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Original Article

Comparison of Real Time PCR and Gel-based PCR for the Diagnosis of Y Chromosome microdeletions in infertile azoospermia males

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Abstract

Infertility is a disorder of the reproductive system characterized by a couple's inability to conceive after at least one year of regular, unprotected sexual activity. Approximately 15% of couples worldwide struggle with infertility. Male infertility accounts for 20–30% of all infertility cases and is defined as the inability of a male to achieve conception in a healthy, fertile female. Azoospermia factor (AZF) genes are located on the long arm of the human Y chromosome and are involved in spermatogenesis. Microdeletions in the AZF region are now recognized as the second most prevalent genetic cause of spermatogenetic failure and male infertility. This study aims to diagnose AZF deletions in 289 males with infertile azoospermia and 50 males with fertile sperm using two PCR techniques, real-time PCR and gel-based PCR, and compare the results of both techniques. In diagnosing AZF deletions, the results of both real-time PCR and gel-based PCR are identical. Generally AZF deletion found in 10 patients (3.5%), three of them (30%) had microdeletions in the AZFc region, 3 of them (30%) had microdeletions in the AZFb region, also other 3 patients had microdeletions in the b and c of AZF (AZF b,c) region, and the final one patient (10%) had microdeletions in the all a, b and c (AZF a,b,c) region. According to the findings of this study, there are no distinctions between both techniques for diagnosing AZF deletion in males with infertility.

Keywords: Male Infertility, Y chromosome, Azoospermia factor, Gel based PCR technique, Real time PCR technique.

INTRODUCTION

Worldwide up to 15% of couples are affected by infertility, which is defined as the inability of a sexually active couple to carry a pregnancy to delivery, after one year of unprotected intercourse. According to the WHO statistical data, around 48 million couples and 186 million individuals suffer from infertility globally. Male infertility refers to a male's inability to make pregnancy in a normal fertile female, and generally, it is responsible for near 20-30 % of all infertility cases [1-3]

The human Y chromosome is genetically dynamic and susceptible to significant variation due to the high proportion of segmental duplications that underlay the wide range of deletions and duplications seen in various locations on this chromosome. Because the Yq locus contains a large number of genes that are transcribed in the testis and plays a specific role in spermatogenesis, loss of these regions would lead to infertility. Microdeletions occur as a result of homologous recombination of identical segments within palindromic sequences [4].

There are three types of microdeletions in the long arm of the human Y chromosome (Yq) known as AZFa, AZFb, and AZFc deletions. Because these deletions are one of the leading causes of spermatogenic failure leading in male infertility, screening for AZF deletions has become part of the diagnostic work-up for infertile men. Yq microdeletions are identified exclusively in men with abnormal spermiogram and would not have been witnessed in large series of fertile men, suggesting that these deletions are the cause of the failure of spermatogenesis, and therefore infertility [5,6].

Various studies have shown that 10% of the men suffering from azoospermia have microdeletions in their three types of azoospermia factor genes. These deletions remove a number of genes responsible for germ cell development in males (spermatogenesis) and its maintenance. The Y chromosome in humans is important for sex determination and male germ cell progress and maintenance [7,8].

Because these deletions are one of the leading causes of spermatogenic failure, screening for AZF deletions has become part of the diagnostic work-up for infertile men. Yq microdeletions are identified exclusively in men with abnormal spermiogram and would not have been witnessed in large series of fertile men, suggesting that these deletions are the cause of the failure of spermatogenesis, and therefore infertility 20 [6].

The SCOS and azoospermia are undoubtedly caused by deletions of the entire AZFa region. Because genes in the AZFa locus are expressed in germ cells prenatally, it is possible that the loss of these genes will result in germ cell death, resulting in Sertoli Cell Only Syndrome (SCOS) [9]. However, partial AZFa deletions are related to phenotypes ranging from azoospermia to normozoospermia, indicating that the amount of genetic content lost is a critical determinant of azoospermia caused by AZFa deletions. Hence, the diagnosis of a complete deletion of the AZFa region suggests that retrieving testicular spermatozoa for intracytoplasmic sperm injection (ICSI) is practically impossible [10].

The genes in the AZFb locus promote sperm growth and maturation and are thought to be essential for the efficient progression of sperm through meiosis into spermiogenesis. Patients with AZFb deletions have a testicular phenotype of maturation arrest, frequently at the spermatocyte stage, with a lack of post-meiotic germ cells in the majority of the tubules [11].

Variable phenotypes have been observed in men with AZFc deletions, ranging from complete azoospermia to mild oligozoospermia [12]. In general, men with AZFc deletions have elongating spermatids in their testis, and sperm can be retrieved in a reasonable number of AZFc deleted infertile males [13]. In most men with AZFc deletion, spermatogenesis is completed, but on a reduced scale resulting in oligozoospermia [10].

By semen analysis, complete AZFc deletions are associated with the drastic reduction in sperm count and most AZFc deleted males are severely oligozoospermic and some may even be azoospermic [6]. There are also rare AZFc deleted males who have naturally produced many children, but the sons have all been founded to be infertile [14]. The AZFc region is particularly prone to NAHR happenings which could promote the formation of both partial deletions and duplications [7,15].

This study was aimed to compare the results of real time PCR with gel-based PCR technique for identification of microdeletions AZF regions of the Y chromosome in a number of infertile azoospermia Kurdish males.

MATERIALS AND METHODS

Patients

This study was conducted on 289 Iraqi Kurdish infertile males in Erbil province. All cases were diagnosed with azoospermia (primary infertility), with median age 35 year. Semen analysis was done according to WHO guidelines [1]. In this study, the control group consisted of 40 healthy males with paternity evidence that did not use assisted reproductive technology (ART), with a median age of 36 years.

MOLECULAR METHODS

Genomic DNA extraction

Every participant gave consent to the collection of two milliliters of venous blood using a sterile syringe, and blood samples were collected in anticoagulant EDTA tubes. Genomic DNA was extracted from blood samples in accordance with manufacturer's instructions (Genomic DNA Mini Kit, Geneaid, Taiwan). The sequential procedures were carried out in accordance with a manufacturing protocol. The NanodropTM 1000 spectrophotometer (Thermo Scientific, USA) was used to assess the purity, amount, and quality of each DNA sample.

Gel-based PCR testing for Y chromosome microdeletions

Molecular screening was done for each patient and control in accordance with the EAA and

EMQN procedure to identify microdeletion on the Y chromosome focusing on the most significant AZF sequence-tagged sites (STS) on the Y chromosome (Krausz et al., 2014). For the identification of the three AZF microdeletions four PCR reactions were prepared in which two PCR reactions multiplex A 1 and A2, the mixture A 1 consisted of ZFX/Y, sY86 (AZFa) primers and the mixture A2 consist of SRY and sY254 (AZFc/DAZ) and sY127 (AZFb) primers. The mixture B1 consisted of ZFX/Y and sY134 (AZFb) primers and the mixture B2 consists of SRY and sY84 (AZFa) and sY255 (AZFc/DAZ) primers. The primer sequences used according the protocol described by [6].

Primer preparation

All specific primers that were used in conventional PCR were purchased in lyophilized form in microcentrifuge vials to obtain a concentration of 100 pmol /µl of each primer. Sterile D.W. was added to each vial for dissolving the lyophilized primers according to manufactures protocol.

Gel based PCR sample preparation

The PCR mixture was prepared with final volume of 25 µl containing 12.5 µl of 2X of Master Mix (AMPLIQON), 1 µl each primer with 10 pM concentration (Macrogen; LIGO) and 5 µl of genomic DNA sample as a template, the mixture compledted to 25 µl with deionized distilled water. 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).

Gel electrophoresis

Using five microliters of each PCR mixture and 45 minutes of electrophoresis time, the amplified PCR product for each sample was separated on a 2% agarose gel (containing 0.5 microliters of ethidium bromide. After electrophoresis completed the gel was inspected under UV light using the gel documentation system (Proxima 2500 Isogene Life science, Netherlands) [16]. To compare and confirm the product size of each amplicon, PCR products were tested with a 100bp DNA ladder (GeneDirex).

Real Time PCR testing for Y chromosome microdeletions

Real-time PCR technique was used to investigate Y chromosome microdeletions for both patients and controls, similar to gel-based PCR, to diagnose same sequence tagged sites (STS) of AZF microdeletions on the Y chromosome, the STS were screened are SY84 (AZFa), SY86 (AZFa), SY127 (AZFb), SY134 (AZFb), SY254 (AZFc/DAZ) and SY255 (AZFc/DAZ) whereas genes that responsible or encoding for human zinc-finger protein (ZFX/Y) that present on the X and Y chromosomes, and sex determining region (genes) located on the Y chromosome (SRY, i.e., STS SY14) used as Control [6]. The Y chromosome microdeletion Real Time PCR Kit (Cat. No. 15R-10-08, SNP Biotechnology, Turkey), which contains eight RT PCR mixtures with sequence-specific primers and probes, was utilized. FAM was the analysis's fluorescence. Additionally, an internal control that is labeled with HEX or JOE is included in each RT PCR mixture. For each region 20 µL of RT PCR mixtures was mixed with 5 µL (10-100ng) of DNA sample, then running with RT PCR machines (Prime Pro 48 Real-Time PCR System-Techne) with the condition program; 95c for 5 Min. one cycle. 95c for 15sec. 60C for 1Min. 40 cycles according to manufactures protocol.

RESULTS

In this study 289 infertile Kurdish males were screened for AZF-microdeletion in their Y chromosomes and 50 fertile males as a control group by both Gel-based PCR and RT-PCR technique. The results of both techniques conventional PCR and RT-PCR technique are exactly similar, as follows: AZF-microdeletion was not detected in the normal group. However, from 289 patients with azoospermia, the AZF-microdeletion was found in 10 patients (3.5%) Figure 1, 2, Table 1. Among the 10 patients that had AZF-microdeletion, three of them (30%) had microdeletions in the AZFc region, three of them (30%) had microdeletions in the AZFb region (Figure 2), other three patients had microdeletions in both b and c of AZF (AZF b,c) regions, and the last patient (10%) had microdeletions in all the a, b and c (AZF a,b,c) regions.

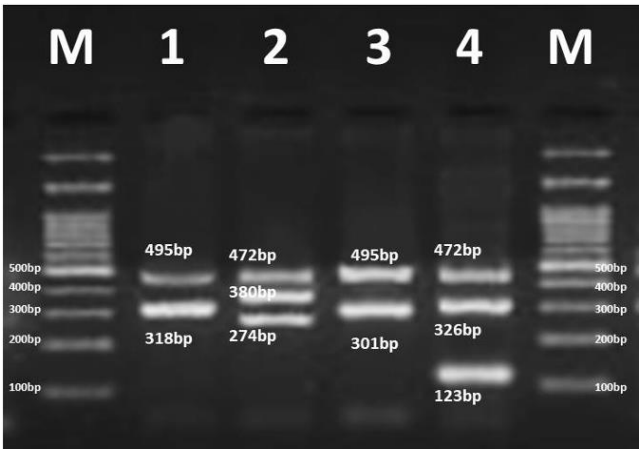

Figure 1. Agarose gel electrophoresis of PCR result (2.5% agarose with 1X TBE) of a normal fertile sample that have all AZF regions: Lane M represent 100bp size DNA ladder marker. Lane 1 represent PCR product of mixture A1 with bands 495bp of ZFX/Y and 318 bp of AZFa (sY86). Lane2 represent PCR product of mixture A2 with bands 472bp of SRY (sY14), 380bp of AZFc (sY254) and 274 bp of AZFb (sY127). Lane3 represent PCR product of multiplex B1 with bands 495bp of ZFX/Y and 301 bp of AZFb (sY134). Lane B2 with bands 472bp SRY, band 326 bp of AZFa (sY84), and 123bp of AZFc (sY255).

Table 1. Frequency of AZF microdeletions types on Y chromosome in 289 Kurdish azoospermia infertile males.

Yq AZF microdeletions	Infertile azoospermia (n)	Total (%)
AZFb	3	(30%)
AZFc	3	(30%)
AZFbc	3	(30%)
AZFabc	1	(10%)

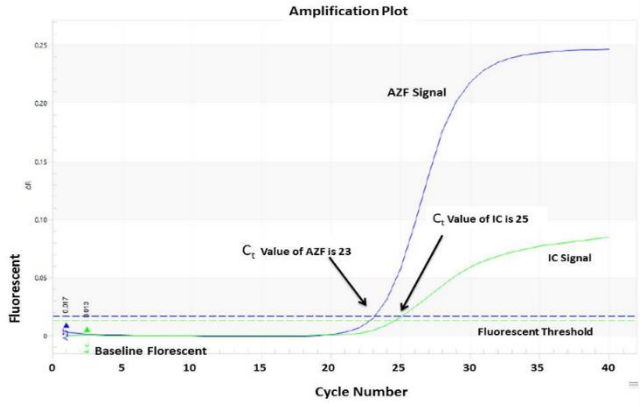

Figure 2. Prime Pro 48 Real Time (Techne) amplification plot. The figure is the fluorescence of the reporter dye is plotted against PCR cycle number of a sample that have one type of AZF region. The experiment consists of 40 cycles. Showing the AZF curve (signal) labeled with fluorescent FAM reporter dye with threshold cycle (Ct) 23 and the internal control (IC) labeled with fluorescent HEX dye have Ct 25.

DISCUSSION

Y-chromosomal microdeletions are the second most common genetic cause of male infertility, after the Klinefelter syndrome. Numerous researchers have documented the presence of microdeletions in infertile individuals worldwide and the molecular identification of deletions has emerged as a key test in the diagnosis of male infertility [13,17]. The EAA and EMQN now routinely recommend screening for Y chromosome microdeletions in individuals with azoospermia and oligozoospermia [6]. Because different STSs procedures and study populations are used by various laboratories, the prevalence of Y chromosomal microdeletions in infertile males differs significantly [18].

In this study, both techniques standard gel-based PCR and Real-Time PCR techniques were used for diagnosing microdeletions in Y chromosomal in 296 infertile Kurdish males and 50 fertile males as a control group. Multiplex PCR is the simultaneous detection of multiple targets in a single reaction well, with a different pair of primers for each target. Generally multiplex PCR is used in life science research, clinical diagnostics, and forensic laboratories. Multiplex PCR and standard gel electrophoresis are genetic technologies commonly used for the detection of microdeletions in Y chromosomal. However, there are some limitations to analyzing multiple PCR products on a gel. For instance, the process takes more time as mentioned in EAA and EMQN protocol, and thus is time-consuming, which is considered a negative point for this technique [6,19,20].

Both gel-based PCR and real-time PCR follow a similar procedure. Both techniques depend on the amplification of targeting nucleic acid by the activity of the DNA polymerase enzyme at the free 3'-OH end of both forward and reverse primers, but in real-time PCR there is another extra oligonucleotide primer located between forward and reverse primers that carry fluorescent probes

when hydrolyzed by the activity of 5-exo-nuclease activity DNA polymerase. The fluorescence activity increased due to the cleaved probe, this probe gives the real-time PCR more specificity and sensitivity property over the gel-based PCR [18,21].

On the other hand, in gel-based PCR the amplified product (end-point analysis) can be seen on agarose gel by running the amplifying product on an agarose gel, after staining with ethidium by using a UV transilluminator (gel documentation). At this point the researcher becomes quite sure that this amplified PCR product (known size band in base pair) is the exact target, but in real-time PCR the researcher is not able to see the size of the amplified product, since real-time PCR allows the tracking and analysis of the accumulation of amplified product during the reaction in real-time. Another advantage of standard gel-based PCR is that it is the only method enabling researchers to sequence a single target [18,21].

In real-time PCR there is no need for additional analysis after PCR, in contrast to conventional gel-based PCR assays, real-time PCR generally provides higher sensitivity, speed, throughput, and prospective automation. Real-time PCR allows the accumulation of amplified product to be detected and monitoring during the reaction progresses, which are all desirable features for epidemiological studies. In addition, closed-tube analysis eliminates the risk of contamination during post-PCR handling steps, Real-time PCR is not only being increasingly used to identify genes related to human diseases in the research laboratory but also has been rapidly establishing itself as a diagnostic technique in the clinical diagnostic laboratory [18].

The important point in choosing one of the two techniques is depending on the availability of instruments in a given laboratory. Nowadays the price of conventional thermal cycler machines is too low compared to high price real time PCR machine. Another point is the price of real time ready to use diagnostic kits is too high compared with the price of primers and PCR mixtures used for specific genetic disease diagnosis.

In conclusion, according to the results of this study no differences between the two techniques real time and gel based PCR for diagnose the microdeletions of Y chromosome in infertile azoospermia patients. The selection one of the technique by clinical diagnostic laboratory is depending on the availability of the each PCR machine and professionals, indeed the real time PCR testing is faster and time efficient but more expensive than gel based PCR technique.

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Conflict of Interest

The authors declare they have no conflicting interests.

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