermiogram**	**Physical Examination /Hormone USG findings Values**	**Pathological Diagnosis**	**Chromosome Analysis**	**YMD Analysis (Other Commercial Kit*)**	**YMD Analysis (Our Genetic protocol)**
20	33	Azoospermia	- FSH:16.1 LH:7.1 TT:5.18	-	46,XY	Deletion (-)	Deletion (-)

21	**45**	**Azoospermia**	**-**	**-**	**Primary infertility**	**46,XY**	**AZFc and AZFd deletion**	**Partial AZFb+c +sY160 deletion**
22	33	Azoospermia	-	-	-	46,XY	Deletion (-)	Deletion (-)
23	**40**	**Azoospermia**	**-**	**-**	**-**	**46, XY**	**AZFc deletion**	**Complete AZFc deletion (sY160+)**
24	33	Azoospermia	Grade 1 varicocele on the left side, a slight decrease in the size of both testicles	FSH:4.75 LH:3.22 TT:3.03	-	46, XY	Deletion (-)	Deletion (-)
25	**32**	**Azoospermia**	**-**	**-**	**-**	**46, XY**	**AZFa+b+c deletion (ZFY/ZFX+, SRY+)**	**Complete AZFa+b+c +sY160 deletion (ZFY/ZFX+, SRY+)**
26	29	Azoospermia	-	FSH:1.6 LH:2.3 TT:2.42	-	46,XY	Deletion (-)	Deletion (-)
27	26	Azoospermia	-	FSH:7.9 LH:7.5 TT:4.09	-	46,XY	Deletion (-)	Deletion (-)
28	26	Azoospermia	-	FSH:21.1 LH:22.5 TT:4.23	Infertility	**47,XXY**	Deletion (-)	Deletion (-)
29	37	Azoospermia	-	-	Primary infertility	46,XY	Deletion (-)	Deletion (-)
30	29	Azoospermia	Grade 1varicocele on the left side	FSH:4.8 LH:8.8 TT:3.72	Primary infertility	46,XY	Deletion (-)	Deletion (-)
31	**34**	**Azoospermia**	**-**	**FSH:31.7 LH:12.1 TT:4.84**	**Infertility**	**46,XY**	**AZFc and AZFd deletion**	**Partial AZFb+c +sY160 deletion**
32	30	Azoospermia	Grade 1 varicocele on the left side	-	-	46,XY	Deletion (-)	Deletion (-)
33	33	Azoospermia	Grade 1 varicocele on the left side	-	-	46,XY	Deletion (-)	Deletion (-)

Table 4. Examination, laboratory, cytogenetic, and molecular genetic results (Continued)

Patient No	**Age**	**Spermiogram**	**Physical Examination USG findings**	**/Hormone Values**	**Pathological Diagnosis**	**Chromosome Analysis**	**YMD Analysis (Other Commercial Kit*)**	**YMD Analysis (Our Genetic protocol)**
34	33	Azoospermia	Grade 1 varicocele on the left side	FSH:8.5 LH:4.1 TT:3.3	-	46,XY	Deletion (-)	Deletion (-)
35	**30**	**Azoospermia**	**-**	**FSH:48 LH:29.2 TT:2.66**	**-**	**46, XX**	**AZFa+b+c deletion (ZFY/ZFX+, SRY+)**	**Deletion (+) Complete AZFa+b+c +sY160 deletion (ZFY/ZFX+, SRY+)**
36	30	Azoospermia	-	-	Primary infertility	46,XY	Deletion (-)	Deletion (-)
37	33	Azoospermia	-	FSH:5 LH:5.6 TT:3.08	-	46,XY	Deletion (-)	Deletion (-)
38	32	Azoospermia	-	FSH:6.3 LH:2.85 TT:2.85	-	46,XY	Deletion (-)	Deletion (-)

39	27	Azoospermia	-	-	-	46,XY	Deletion (-)	Deletion (-)	
40	20	Azoospermia	-	-	-	46,XY	Deletion (-)	Deletion (-)	
41	46	Azoospermia	-	-	Primary infertility	46,XY	Deletion (-)	Deletion (-)	
42	35	Azoospermia	-	-	-	46,XY	Deletion (-)	Deletion (-)	
43	42	Azoospermia	-	-	Primary infertility	46,XY	Deletion (-)	Deletion (-)	
44	33	Azoospermia	-	-	Primary infertility	46,XY	Deletion (-)	Deletion (-)	
45	28	Azoospermia	-	-	-	46,XY	Deletion (-)	Deletion (-)	

*Abbreviations; AZF: Azoospermia Factors, FSH: Follicle stimulating hormone, LH: Luteinising hormone, TT: Testosterone, USG: Ultrasonography, YMD: Y microdeletion. Normal values; FSH: 1.5-12.4 mlU/mL, LH: 1.8-8.6 mIU/mL, Testosterone: 3-10 ng/mL. *****For some ethical and legal constraints, the manufacturer's name of the commercial kit used in the study is not shared in our current publication.*

In our study, sY1206 (394 bp), sY86 (320 bp), sY117 (262 bp), sY130 (173 bp), sY182 (125 bp) for Mix A, ZFY/ZFX (495 bp), sY254 (380 bp) for Mix B, sY84 (328 bp), sY127 (274 bp), sY81 (209 bp), sY153 (139 bp), SRY(sY14) (472 bp), sY134 (301 bp), sY160 (236 bp), sY255 (124 bp) primers were used for C Mix. Figure 2 shows agarose gel images of the first-step PCRs of reaction mixtures A, B, and C of Patients studied from 21 to 36. Patients 21 and 31 had deletions in sY1206 in Mix A, sY153, and sY254 in Mix B, and sY255 and sY160 in Mix C. According to the results, the deleted primer regions in Patients 21 and 31 belonged to the AZFc region. The heterochromatin region of the Y chromosome (sY160 primer) was not amplified in either patient, suggesting that the chromosome's terminal end had been deleted. In patient 23, a deletion suggestive of AZFc deletion was detected with loss of sY1206 in Mix A, sY153, and sY254 in Mix B, and sY255 in Mix C. However, unlike the previous two patients, the heterochromatin region of the Y chromosome (Sy160) was present in Patient 23 (Figure 2, C Mix). In Patient 25 and Patient 35, deletions were found in all primers in Mix A, all primers except ZFY/ZFX primer in Mix B, and in all primer regions except SRY (sY14) primer in Mix C suggesting complete AZFa+b+c deletion.

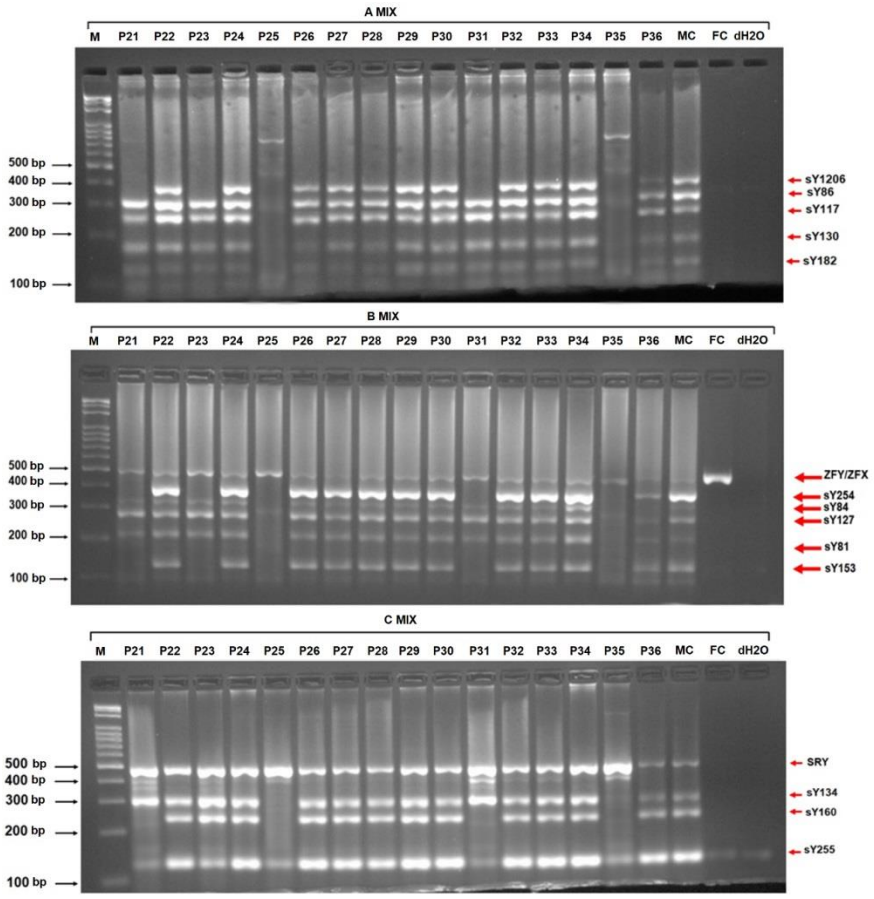

Figure 2. Agarose gel images of A, B, and C mixes of Patient 21 through Patient 36. The black arrow indicates the band positions of DNA Markers (M), while the red arrow indicates the band positions of PCR products with STS primers. M - DNA Marker (1 kb), MC - Male control, FC - Female control, dH2O - DNA-free water control.

In the C-mix in Figure 2, the sY255 primer band is quite bright in patients without deletion and weak in patients with deletion. This is because the sY255 primer band with a product length of 124 bp and the primer dimers that do not bind in the reaction are observed in the same alignment. The primer dimer structures in the female control and DNA-free water control samples also support this conclusion. However, to be sure, the presence of the sY255 deletion in Patients 21, 23, 25, and 31 was confirmed by a second PCR. As shown in Figure 3A, the sY255 primer region was deleted in Patients 21, 23, 25, and 31, except for the fertile male control. The Sy134 primer was used as an internal control primer, which is expected to be present in patients other than Patient 25 with complete deletions, obtained in the first-step of PCR. Sy1161 primer is another primer region that is expected to be present in Patients 21, 23, and 31. According to the results, the sY1161 primer region was consistent with a deletion in Patients 21 and 31, while it was present in Patient 23 (Figure 3A).

In the first-step PCR, in Patients 21, 23, and 31, all primer regions from the p arm of the Y chromosome to sY134 in the q arm AZFb region of the Y chromosome were present, and a deletion including sY153 in the AZFc region was detected. Therefore, to determine the breakpoints of deletions and to differentiate complete/partial deletions, we performed second-step PCR in 3 samples using primer pairs sY1258, sY1161, sY1192, sY1191, sY1161, sY1192, sY1191 between sY134 primer and sY153. The result of the second-step PCR for sY1258 primer in Patients 21, 23, and 31 is shown in Figure 3B. sY134 primer was used as an internal control primer in all patients with expected positivity. Fertile male control and DNA-free water control were also included in the study. As seen in Figure 3B, in Patients 21 and 31, the sY1258 region was found to be deleted, but in Patient 23, the sY1258 region was present.

Figure 3C shows the results of the second-step PCR performed with sY1161, sY1192, and sY1191 primer pairs in Patients 21, 23, and 31. In the same

study, sY153 primer was also used as a deletion control in the relevant patients. Fertile male control and DNA-free water control were also included in the study. As seen in Figure 3C, deletions were detected for all primers in Patients 21 and 31, while in Patient 23 the sY1161 primer was present and there were deletions in the sY1192, sY1191, and Sy153 primers.

Finally, since the terminal heterochromatin region of the q arm of the Y chromosome (sY160) was present in Patient 23 in the first-step PCR, but the Sy1206 primer in front of it was deleted, we performed a further second-step PCR with the sY1201 primer between both primers. Since the terminal region (Sy160) was already deleted in Patients 21 and 23, we expected that the sY1201 primer region would also be deleted. For sY1201, we employed both patients as deletion controls. We also used the sY134 primer as an internal control primer that we expected to detect in every patient (Figure 3D). Even though nonspecific bands are present in the figure, the sY1201 region was found to be deleted in Patients 21, 23, and 31.

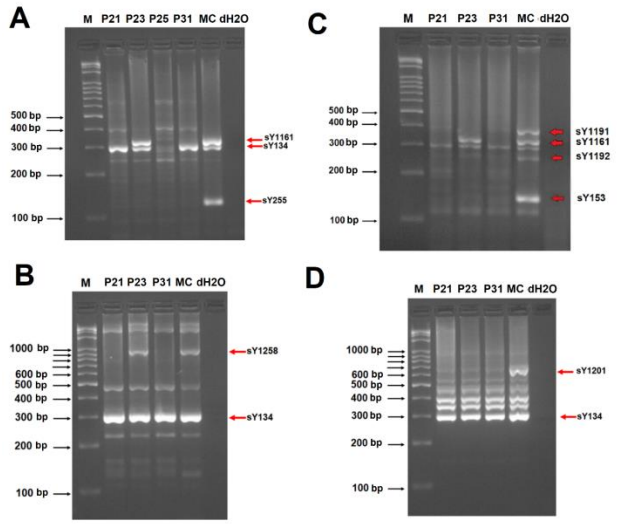

Figure 3. Gel images of the second-step PCR of Patients 21, 23, 25, and 31 with deletion. The black arrow indicates DNA Marker (M) band positions and the red arrow indicates the band positions of PCR products made with STS primers. M - DNA Marker (1 kb), MC - Male, control, dH2O - DNA-free water control. **A.** Confirmation of the presence of sY255 deletion in Patients 21, 23, 25, and 31 by the second study. **B.** Results of the second-step PCR to detect the presence of the sY1258 primer region in Patients 21, 23, and 31. **C.** Second-step PCR result for the detection of sY1161, sY1192, and sY1191 primer regions in Patients 21, 23, and 31. **D.** Second-step PCR result for the detection of the presence of sY1201 primer region in Patients 21, 23, and 31.

Figure 4 shows the results of our Y microdeletion protocol, which was optimized and tested for specificity and reliability, in 45 infertile male patients. The primers used in the first-step are circled in red. The presence of deletion in the primer region studied for the patients is indicated by the '-' symbol, and the absence of deletion is indicated by the '+' symbol. As seen in Figure 4, a deletion starting from sY1258 and including the sY160 heterochromatin terminal region was detected in Patients 21 and 31. In Patient 23, a deletion was detected starting from sY1192 and including sY1201, but the terminal heterochromatin region (sY160) was preserved. In Patients 25 and 35, ZFY/ZFX and SRY regions were intact, while other primer regions were deleted.

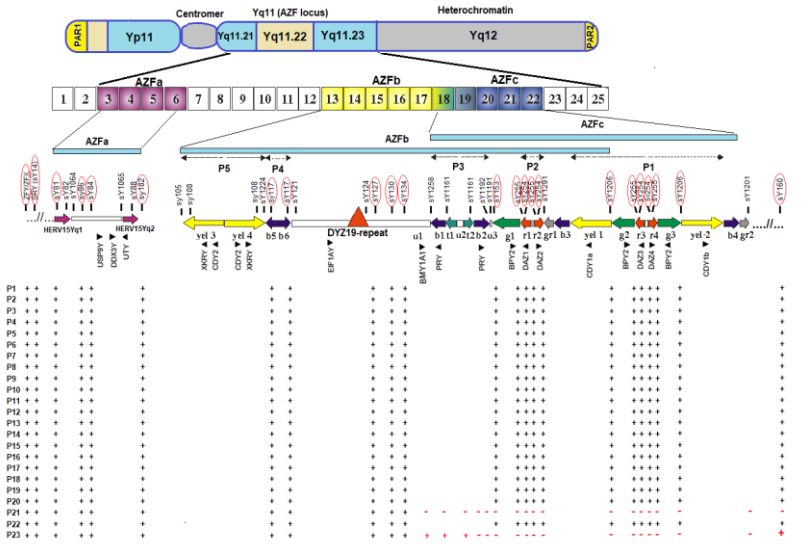

Figure 4. Results of primers used for first and second-step PCR on 45 patients studied.

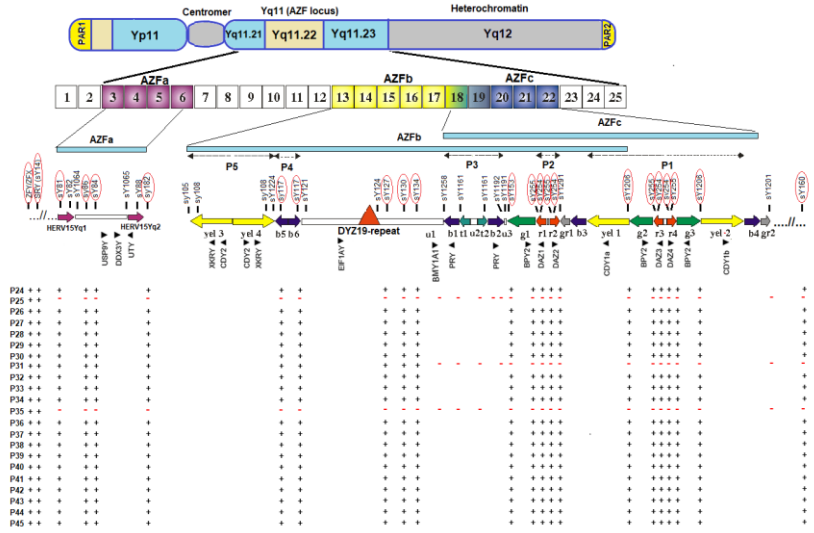

Figure 4. Results of primers used for first and second-step PCR on 45 patients studied (continued).

4. Discussion and Conclusion

Infertility, which is defined as the failure to achieve pregnancy following 1 year of consistent and unprotected vaginal intercourse, is an extremely emotionally, physically, and financially stressful condition for patients (2). For a man to be fertile, he must have appropriate spermatogenesis, successful epididymal maturation and sperm storage, and timely sexual activity. Men are responsible for approximately 20% of infertility cases (23), and an estimated 15–30% of male-related causes are assumed to be genetic (12). The most common genetic causes are YMCs and sex chromosome abnormalities (24). YMCs are the second most common inherited cause of male infertility after Klinefelter syndrome (25).

The diagnosis of AZF deletions has prognostic significance and greatly influences the patient's treatment options. Testicular sperm extraction (TESE) or (ICSI) applications for sperm in the ejaculate of patients with complete AZFa or AZFb deletion are not recommended (18–20), however viable sperm can be found in cases of partial deletion. In cases of partial AZFa and AZFb and complete AZFc deletion, a successful TESE or ISCI

procedure can be performed (10). The importance of partial AZFc deletions in terms of assisted reproductive techniques is controversial (21). The cost of treatment options for infertile male patients is quite high. Therefore, karyotype analysis and YMC tests should be performed before assisted reproductive techniques for these patients (22). Considering the recommendations of the EAA/EMQN 2023 guideline, it was noted that they were insufficient or limited in detecting AZFa, AZFb, and AZFc partial deletions, especially in terms of regional coverage (10).

In the literature, although different methodologies are applied to detect AZF deletions, the gold standard protocol currently used in the genetic diagnosis of the disease is the multiplex PCR methodology. In line with the new recommendations of the EAA/EMQN 2023 guideline (10), 2-step PCR is recommended for the efficient determination of deletions occurring in AZFa, b, and c in Y microdeletion analysis. In the first-step of PCR, six primer regions should be analyzed (sY84, sY86, sY127, sY134, sY254, and sY255). In the manual, it is stated that if one of the primers does not work, the reaction should be repeated. Because there may be a problem due to PCR conditions. If deletions were detected in 2 or more of these primers, it was recommended to proceed to second-step of PCR and determine the breakpoint of the deletion. In our study, we ordered a total of 30 primer pairs containing SRY, ZFX/ZFY, AZFa, b, c, and Y distal heterochromatin regions, taking into account the recommendations of the EAA/EMQN 2023 guideline. By utilizing as many STS markers as possible, the risk of false negative results was decreased and deletion sites were more accurately and highly sensitively identified.

Most of the commercial kits currently widely used in our country are foreign-dependent. Some local Y microdeletion kits developed in our country were also found. Some of these kits work with fragment analysis and some with real-time PCR methodology. Fragment analysis is a high-sensitivity genetic analysis technique that involves fluorescently labeling DNA fragments, capillary electrophoresis, comparison with an internal standard, and size-based differentiation (26). Although fragment analysis is a sensitive methodology compared to multiplex PCR due to the need for fluorescently labeled primers and capillary electrophoresis like ABI, which requires expensive equipment, the reaction cost is considerably increased, and not all centers have fragment analysis devices. Some Y microdeletion kits readily available in the literature are analyzed with real-time PCR methodology, which is a sensitive method. Real-time PCR is a molecular biology laboratory technique based on PCR. It monitors the amplification of a specific target DNA molecule during PCR (i.e., in real time) rather than at the end as in traditional PCR (27). Real-time PCR methodology is costly compared to the requirements of fluorescently labeled probes (TaqMan probes, Scorpion probes, LightCycler probes, etc.) and classical thermal cycling devices. In the literature, methodologies using SYBR GREEN-based real-time PCR methodology instead of high-cost labeled probes are also found. At the beginning of the SYBR Green procedure, double-stranded DNA, primers, and SYBR Green fluorescent dye are added to the reaction mixture. When the primers bind and elongation begins, the SYBR Green dye attaches itself to the double-stranded DNA, initiating fluorescence emission. The amount of fluorescent signal emitted and read by the real time PCR equipment monitor increases with the amplification of DNA (28,29). Since this methodology uses a methodology based on the binding of a fluorescently labeled molecule to double-stranded DNA, its cost is relatively low compared to other probe-based methodologies. However, the cost of SYBR Green-based real-time PCR methodology remains high compared to multiplex PCR. In multiplex PCR, where multiple targets are amplified, it is difficult to distinguish between different amplicons as they all produce the same fluorescent signal. In addition, real-time PCR equipment is not available in every center. When the literature was examined, it was realized that some kits use multiplex PCR methodology. However, in general, when we analyzed the available kits, we found that the majority of these kits were inadequate in detecting AZFa, AZFb, and AZFc partial deletions. In addition, sY83 and sY143 primers, which are included in old kits that are outdated in the literature, are no longer recommended for the determination of deletion breakpoints. For the efficient discrimination of complete and partial AZFa deletions, it is strongly recommended to use sY1064 at the AZFa proximal border instead of sY83, and for the successful identification of AZFb complete and partial deletions, sY143 should no longer be used and sY1192 should be used instead. AZFa proximal border markers sY83 and sY1064 and AZFb distal border markers sY143 and sY1192 cannot be used interchangeably. In addition, the Y chromosome heterochromatin region primer (sY160) is not available in many ready-to-use kit protocols. The

guideline strongly recommends testing the sY160 heterochromatin marker, which also enables the detection of terminal deletions (10).

In our study, 15 pairs of primers were used in multiplex sets in the front panel with first-step PCR. Since the results were expected to be negative in most of the individuals studied (approximately 80–90%), second-step PCR was not required. Out of 45 male patients diagnosed with male infertility, the Y chromosomal microdeletion rate was found in 5 individuals (11.1%) in our study. This result is similar to the literature in Turkey. In a study conducted on 437 infertile male patients in Turkey, it was reported that YMC was found in 44 patients (10.06%) (22). It was observed that the reported frequency of YMCs in infertile male groups varies significantly in the literature according to ethnic origin and geographical location. The prevalence has been reported to be less than 2% in countries such as Germany and Austria, 12% in the United States of America, and 24.2% in Iran (30). This discrepancy may potentially be impacted by the clinical definitions of the patients, the inclusion criteria of the study participants, and incorrect deletion detection.

Partial AZFb+c deletion was found in 2 (4.4%) (Patients 21 and 31), complete AZFa+b+c deletion in 2 (4.4%) (Patient 25 and Patient 35), and complete AZFc deletion in one (2.2%) (Patient 23) of the 45 patients studied. In the literature, in 437 male infertile patients, AZFc partial gr/gr deletion was found in 17 (3.9%), partial AZFb+c in 9 (2.1%), partial AZFc in 8 (1.8%), complete AZFb+c deletion in 4 patients, complete AZFc (0.5%) deletion in 2 (0.5%), and complete AZFa deletion in 1 patient (22). In our study, second-step PCR was not required in individuals with complete AZFa+b+c deletion (Patients 25 and 35). For Patients 21, 23, and 31 with AZFc deletion, second-step PCR analysis was performed by using primer pairs sY1258, sY1161, sY1192, and sY1191 in front of the deletion site to detect the exact breakpoints of the deletions. In the literature, it has been reported that AZFc region deletions are the most common YMC and constitute 60-80% of all reported deletions (10).

In our study, no deletions involving AZFa or AZFb regions alone were detected in any of the patients studied, and there was no need to use the second-step primers ordered for these regions. Therefore, in our novel genetic protocol, most of the cost was incurred by the 3 multiplex set PCRs performed in the first-step. In the literature, it is known that complete deletion of the AZFa region causes ‘Sertoli Cell Only (SCO)’ syndrome, a disease in which germ cells are absent and seminiferous tubules contain only Sertoli cells as a result of loss of *DDX3Y* and *USP9Y* genes (31). AZFa partial deletions are associated with phenotypes ranging from azoospermia to normozoospermia (20). The two STS markers sY84 and sY86 used in the molecular analysis of the AZFa region are located in front of the *USP9Y* and *DDX3Y* genes. According to the pathogenic mechanism of the deletion and available data, when a deletion is detected in both sY84 and sY86, the probability of encountering a complete deletion for AZFa is very high. However, it is possible (although rare) to delete both markers without affecting the two AZFa genes or to affect only *USP9Y* (32,33). Therefore, it is crucial to test deletion breakpoints with additional primers anterior and posterior to AZFa deletions. In AZFa complete deletion, sY82 (present), and sY1064 (absent) are used for the proximal border, and sY1065 (absent), and sY88 (present) are used for the distal border (10). If only sY84 or sY86 are found to be deleted (and amplification failures can be excluded), the AZFa region should be analyzed in more detail (13). However, this event is currently thought to be extraordinarily rare. The average frequency of partial AZFa deletion has been reported to be 5.3% (34). In contrast to our novel genetic protocol, the other studied commercial kit did not include primers sY81, sY82, sY1064, sY1065, and sY88, and was expected to miss partial AZFa deletions if present.

In our study, complete AZFb deletion was not detected. However, partial AZFb+c deletion starting from the P3 palindrome of the AZF region and continuing along AZFc, including the terminal part of the Y chromosome, was found in 2 patients (Patients 21 and 31). In contrast to our novel genetic protocol, other commercial kit results using STS primers sY127, sY130, and sY131 for the AZFb region; sY152 and sY153 for the AZFd region; and sY254 and sY255 for the AZFc region reported AZFc and AZFd deletions in the same 2 patients. In the current literature, sY153 is used as the distal border of the AZFb region and is expected to be present in the presence of AZFb deletion. In addition, sY152 is known to map to DAZ genes, similar to sY255 and sY254, and it has been reported that the “AZFd” region defined upon the suggested absence of sY152 is not present and is not recommended for use (10). In our new genetic protocol, we could also capture partial AZFb deletion by using additional markers sY1258, sY1161, sY1192, and sY1191. Therefore, when the

deletion is detected in the first-step of PCR, it is very important to use additional markers to identify the exact breakpoints of the deletion in a second PCR. In the literature, complete deletions of AZFb have been associated with the meiotic arrest of spermatogenesis and the absence of postmeiotic germ cells. Non-classical partial AZFb deletions occurring in the AZFb region have also been associated with various testicular pathologies, including meiotic arrest, cryptozoospermia, severe oligozoospermia, or oligoasthenoteratozoospermia (OAT syndrome) (35). sY127 and sY134 markers are located in the median and distal parts of the AZFb region. According to available data, in most cases, deletion of both markers indicates complete deletion of the AZFb region. To determine the prognostic value of the deletion in terms of the TESE result, a mandatory deletion elongation analysis with additional markers is required. These are sY105 (present) sY121 and sY1224 (absent) for the proximal border and sY1192 (absent) and sY153 (present) for the distal border. The EAA/EMQN 2023 guideline states that sY1192 has prognostic value for TESE outcomes. Recent data have also shown that sY1224, which was recommended as equivalent to sY121 in previous versions of the guidelines, may still be conserved in a notable number of complete AZFb and AZFbc deletions. Although the phenotypic consequences of this variation have yet to be clarified, the use of both sY121 and sY1224 (in the absence of sY121) is strongly encouraged (10). Similar to complete AZFa deletions, it is almost impossible to obtain sperm by TESE in men with complete AZFb deletions (6,36). It has been reported that TESE may be attempted in azoospermic carriers of atypical AZFb or AZFbc deletions characterized by a proximal breakpoint at the Y chromosome P4 palindrome (instead of P5). Indeed, a smaller deletion with a proximal deletion point at P4 may be associated with the retention of additional AZFb gene copies such as *XKRY*, *CDY2*, and *HSFY* and thus a less severe, TESE-positive outcome (36–38). Similarly, partial AZFb deletions defined by the positivity of the sY1192 distal marker (part of the deletion extension analysis) may also be compatible with residual spermatogenesis. The presence of sY1192 in this patient's deletion model strongly suggests that additional copies of *RMBY1* remain intact, possibly leading to a less severe testicular phenotype (39). Another recent study reached a similar conclusion and emphasized the importance of differentiating between complete (P5/proximal P1) and partial AZFb deletions. Comprehensive analysis of P3 palindrome in the AZF region and overlapping regions of AZFb and AZFc regions is necessary for the clinical evaluation of these patients (40).

In our study, 1 patient (Patient 23) had a result compatible with AZFc complete deletion (b2/b4), including loss of sY1192, sY1191, sY153, sY255, sY254, Sy1291, and sY1206 regions, respectively. However, the heterochromatin terminal region (sY160) was discovered to be preserved in our patient. It is known that the DAZ gene family with a 4-copy polymorphic structure in the r1, r2, r3, and r4 regions in the AZFc region is effective in male germ cell formation and protection (41,42). Deletions occurring in the AZFc region have been associated with sperm maturation defects (43). In complete and partial AZFc deletions, the clinical phenotype may be variable (22). AZFbc deletions occur as a result of intrachromosomal homologous recombination between P5/distal P1 (7.7 Mb) or between P4/distal P1 (7.0 Mb) (44). The two pairs of primers sY254 and sY255 used for the AZFc region are specific for the DAZ gene, located in four copies organized in two clusters on the reference Y chromosome sequence. Based on the available data, deletion of only one of these 2 markers is extremely unlikely and should be considered a technical error. Deletion analysis using the sY160 heterochromatin marker will allow us to understand whether a complete AZFc deletion (b2/b4, sY160 present) is accompanied by a terminal deletion (sY160 absent). Terminal deletions and b2/b4 deletions may be associated with a mosaic karyotype (46,XY/45,X) (45,46), and therefore karyotype analysis is mandatory for these patients for TESE prognostic reasons. In Patient 23, sY160 primer was present, and complete AZFc deletion was not accompanied by the terminal deletion. The other commercial kit we tested did not include the STS primer sY160, which maps to the heterochromatin region of the Y chromosome and missed the deletion of that region. Partial AZFc deletion was not found in our study. However, in our new genetic protocol, there were sufficient additional STS primers (sY1161, sY1191, sY1291, sY1206, and sY1201) to capture partial AZFc deletions. In the literature, sY1291 and sY1191 primers are used for gr/gr partial AZFc deletion (44). The diagnosis is based on the absence of the sY1291 marker and the presence of sY1191. It should be noted that a false deletion rate of 5% was detected in a multicentre study (47), highlighting the importance of optimization of PCR conditions and additional validation steps. The b1/b3 partial deletion is based on the absences of sY1161,

sY1191, and sY1291. The lack of sY1191 identify the b2/b3 partial deletion (48). The other commercial kit we used in the study did not have additional STS primers for the identification of partial AZFc and would have missed partial AZFc deletions anyway.

In our study, ZFY/ZFX and SRY(sY14) primers were included in both the commercial kit and our novel genetic protocol, and regions were present in Patients 25 and 35 with complete AZFa+b+c deletions. The karyotype analysis result of Patient 35 was 46,XX male. In the literature, deletions detected as AZFabc are generally associated with an abnormal karyotype such as 46,XX male, or iso(Y) (10,46). Men with 46,XX karyotype have testicular abnormalities characterized by a genital structure ranging from normal genital structure to ambiguous genitalia. Although pubic hair and penis size are normal during puberty, most individuals with 46,XX testicular disease have gynecomastia, small testicles, and infertility due to azoospermia (49). Individuals with a positive *SRY* gene are followed up after puberty because of small testicles, gynecomastia, azoospermia, and sometimes short stature and atypical genital structures. Gynecomastia is relatively uncommon in these people. In SRY-negative 46,XX male patients, the genitals are more likely to be ambiguous from birth, as in penoscrotal hypospadias and undescended testes. Gynecomastia is common in adolescence if treatment is not received (50).

In conclusion, this study is an important step toward the investigation of Y microdeletions in male infertile patients with a unique genetic protocol. The diagnosis of AZF deletions has prognostic significance and greatly affects the patient's treatment options. Depending on whether the deletion is complete or partial, the sperm production of the patient may be variable, and the success of assisted reproductive techniques may be affected. Chromosome analysis and Y chromosome microdeletion tests should be performed before assisted reproductive techniques are utilized in infertile male patients. The most important technical limitation of our study is the low sample size. Due to the low sample size, the patients with AZFa and AZFb deletions were not detected. However, we believe that the second set of AZFa and AZFb primers we ordered will also work effectively in multiplex PCR sets and discriminate the complete AZFb and complete AZFc. Future studies with larger sample groups and other clinical factors will provide a better understanding of the effects of Y microdeletion on male infertile patients. Such studies will contribute to improving the treatment processes of male infertile patients and developing treatment approaches.

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