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

Tung Nguyen Thanh¹, Sang Trieu Tien², Phong Nguyen Van², Son Dang Thai³, Thuc Luong Cong⁴, Tuan Dinh Le⁵, Son Tien Nguyen⁵, Tuan Tran Van⁶, Hoang Huy Duong⁶, Tien Minh Bui⁷, Kien Trung Nguyen⁷

¹Military Institute of Clinical Embryology and Histology, Vietnam Military Medical University, Hanoi, 100000, Vietnam; ²Department of Biology and Medical Genetics, Vietnam Military Medical University, Hanoi, 100000, Vietnam; ³Institute of Biological and Food Technology, Hanoi Open University, Hanoi, 100000, Vietnam; ⁴Cardiovascular Center, Military Hospital 103, Vietnam Military Medical University, Hanoi, 100000, Vietnam; ⁵Department of Rheumatology and Endocrinology, Military Hospital 103, Vietnam Military Medical University, Hanoi, 100000, Vietnam; ⁶Department of Neurology, Thai Binh University of Medicine and Pharmacy, Thai Binh, 410000, Vietnam; ⁷Department 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 5×10⁶ 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 5×10⁶ 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 AZFa,b,c 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 AZF,a,b,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.¹² According to estimated data worldwide, the prevalence of male infertility ranges from about 50% or nearly 30 million men.¹³ 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.⁴⁵ 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.⁶⁷ 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...
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. In 1995, Reijo et al determined the location of the AZF region in the distal part of Yq11 and named it the AZF region. 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. 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. 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 feasible 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 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

<table>
<thead>
<tr>
<th>Order</th>
<th>Size (bp)</th>
<th>STS/Gene</th>
<th>Region</th>
<th>Forward (5'-3') and Reverse (3'-5') Primer Sequences</th>
<th>Complete Mutation Deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>495</td>
<td>ZFX/Y</td>
<td>ZFX/Y</td>
<td>ACCRCTGTACTGACTGATTACAC GCAYCTTTGGTATCYGAGAAAGT</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>472</td>
<td>SRY</td>
<td>SRY</td>
<td>GAATATTTTCCGCTTCCCAGA GCTGGTGCTCCATTCTTGAG</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>125</td>
<td>sY152</td>
<td>AZFc</td>
<td>AAGACAGTCTGCATGGTTCA ACAGGAGGCTTACTGACTGAGT</td>
<td>Positive</td>
</tr>
<tr>
<td>4</td>
<td>320</td>
<td>sY86</td>
<td>AZFa</td>
<td>GTGACACACAGACTATGCTTC ACACACACAGACGACAGCT</td>
<td>Positive</td>
</tr>
<tr>
<td>5</td>
<td>274</td>
<td>sY127</td>
<td>AZFb</td>
<td>GGCTCACAAGAAGAAAAGA CTGCAGGCTAATAAGGA</td>
<td>Negative</td>
</tr>
<tr>
<td>6</td>
<td>350</td>
<td>sY254</td>
<td>AZFc</td>
<td>GGGTGTACAGAAGGCAAA GAACCGATCTACCAAGCAGC</td>
<td>Positive</td>
</tr>
<tr>
<td>7</td>
<td>326</td>
<td>sY84</td>
<td>AZFa</td>
<td>AGAAGGGTTCTGAAAGCAGT GCCTACTACGTGAGGCT</td>
<td>Positive</td>
</tr>
<tr>
<td>8</td>
<td>301</td>
<td>sY134</td>
<td>AZFb</td>
<td>GTCTGCTCACCATAAAACG ACCACTGCCAAAACCTTCA</td>
<td>Positive</td>
</tr>
<tr>
<td>9</td>
<td>126</td>
<td>sY255</td>
<td>AZFc</td>
<td>GTTACAGGATCGCCGTGAT CTCGTATCTGACGCGCC</td>
<td>Positive</td>
</tr>
<tr>
<td>10</td>
<td>264</td>
<td>sY82</td>
<td>AZFa</td>
<td>ATCTGCCCCTCTGAAATCTC CAGTGTACGTGAGATGA</td>
<td>Negative</td>
</tr>
<tr>
<td>11</td>
<td>275–277</td>
<td>sY83</td>
<td>AZFa</td>
<td>CTTGACTAAGAAAGGCCT CTATTGGTGGTGCTGTC</td>
<td>Positive</td>
</tr>
<tr>
<td>12</td>
<td>110</td>
<td>sY1064</td>
<td>AZFa</td>
<td>GGGTGGTGCTGACCTAAATAG GAACCTAGAGAGGAA</td>
<td>Positive</td>
</tr>
<tr>
<td>13</td>
<td>239</td>
<td>sY1065</td>
<td>AZFa</td>
<td>TCAGGTACTGTGATGCCC TGAAAGAGGACCAAGGGAAA</td>
<td>Positive</td>
</tr>
<tr>
<td>14</td>
<td>247</td>
<td>sY1182</td>
<td>AZFa</td>
<td>ATGGCTCTACCCCAACTGAG CATTGCCGCTCCCCTGAGCT</td>
<td>Positive</td>
</tr>
<tr>
<td>15</td>
<td>123</td>
<td>sY88</td>
<td>AZFa</td>
<td>TTGAATCCCAAATACATGGGC CACCCGACATTGTTTTAC</td>
<td>Negative</td>
</tr>
<tr>
<td>16</td>
<td>301</td>
<td>sY105</td>
<td>AZFb</td>
<td>AAGGCGTCTCCTCTTTGCTTC AGGGAGCTTAACTCAACT</td>
<td>Negative</td>
</tr>
</tbody>
</table>

(Continued)
Table 1 (Continued).

<table>
<thead>
<tr>
<th>Order</th>
<th>Size (bp)</th>
<th>STS/Gene</th>
<th>Region</th>
<th>Forward (5'-3') and Reverse (3'-5') Primer Sequences</th>
<th>Complete Mutation Deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>190</td>
<td>sY121</td>
<td>AZFb</td>
<td>AGTTCACAGAATGGAGCCCTG  CAGTACACAGGCTTTCGTGGTC</td>
<td>Positive</td>
</tr>
<tr>
<td>18</td>
<td>690</td>
<td>sY1224</td>
<td>AZFb</td>
<td>GGCTTAACTTTGGAGGGTG  GCTGGACGCTAACAGCA</td>
<td>Variable</td>
</tr>
<tr>
<td>19</td>
<td>311</td>
<td>sY143</td>
<td>AZFb</td>
<td>GCAGGATGAGAAGCAGGTAG  CGTGCTGCTGGAGACTAATC</td>
<td>Positive</td>
</tr>
<tr>
<td>20</td>
<td>255</td>
<td>sY1192</td>
<td>AZFb</td>
<td>ACTACCATTTCGGAAGCCG  CTCCATTGCTTATGCCATT</td>
<td>Positive</td>
</tr>
<tr>
<td>21</td>
<td>139</td>
<td>sY153</td>
<td>AZFb</td>
<td>GCATCCTCATTTATGTCCA  CACCCAAAAAACACTGAGTA</td>
<td>Negative</td>
</tr>
<tr>
<td>22</td>
<td>527</td>
<td>sY1291</td>
<td>gr/gr</td>
<td>TAAAAGGCGACAATGACAGG  GGGAGAAAAAGTCTGCAACG</td>
<td>Positive</td>
</tr>
<tr>
<td>23</td>
<td>385</td>
<td>sY1191</td>
<td>gr/gr</td>
<td>CCAGACGTCTACCCCTTCG  GAGGCGAGTCCGATGTTACCA</td>
<td>Negative</td>
</tr>
<tr>
<td>24</td>
<td>236</td>
<td>sY160</td>
<td>Heterochromatin</td>
<td>TACGGGTCTCGAATGGAATA  TCATTGCATTCCCTTCCATT</td>
<td>Negative</td>
</tr>
</tbody>
</table>

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.

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).

![Diagram of AZF deletion analysis using M-PCR](https://example.com/diagram.png)

Figure 1 Procedure for determining AZF deletion using M-PCR.
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. 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. Chris Tyler-Smith et al showed that the ubiquitin-specific USP9Y gene is not necessary for the process. normal spermatogenesis. Detecting deletions directly on candidate genes becomes difficult due to large gene sizes and unclear phenotypic correlations. Therefore, sY84 and sY86 with simple sequences
and high sensitivity in detecting AZFa microdeletion and azoospermia phenotype have been most widely used to diagnose AZFa microdeletion. S84 deletion without sY86 deletion has been observed in infertile men with normal sperm counts as well as in healthy men. Partial deletion of AZFa (fragment sY86) may not affect sperm production,
sperm ejaculation and human fertility. 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.

With deletions in the AZFb region, many large hospitals worldwide have employed the sY127 and sY134 sequences to confirm the diagnosis. 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.

Regarding deletions in the AZFc region, sY54 and sY255 are recommended by EAA/EMQN as representatives of the AZFc region. 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. 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-).

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. gr/gr deletion carriers exhibit phenotypes ranging from azoospermic to normal. Furthermore, the gr/gr deletion is a risk factor for testicular germ cell tumors (TGCT). 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.

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%). 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%. 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).

<table>
<thead>
<tr>
<th>Deletion region</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZFa alone</td>
<td>2</td>
<td>6.25%</td>
</tr>
<tr>
<td>AZFb alone</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>AZFc alone</td>
<td>3</td>
<td>9.38%</td>
</tr>
<tr>
<td>AZFa,c</td>
<td>3</td>
<td>9.38%</td>
</tr>
<tr>
<td>AZFb,c</td>
<td>18</td>
<td>56.24%</td>
</tr>
<tr>
<td>AZFa,b,c</td>
<td>6</td>
<td>18.75%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>32</td>
<td>100%</td>
</tr>
</tbody>
</table>

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

Abbreviation: AZF, Azoospermia factors.
Many techniques are used to diagnose AZF deletions such as real-time PCR, MLPA developed by MRC-Holland, and QF-PCR technique etc. 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. 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). Some M-PCR techniques were developed with too much STS and followed outdated EMQN recommendations, which is unsuitable for clinical practice today. 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.

**Acknowledgments**

We thank the staff in the the Department of Biology and Medical Genetic, Military institute of clinical Embryology and Histology and Military Hospital 103 for collecting the samples and supporting the study.

**Funding**

The authors received no financial support for the research, authorship, and/or publication of this article.

**Disclosure**

The authors declared no potential conflicts of interest to the research, authorship, and/or publication of this article.
References


