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# A COMPREHENSIVE REVIEW ON THE ROLE OF GENETIC TESTING IN MALE INFERTILITY

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## ABSTRACT

According to the World Health Organisation (WHO) globally 1 in 6 people is affected by infertility making it a health challenge worldwide. Figuring out the exact number of males affected by infertility around the globe has yet to be discovered due to a lack of data and underreporting. However, the available statistics likely based on global data show that the male factor accounts for 50% of infertility cases. Major causes are chromosomal aneuploidies, Y chromosome microdeletions, and genetic mutations such as CFTR and CATSPER2 gene mutation. These errors in the genome can affect the process of spermatogenesis resulting in abnormal sperm production. Hence, diagnosing these conditions for the disease's exact cause and clinical management becomes necessary. This review deals with the significance of biochemical, metabolomics, cytogenetics and molecular analysis including next-generation sequencing (NGS) and competitive microarray analysis for diagnosing male infertility. These comprehensive analytical techniques enable us to identify a lot many

potential genetic variants, markers, imbalances, factors and faulty gene expression patterns associated with male infertility.

## INTRODUCTION

The condition of male infertility involves complex factors including a strong genetic and epigenetic influence.[1] About 10% of married couples experience infertility issues, which increases every year in male predominance. [2] Many chromosomal abnormalities and genetic disorders are associated with male infertility including azoospermia (AZ), severe oligozoospermia (OL), or normozoospermia, and genetic disorders such as Klinefelter syndrome or Y chromosomal deletions, which can impact the phenomenon of spermatogenesis. If such abnormalities are identified then proper diagnosis and treatment should be done.[3] Male factors contribute to approximately 30–50% of infertility cases, evaluation of these cases includes detailed history taking, focused physical examination, selective laboratory testing[4] karyotyping analysis, Y-chromosome microdeletion screening and CFTR gene mutation tests are performed to investigate genetic aetiology in patients with AZ and severe OL and more.[1] Various genetic disorders can be analysed through karyotyping. The most common sex chromosome disorder is Klinefelter syndrome having XXY karyotype a total of 47 chromosomes. Chromosomal translocations harm the fertility of males as they can result in either equal (balanced translocation) or unequal distribution of genetic material (unbalanced or Robertsonian translocation). Due to this carriers are at increased risk of sperm aneuploidy, which could result in miscarriage or babies with translocated trisomy. Structural abnormalities in Y chromosomes such as microdeletions (del(Yq), ring Y) and duplication of Y chromosomes (dup(Y) are also responsible for male infertility.[5]

### Causes for male infertility

Some of the genetic causes underlying male infertility are listed and briefly described below:-

**1. Chromosomal aneuploidies**

**(a) Klinefelter syndrome (47, XXY)**

Klinefelter syndrome is one of the most common sex chromosome disorders in which a higher number of X chromosomes are present as compared to one X chromosome that is present in normal XY karyotype. This abnormality results in male infertility. Although it occurs in about 1 in 600 newborn males, many cases are not diagnosed properly because there is a huge variation in phenotypical changes. Hence, there is a need for an appropriate

diagnosis that should be made by checking the patient's medical history and further by conducting a physical exam, semen analysis, and finally by karyotype testing. Therefore, genetic testing should become more common as it will increase the likelihood of identifying more Klinefelter syndrome cases.[6]

**(b) Jacob's syndrome**

Jacob's syndrome, also known as 47, XYY, is a genetic condition where males have an extra Y chromosome. It occurs in about 1 in 1,000 male births. Although some may experience developmental delays, language difficulties, and behavioural issues more often than not (i.e. approximately 85%) it is not detected because symptoms may be absent in most cases unless and until they start showing fertility problems.[7,8]

**(c) 46, XX male syndrome**

The 46, XX male syndrome which is also called the Chapelle Syndrome affects about 1 in 20,000 males and is responsible for approximately 2% of male infertility cases. It occurs when an individual with 46, XX karyotype (A karyotype typically found in females) starts showing characteristics of a male phenotype. Early diagnosis becomes difficult as 90% of the cases show a typical male phenotype. Diagnosis usually happens during puberty certain symptoms like underdeveloped sex organs (Hypogonadism) or breast development in males (Gynecomastia) begin to show or it might be identified even later when infertility issues arise as all cases lack sperm production (Aspermatogenesis).[9]

**2. Y chromosome microdeletions (YCMDs)**

The Y chromosome plays an important role in normal male genitalia development and sperm production. But its long arm is prone to deletions which can lead to male infertility. YCMDs are the second leading genetic cause of male infertility, after Klinefelter syndrome. As a result, testing for these deletions is necessary for men who are showing symptoms of infertility.[10]

Y chromosome is acrocentric, having a short p arm and a long q arm designated as Yp and Yq respectively, separated by a centromeric region. The Euchromatin region constitutes the short arm Yp, centromere and proximal long arm, while the heterochromatin region is the distal long arm (Yq). Heterochromatin and Euchromatin region together form a male-specific region (MSY), approximately 95% of the Y chromosome while the remaining 5% are Pseudoautosomal regions (PAR).[11,12]

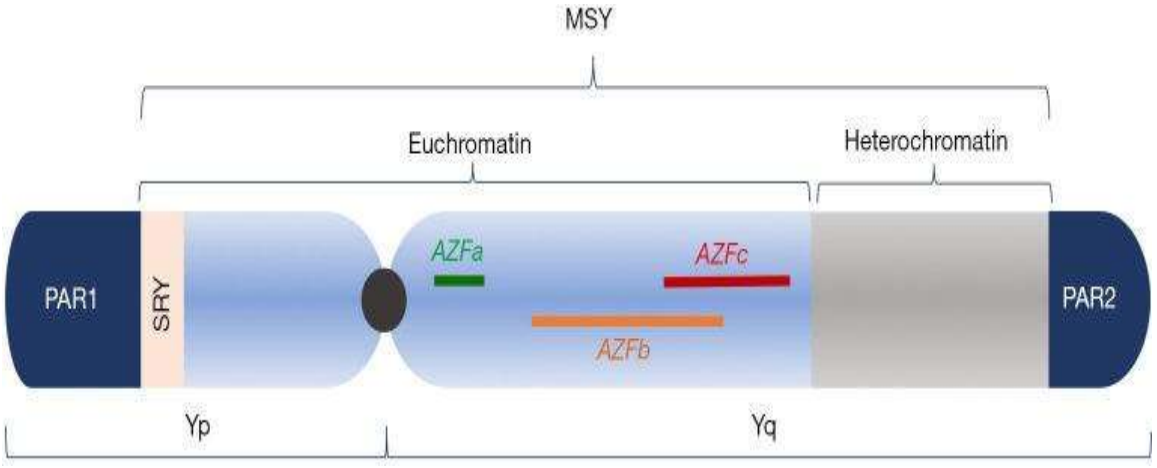


**Figure 1: Different regions of Y-chromosome.**[13]

The PAR on the Y chromosome play essential roles in various physiological processes, they are divided into PAR1 and PAR2. PAR1 is present at the end of the short arm (Yp), crucial for the pairing and exchange of genetic material between the Y and X chromosomes during male meiosis, the SHOX gene of PAR1 is crucial for bone growth and development, with mutations leading to disorders like Léri-Weill dyschondrosteosis. The ASMT gene belonging to PAR1, is involved in melatonin synthesis, influencing sleep-wake cycles.[14]

Whereas PAR2 is present at the end of the long arm (Yq). PAR1 and PAR2 span approximately 2600 and 320 kilobases (kb) of DNA, respectively. Genes in these regions are inherited like autosomal genes. Although PAR1 and PAR2 together account for about 5% of the Y chromosome, the remaining 95% is known as the "Non-Recombining Y" (NRY).[15]

MSY does not undergo recombination with the X chromosome during meiosis. This region has remained largely unchanged in primate and eutherian genomes for millions of years. It includes amplicon sequences that are primarily expressed in the testis. In humans, about 25% of the MSY euchromatin consists of these amplicon regions, organized into eight large, nearly identical palindromic sequences. These sequences frequently undergo gene conversion, which helps prevent genetic decay. During meiosis, these large amplicon regions can rearrange within the chromosome through a process called non-allelic homologous recombination. This can lead to deletions, duplications, or inversions within the Y chromosome, potentially affecting genes essential for fertility.[16], [11], [17]

The AZFactor (AZF) region is particularly important, as it is a crucial segment of the Y chromosome and plays a vital role in spermatogenesis and male fertility. The AZF region is divided into three sub-regions:

**Table 1: AZF loci associated with spermatogenesis, Mutations or deletions in the AZF region can lead to AZ or severe OL, resulting in male infertility.[18-23]**

| AZF Regions | location | Size | Genes | Functions | Reasons |
|---|---|---|---|---|---|
| (a)AZFa | Proximal Yq11 region | 1.5 Mb | DBY, USP9Y | Essential for spermatogonial proliferation and differentiation | AZFa region deletions account for 0.5–4% of all Yq-microdeletions. Complete deletions of the AZFa region result in AZ and Sertoli cell-only syndrome (SCOS) |
| (b)AZFb | Middle Yq11 region | 3.5 Mb | RBMY, PRY | Meiosis and sperm maturation | Complete deletions of AZFb occur at a frequency of 1–5% of all Yq-microdeletions and lead to similar results as AZFa deletion, namely SCOS or spermatogenic arrest resulting in AZ. |
| (c) | Distal Yq11 regionAZFc | 2.5 Mb | DAZ, CDY | Regulates spermatic gene expression and chromatin remodelling. | In contrast to the complete deletions mentioned earlier, complete AZFc deletions are linked to a range of clinical and histological outcomes. These can vary from AZ to residual spermatogenesis and OL. |

According to the Practice Committee of the American Society for Reproductive Medicine (ASRM), YCMDs may occur in up to 2% of the general population. However, the European Academy of Andrology (EAA) estimates a much lower prevalence, around 1 in 4000 (0.025%) in unselected men.[24] The proposed role of the AZF region in spermatogenesis suggests that YCMs are significantly more common among infertile men or those with

abnormal semen parameters. The Practice Committee of the ASRM estimated in 2015 that YCMDs occur in up to 16% of men with severe or azoospermia. A 2016 review by the European Association of Urology reported YCMDs prevalence to be 8–12% in azoospermic men and about 3–7% in men with severe OL.[20]

### 3. Cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations

CFTR gene variations, apart from being responsible for cystic fibrosis, are also known to induce male infertility by causing Congenital Absence of the Vas Deferens (CAVD). Genetic variations in the CFTR gene during foetal development can cause disruptions in fluid transport within the Wolffian duct. This will prevent the differentiation of the Wolffian duct into the epididymis and vas deferens. Hence, CFTR gene mutations are believed to be responsible for the CAVD and also the absence of distal epididymis. It has also been suggested that CFTR is responsible for spermatogenesis and mutations in the CFTR gene can affect sperm production. For example, CFTR gene mutations might disrupt the body's response to follicle-stimulating hormone (FSH) and they can also affect the cAMP-response element binding protein (CREB) pathway. Both of these can lead to impaired sperm production.[25]

CAVD is a genetic condition in which the vas deferens are affected and it is primarily responsible for male infertility. The vas deferens is a tube-like structure that carries sperm from the epididymis (where sperm mature) to the ejaculatory ducts. Based on the abnormalities observed in the male reproductive system, CAVD is categorized into three types: congenital bilateral absence of the vas deferens (CBAVD), congenital bilateral partial aplasia of the vas deferens (CPAVD), and congenital unilateral absence of the vas deferens (CUAVD). CBAVD is the most prevalent form, affecting 1-2% of infertile men, while CUAVD is less common. However, it could be because in most cases, CUAVD is asymptomatic and remains undetected.[26]

In CBAVD both the vas deferens are absent from birth and although testes retain the ability to produce sperm, the absence of the vas deferens prevents sperm from being transported to the ejaculatory ducts, resulting in AZ (no sperm in the semen) which ultimately renders the male infertile. Men with CBAVD often have other reproductive system abnormalities and may show symptoms similar to cystic fibrosis.[27]

Mutations in the *CFTR* gene, present on chromosome 7, are primarily responsible for CBAVD. The *CFTR* gene produces a Cl− selective channel protein which plays an important role in maintaining the levels of chloride and bicarbonate that is essential for sperm maturation that occurs during its travel through the epididymis. Recently, the adhesion G-protein-coupled receptor G2 (*ADGRG2*) gene located in Xp22.13 was also found to be playing a role in CBAVD. Mutations in this gene were found to disrupt fluid balance within the testes, causing sperm to become stagnant. This ultimately leads to a condition known as obstructive AZ, where sperm are blocked from being transported normally.[28]

Several leading health organizations, like the World Health Organization, the American Society of Reproductive Medicine, and the American Urological Association, advise that all males who have or are showing symptoms of cystic fibrosis (CF) or CBAVD should undergo testing for *CFTR* gene mutations.[29]

### 4. Environmental and living factors

Humans are subjected to numerous environmental and lifestyle factors that significantly contribute to male infertility. The male reproductive system is highly suspectable to these environmental and lifestyle choices that lead to infertility and compromise semen quality which contributes to sperm quality, decreased sperm concentration, motility and viability, as well as abnormal sperm morphology and sperm DNA fragmentation ultimately resulting in male infertility.[30] Environmental contaminants include heavy metals, pesticides, polychlorinated hydrocarbons, radicals, DDT, Dioxins and air pollution. The lifestyle factors are the adaptable habits which affect the overall health and fertility of the human Lifestyle factors include age, radiation, heat, obesity, consumption of alcohol, smoking, recreational drugs, use of mobile phones and many other variables that influence male fertility.[31] However, it is possible to prevent or alter all of these factors which enables us to reduce the associated risk. All the factors mentioned, such as chemicals, air pollutants, heat exposure and making beneficial changes in our daily routines can contribute to reducing the overall occurrence of male infertility.[30]

## Diagnostic techniques

### 1. Metabolomic analysis

In accordance with the WHO guidelines, diagnosis of male infertility is primarily conducted by semen analysis. Although semen analysis can explain the qualitative [asthenozoospermia (AS), teratozoospermia (TE), and necrospermia] and quantitative [AZ, cryptozoospermia, and

oligoasthenozoospermia (OA)] conditions of sperm however, it is unable to provide decisive results. Hence, along with semen analysis, metabolomics approaches can also be used that may provide more accurate diagnosis and treatment for male infertility. During metabolomic analysis seminal plasma, urine or blood samples of subjects are analysed for potential metabolite biomarkers associated with male infertility these include oxidative stress (OS) related metabolites, antioxidants and reactive oxygen species (ROS). OS can be a major factor in ROS production and impairment of the antioxidant defence mechanism that contributes to spermatogenic abnormalities. Researchers incorporated untargeted metabolic profiling via LC-MS to analyse healthy control (HC) participants and individuals suffering from different conditions of infertility. The comparison resulted in the detection of 63 potential biomarkers including L-carnitine`, ALCs, amino acids, fatty acids, dipeptides, PEs, SM and purines.[32]

The study conducted by Ma et al. sought to identify blood plasma metabolomics to differentiate (HC) from TE, AS, AZ and OL patients. The blood plasma samples of different subjects were profiled with the help of GC-MS and a typical ion chromatogram (TIC) was obtained. Fifty-three metabolites (amino acids, carbohydrates, fats, lipids and urea) were detected. The R-match scoring system was used to compare the similarity between the mass spectrum of metabolites extracted and the spectrum of known metabolites available in the library of the National Institute of Standards and Technology (NIST) database. In the case of semen abnormality (SA), research suggests that several carbohydrate metabolic pathways were disturbed, an increase in pyruvate metabolism (PM), glyoxylate and dicarboxylate metabolism (GDM), tricarboxylic acid cycle (TCA) whereas, decrease in inositol phosphate metabolism (IPM) is observed. Talking of amino acid metabolism, the SA group exhibits an increase in glutathione metabolism (GM), and glycine, serine & threonine metabolism (GSTM). Increased concentration of two saturated fatty acids (SFA) estres i.e. glyceryl palmitate and glycerol monostearate in SA patients is because of disrupted lipid metabolism.[33]

## 2. Karyotype analysis

The word "karyotype" originates from the Greek word "kernel" and refers to the contents of the nucleus. There are 22 pairs of autosomal chromosomes and 1 pair of sex chromosomes in humans.[34] Karyotyping analysis is an approach used in cytogenetics as it specifically helps

to visualise the chromosomes for structural abnormalities and aneuploidies that might be hindering the process of spermatogenesis.[35]

### 2.1 Conventional karyotyping

Conventional karyotyping is a cytogenetic technique involving the cultivation of blood cells in RPMI 1640 media and their subsequent arrest at metaphase with the help of colcemid The visualization of chromosomes after Geimsa staining enables the analysis of chromosomal aneuploidies. In G banding Giemsa binds to DNA resulting in the formation of bands with dark and light intensities. It has become the most widely used technique for banding chromosomes as unique banding patterns can be seen on each chromosome which makes the identification of chromosomes easier. The structure analysis of chromosomes is done by visualising the metaphases present on the slide under a microscope.[34] Various advanced microscopes and software will be used to evaluate the karyotype for future aspects.

### 2.2 Chromosomal painting techniques

#### 2.2.(a) Multiplex fluorescent in situ hybridisation (M-FISH)

While conventional karyotyping can identify some chromosomal abnormalities, Fluorescent in situ hybridisation (FISH) is more sensitive in detecting small genetic variations, offering a more comprehensive analysis.[36] FISH is one of the methods used to visualise the aneuploidy frequencies and chromosome abnormalities[deletion, duplications and translocations] in sperm cells. This method utilises multiple fluorescently labelled DNA and RNA probes.[37]

Chromosomes 13,18,21, X and Y show higher rates of aneuploidy in testicular sperm. The tightly packed chromatin in the sperm's head is decondensed first to make it available for the hybridisation of probes. Fluorescently labelled DNA probes, specific for each chromosome are allowed to hybridise with their complementary DNA sequences. These complementarities emit a fluorescent signal which is further determined by special fluorescence microscopes having narrow bandpass filter sets sensitive for stains FITC(green), Texas Red, and a DAPI/FITC/Texas Red (combination of blue, green, and red). The results can be interpreted both quantitatively and qualitatively and identify men at risk for genetic abnormalities. The quantitative approach assigns a numerical score value to evaluate sperm quality while, the qualitative approach deals only with a limited set of chromosomes (21, X and Y).[38] FISH usually produces results in 24-48 hours with a resolution of approximately 3 megabases and is also capable of identifying aneuploidies, as low as 7%.[34] Sperm examination through

FISH can detect abnormal sperm conditions, which are one of the reasons behind implantation failure and miscarriage.[37]

Male infertility kits can also be used in the Y-chromosome microdeletion analysis and the CFTR analysis. Multiplex quantitative fluorescent-polymerase chain reaction is used to quantify the sequence of specific areas of DNA with fluorescently labelled probes for the determination of sex chromosome aneuploidies.[39]

### 2.2. (b) Spectral imaging karyotyping (SKY)

Spectral imaging karyotyping is another unique technique dependent on fluorescence in situ hybridisation that allows the visualization of all human chromosomes. individually and more precisely. SKY detects the chromosomal abbreviations even with complex atypical karyotypes such as abnormalities based on number, structure, banding patterns and evolutionary relationships of numerous diseases and birth defects.[40]

In SKY, painting probes assign specific colours to each pair of homologous chromosomes (22 pairs of autosome, X and Y)chromosome. This specification is achieved by mixing two different fluorescent dyes, chosen from the pool of five available dyes: spectrum orange, Texas red, Cy5, spectrum green, and Cy5.5.[41] A combination of epifluorescence microscopy, charge-coupled device (CCD) imaging, and Fourier spectroscopy captures the image that gives a full-colour spectrum at every point in the image with a single exposure.[42]

SKY illustrates the differentiation of chromosomes according to their spectral properties.[43] The advantage of SKY is the rapid identification of interchromosomal abbreviations i.e. translocations and rearrangements.[44] If the specific chromosome is misplaced or translocated, other chromosomes may be involved in the translocation.[45] SKY can visualise the entire set of chromosomes in 3D, and the image captured is examined for chromosomal abnormalities caused by any deviations in chromosomal number, structure integrity and overall morphology.[40]

## 3. Quantitative fluorescent polymerase reaction (QF-PCR)

QF-PCR is a powerful molecular technique used to detect chromosomal aneuploidies. Fluorescently labelled primers amplify the genetic markers of interest (target sequence), which are then separated based on size using an automated genetic analyser. Computer software evaluates the peak generated and their area ratio can determine the relative copy

number of chromosomes. This is a high-efficiency and cost-effective technique, giving results within 24 hours. (46) QF-PCR analysis shows results from several markers present on chromosomes 13, 18, 21, X and Y. *AMEL*, *ZFX*, *T1* and *T3* are non-polymorphic markers. *AMEL* and *ZFX* are present on both X and Y chromosomes, whereas *T1* and *T3* are X chromosome counting markers present only on the X chromosome. *X1*, *X3*, and *X9* are polymorphic STR markers present on the X chromosome only. *XY2* and *XY3* are other polymorphic STR markers on both X and Y chromosomes.

Our QF-PCR analysis shows 2 peaks for *AMEL* and *T3* markers and a single peak for *X1*. Figure (2) In another case, two peaks for *ZFYX* and a single peak for the *SRY* marker can be observed. Figure (3)

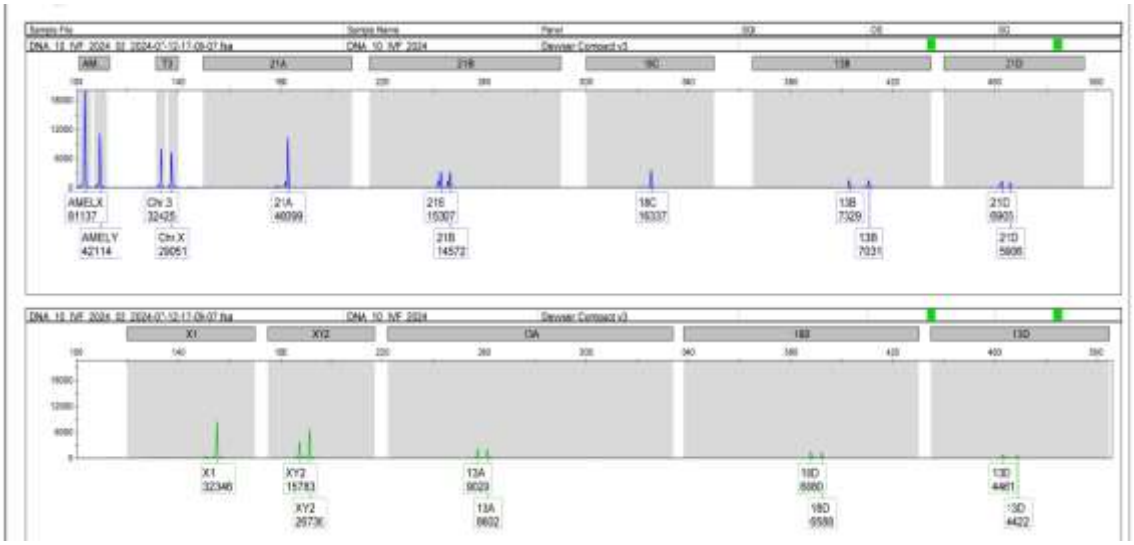

**Figure 2: Electrophoretogram for *AMEL, T3* and *X1* markers.**

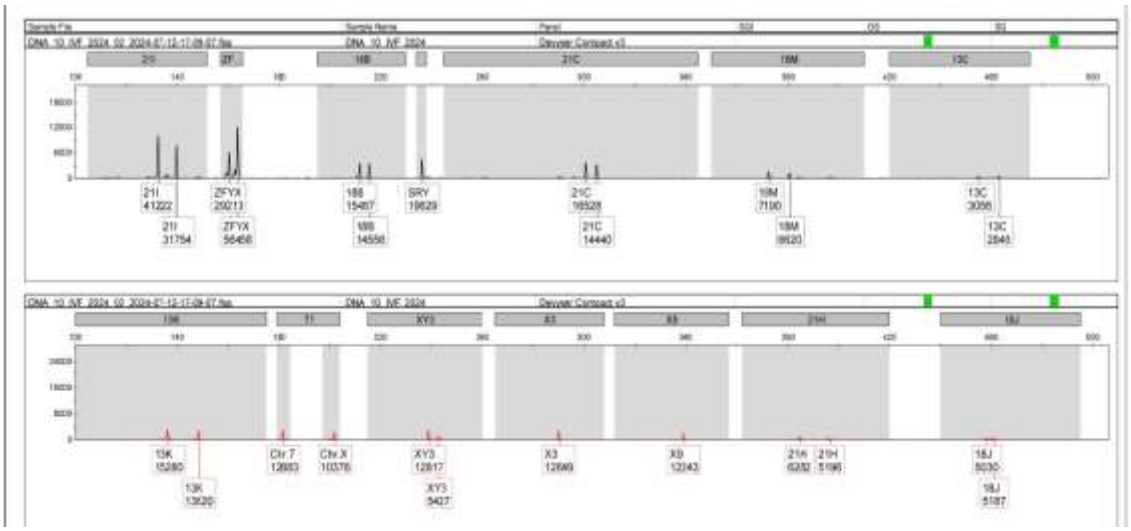

**Figure 3: Electrophoretogram for *ZFYX* and *SRY* markers.**

A study was conducted using 14 plex QF-PCR to detect the absence of the sex-determining region of the Y chromosome (SRY) gene in a 33-year-old infertile male. 14 markers *[AMEL, T3, sY84, sY86, sY127, sY134, sY254, sY255, sY1191, sY1192, sY1291, SRY, CDY and DAZ]* have been used for the analysis. *AMEL* and *T3* are non-polymorphic markers. *AMEL* is present on both X and Y chromosomes, whereas *T3* is an X chromosome counting marker present only on the X chromosome. The Y chromosome-specific markers *sY84, sY86, sY127, sY134, sY254, sY255, sY1191, sY1192, sY1291* and *SRY* are non-polymorphic STR markers therefore they are expected to give a single peak for male genotype who has a single Y chromosome. However, the electrophoretogram demonstrated an area of 2:2 ratio for the T3 peak, consistent with normal female genotype and no detectable peaks for all the Y chromosome-specific and *CDY* & *DAZ* markers. This indicates the presence of 2 X chromosomes and the complete absence of a Y chromosome leading to a rare condition known as SRY negative 46 XX male syndrome.[47]

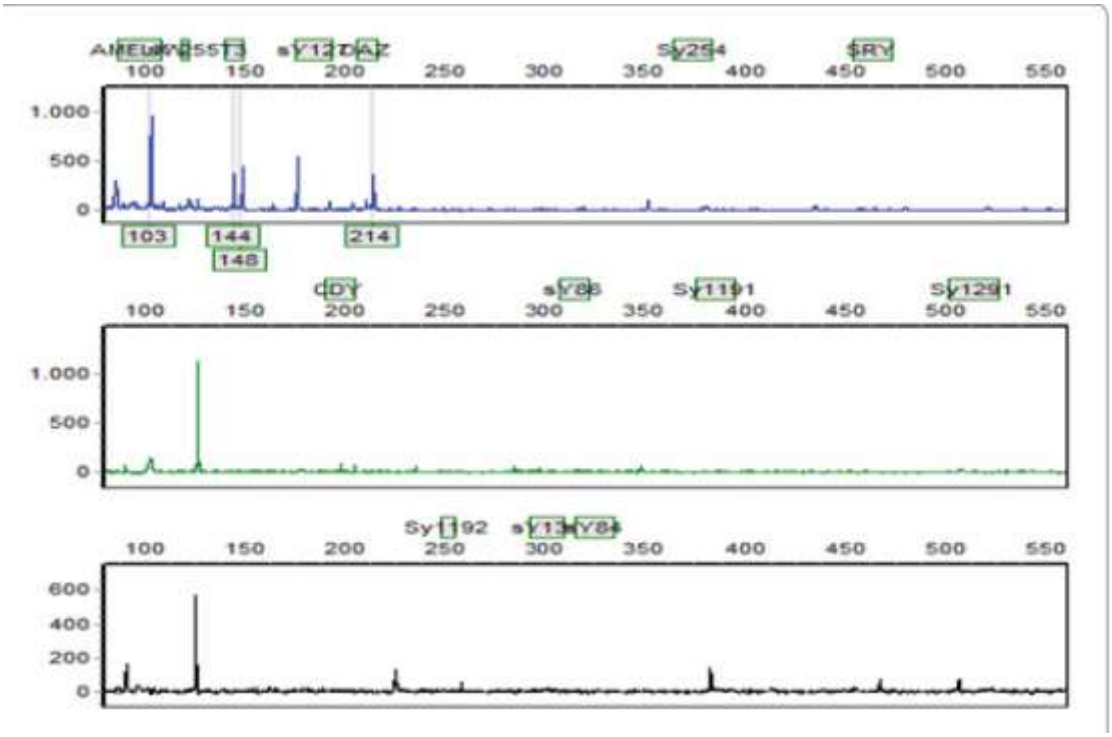


**Figure 4: Electrophoretogram of the 14-plex QF-PCR showing the SRYnegative 46, XX male.[47]**

### 4. Sequence-tagged site polymerase chain reaction (STS-PCR)

Since Y chromosome microdeletions (YCMDs) are too small to be detected with karyotyping, polymerase chain reaction (PCR) amplification must be used. As per the guidelines from the European Academy of Andrology (EAA) and the European Molecular

Genetics Quality Network (EMQN), the PCR reaction should use sequence-tagged sites (STS) primers that target non-polymorphic regions of the Y chromosome commonly deleted in oligospermic and azoospermic men. To enhance the sensitivity and specificity of the analysis, two STS loci should be amplified in each AZF region. The EAA/EMQN guidelines recommend the following set of primers for detecting microdeletions in the region of Y-chromosome sY84 & sY86 for the AZFa region; sY127& sY134 for the AZFb region; sY254 & sY255 for the AZFc region.[20]

Ideally, the reactions should be conducted in a multiplex format that includes appropriate positive (SRY) and negative (ZFX/ZFY X-chromosome genes) internal controls. Additionally, a female sample and a blank sample (substituting DNA with water) should be run simultaneously to check for DNA contamination. This combination of primers and quality control methods has consistently produced accurate results. However, repetitive testing may be required in cases of inconclusive outcomes or suspected technical issues. Adhering to these guidelines, standardising laboratory procedures, and undergoing annual external quality assessments (EQA/EMQN) have reduced diagnostic errors from about 8% to 1–2% (as of 2014).[20] [16]

**Interpretation of results**

The interpretation involves checking if specific regions of the Y chromosome are present or absent. If the PCR products for the sequence-tagged sites (STS) are there, it means those regions are intact. If they're missing, it suggests deletions in those regions. Internal controls play a crucial role. The SRY gene, a positive control, ensures that the PCR reaction is functioning correctly. On the other hand, the ZFX/ZFY genes, which serve as negative controls, help verify the DNA sample's quality and confirm that the reaction conditions are appropriate.

Additionally, female and blank controls are used. The female control should only show amplification for the ZFX/ZFY genes, indicating no contamination with Y chromosome DNA. The blank control should show no amplification at all, ensuring there's no DNA contamination in the reagents or equipment.[48]

**5. Multiplex ligation probe-dependent amplification (MLPA)**

STS-PCR design is limited to identifying only specific Y-chromosome deletion it cannot identify other CNVs and duplications that interrupt spermatogenesis. Hence, multiplex

ligation probe-dependent amplification (MLPA) is a superior alternative that offers a broader spectrum of diagnoses. The assay utilises 53 multiple oligonucleotide probes 12 were autosomal chromosomes specific (for internal control reaction), and 43 were Y-chromosome AZF region-specific probes (16 probes for AZFa, 15 probes for AZFb, and 12 probes for AZFc regions). A total of 84 men, aged 18 to 50 were examined out of which 43 were suffering either from AZ or OL and 41 were fertile controls. Three patients were detected with the same AZFc duplication located at BPY2, DAZ, and CDY1B genes. One patient was detected with duplication in the SRY and AZFa regions, deletion in AZFb and complete deletion in the AZFc region. The peaks obtained compared with control results were considered abnormal if the relative peak-height ratio was less than 0.75 (deletion) or greater than 1.25 (duplication).[38]

### 6. Genomic microarray analysis

With more understanding of genetic disorders, it is necessary to analyse DNA, mRNA, proteins, and other biological compounds.[39] The principle behind the microarray technique is nucleic acid hybridisation. The “DNA chips" contain fluorescently labelled probes complementary to the target DNA sequence. The target DNA binds to probes through complementary base pairing, and unbound fragments are washed away.

For obtaining the cDNA, mRNA acts as an intermediary molecule that carries the genetic information from the nucleus to the cytoplasm for protein synthesis. So, through the mRNA, the organism's DNA can be easily accessed. Because of the unstable nature of mRNA, it is important to convert mRNA to cDNA. The cDNAs are labelled by fluorescent markers Cy3 (Green) and Cy5 (Red).[40]

**Microarray can be used in the following ways**

a) Gene expression analysis: To check expressions of genes responsible for sperm infertility. Globozoospermia was found where there was a mutation in a gene called *DPY19L2* using a 250 k SNP array.[13]

b) Genotyping: For detecting Single-Nucleotide Polymorphism. The point mutations in the nitrogenous bases of the DNA (A, T, G or C) can be detected through allelic discrimination i.e. by differentiating between two or more alleles of a gene at a specific genetic locus.[52]

c) For observing DNA mutations: Microarrays are being used to check which gene is responsible for genetic disorders.

d) To study genomic gains and losses: To identify duplication & deletion associated with any chromosome in the genome.

### 6.1. Comparative genomic hybridization

Comparative genomic hybridization is a technique used to detect fluorescently labelled DNA to study copy number variations (CNVs). Like in microarray, fluorescently labelled DNA is used to bind the target DNA to determine the quantity of complementary DNA between subjects by visualising the colour of each probe, and three subjects by visualising the colour of each probe.[13]

### 6.2. Microarray for detection of male infertility

A study detected a total of 227 CNVs in the patient group even after the elimination of CNVs present in the control group or CNVs that are not linked to male infertility. After that 11 regions were closely investigated and an expression pattern of seven genes was found, for which if in one area, one gene was not expressed in the testis, then that region was eliminated. After these 10 promising regions were closely studied and quantitative PCR was carried out under controlled populations to check for the genes affected by male infertility.[53]

Another study assessed twenty-one DNA cases to look into CNV involving Y-chromosomes. All cases included *AZF* deletions: *seven AZFb/c deletions, six AZFa/b/c deletions, four AZFc deletions, two AZFb deletions, one AZFa deletion, and one AZFa/b deletion.* Out of the 21 cases, 20 cases were cordial cases showing AZF deletion and the remaining 11 cordial cases showed additional sex chromosome CNVs detected by chromosomal microarray and confirmed by multimix PCR.[54]

A paper was published where a microarray analysis was used to check the microRNA expression pattern in the semen of infertile men. 86 infertile men [B] and 86 fertile men [H] as control were used to conduct the microarray. The miRNA expression patterns were compared between infertile males [B] and fertile males [H]. The miRNA contained 2844 oligonucleotide probes (1823 human, 648 mouse and 373 rat) complementary to the mammalian miRNA. It was found out 51 miRNAs were differentially expressed between

infertile and fertile males, 21 miRNAs were overexpressed significantly and 31 miRNAs were underexpressed significantly.[55]

### 7. Sanger and Customised Next-Generation Sequencing

According to the Online Mendelian Inheritance in Man (OMIM) database more than 200 genetic disorders are related to male infertility. Up to 2300 genes are involved in human spermatogenesis so it becomes essential to identify the responsible ones for male infertility.[56]

Patel et al conducted a retrospective study which stated that the main reasons behind male infertility are chromosomal alterations, inversions, translocations, YCMDs, and gene mutations (for example single-nucleotide variants (SNVs) in the *CFTR* gene). Previously, the *CFTR* IVS8-5T mutation, YCMDs and sex chromosome aneuploidy were detected by running multiplex PCR, PCR of sequence-tagged sites, and traditional karyotyping or microarray analysis, respectively. With the help of NGS, we can identify all of them on a single platform with exquisite sensitivity and specificity. NGS along with bioinformatics algorithms can analyze gene mutations, alterations and multiple variants simultaneously associated with male infertility.

A male gene panel consisting of YCMDs, CFTR mutations, and sex chromosome aneuploidies is made. The workflow then sequenced DNA samples using NGS technology, YCMDs, sex chromosome aneuploidies, CFTR IVS8-5T polymorphism, indels, and SNVs are called which are further annotated by domain specialists. In case of any differences between the received and expected NGS result orthogonal analysis is also performed. To ensure the accuracy of clinical sensitivity, YCMDs samples with known variants were checked and NGS could detect 15/16 YCMDs correctly.

a) YCMDs of smaller size were found in three cases (NA20435, NA18333, and NA22031).
b) NGS result of one case (NA20434) located a microdeletion of 1.17Mbp downstream of the previously known location.
c) NA12662 were detected with X chromosome duplication i.e. sex chromosome aneuploidy but it missed Y chromosome microdeletion.

The sensitivity for detecting YCMD and sex chromosome aneuploidies is 93.75% and 100% respectively. NGS results were compared with previously known data and microanalysis was performed as a confirmatory test.[57]

Another customised gene panel consisting of a total of 175 genes correlating with male infertility was described by Online Mendelian Inheritance in Man (OMIM), GeneReviews, and primary literature. 110 genes were categorised as diagnostic (variations in these genes have a direct relationship with infertility), and 65 genes were categorised as pre-diagnostic/ informative (variants are associated with infertility but a casualty link has not been established yet). The gene panel generated an average sequencing depth of 359X, however, 98% of the target region sequence has a sequencing depth of at least 25X.

Percone et al. showed that infertile subjects who were found negative against the NGS diagnostic gene panel but are clinically suspects for primary defects of spermatogenesis were screened against the NGS pre-diagnostic gene panel afterwards and detected with the variants of informative genes, almost half of these variants belong to the category of cytoplasmic dynein genes. Genetic variants detected in 12 of the 65 pre-diagnostic genes analysed [DNAH11, DNAH10, DNAH5, DNAI1, CCDC40, CFTR, GALNTL5, AMELY, KLK4, KLK14, CATSPER2, and ADCY10].

The CATSPER2 gene sequence when evaluated by MutationTaster detected a variant c.842+1G>C in the CATSPER2 gene that disturbs the splice site which in turn causes the activation of the cryptic splice site which results in the introduction of a premature stop codon that is considered disease-causing.[56]

In a study, researchers examined 38 individuals with CBAVD and used advanced genetic sequencing to detect changes in the *CFTR* and *ADGRG2* genes. Out of 38, they found that 27 patients had at least one likely pathogenic or pathogenic mutation in *CFTR* or *ADGRG2*. Besides the well-known IVS9-5T variant, they identified 15 mutations in the *CFTR* gene and one in the *ADGRG2* gene, four of which were previously unknown.

5 T allele is known to be a common mutation in patients with CBAVD. A study on Chinese men with CAVD has shown that this specific allele is particularly frequent, occurring in 25% of cases.[28]

Another study carried out Whole-exome sequencing and Sanger sequencing and identified a new mutation (c.G118T: p.Glu40*) in *ADGRG2* in two patients diagnosed with CBAVD. Moreover, further analysis showed that because of this mutation, the gene lost its ability to

express and produce *ADGRG2* proteins that could otherwise be seen in healthy individuals.[58]

Custom-made NGS gene panel demonstrates significant potential for improving the genetic diagnosis and clinical management of male infertility. The efficacy of NGS will able to help in identifying the aetiology behind male infertility and conducting meta-analysis. These kinds of customized gene panels can be used to identify genes that are directly and indirectly linked to male infertility.

**Genetic counselling**

The genetic basis of infertility remains a complex and evolving field as the genes involved are being discovered constantly. Genetic counselling provides crucial information about the potential genetic cause of infertility and possible treatment options available. This makes patients understand and adapt to the genetic aetiology of the condition. Genetic counselling includes interpreting 3 generation pedigree analysis for birth defects, miscarriages and genetic conditions to estimate the occurrence and chance of inheriting the condition to the future generation. Genetic counselling also provides us with management, prevention and options available for addressing genetic conditions. Generally, couples/males suffering from infertility opt for Assisted reproductive technology (ART) procedures through Intracytoplasmic sperm injection (ICSI). These techniques may lead to a successful birth but also have some risks associated such as what if the underlying genetic cause of infertility is inherited to the progeny. It may result in the birth of offspring who are infertile (same genetic condition as parents) because the underlying genetic cause of infertility has not been treated. Another risk could be that the ICSI may cause damage to sperm DNA or the egg. That is why genetic counselling becomes crucial to help provide all the information including risks, disadvantages, transmitting genetic disorders to offspring if any and reproductive options available. Hence, genetic counselling is all about informed choices and psychosocial support.[59,60]

## CONCLUSION

For male infertility cases that are caused due to underlying genetic variations, genetic testing in the initial stages becomes crucial for early and accurate detection of the disorder. As more genetic diagnostic techniques are employed, a wider range of the still unknown genetic mutations responsible for male infertility and specific sperm production defects could be discovered. This will not only improve our understanding of the diseases but will also pave

the way for better treatment options, allowing for a more targeted therapeutic approach. It will also contribute to the further development of diagnostic techniques that will facilitate the early and accurate detection of male infertility in the future. Ultimately, genetic counselling is the cornerstone of healthcare promoting well-being and preventing genetic diseases.

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