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ORIGINAL RESEARCH

Optimization of Multiplex-PCR Technique To Determine Azf Deletions in infertility Male Patients

Tung Nguyen Thanh[1], Sang Trieu Tien[2], Phong Nguyen Van[2], Son Dang Thai[3], Thuc Luong Cong[4], Tuan Dinh Le[5], Son Tien Nguyen[5], Tuan Tran Van[1], Hoang Huy Duong[6], Tien Minh Bui[7], Kien Trung Nguyen[7]

[1]Military Institute of Clinical Embryology and Histology, Vietnam Military Medical University, Hanoi, 100000, Vietnam; [2]Department of Biology and Medical Genetics, Vietnam Military Medical University, Hanoi, 100000, Vietnam; [3]Institute of Biological and Food Technology, Hanoi Open University, Hanoi, 100000, Vietnam; [4]Cardiovascular Center, Military Hospital 103, Vietnam Military Medical University, Hanoi, 100000, Vietnam; [5]Department of Rheumatology and Endocrinology, Military Hospital 103, Vietnam Military Medical University, Hanoi, 100000, Vietnam; [6]Department of Neurology, Thai Binh University of Medicine and Pharmacy, Thai Binh, 410000, Vietnam; [7]Department of Obstetrics and Gynecology, Thai Binh University of Medicine and Pharmacy, Thai Binh, 410000, Vietnam

Correspondence: Sang Trieu Tien, Department of Biology and Medical Genetics, Vietnam Military Medical University, Hanoi, 100000, Vietnam, Email trieusangk83@yahoo.com.vn

Background: To optimize the multiplex polymerase chain reaction (M-PCR) technique to diagnose microdeletions of azoospermia factors (AZF) on the Y chromosome and initially apply the technique to diagnose male patients with sperm density less than 5×10^6 million sperm/mL was assigned to do a test to check for AZF microdeletions on the Y chromosome.

Methods: Based on the positive control samples which belong to male subjects who have had 2 healthy children without any assisted reproductive technologies, the M-PCR method was developed to detect simultaneously and accurately AZF microdeletions on 32 male patients with sperm densities below 5×10^6 million sperm/mL of semen at the Department of Biology and Medical Genetics – Vietnam Military Medical University.

Results: Successful optimization of the M-PCR technique including 7 reactions arranged according to each AZFabc region using 24 STS/gene on the Y chromosome. Initial application to diagnose AZF deletion on 32 azoospermic and oligospermic men reveals that AZFa deletion accounts for 6.25% (2/32); deletion of all 3 regions AZFa,b,c with 18.75% (6/32 cases); The combined deletion rate of AZFb,c is highest, accounting for 56.24% (18/32 patients).

Conclusion: Successfully optimized the M-PCR technique in identifying AZF microdeletions using 24 sequence tagged sites (STS)/gene for azoospermic and oligozoospermic men. The M-PCR technique has great potential in the application of AZF deletion diagnosis.

Keywords: male infertility, azoospermia factors, AZF, multiplex polymerase chain reaction, M-PCR, sequence tagged sites, STS

Introduction

Approximately 15% of couples globally, or 48.5 million couples, experience infertility.[1,2] According to estimated data worldwide, the prevalence of male infertility ranges from about 50% or nearly 30 million men.[1,3] Modifiable factors affecting male infertility have been shown in many studies, such as infections, chronic illnesses, body mass index, environmental toxin exposures, dietary factors and alcohol use.[4,5] In addition, genetic factors are one of the most influential factors in infertility treatment. Accurate diagnosis of the genetic cause of infertility in men remains a formidable task because the spermatogenesis process is governed, controlled, and precisely coordinated by thousands of genes. Small deletions on the Y chromosome, in conjunction with Klinefelter syndrome, are recognised as a common genetic cause of male infertility.[6,7] Small deletions that occur mainly in a specific region on the long arm of the Y chromosome (Yq). In 1976, Tiepolo et al, when studying chromosomal images of male infertility patients, believed that

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there must be a genetic factor that causes azoospermia, which they called the "azoospermia factor" – AZF) is located on the long arm of chromosome Y.[8] In 1995, Reijo et al determined the location of the AZF region in the distal part of Yq11 and named it the AZF region.[9] From the molecular perspective, the spermatogenesis ability is regulated by genes located in the AZF region and is divided into 3 smaller regions: AZFa, AZFb, and AZFc.[6] A special feature of the boundaries of the AZF regions is the presence of homologous sequences Repeats are susceptible to deletion or duplication through a mechanism called non-allelic homologous recombination (NAHR). Depending on the specific type of AZF deletion, patients will have clinical manifestations of reduced sperm count in semen, ranging from complete azoospermia (non-obstructive azoospermia) to slightly reduced sperm count (< 15 million/mL).

Along with the development of technology, assisted reproductive techniques (ART) interventions such as intracytoplasmic sperm injection (ICSI) and testicular sperm extraction (TESE) have currently made parenthood possible for many people. However, these procedures carry the risk of increasing genetic abnormalities in the next generation, while the genetic landscape of male infertility is inherently complex because the histological phenotypes of semen and testicles are extremely unusual identical and have many genes related to spermatogenesis. Therefore, diagnosing genetic causes of male infertility, especially detecting basic deletions and microdeletions in the extended region of AZF, is of great clinical significance, not only in guiding clinicians in the diagnosis, treatment, and prognosis of male infertility patients, but also as a basis for researching genetic characteristics and population genomics, from which to develop strategies to develop and protect genetic resources.

A battery of modern technologies have been applied to detect small deletions in the AZF region such as polymerase chain reaction (PCR), real-time PCR, multiplex ligation-dependent probe amplification (MLPA), and quantitative fluorescent (QF)-PCR techniques worldwide.[10] However, there is a big challenge having the ability to widely apply these tests in diagnosis when the cost of testing is still high, requires modern equipment, and requires a high level of professional capacity, especially in small areas in developing countries like Vietnam. Arise from the practical issues, with the desire to develop a technique to detect small deletions in the AZF region that is easy to manipulate, saves time, and is feasibly applicable in practice, we aim to optimize the multiplex (M)-PCR technique and initially apply the diagnosis of small microdeletions in the extended region on the Y chromosome of azoospermia and oligospermia patients using 24 specific and highly sensitive primer pairs to identify sequence tagged sites (STS) and genes.

Subjects and Methods

Study Population

We screened 285 infertile male patients who were tested for AZF deletions, with 32 deletion cases from November 2021 to December 2022. Peripheral venous blood samples anticoagulated with EDTA from 32 male patients who were non-obstructive azoospermia or oligospermia with sperm density with sperm density less than 5×10^6 million sperms/mL were assigned to test for AZF microdeletions on the Y chromosome at the Department of Biology and Medical Genetics - Vietnam Military Medical University.

Positive control samples are men who have had 2 healthy children without any reproductive interventions.

Method

DNA Extraction

Peripheral venous blood samples were aspirated to extract DNA using the G-spin™ Total DNA Extraction Kit (Korea) according to the manufacturer's instructions. The extracted DNAs were measured for purity and concentration using a SpectraMax QuickDrop machine; and then diluted with deionized water to achieve a concentration of approximately 20 ng/μL, ensuring A260/280 purity of 1.8–2.0 and stored at −20°C.

Primer Selection and Design

Amplified primer pairs 22 STSs located in the AZF region and 2 internal control sequences (SRY and ZFX/Y) to detect microdeletions in the AZF region on the Y chromosome according to the instructions of the European Academy of Andrology/European Molecular Genetics Quality Network (EAA/EMQN) (Table 1). STSs to detect AZFa base deletions include sY84 and sY86; AZFb includes sY127 and sY134; AZFc includes sY254, sY255, and sY1524. Extended region

Table 1 Primer Pairs to Detect Microdeletions of the AZF Region on the Y Chromosome

Order	Size (bp)	STS/Gene	Region	Forward (5'-3') and Reverse (3'-5') Primer Sequences	Complete Mutation Deletion
1	495	*ZFX/Y*	*ZFX/Y*	ACCRCTGTACTGACTGTGATTACAC	Negative
				GCACYTCTTTGGTATCYGAGAAAGT	
2	472	*SRY*	*SRY*	GAATATTCCCGCTCTCCGGA	Negative
				GCTGGTGCTCCATTCTTGAG	
3	125	sY152	AZFc	AAGACAGTCTGCCATGTTTCA	Positive
				ACAGGAGGGTACTTAGCAGT	
4	320	sY86	AZFa	GTGACACACAGACTATGCTTC	Positive
				ACACACAGAGGGACAACCCT	
5	274	sY127	AZFb	GGCTCACAAACGAAAAGAAA	Negative
				CTGCAGGCAGTAATAAGGGA	
6	350	sY254	AZFc	GGGTGTTACCAGAAGGCAAA	Positive
				GAACCGTATCTACCAAAGCAGC	
7	326	sY84	AZFa	AGAAGGGTCTGAAAGCAGGT	Positive
				GCCTACTACCTGGAGGCTTC	
8	301	sY134	AZFb	GTCTGCCTCACCATAAAACG	Positive
				ACCACTGCCAAAACTTTCAA	
9	126	sY255	AZFc	GTTACAGGATTCGGCGTGAT	Positive
				CTCGTCATGTGCAGCCAC	
10	264	sY82	AZFa	ATCCTGCCCTTCTGAATCTC	Negative
				CAGTGTCCACTGATGGATGA	
11	275–277	sY83	AZFa	CTTGAATCAAAGAAGGCCCT	Positive
				CAATTTGGTTTGGCTGACAT	
12	110	sY1064	AZFa	GGGTCGGTGCACCTAAATAA	Positive
				TGCACTAAAGAGTGATAATAAATTCTG	
13	239	sY1065	AZFa	TCAGGTACTGTGATGCCGTT	Positive
				TGAAGAGGACACAAAGGGAAA	
14	247	sY1182	AZFa	ATGGCTTCATCCCAACTGAG	Positive
				CATTGGCCTCTCCTGAGACT	
15	123	sY88	AZFa	TTGTAATCCAAATACATGGGC	Negative
				CACCCAGCCATTTGTTTTAC	
16	301	sY105	AZFb	AAGGGCTTCTTCTCTTGCTT	Negative
				AGGGAGCTTAAACTCACCGT	

(*Continued*)

Table 1 (Continued).

Order	Size (bp)	STS/Gene	Region	Forward (5'-3') and Reverse (3'-5') Primer Sequences	Complete Mutation Deletion
17	190	sY121	AZFb	AGTTCACAGAATGGAGCCTG	Positive
				CCTGTGACTCCAGTTTGGTC	
18	690	sY1224	AZFb	GGCTTAAACTTGGGAGGGTG	Variable
				CAAAGAGCCTCCCAGACCA	
19	311	sY143	AZFb	GCAGGATGAGAAGCAGGTAG	Positive
				CCGTGTGCTGGAGACTAATC	
20	255	sY1192	AZFb	ACTACCATTTCTGGAAGCCG	Positive
				CTCCCTTGGTTCATGCCATT	
21	139	sY153	AZFb	GCATCCTCATTTTATGTCCA	Negative
				CAACCCAAAAGCACTGAGTA	
22	527	sY1291	gr/gr	TAAAAGGCAGAACTGCCAGG	Positive
				GGGAGAAAAGTTCTGCAACG	
23	385	sY1191	gr/gr	CCAGACGTTCTACCCTTTCG	Negative
				GAGCCGAGATCCAGTTACCA	
24	236	sY160	Heterochromatin	TACGGGTCTCGAATGGAATA	Negative
				TCATTGCATTCCTTTCCATT	

Abbreviation: AZF, Azoospermia factors.

STS with AZFa includes sY82, sY83, sY1064, sY1065, sY1182, and sY88; The extended region with AZFb includes sY105, sY121, sY1224, sY143, sY1192, and sY153; and the extended region with AZFc includes sY160, sY1191, sY1291. In addition, we designed the sY152 amplification sequence to detect AZFc deletion.

PCR Reaction to Determine the Optimal Primer Annealing Temperature

Based on the melting temperature of the primer pairs, we select the temperature range to perform the single-primer gradient-PCR reaction. PCR products were checked by 3% agarose gel electrophoresis, soaked with 1 µL/mL Ethidium bromide dye, and then gel captured. We then analyze and select the optimal annealing temperature for each primer pair.

The single-primer PCR reaction was optimized with the following reaction ingredients: 6.25µL of GoTaq Green Mastermix 2X, 0.25µL of each forward and reverse primer (according to Table 1), 1µL of control DNA template and nuclease-free water for just enough 12.5µL. Thermal cycling for single-primer PCR: 95°C for 10 minutes, 35 cycles with 94°C - 30s, 57°C (with primer pair 1–23) or 60°C (with primer pair 24) - 30s, 72°C - 30s and 72°C - 10 minutes and store at 4°C.

Optimization of M-PCR Reaction

After successfully amplifying 24 primer pairs, pair the M-PCR reaction to simultaneously detect deletions to save costs and reduce testing time. Seven M-PCR reactions were arranged based on the size and position of the AZFabc regions combined with internal controls including:

- Basic AZFabc region determination reaction: Set 1: primer pairs 1-3-4-5-6 and Set 2: primer pairs 2-7-8-9.

- Reaction to determine the extended AZFabc region: Set 3: primer pairs 10–12-13; Set 4: primer pairs 11–14-15; Set 5: primer pairs 16–18-20; Set 6: primer pairs 17–19-21; Set 7: primer pairs 22–23-24.

The M-PCR reaction was optimized in 12.5 µL reaction with primer pair concentration (10 pmol/µL) along with 6.25 µL GoTaq Green Mastermix 2X, 1.5 µL DNA template, and clean water for sufficient reaction. Thermal cycling for M-PCR reaction with Set: 2,3,4,5,6 at 95°C for 10 minutes, 35 cycles with 94°C - 30s, 59° - 30s, 72°C - 45s and 72°C - 10 minutes and stored at 4–8°C. For sets 1 and 7, due to the presence of many byproducts, Touchdown-PCR reaction with thermal cycle: 95°C for 5 minutes, 35 cycles with 94°C - 30s, 61°C at 5 first cycle and 59°C at 30 last cycle - 60s, 72°C - 30s and 72°C - 5 minutes and stored at 4°C.

PCR products were evaluated by electrophoresis on a 3% agarose gel for 35 minutes with a current of 250V and stained with 1 µL/mL Ethidium bromide.

The procedure for determining AZF deletions using M-PCR is designed by us according to the diagram in Figure 1 (Figure 1).

Data Analysis and Statistical Analysis

Agarose gel electrophoresis results were used to determine the type of AZF microdeletion for each sample. With the obtained data, we created a statistical table using Microsoft Excel software (Microsoft Corp., Washington, DC, USA) and calculated the number and proportion of each specific type of deletion.

Results

Single-Primer PCR Reaction

With the established reaction conditions and primer annealing temperature of 57°C for primer pairs 1 to 23 and 60°C for primer pair 24, the single-primer PCR reaction successfully amplified PCR products containing the size is correct as announced on National Center for Biotechnology Information (NCBI), the electrophoresis tape does not appear by-products, and the image is clear. The reaction results show that the primer sequence has high specificity, accurately identifying STS/genes (Figure 2).

Optimize Multiplex PCR Reaction

After successfully standardizing the single-primer PCR method, selecting paired STSs based on each AZFabc region will help to ease deletion control by region (Figure 3): Reaction 1: control Y-sY152-sY86-sY127-sY254 (Figure 3A); Reaction 2: control (Y)-sY84-sY134-sY255 (Figure 3B); Reaction 3: sY82-sY1064-sY1065 (Figure 3C); Reaction 4: sY83-sY1182-sY88 (Figure 3D); Reaction 5: sY105-sY1224-sY1192 (Figure 3E); Reaction 6: sY121-sY143-sY153 (Figure 3F); Reaction 7: sY1291-sY1191-sY160 (Figure 3G).

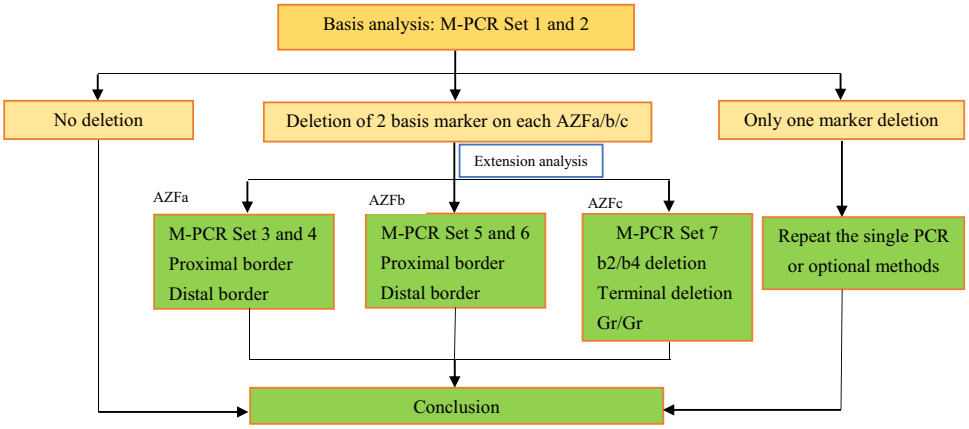

Figure 1 Procedure for determining AZF deletion using M-PCR.

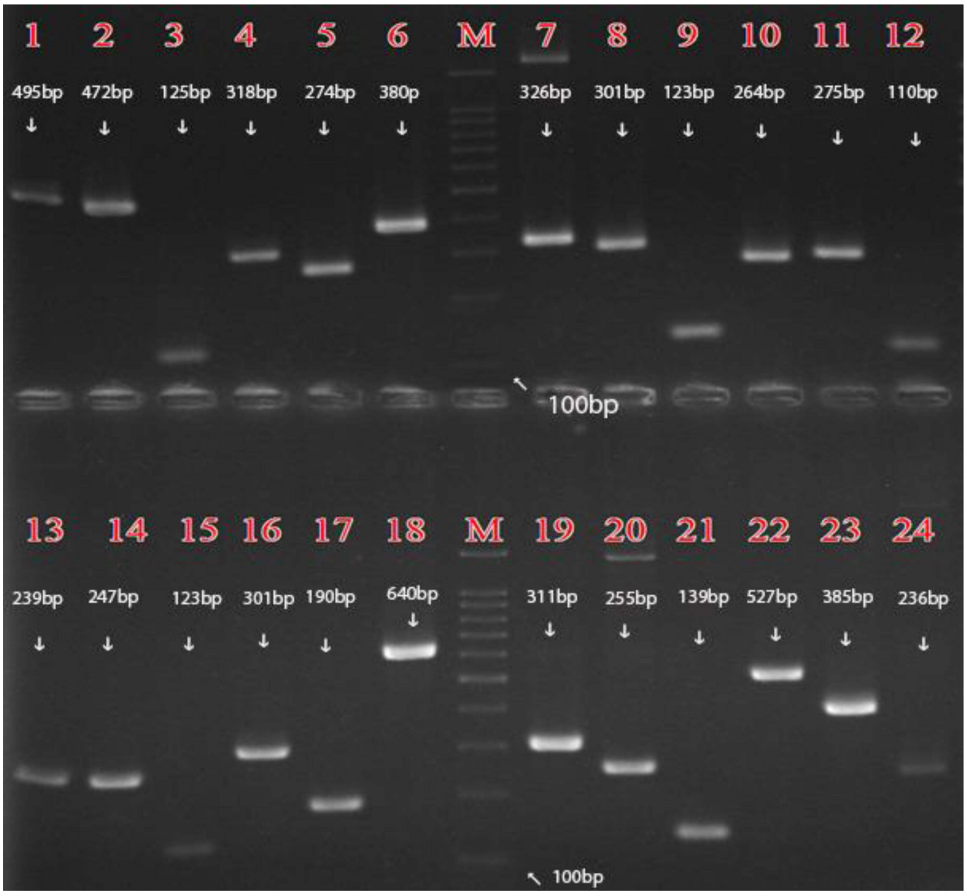

Figure 2 Electrophoresis results of single-primer PCR products of 22 STS and 2 internal controls.

Regarding the M-PCR reaction after optimization, the electrophoretic image of the product shows that the band is separated, with similar results between the single-primer PCR reaction and M-PCR. The banded appearance of the control gene (SRY) confirmed that the patient sample was male. The negative result of the control reaction shows that there is no cross-contamination during the process. Each M-PCR of the reactions showed successful STS pairing.

Initial Results of Applying M-PCR Reaction in Diagnosing AZF Deletion

AZF microdeletion testing on 32 patients are classified by region and number of patients in Table 2. Electrophoresis results showed that 32 patients had AZF deletions combining both basic and extended regions, of which AZFa deletions were only 2/32 cases, accounting for 6.25%. No isolated AZFb deletion was detected, the rate of AZFb,c deletion was 18/32 cases, accounting for 56.24%. There were 6/32 patients (accounting for 18.75%) with a combined loss of all 3 AZFabc regions. Notably, loss of sY152 appeared in most cases (30/32) (Table 2).

Discussion

The European Academy of Andrology and the European Molecular Genetics Quality Network (EAA/EMQN) recommended widely used two STS namely sY84 and sY86 for the detection of AZFa microdeletions in infertility males. The genes sY84 and sY86 are located upstream of the USP9Y and DDX3Y genes, respectively. USP9Y is one of the candidate genes of AZFa that plays an important role in the development of male germ cells.[11–14] However, there have been studies that refute this perspective, based on pieces of evidence that men with USP9Y deletion could have normal sperm as well as the ability to give birth.[15–17] Chris Tyler-Smith et al showed that the ubiquitin-specific USP9Y gene is not necessary for the process. normal spermatogenesis.[15] Detecting deletions directly on candidate genes becomes difficult due to large gene sizes and unclear phenotypic correlations. Therefore, sY84 and sY86 with simple sequences

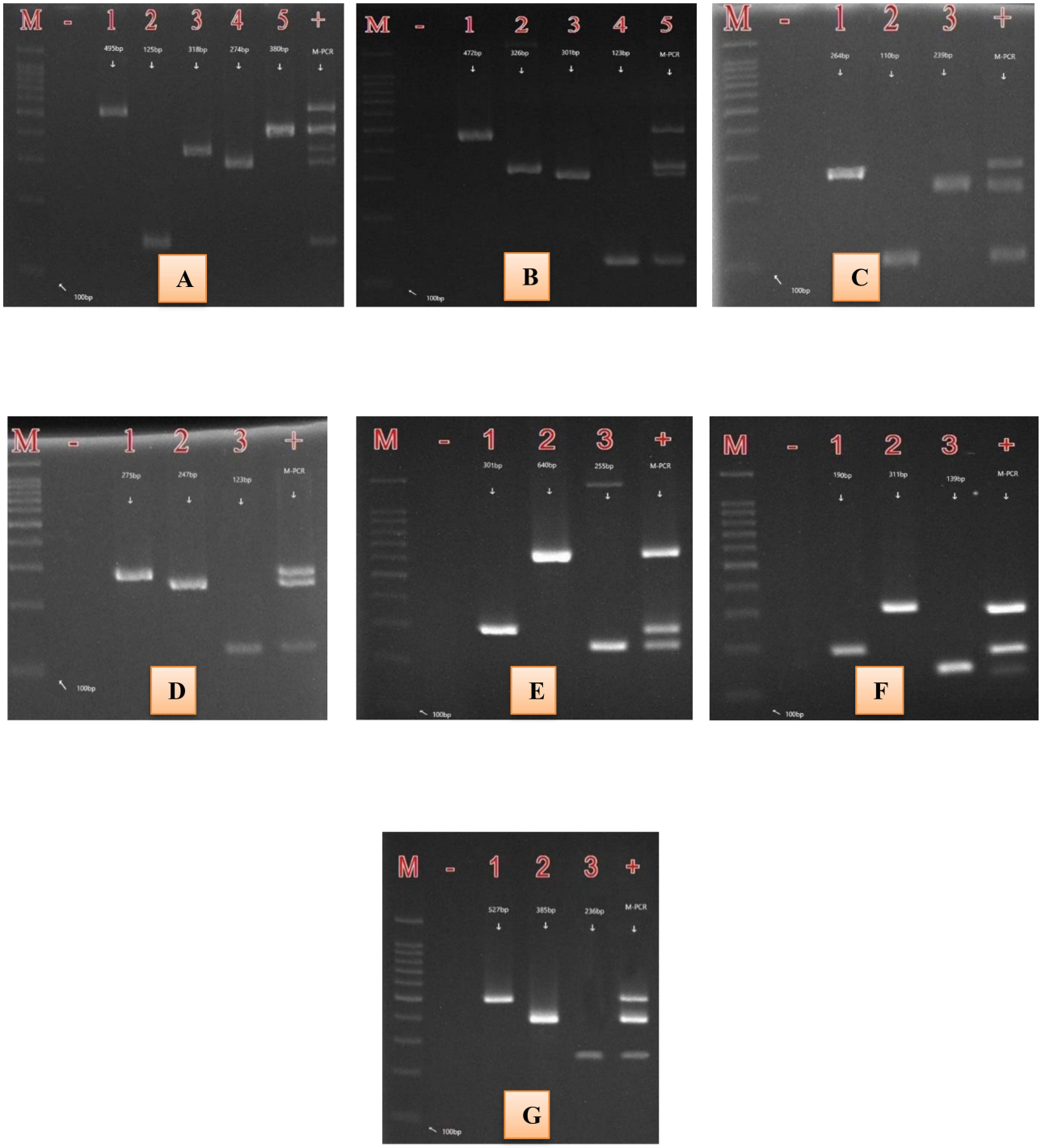

Figure 3 Electrophoresis results of M-PCR **A**. Electrophoresis results of M-PCR reaction 1 M Ladder 100 bp; (-): negative control; (+): M-PCR reaction; 1: ZFY (control); 2: sY152; 3: sY86; 4: sY127; 5: sY254. **B**. Electrophoresis results of M-PCR reaction 2 M Ladder 100 bp; (-): negative control; (+): M-PCR reaction; 1: SRY (control); 2: sY84; 3: sY134; 4: sY255. **C**. Electrophoresis results of M-PCR reaction 3 M Ladder 100 bp; (-): negative control; (+): M-PCR reaction; 1: sY82; 2:sY1064; 3: sY1065. **D**. Electrophoresis results of M-PCR reaction 4 M Ladder 100 bp; (-): negative control; (+): M-PCR reaction; 1: sY83; 2:sY1182; 3: sY88. **E**. Electrophoresis results of M-PCR reaction 5 M Ladder 100 bp; (-): negative control; (+): M-PCR reaction; 1: sY105 2:sY1224; 3: sY1192. **F**. Electrophoresis results of M-PCR reaction 6 M Ladder 100 bp; (-): negative control; (+): M-PCR reaction; 1: sY121; 2:sY143; 3: sY153. **G**. Electrophoresis results of M-PCR reaction 7 M Ladder 100 bp; (-): negative control; (+): M-PCR reaction; 1: sY1291; 2:sY1191; 3: sY160.

and high sensitivity in detecting AZFa microdeletion and azoospermia phenotype have been most widely used to diagnose AZFa microdeletion.[18] sY84 deletion without sY86 deletion has been observed in infertile men with normal sperm counts as well as in healthy men.[19] Partial deletion of AZFa (fragment sY86) may not affect sperm production,

Table 2 Number and Percentage of Male Infertility with AZF Deletion by Region

Deletion region	n	%
AZFa alone	2	6.25%
AZFb alone	0	0%
AZFc alone	3	9.38%
AZFa,c	3	9.38%
AZFb,c	18	56.24%
AZFa,b,c	6	18.75%
Total	32	100%

Abbreviation: AZF, Azoospermia factors.

sperm ejaculation and human fertility.[20] Therefore, according to EAA/EMQN recommendations, if a deletion occurs only on sY84 or sY86, the single-primer PCR reaction should be performed again or confirmed with another diagnostic methods (eg other PCR variants or Sanger sequencing). If both sY84 and sY86 are deleted, extension analyses must be performed on all of the following loci: sY82, sY83, or sY1064 for the proximal border and sY1065 or sY1182 and sY88 for the distal border.[21]

With deletions in the AZFb region, many large hospitals worldwide have employed the sY127 and sY134 sequences to confirm the diagnosis.[22–30] Similar to deletions in the AZFa region, according to the recommendations of EAA/EMQN, the complete loss of sY127, sY134 of AZFb require further identification of additional extended microdeletions: sY105 and sY121 or sY1224 for the proximal border and sY143 or sY1192 and sY153 for the distal border.[21]

Regarding deletions in the AZFc region, sY54 and sY255 are recommended by EAA/EMQN as representatives of the AZFc region.[21] We added sY152 to represent the AZFc region because sY152 is a common STS deletion site in men with oligospermia and is considered a candidate for future diagnosis of AZF microdeletions. Loss of sY152 might not affect semen quality and clinical outcomes but causes lower fertilization success rates after ICSI treatment, which adds prognostic value to patients with loss of sY152 segment for AZF.[31] Deletions occurring in one of the two STSs (sY54 and sY255) are rare and generally considered technical errors. Complete deletions of sY54 and sY255 in AZFc require the identification of additional microdeletions extending sY160 to differentiate between AZFc's complete b2/b4 deletions (b2/b4, sY160+) and terminal deletions (sY160-).[21]

In addition, we simultaneously determined whether partial AZFc deletions (gr/gr) occurred by using two STS, namely sY1291 and sY1191. This loss of gr/gr causes a deletion of about half of the AZFc region.[32] gr/gr deletion carriers exhibit phenotypes ranging from azoospermic to normal.[33–39] Furthermore, the gr/gr deletion is a risk factor for testicular germ cell tumors (TGCT).[40] There have been reports on the impact of gr/gr deletion on TGCT as an independent factor, and regular tumor screening is recommended in infertile gr/gr deletion carriers and their male family members.[41]

By initially applying the M-PCR technique on 32 azoospermic and oligospermic men, we found that the most common deletion rate was at AZFb,c (18/32 patients, accounting for 56.24%). The results of this study are similar to a study by Yuan Pan et al on 190 people with Y chromosome abnormalities, of which 35 patients had AZF microdeletions with the most common loss of AZFb,c (17 patients accounted for 48.57%).[23] A study by Trinh The Son in 153 Vietnamese patients with azoospermia and oligospermia have microdeletions of AZF showed that small deletions in the AZFc region are the most common (49.67%), deletions in the AZFb,c,d, and AZFb,c regions are respectively is 28.10% and 7.84%.[42] Although the author used the M-PCR technique to detect the deletion of basic AZF fragments and single-primer PCR for the deletion of extended AZF fragments, their result is different from ours. It is due to the small sample size of the study we conducted and may be due to differences in opinion in classifying the AZF region (Add AZFb deletion combinations c,d; AZFb,d; AZFc,d; AZFd).

Many techniques are used to diagnose AZF deletions such as real-time PCR, MLPA developed by MRC-Holland, and QF-PCR technique etc.[43–45] These techniques are highly sensitive but expensive, and modern technical equipment and high-quality expertise demanding. Therefore, the conventional PCR method still shows many advantages such as ease of implementation and high applicability, which is suitable for the conditions of a developing country like Vietnam. In Vietnam, almost M-PCR techniques for identifying AZF microdeletions only optimize M-PCR reactions to identify the basic AZFabc region. The extended AZFabc region must thereby be performed using single-primer PCR. This also happened in another study in the world.[46] Other M-PCR techniques were developed with many STS in four or five M-PCR reactions. In these methods, the STS primer pairs for identifying extended AZFabc region are paired randomly, leading to unnecessary identification of other extended microdeletions in case of only one deletion of the basic region (AZFa, AZFb or AZFc).[47,48] Some M-PCR techniques were developed with too much STS and followed outdated EMQN recommendations, which is unsuitable for clinical practice today.[49] The M-PCR technique optimized by us includes reactions arranged according to AZF region (AZFa, AZFb, or AZFc; basis or extended), making it easier to control deletions by region and allowing us to work step by step efficiently. Also, we could detect AZF deletions for about 6 hours using simple procedures. The low cost of performing this M-PCR will create great potential in the application of AZF deletion diagnosis in hospitals in Vietnam.

Our study only initially investigates the frequency of AZF microdeletions on a sample size of 32 patients without collecting additional data on clinical outcomes, so the sensitivity and specificity of the technique cannot be evaluated, and further data are not discussed. Further studies with larger study population need performing to evaluate the specificity and sensitivity of the technique.

Conclusion

We have successfully optimized the M-PCR technique to identify AZF deletions using 24 STS/gene, thereby initially successfully applying the diagnosis of AZF deletions to 32 male subjects with azoospermia or oligospermia. The M-PCR technique shows great potential for establishing the diagnosis of AZF deletion.

Abbreviations

AZF, Azoospermia factor; STS, Sequence tagged-sites; DNA, Deoxyribonucleic acid; PCR, Polymerase chain reaction; M-PCR, Multiplex polymerase chain reaction; EAA/EMQ, The European Academy of Andrology and the European Molecular Genetics Quality Network.

Data Sharing Statement

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Statements

All participants were provided with written informed consent, and the protocol was approved by the Institutional Review Board of the Vietnam Military Medical University, (No.1068/2019/VMMU-IRB). The study was also conducted using good clinical practice following the Declaration of Helsinki.

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Disclosure

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References

1. Agarwal A, Mulgund A, Hamada A, Chyatte MR. A unique view on male infertility around the globe. *Reprod Biol Endo*. 2015;13(1):1–9. doi:10.1186/s12958-015-0032-1
2. Tüttelmann F, Ruckert C, Röpke A. Disorders of spermatogenesis: perspectives for novel genetic diagnostics after 20 years of unchanged routine. *Med Gene*. 2018;30(1):12–20. doi:10.1007/s11825-018-0181-7
3. Elsaid HOA, Gadkareim T, Abobakr T, et al. Detection of AZF microdeletions and reproductive hormonal profile analysis of infertile Sudanese men pursuing assisted reproductive approaches. *BMC Urology*. 2021;21(1):69. doi:10.1186/s12894-021-00834-3
4. Rossi BV, Abusief M, Missmer SA. Modifiable risk factors and infertility: what are the connections? *Ameri J Life Med*. 2016;10(4):220–231. doi:10.1177/1559827614558020
5. Napolitano L, Barone B, Crocetto F, et al. The COVID-19 pandemic: is it a wolf consuming fertility? *Interna J Ferti Steril*. 2020;14(2):159.
6. Alonso RL, Bailo PS, Márquez MÁC, et al. AZF gene microdeletions in azoospermic–oligozoospermic males. *Med Clíni*. 2023;160(4):151–155. doi:10.1016/j.medcli.2022.06.016
7. Seshagiri PB. Molecular insights into the causes of male infertility. *J Biosci*. 2001;26(4):429–435. doi:10.1007/BF02704745
8. Vogt PH. AZF deletions and Y chromosomal haplogroups: history and update based on sequence. *Human Rep Update*. 2005;11(4):319–336. doi:10.1093/humupd/dmi017
9. Reijo R, Lee T-Y, Salo P, et al. Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA–binding protein gene. *Nature Genet*. 1995;10(4):383–393. doi:10.1038/ng0895-383
10. Navarro-Costa P, Plancha CE, Gonçalves J. Genetic dissection of the AZF regions of the human Y chromosome: thriller or filler for male (in) fertility? *Biomed Res Int*. 2010;2010:936569. doi:10.1155/2010/936569
11. Kishi K, Uchida A, Takase HM, et al. Spermatogonial deubiquitinase USP9X is essential for proper spermatogenesis in mice. *Reproduction*. 2017;154(2):135–143. doi:10.1530/REP-17-0184
12. Sun C, Skaletsky H, Birren B, et al. An azoospermic man with a de novo point mutation in the Y-chromosomal gene USP9Y. *Nature Genet*. 1999;23(4):429–432. doi:10.1038/70539
13. Lee KH, Song GJ, Kang IS, et al. Ubiquitin-specific protease activity of USP9Y, a male infertility gene on the Y chromosome. *Reprod Fertil Dev*. 2003;15(2):129–133. doi:10.1071/RD03002
14. Brown GM, Furlong RA, Sargent CA, et al. Characterisation of the coding sequence and fine mapping of the human DFFRY gene and comparative expression analysis and mapping to the sxrb interval of the mouse Y chromosome of the Dffry gene. *Human Mole Gene*. 1998;7(1):97–107. doi:10.1093/hmg/7.1.97
15. Tyler-Smith C, Krausz C. The will-o'-the-wisp of genetics—hunting for the azoospermia factor gene. *New Engl J Med*. 2009;360(9):925. doi:10.1056/NEJMe0900301
16. Luddi A, Margollicci M, Gambera L, et al. Spermatogenesis in a man with complete deletion of USP9Y. *N Engl J Med*. 2009;360(9):881–885. doi:10.1056/NEJMoa0806218
17. Colaco S, Modi D. Genetics of the human Y chromosome and its association with male infertility. *Reprod Biol Endo*. 2018;16(1):1–24. doi:10.1186/s12958-018-0330-5
18. Simoni M, Bakker E, Krausz C. EAA/EMQN best practice guidelines for molecular diagnosis of y-chromosomal microdeletions. state of the art 2004. *Int JAndrology*. 2004;27(4):240–249. doi:10.1111/j.1365-2605.2004.00495.x
19. Wu Q, Chen G-W, Yan T-F, et al. Prevalent false positives of azoospermia factor a (AZFa) microdeletions caused by single-nucleotide polymorphism rs72609647 in the sY84 screening of male infertility. *Asian J Andro*. 2011;13(6):877. doi:10.1038/aja.2011.51
20. Jia Y, Niu Z-G, W-Y L, et al. A fertile male with a single sY86 deletion on the Y chromosome. *Asian J Andro*. 2020;22(3):333. doi:10.4103/aja.aja_94_19
21. Krausz C, Navarro-Costa P, Wilke M, Tüttelmann F. EAA/EMQN best practice guidelines for molecular diagnosis of Y-chromosomal microdeletions: state of the art 2023. *Andrology*. 2023. doi:10.1111/andr.13514
22. Dada R, Gupta N, Kucheria K. Molecular screening for Yq microdeletion in men with idiopathic oligozoospermia and azoospermia. *J Biosci*. 2003;28(2):163–168. doi:10.1007/BF02706215
23. Pan Y, H-g Z, Xi Q, et al. Molecular microdeletion analysis of infertile men with karyotypic Y chromosome abnormalities. *J Int Med Res*. 2018;46(1):307–315. doi:10.1177/0300060517719394
24. Dada R, Gupta N, Kucheria K. AZF microdeletions associated with idiopathic and non-idiopathic cases with cryptorchidism and varicocele. *Asian J Andro*. 2002;4(4):259–263.
25. Gao X, Chen G, Huang J, et al. Clinical, cytogenetic, and molecular analysis with 46, XX male sex reversal syndrome. *J assi rep gene*. 2013;30(3):431–435. doi:10.1007/s10815-013-9939-7
26. Sheikhha MH, Zaimy MA, Soleimanian S, et al. Multiplex PCR Screening of Y-chromosome microdeletions in azoospermic ICSI candidate men. *Iran J Reprod Med*. 2013;11(4):335.
27. Elfateh F, Rulin D, Xin Y, Linlin L, Haibo Z, Liu R-Z. Prevalence and patterns of Y chromosome microdeletion in infertile men with azoospermia and oligzoospermia in Northeast China. *Iran J Reprod Med*. 2014;12(6):383.
28. Zhang Y-S, L-L L, Xue L-T, Zhang H, Zhu -Y-Y, Liu R-Z. Complete azoospermia factor b deletion of Y chromosome in an infertile male with severe oligoasthenozoospermia: case report and literature review. *Urology*. 2017;102:111–115. doi:10.1016/j.urology.2016.07.022
29. N-h SONG, H-f W, Zhang W, et al. Screening for Y chromosome microdeletions in idiopathic and nonidiopathic infertile men with varicocele and cryptorchidism. *Chinese Med J*. 2005;118(17):1462–1467.
30. Piña-Aguilar RE, Martínez-Garza SG, Kohls G, et al. Y chromosome microdeletions in Mexican males of couples with idiopathic recurrent pregnancy loss. *J Obstetrics Gynaecol Res*. 2012;38(6):912–917. doi:10.1111/j.1447-0756.2011.01809.x
31. Zhu Y, Wu T, Li G, et al. The sperm quality and clinical outcomes were not affected by sY152 deletion in Y chromosome for oligozoospermia or azoospermia men after ICSI treatment. *Gene*. 2015;573(2):233–238. doi:10.1016/j.gene.2015.07.051
32. Lange J, Skaletsky H, van Daalen SK, et al. Isodicentric Y chromosomes and sex disorders as byproducts of homologous recombination that maintains palindromes. *Cell*. 2009;138(5):855–869. doi:10.1016/j.cell.2009.07.042

33. Krausz C, Giachini C, Xue Y, et al. Phenotypic variation within European carriers of the Y-chromosomal gr/gr deletion is independent of Y-chromosomal background. *J Med Genet*. 2009;46(1):21–31. doi:10.1136/jmg.2008.059915
34. Giachini C, Guarducci E, Longepied G, et al. The gr/gr deletion (s): a new genetic test in male infertility? *J Med Genet*. 2005;42(6):497–502. doi:10.1136/jmg.2004.028191
35. Ferlin A, Tessari A, Ganz F, et al. Association of partial AZFc region deletions with spermatogenic impairment and male infertility. *J Med Genet*. 2005;42(3):209–213. doi:10.1136/jmg.2004.025833
36. Fernandes S, Huellen K, Goncalves J, et al. High frequency of DAZ1/DAZ2 gene deletions in patients with severe oligozoospermia. *Mol Hum Reprod*. 2002;8(3):286–298.
37. Yang Y, Ma M, Li L, et al. Differential effect of specific gr/gr deletion subtypes on spermatogenesis in the Chinese Han population. *Int JAndrology*. 2010;33(5):745–754. doi:10.1111/j.1365-2605.2009.01015.x
38. Sin H-S, Koh E, Shigehara K, et al. Features of constitutive gr/gr deletion in a Japanese population. *Hum Reprod*. 2010;25(9):2396–2403. doi:10.1093/humrep/deq191
39. Repping S, Skaletsky H, Brown L, et al. Polymorphism for a 1.6-Mb deletion of the human Y chromosome persists through balance between recurrent mutation and haploid selection. *Nature Genet*. 2003;35(3):247–251. doi:10.1038/ng1250
40. Nathanson KL, Kanetsky PA, Hawes R, et al. The Y deletion gr/gr and susceptibility to testicular germ cell tumor. *Am J Hum Genet*. 2005;77(6):1034–1043. doi:10.1086/498455
41. Moreno-Mendoza D, Casamonti E, Paoli D, et al. gr/gr deletion predisposes to testicular germ cell tumour independently from altered spermatogenesis: results from the largest European study. *Eur J Hum Genet*. 2019;27(10):1578–1588. doi:10.1038/s41431-019-0420-7
42. Trinh S, Nguyen NN, Thi Thu Le H, et al. Screening Y chromosome microdeletion in 1121 men with low sperm concentration and the outcomes of microdissection testicular sperm extraction (mTESE) for sperm retrieval from azoospermic patients. *App Clini Gene*. 2023;Volume 16:155–164. doi:10.2147/TACG.S420030
43. Plaseski T, Noveski P, Trivodalieva S, et al. Quantitative fluorescent-PCR detection of sex chromosome aneuploidies and AZF deletions/duplications. *Genet Test*. 2008;12(4):595–605. doi:10.1089/gte.2008.0068
44. Saito K, Miyado M, Kobori Y, et al. Copy-number variations in Y-chromosomal azoospermia factor regions identified by multiplex ligation-dependent probe amplification. *J Human Gene*. 2015;60(3):127–131. doi:10.1038/jhg.2014.115
45. Torres TP, Rojas XB, Narváez MB, Montanero EL, Sarasti AN. Triplex real-time polymerase chain reaction optimization for AZF Y-chromosome microdeletion analysis. *JBRA Assis Rep*. 2015;19(2):37–43. doi:10.5935/1518-0557.20150011
46. Mitra A, Dada R, Kumar R, et al. Screening for Y-chromosome microdeletions in infertile Indian males: utility of simplified multiplex PCR. *Indian J Med Res*. 2008;127(2):124–132.
47. Suganthi R, Vijesh VV, Jayachandran S, et al. Multiplex PCR based screening for microdeletions in azoospermia factor region of Y chromosome in azoospermic and severe oligozoospermic south Indian men. *Iran J Reprod Med*. 2013;11(3):219.
48. Zheng HY, Li Y, Shen FJ, et al. A novEl Universal multiplex PCR improves detection of AZFc Y-chromosome microdeletions. *J assi rep gene*. 2014;31(5):613–620. doi:10.1007/s10815-014-0204-5
49. Vogt PH, Bender U. Human Y chromosome microdeletion analysis by PCR multiplex protocols identifying only clinically relevant AZF microdeletions. *Spermatogenesis*. 2013;927:187–204.doi:10.1007/978-1-62703-038-0_17