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RESEARCH PAPER

Molecular Diagnosis of Y-Chromosome Microdeletions in Some Kurdish Infertile Males: EAA/EMQN PCR Protocol Optimization.

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A B S T R A C T:
Globally about 15% of couples don't achieve pregnancy within one year and look for medical treatments to with their infertility. Infertility is the incapability of couples to conceive a child after one year of unprotected intercourse. Male factors are responsible for about 50% of all infertility cases and genetic factors are diagnosed in about 15–20% of infertile males causing azoospermia or severe oligozoospermia. Several genes named azoospermia factor (AZF) present on the long arm of the human Y-chromosome are participated in spermatogenesis, and microdeletions in these genes have been recognized to be the second genetic cause of spermatogenetic failure after Klinefelter syndrome resulting in male infertility. In this current study, a simple PCR format is investigated for AZF microdeletion screening. This present study performed on 296 infertile Kurdish males, 289 patients with azoospermia (97.6%) and, 7 patients with severe azoospermia (2.4%), and 50 healthy fertile men as the control group in Erbil governorate/Iraq. Results showed that AZF deletions were found in 10 of 289 patients (3.5%), 3/10 (30%) had microdeletions in their AZFc region, 3/10 (30%) had microdeletions in the AZFb region, 3/10 (30%) in AZFb,c region, and the final one (10%) had microdeletions in all regions AZFa,b,c. No microdeletions were found both oligozoospermias and the normal group.

KEY WORDS: Male Infertility, Y-Chromosome Microdeletions (YCMD), Azoospermia Factor (AZF), PCR Technique.
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1. INTRODUCTION:

Worldwide up to 15% of couples are affected by infertility, which is defined as the inability of sexually active couple to carry a pregnancy to delivery, after one year of unprotected intercourse. Generally, male factors are responsible for about 50% of all infertility cases (Rowe et al., 2000, Organization, 2010, Jungwirth et al., 2019) .

Genetic factors are diagnosed in about 15–20% of infertile males that causes azoospermia or severe oligozoospermia in affected male (Krausz and Riera-Escamilla, 2018, Tournaye et al., 2017). As shown in Figure 1, the human Y chromosome is acrocentric consists of two arms namly, a short arm (Yp) and a long arm (Yq). In the end, part of each arm there's regions called pseudoautosomal regions (PARs). PAR1 in the short arm, and PAR2 is located in the long arm, and the two both arms are separated by a centromere (Colaco and Modi, 2018).

The regions that specify maleness in humans are located in the Y-chromosome called male-specific region of the Y chromosome (MSY) consisting 95% of the chromosome's length and is flanked by PAR1 and PAR2 regions (Skaletsky et

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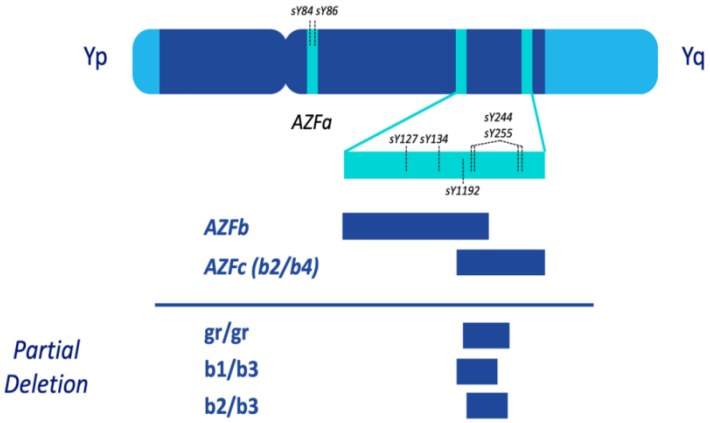

Figure 1. Schematic representation of the Y-chromosome and male-specific region microdeletions. Pseudoautosomal regions (PAR1 and PAR2) are represented by light blue areas. Locations of STS primers presently recommended for microdeletion identification (ie sY84) are indicated by vertical dashed lines. Partial deletions of the AZFc (b2/b4) subregion are denoted by gr/gr, b1/b3, and b2/b3 (Rabinowitz et al., 2021)

al., 2003). The azoospermia factor (AZF) regions are located on the long arm of the Y-chromosome within the MSY region, which contains genes necessary for spermatogenesis (Hotaling and Carrell, 2014). These regions contain repeated homologous sequences that are vulnerable to mutations such as deletions or duplications as a subsequence of non-allelic homologous recombination (NAHR) (Krausz et al., 2014). Various studies have shown that 10% of men that suffering from azoospermia have been diagnosed with microdeletions in their three types of azoospermia factor, AZF a (the most proximal segment), AZF b (middle), and AZF c (distal), these deletions remove several genes that are responsible for germ cell development in males (spermatogenesis) and their maintenance (Witherspoon et al., 2021, Kuroda-Kawaguchi et al., 2001).

Normal AZFa region is about 1,100 kb size which contains only two single-copy genes: Ubiquitin Specific Peptidase 9 Y-linked (USP9Y) gene that encodes for the Probable ubiquitin carboxyl-terminal hydrolase (FAF-Y) and DEAD-Box Helicase 3 Y-linked (DDX3Y) that encodes ATP-dependent RNA helicase. The complete deletion of the AZFa region deleting about 792 kb that include both two genes USP9Y and DDX3Y (Krausz et al., 2014).

The AZFb region which partly overlaps with the AZFc area is structurally complex. Within the AZFb region, there are 14 amplicons (multicopy DNA sequence units), seven of them are specific to the AZFb region, while the others are participate with AZFc. The AZFb also contains the proximal part of P1 and palindromes from P2–P5. In the case of the complete deletion of AZFb, about 6.2 Mb regions will be missed that wich encompasses 32 gene copies and transcription units (Krausz et al., 2014, Kuroda-Kawaguchi et al., 2001). Complete deletions of AZFb about 1-5% of all Y-microdeletions lead to Sertoli Cell Only Syndrome (SCOS) or spermatogenic arrest and azoospermia (Krausz et al., 2014, Krausz et al., 2000, Lange et al., 2009, Witherspoon et al., 2021).

The most frequent YCMD type is occurred in AZFc region which is comprised up to 80% of all YCMD (Krausz et al., 2014, Lange et al., 2009). In case of AZFc complete deletion, about 3.5 Mb of the long arm of the Y chromosome is removed, leading to detaching and remove 21 copies of genes and transcription units (Kuroda-Kawaguchi et al., 2001). Diagnosis of AZF Microdeletions can't be detected by a classic technique such as karyotyping. Both agencies the EAA and the EMQN published guidelines for molecular diagnosis of YCMD, AZF microdeletions can be currently recognized by multiplex polymerase chain reaction (PCR) technique (Krausz et al., 2014, Zhu et al., 2017).

This present study aimed to ascertain and screen the YCMD types and their frequencies in the AZF area in 296 infertile Kurdish males that they have been diagnosed with azoospermia and severe oligospermia in Erbil province northern of Iraq. The screening was carried out through optimizing the multiplex polymerase chain reaction PCR technique protocol. The optimization protocol was done according to the recommendations and practice guidelines by EAA/ EMQN (Krausz et al., 2014).

2. MATERIALS AND METHODS

2.1 Patients and Control:

The current study was approved by the Research Ethics Committee at College of Science, Salahaddin University-Erbil (SUE), Iraq and informed consent was obtained from each subject (participants). This current study was conducted on 296 Iraqi Kurdish infertile males in Erbil province during December 2019 and December

2020. The selection of patients who participated in this study was based on their medical histories that have been recorded in public and private hospitals and infertility centers. All cases were diagnosed with primary infertility and didn't have obstructive azoospermia. The majority of patients were diagnosed or characterized with azoospermia (n=289) and only seven of them with severe oligozoospermia (sperm count $<5\times10^6$/ml) based on semen fluid analysis results. Patients were aged from 20-58 years. Seminal fluid analysis was done according to standard parameters using World Health Organization criteria (WHO, 2021). The control group in this study was 50 healthy males with proven paternity without Assisted Reproductive Technology (ART) like invitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) or gamete intrafallopian transfer (GIFT). All patients were interviewed with a male infertility questionnaire form prepared in this study; the questionnaire form contained many fields started from Fertility History, Sexual History, Environmental Exposures, Past Medical and Surgical History, and Family infertility History.

2.2 Molecular Methods:

2.2.1. Genomic DNA Extraction

Two ml of venous blood using a sterile syringe was taken from each patient and blood samples were collected in an anticoagulant EDTA tubes. Genomic DNA was isolated from blood samples according to (Genomic DNA Mini Kit, Geneaid, Taiwan) the manufacture's instruction, the sequential procedures were done according to a manufacturing protocol. The quality, integrity and quantity of each DNA sample were determined by NanodropTM 1000 spectrophotometer (Thermo Scientific, USA).

2.2.2 Molecular analysis:

2.2.2.1. Study design

To Diagnose microdeletion on the Y-chromosome, molecular screening was performed for each patient and controls according to the EAA and EMQN protocol (Krausz et al., 2014) targeting the most important sequence-tagged sites (STS)s of AZF regions on the Y chromosome.

In the first format two multiplex PCR reactions (multiplex PCR A and B) with two replicates (technical replicates) were prepared as mentioned in the EAA and EMQN protocol for the detection of the three AZF microdeletions. Each multiplex reaction was contained five amplicons of three AZF loci (AZFa, b, and c) and SRY and ZFX/Y. For each AZF loci, two AZF subregions were screened, in which in multiplex A the AZF subregion are sY254, sY86, and sY127; in PCR B, AZF subregion is sY255, sY84, and sY134, whereas genes that are responsible or encoding human zinc-finger protein (ZFX/Y) and sex-determining region (genes) (SRY, i.e., STS SY14) used as control and used for both multiplex PCR A and B. The primer sequences for multiplex A and multiplex B and their amplicons are shown in table 1.

2.2.2.2 PCR sample preparation

The PCR mixture was prepared with final volume of 20 µl containing 10 µl of 2X of Master Mix (AMPLIQON), 0.7 µl each primer with 10 pM concentration (Macrogen; LIGO) and 3 µl of genomic DNA sample as a template. The PCR condition was as follows: initial denaturation for 5 min at 95°C; 35 cycles, denaturation 30 s at 94°C; annealing 30 s at 56°C; and elongation 45s at 72°C and a final elongation step of 10 min at 72°C.

In the second format: four PCR reactions were prepared in which two PCR reactions for multiplex A as follows: multiplex A 1 and A2, the format A 1 consisted of ZFX/Y, sY86 primers and the multiplex A2 consist of SRY and sY254 and sY127 primers. The multiplex B1 consisted of ZFX/Y and sY134 primers and the multiplex B2 consists of SRY and sY84 and sY255 primers. The PCR condition was as follows: initial denaturation for 5 min at 95°C; 35 cycles, denaturation 30 s at 94°C; annealing 30 s at 60°C; and elongation 30s at 72°C and a final elongation step of 10 min at 72°C by using the thermal cycler machine (Alpha thermal Cycler; code: AC196).

2.2.2.3 Gele Electrophoresis

For each sample, the amplified PCR product was separated on a 2.0% agarose gel

Table 1: The table shows the locus of AZF types**,** primer name and primer sequence, PCR product size and status in case of deletions (Krausz et al., 2014).

Locus	Primer	Sequence	Product size (bp)	Status in classic, complete deletion
Multiplex A and B ZFX/Y	ZFX/Y-F ZFX/Y-R	5′-ACC RCT GTA CTG ACT GTG ATT ACA C-3′ 5′-GCA CYT CTT TGG TAT CYG AGA AAG T-3′	495	Present
SRY	sY14-F sY14-R	5′-GAA TAT TCC CGC TCT CCG GA-3′ 5′-GCT GGT GCT CCA TTC TTG AG-3′	472	Present
Multiplex A AZFa	sY86-F sY86-R	5′-GTG ACA CAC AGA CTA TGC TTC-3′ 5′ - ACA CAC AGA GGG ACA ACC CT - 3′	318	Absent
AZFb	sY127-F sY127- R	5′-GGC TCA CAA ACG AAA AGA AA-3′ 5′-CTG CAG GCA GTA ATA AGG GA-3′	274	Absent
AZFc	sY254-F sY254-R	5′-GGG TGT TAC CAG AAG GCA AA-3′ 5′-GAA CCG TAT CTA CCA AAG CAG C-3′	380	Absent
Multiplex B AZFa	sY84- F sY84-R	5′-AGA AGG GTC CTG AAA GCA GGT-3′ 5′-GCC TAC TAC CTG GAG GCT TC-3′	326	Absent
AZFb	sY134-F sY134-R	5′-GTC TGC CTC ACC ATA AAA CG-3′ 5′-ACC ACT GCC AAA ACT TTC AA-3′	301	Absent
AZFc	sY255-F sY255-R	5′-GTT ACA GGA TTC GGC GTG AT-3′ 5′-CTC GTC ATG TGC AGC CAC-3′	123	Absent

(containing 0.5 µl ethidium bromide) using 5 µl of each PCR mixtures with 50 minutes electrophoresis duration. After electrophoresis, the gel was visualized under UV light using gele documentation system (Proxima 2500 Isogene Life science, Netherland) (Brown, 2020) . PCR products were runs with a 100bp DNA ladder (GeneDirex) to compare and verify the product size of each amplicon.

3. RESULTS:

3. 1. PCR testing

In present-study, 296 infertile Kurdish males were screened for AZF-microdeletion in their Y chromosomes and 50 fertile males as a control group. No AZF-microdeletion was detected in both severe oligozoospermias and the normal group (Figure 2). However, from 289 patients with azoospermia, the YCMD were found in 10 patients (3.5%) (Table 2). Among the 10 patients that had YCMD, three of them (30%) had microdeletions in the AZFc region (Figure 3), three of them (30%) had microdeletions in the AZFb region (Figure 4), other three patients had microdeletions in both b and c of AZF (AZF b,c) regions (Figure 5), and the other final patient (10%) had microdeletions in the all a, b and c (AZF a,b,c) regions (Figure 6).

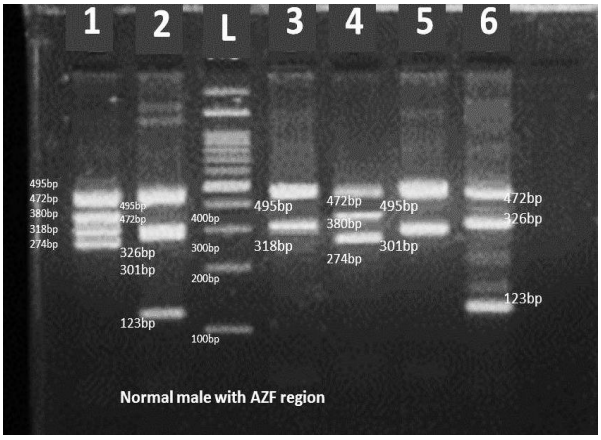

Figure 2: 1X TBE 1.5% agarose gel electrophoresis of PCR result of normal male that have all locus in AZF regions: Lane 1 represent PCR product of multiplex A with bands 495bp of ZFX/Y and 472bp SRY as two control gene and band 318 bp of AZFa (sY86), 274 bp of AZFb (sY127) and 380bp of AZFc (sY254) and Lane 2 represent PCR product of multiplex B with bands 495bp of ZFX/Y and 475bp of SRY as control gene and band 326 bp of AZFa (sY84), 301 bp of AZFb (sY134) and 123bp of AZFc (sY255). Lane L represent 100bp size DNA ladder marker.

Lane 3 represent result of PCR mixture A1 with bands 495bp of ZFX/Y and 318 bp of AZFa (sY86), Lane 4 represents result of PCR mixture A2 with three bands; 475bp of SRY, 380bp of AZFc (sY254) and, 274 bp of AZFb (sY127). Lane 5 represents result of PCR mixture B1 with two bands; 495bp of ZFX/Y and 301 bp of AZFb (sY134). Lane 6 represents result of PCR mixture B2 with three bands; 472bp of SRY, 326 bp of AZFa (sY84) and 123bp of AZFc (sY255).

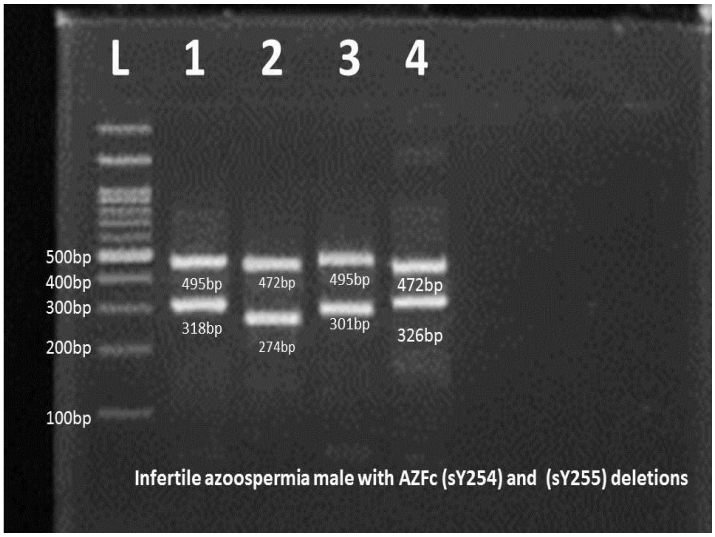

Figure 3: 1X TBE 1.5% agarose gel electrophoresis of PCR result of infertile azoospermia male with AZFc region deletion: Lane L represent 100bp size DNA ladder marker, Lane 1 represent result of PCR mixture A1 with two bands of ZFX/Y and AZFa (sY86), Lane 2 represents result of PCR mixture A2 with three bands of SRY and AZFb (sY127) but without AZFc (sY254). Lane 3 represents result of PCR mixture B1 with two bands of ZFX/Y and AZFb (sY134). Lane 4 represents result of PCR mixture B2 with two bands of SRY, and AZFa (sY84) but without AZFc (sY255).

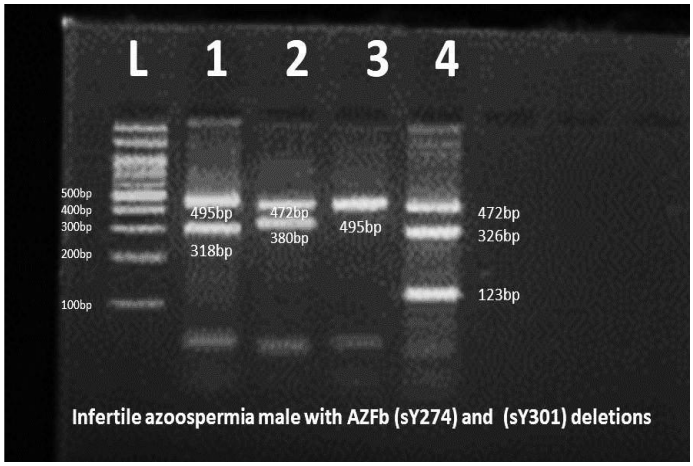

Figure 4: 1X TBE 1.5% agarose gel electrophoresis of PCR result of infertile azoospermia male with AZFb region deletion: Lane L represent 100bp size DNA ladder marker, Lane 1 represent result of PCR mixture A1 with two bands of ZFX/Y and AZFa (sY86), Lane 2 represents result of PCR mixture A2 with two bands of SRY and AZFc (sY254) but without AZFb (sY127). Lane 3 represents result of PCR mixture B1 with only one band of ZFX/Y with deletion of AZFb (sY134). Lane 4 represents result of PCR mixture B2 with three bands of SRY, and AZFa (sY84) and AZFc (sY255).

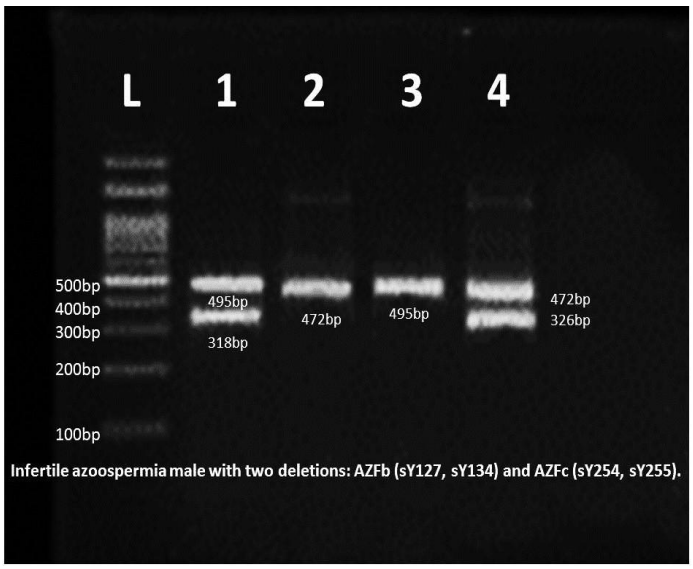

Figure 5: 1X TBE 1.5% agarose gel electrophoresis of PCR result of infertile azoospermia male with AZFb,c regions deletion: Lane L represent 100bp size DNA ladder marker, Lane 1 represent result of PCR mixture A1 with two bands of ZFX/Y and AZFa (sY86), Lane 2 represents result of PCR mixture A2 with one band of SRY but without AZFc (sY254) and AZFb (sY127). Lane 3 represents result of PCR mixture B1 with only one band of ZFX/Y with deletion of AZFb (sY134). Lane 4 represents result of PCR mixture B2 with two bands of SRY, and AZFa (sY84) but without AZFc (sY255).

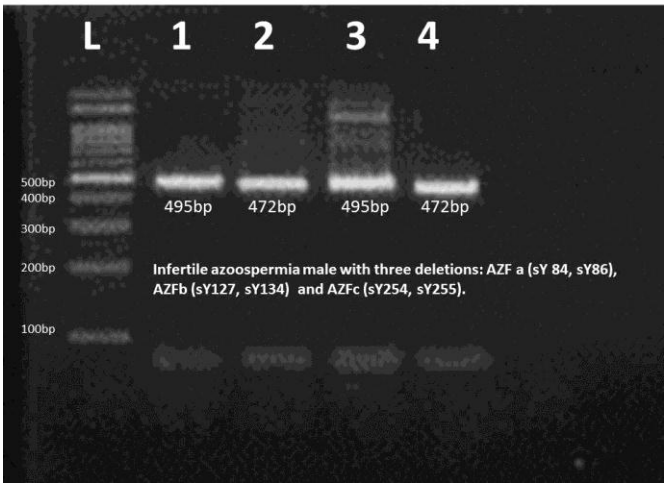

Figure 6: 1X TBE 1.5% agarose gel electrophoresis of PCR result of infertile azoospermia male with deletions in all regions AZFa,b,c: Lane L represent 100bp size DNA ladder marker, Lane 1 represent result of PCR mixture A1 with one band of ZFX/Y without AZFa (sY86), Lane 2 represents result of PCR mixture A2 with one band of SRY but without AZFc (sY254) and AZFb (sY127). Lane 3 represents result of PCR mixture B1 with only one band of ZFX/Y with deletion of AZFb (sY134). Lane 4 represents result of PCR mixture B2 with one band of SRY, but without AZFa (sY84) and AZFc (sY255).

Table 2. Frequency and types of AZF microdeletions on Y-chromosome in Kurdish infertile males

AZF microdeletions types	Azoospermia (n) patients	Total n (%)
AZFb	3	(30%)
AZFc	3	(30%)
AZFbc	3	(30%)
AZFabc	1	(10%)

4. DISCUSSION

This present study is the first molecular study depends on conventional PCR for screening YCMD in infertile males diagnosed with azoospermia and oligozoospermia in the Kurdish population in Erbil city.

The EAA and EMQN provide a useful protocol for checking YCMD in infertile males that has been depended in this study (Krausz et al., 2014, Simoni et al., 2004). Both organizations state that each laboratory must prepare and validate its protocol (Krausz et al., 2014).

In the first step as the EAA and EMQN recommended, two multiplex PCR reactions (multiplex PCR A and B) were prepared for the analysis of the three AZF deletions on the Y chromosome and the two control genes SRY and ZFX/Y and the final volume of each PCR mixtures were 50 µl, in which in each multiplex there're five different amplicons (bands) that should be separated in agarose gel electrophoresis. The amplicons regarding SRY and ZFX/Y gene are 495 bp and 472 bp respectively, they are too close to each other, only by 27bp they vary and also in case of AZFa sY84 and AZFb sY134 that have amplicons 326 bp and 301 bp respectively, they vary only by 25bp; separation or discrimination between those too close amplicons are difficult and time-consuming, waiting for overnight by agarose gel electrophoresis process.

For solving those problems, in this study, a new PCR format was optimized and presented for molecular screening of Y-chromosome microdeletions in infertile males that diagnosed as azoospermia and oligozoospermia, in which instead of two PCR reactions, four PCR reactions were prepared (multiplexA1, A2 and multiplexB1, B2) with 20 µl PCR mixture as final volume for each multiplex after amplification completed the amplified PCR products separated by 1.5 agarose gel for 50 minutes. As shown in Figure 2 the bands are discriminated well because there're just three bands in multiplexA1 and multiplexB1 and only two bands in multiplexA2 and multiplexB2, and the molecular size of each band is so different easily separated in agarose gel electrophoresis not overlapped like two-band regarding SRY and ZFX/Y.

After optimizing the PCR condition, initially, 10 samples were tested (as a trial) with two PCR formats to compare between them and validate the present format, both formats gave the same result regarding successfulness PCR condition and primer concentrations as illustrate in Figure 2. Then all samples were screened by the presented PCR format to finding the AZF-microdeletion in participating males.

Microdeletions are as high among infertile men after Klinefelter syndrome especially in Azoospermic men and it is the second-largest genetic cause of infertility in males and the frequency of deletions found in different laboratories may vary from 2-10% worldwide. (Krausz et al., 2014, Zhu et al., 2017, Özdemir et al., 2020, Beg et al., 2019, Arumugam et al., 2021).

The main purpose for Y-chromosome microdeletion screening in infertile males (azoospermia and oligozoospermia) is to determine whether the patient carry deletions in his Y-chromosome, who may be tried to conceive with his wife through (ART) who candidate for testicular sperm extraction (TESE) or intracytoplasmic sperm injection (ICSI); if it is found that a male have a complete deletion of the AZFa region or AZFa,b and c, in this case, TESE should not be recommended. Similarly, TESE on azoospermic carriers of AZFb or AZFb and c deletions is not advisable because the chance to retrieve spermatozoa is close to zero (Zhu et al., 2017).

In conclusion, in this study a simple PCR protocol was presented for molecular screening and ascertaining of Y-chromosome microdeletion types in azoospermia and oligozoospermia males, by using four PCR mixtures. Molecular diagnosis for Y-microdeletions is a necessary test in counseling patients about possible treatment options as well as for possible effects on next generations. The present study demonstrate the occurrence of Y-chromosomal microdeletion types in infertile Kurdish males and the results are within the range of worldwide data. Additionally, future high-throughput DNA sequencing can be utilized to detect and exhibit AZF microdeletion region from Y-chromosomal microdeletion types in infertile participants' males.

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Conflict of Interest

The authors declare no conflicts of interest

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