Detection of Microdeletions in the AZF Region of the Y-chromosome in Azospermic Palestinian Males

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Detection of AZF Microdeletions on the Y-chromosome in Azospermic Palestinian Males

الكشف عن طفرات (AZF)على كرموسوم Y عند الرجال الفلسطينين العديمي الحيوانات المنوية

By

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Abstract

Infertility is a global concern that affects approximately 10-15% of couples. Over 30 million males worldwide are infertile—up to 30% of which are azospermic. Y chromosome microdeletions are one of the most common genetic causes of non-obstructive azospermia. The aim of this study was to screen a group of infertile males from the West Bank of Palestine to identify the frequency of Y microdeletions and assess the significance of this testing for intracytoplasmic sperm injection candidates. DNA extracted from blood samples of 41 azospermic males, 50 normospermic males and 2 females were analyzed by PCR to detect 17 sequence tagged sites (STS) along the long arm of the Y chromosome. The primers were specific to the Azospermia Factor (AZF) regions, AZFa, AZFb and AZFc, where a missing STS indicated a microdeletion. The results showed that 4/41 cases (~9.8%) were deleted in seven different AZFb and AZFc locations, whereas no microdeletions were found in any of the fertile control samples. Results indicated that the frequency in the Palestinian population is similar to that of other world populations and suggests the need for AZF screening in Palestine, in particular for azospermic males undergoing testicular sperm retrieval for assisted reproduction.

Keywords: AZF, Azospermia, microdeletion, Y chromosome

Declaration of Authorship

I declare that this Master's Thesis is my original, independent work and that all literature, images and ideas adapted and included in the text have been rightfully acknowledged. I hereby certify that all research contained in this document is my own, submitted in partial fulfillment of the requirements for the master degree in clinical laboratory science at Birzeit University. This thesis has not been submitted to any other institution for examination, nor was it previously published.

Electronic signature: Shaden Taha

Date: September 2016

Dedication

To my wonderful parents, my little sisters and my darling husband

For all the love, patience, support and inspiration

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Abbreviations

AZF Azospermia Factor

FISH Fluorescent In Situ Hybridization

FSH Follicle Stimulating Hormone

ICMART International Committee for Monitoring Assisted Reproductive Technology

ICSI Intracytoplasmic Sperm Injection

IVF In-Vitro Fertilization

LH Luteinizing Hormone

MSY Male-specific Region of Y chromosome

NOA Non-obstructive Azospermia

PAR Pseudoautosomal Region

PCR Polymerase Chain Reaction

SCOS Sertoli Cell Only Syndrome

STS Sequence Tagged Site

TEFNA Testicular Fine Needle Aspiration

TESE Testicular Sperm Extraction

UMI Unexplained Male Infertility

WHO World Health Organization

Yq Long arm of Y chromosome

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INTRODUCTION

Infertility is a global health concern that the World Health Organization (WHO) and International Committee for Monitoring Assisted Reproductive Technology (ICMART) describe as the "failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse" (Zegers-Hochschild, et al., 2009). As of 2010, approximately 48.5 million couples worldwide were unable to naturally have a child after trying for five years (Mascarenhas, Flaxman, Boerma, Vanderpoel, & Stevens, 2012). Presently, infertility is estimated to affect 10-15% of couples from all regions of the world (Rachid, Lamia, & Amine, 2015) (Agarwal, Mulgund, Hamada, & Chyatte, 2015). Infertility may be due to the male or female partner alone or the contribution from both. Large-scale studies generally cite that 50% of cases are due solely to female infertility, 20-30% male factor-only and 20-30% is due to both (Sharlip, et al., 2002). A meta-analysis review conducted by Agarwal et. al, found that the male-factor actually contributes to a large distribution of 20-70% of all infertility cases, which is significantly wider than previously noted (Agarwal, Mulgund, Hamada, & Chyatte, 2015). However, this very broad interval does not accurately represent all regions of the world, the possible reasons for which are discussed below.

The generation of accurate statistics has been a challenge for researchers due to a few problematic limitations such as bias, inconsistencies in data collection and cultural restrictions. Particularly in the patriarchal populations of the Middle East and Africa, the recognition of a man as infertile is perceived as an insult to his masculinity and social status. For this reason, it is very common for male partners to refuse fertility evaluation or withhold critical information from research questionnaires. In such regions, data on the male-factor is often deduced from existing female factor prevalence (Agarwal, Mulgund, Hamada, & Chyatte, 2015). This leads to

underreported male-factor infertility rates and consequently increased variability between populations. On the other hand, higher rates may be reported when exclusion criteria of cases are too specific and samples are subjected to selection bias. Geographic distribution and environmental health also play an important role in the varying frequencies of male infertility. Standardized, region specific studies of male infertility are needed to provide accurate prevalence on a global scale.

Reduced male fertility is caused by acquired or congenital abnormalities affecting sperm quality, semen transport, hormone regulation or unknown factors. Infertility may be caused by Klinefelter's syndrome, mutations of the Y-chromosome, obstruction in the reproductive tract, cryptorchidism, chemotherapy, testicular tumors and endocrinopathies (Dohle, et al., 2005) (Nieschlag, 2000). Other factors that impair fertility range from malnutrition and lifestyle to chronic disease and exposure to toxic chemicals (Hamada, Esteves, & Agarwal, 2011). Infertility of unknown origins may be referred to as idiopathic or unexplained. "Unexplained male infertility" (UMI) is infertility marked by normal semen and medical historyvafter the female factor has been ruled out (Dohle, et al., 2005) . Idiopathic infertility presents when semen analyses show decreased sperm concentrations but hormone profiles, biopsies and physical exams cannot lead to a diagnosis. This occurs in 30-40% of male-factor cases (Jungwirth, et al., 2014). A significant portion of idiopathic cases may be explained by genetic factors alone or with the contribution of environmental factors, that act upon testicular development or spermatogenesis (Hamada, Esteves, & Agarwal, 2011) (Krausz, 2008) (Sharp & Irvine, 2004). The resulting qualitative and quantitative reduction of sperm leads to conditions such as teratozoospermia (abnormally shaped sperm) and azospermia (the complete absence of sperm in

the ejaculate). This study focuses on idiopathic cases of non-obstructive azospermia (NOA), in which no sperm is found in the ejaculate and almost always absent from the testes.

The most common genetic causes of male infertility due to azospermia are chromosomal aberrations such as Klinefelter's syndrome (males with an extra chromosome: 47, XXY) and variations of this disorder. Chromosomal aberrations are characterized by numerical disorders (extra or missing chromosomes) or structural disorders, where fragments of a chromosome(s) are missing, duplicated or rearranged. The second most common genetic cause of azospermia due to testicular failure is Y-microdeletions, which are caused by recombination events that remove essential spermatogenesis genes from the Y chromosome (Cocuzza, Alvarenga, & Pagani, 2013) (Krausz, Hoefsloot, Simoni, & Tuttelmann, 2014). Microdeletions are relatively small deletions that remove up to 5 Mb of DNA. The terms microdeletion and deletion are used interchangeably as most Y chromosome deletions typically span up to 7 Mb or less. Mutations that cause a deletion of more than 5 Mb are termed large microdeletions. Both chromosomal anomalies and gene mutations affecting the Y-chromosome may cause a disturbance in sperm production and testicular development. The phenotypic effects of disrupted spermatogenesis range from males with mild oligospermia (sperm concentration of 15 million sperm/mL or less) to males with severe azospermia (World Health Organization, 2010).

The male-specific region of the Y-chromosome (MSY) contains a discrete region termed Azospermia Factor (AZF). This region contains numerous spermatogenesis genes and non-coding sequences, subdivided into AZFa, AZFb and AZFc (Skaletsky, et al., 2003). A fourth region, AZFd, was proposed by Kent-First, et. al in 1999, however the complete sequencing of MSY in 2003, and the understanding of microdeletion mechanisms has demonstrated that the region does not exist (Simoni, Bakker, & Krausz, 2004) (Skaletsky, et al., 2003) (Kent-First, et

al., 1999). Classic or complete deletions occur when one entire AZF sub region, or a combination of whole regions is removed; this type of deletion is the most clinically important and causes varying degrees of non-obstructive azospermia and oligospermia (Atia, Abbas, & Ahmed, 2015) (Suganthi, Vijesh, Jayachandran, & Benazir, 2013). Partial deletions, in the context of Y-chromosome infertility, only remove portions of one or more AZF regions. AZF microdeletions are screened for by using PCR and sequence tagged sites (STS): short DNA segments of known locations, which have been mapped along the long arm (Yq) of the Y chromosome (Krausz, 2008). STS that fail to be amplified indicate a deletion at that locus. Screening for Y microdeletions provides essential information for advising azospermic males about the likelihood of sperm retrieval (Krausz, Hoefsloot, Simoni, & Tuttelmann, 2014). Sperm that is extracted from azospermic males often shows low motility and abnormal morphology, however it is unknown what causes the abnormality (Silber & Disteche, 2012). Complete deletions of AZFa, AZFb or AZFb+c regions imply that the chance of sperm retrieval for in vitro fertilization (IVF) is virtually zero (Kleiman, et al., 2011) (Hopps, et al., 2003) (Brandell, et al., 1998). On the other hand, deletions of AZFc alone present a variable clinical picture ranging from oligospermia to azospermia and therefore show varied rates of successful assisted reproduction. In AZFc-deleted azospermic males, the chance for sperm retrieval is approximately 50%, though all sons will inherit the deletion and may develop a more severe phenotype (Van Golde, et al., 2001). The detection of Y-microdeletions is therefore significant not only for diagnostic use, but also for providing prognosis and genetic counseling.

Problem Statement and Objectives

Since the first IVF center was established in Palestine in 1995, numerous others became available in all regions of the West Bank and Gaza. Most of these clinics have the same

principles and diagnostic practices for assessing male infertility, which include two or more semen analyses, physical examination and hormone profiles. Additional testing may include ultrasonography, testicular fine-needle aspiration and testicular sperm extraction. However, molecular and cytogenetic analysis is not requested in the typical lab workup. This may be due to the fact that reliable genetic labs are lacking, testing can be time-consuming and expensive, or that doctors do not find such tests necessary. The incidence of Klinefelter's syndrome and other chromosomal anomalies was found to be highly significant (37.8%) in azospermic Palestinian males, affirming the need for cytogenetic testing (Qumsiyeh, Borqan, & Obeid, 2014). No complete Y-microdeletions were previously detected in the West Bank, although partial deletions were found at a frequency of 5.6% in a study of azospermic and oligospermic Palestinian males in Gaza (Qumsiyeh, Borqan, & Obeid, 2014) (Shaqalaih, 2007). When it comes to the investigation of idiopathic male infertility or providing an accurate prognosis for ICSI, genetic tests and counseling should be included. A distinction of the present study from others in Palestine is that only azospermic males will be examined. This study aims to assess the frequency of AZF deletions and weigh its significance in the assessment of infertility in Palestinian males.

Specific Objectives

- To screen for and establish the frequency of complete and partial Y-chromosome microdeletions in infertile, azospermic Palestinian males.
- To compare the frequency of AZF deletions in Palestine with that of other populations.
- To assess the significance of microdeletion analysis in relation to assisted reproduction
- To provide essential information for genetic counseling before ICSI and add prognostic value to evaluating offspring of azospermic men

LITERATURE REVIEW

Infertility

Infertility is the inability of a couple to reach a clinical pregnancy within one year of trying by regular intercourse (American Society for Reproductive Medicine, 2014). It is categorized as primary or secondary, based on a couple's history of conception. Couples with primary infertility have never conceived, whereas those with secondary infertility were previously fertile with at least one child or abortion, but are unable to conceive again by natural means (Mascarenhas, Flaxman, Boerma, Vanderpoel, & Stevens, 2012). Male infertility affects at least 30 million males worldwide, 30% of which are azospermic (Esteves & Agarwal, 2013); the highest infertility rate, where the male factor is a sole or contributory factor, is 60-70% in the Middle East (Agarwal, Mulgund, Hamada, & Chyatte, 2015).

Infertility in Palestine

Literature on male infertility in Palestine is currently lacking. Few male-factor infertility studies are available, however most research has been focused on female infertility. A prospective cohort study conducted in villages near Hebron, Palestine found that 13.4% of newly married couples were not able to conceive after one year of trying; overall fecundability, the capacity to reproduce or the probability that a single monthly cycle results in pregnancy, was found to be 0.17 which is considered low in comparison to the range of Western, developed countries (.20 or more) (Issa, Sallmen, Nijem, Bjertness, & Kristensen, 2010). A retrospective study of 627 infertile Palestinian males' examined medical records taken from an IVF center to identify etiologies; results confirmed that for those couples where male infertility was involved, the most common diagnosis was idiopathic infertility (AbuAl-Haija, 2011). Such findings indicate the need for genetic and environmental studies of male infertility in Palestine to find the underlying

causes of idiopathic cases. This is necessary in order to reach more conclusive diagnoses and develop treatment or prevention plans. The significance of genetic testing was reinforced by cytogenetic studies that have shown genetic factors play a major role in idiopathic male cases. In the southern Palestinian population, Klinefelter's syndrome and other chromosomal aberrations were found at a frequency of 37.8% (Qumsiyeh, Borqan, & Obeid, 2014). The first incidence of a 48,XXXY azospermic male in Palestine was also found in the same study. Y-chromosome microdeletions were found in 7/125 (5.6%) randomly selected, infertile males from Gaza (Shaqalaih, 2007). The deletions were partial rather than completely deleted AZF regions, but were not found to be statistically different than controls (4.8%). This indicated that partial deletions may only be risk factors or that this region's Y-haplogroup (a group of inherited population-specific alleles on the Y-chromosome) may have a protective effect against severe microdeletions. A large proportion of Palestinians in Israel and the West Bank were found to be in Y-haplotype J—one which may be less vulnerable to complete AZF-microdeletions (Nebel, et al., 2001).

Etiology

Infertility results from a cessation, interruption or disorder of the reproductive system (ASRM, 2008). Causes may be related to problems with spermatogenesis, blockage of sperm transport, hormonal abnormalities or erection and ejaculation problems. Some studies suggest that reactive oxygen species, sperm antibodies and various environmental substances may also play a role in causing male infertility (Cui, et al., 2015) (Ko, Sabanegh, & Agarwal, 2014) (Oliva, Spira, & Multigner, 2001). In cases of non-obstructive azospermia due to Y-microdeletions, the problem lies in spermatogenesis disruption, arrest or decreased production of sperm. Specific diagnosis is critical with non-obstructive azospermic males, because the success rate of ART may be virtually

zero in some cases. The histological picture and sperm maturation processes must be understood before attempting assisted reproduction or potential therapy development.

Spermatogenesis Mechanism

Spermatogenesis is the process by which complex populations of germ cells develop and mature into spermatozoa (Holstein, Schulze, & Davidoff, 2003) . It begins when a male reaches puberty. Three distinct stages are involved: spermatogoniogenesis, spermatocyte maturation, and spermiogenesis. Fig. 1 shows all the stages and resulting cells. Spermatogenesis takes place in the testes' convoluted seminiferous tubules, within tight junctions between nursing cells of Sertoli. These cells are somatic, epithelial cells that provide complete nourishment to developing germ cells. Sertoli cells have numerous roles and functions in spermatogenesis including the production of hormone-stimulating proteins, formation of the blood-testes barrier, phagocytosis of excess cytoplasm and the secretion of various substances necessary to germ cell growth.

Diploid spermatogonia of three types: pale type-A, dark type-A, and type B continuously multiply by mitosis. Type-A cells make up the stem cell pool, whereas type-B cells include intermediate cells that develop into spermatids. Type-A spermatogonia divide mitotically to create type-B spermatogonia and simultaneously maintain the type-A cell pool that restores the germinal cell supply. Type-B spermatogonia undergo clonal expansion, to create primary spermatocytes. The stem cell pool and primary spermatocytes are formed within 16 days. Spermatocyte maturation occurs when a newly formed primary spermatocyte enters meiosis I, which is manifested by chromatin configuration changes that yield two secondary spermatocytes. This process takes 24 days to complete, due to the prolonged DNA reduplication and recombination steps required.

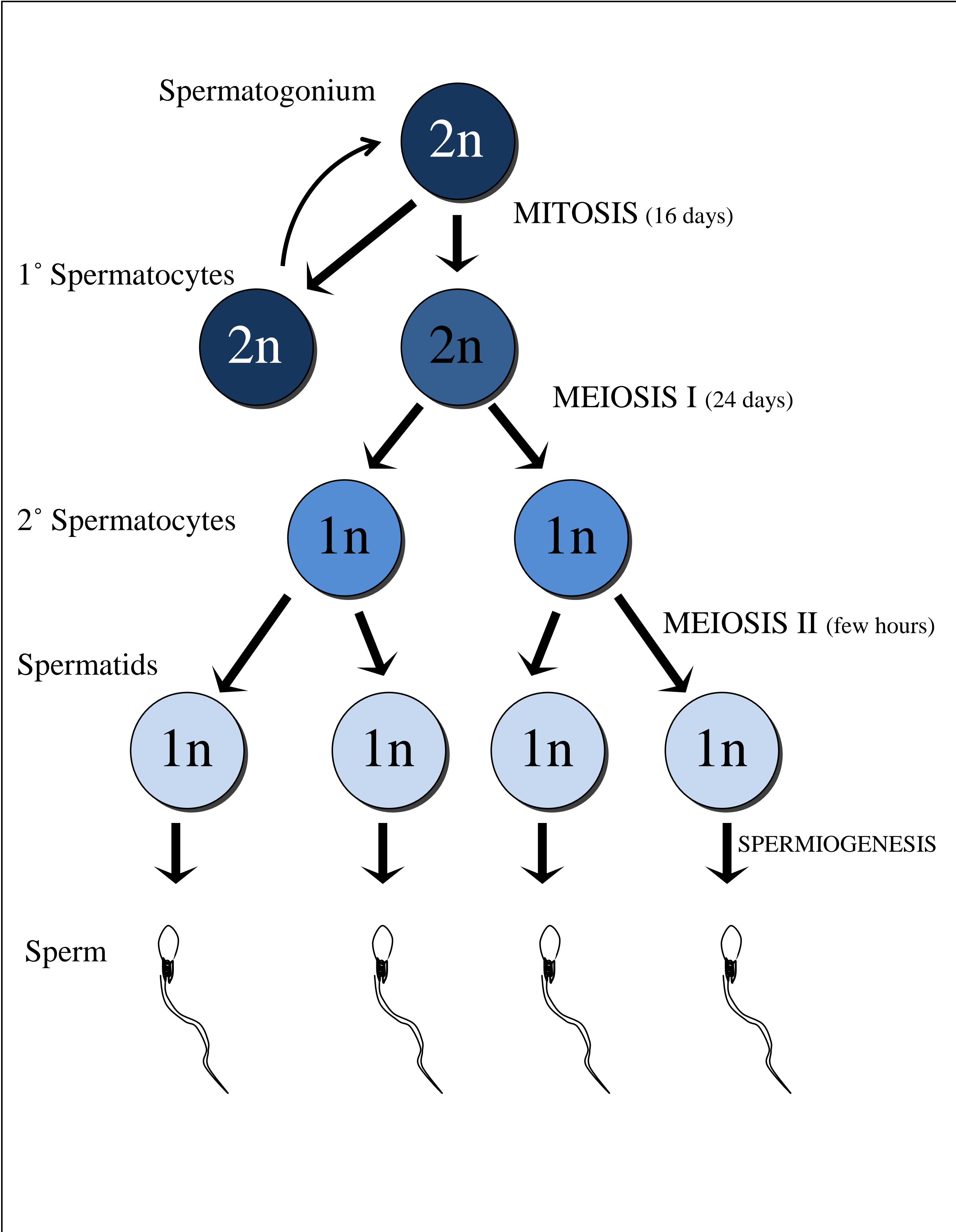


Figure 1: Stages of spermatogenesis, showing cells and chromosome number of each stage.

Secondary cells then undergo meiosis II to create spermatids within five hours only, since there is no complex DNA rearrangement in this step. Each secondary spermatocyte yields two haploid spermatids, which contain half of the original genetic material. Up until this stage, developing cells are radially symmetric.

The final phase of sperm production is spermiogenesis, which lasts approximately 24 days and initiates when spermatids begin to develop polarity in four stages, the Golgi phase, acrosome phase, tail formation, and the maturation phase. During these stages, spermatid DNA is packaged tightly to condense the nucleus for better mobility of the sperm. The Golgi apparatus then surrounds the nucleus and produces enzymes, to form the acrosomal cap around the head of the sperm (acrosomes contain enzymes essential for egg penetration). The tail is formed by elongation of a single centriole, while the midpiece forms as mitochondria wrap in a spiral form around the core of the tail. Fig. 2 shows a diagram of a complete, mature sperm cell. Once the structures are completely developed, excess cytoplasm is phagocytosed by surrounding Sertoli cells, resulting in mature but non-motile sperm. Mature sperm are then released from Sertoli cells into the lumen of seminiferous tubules to remove any traces of cytoplasm or residual organelles.

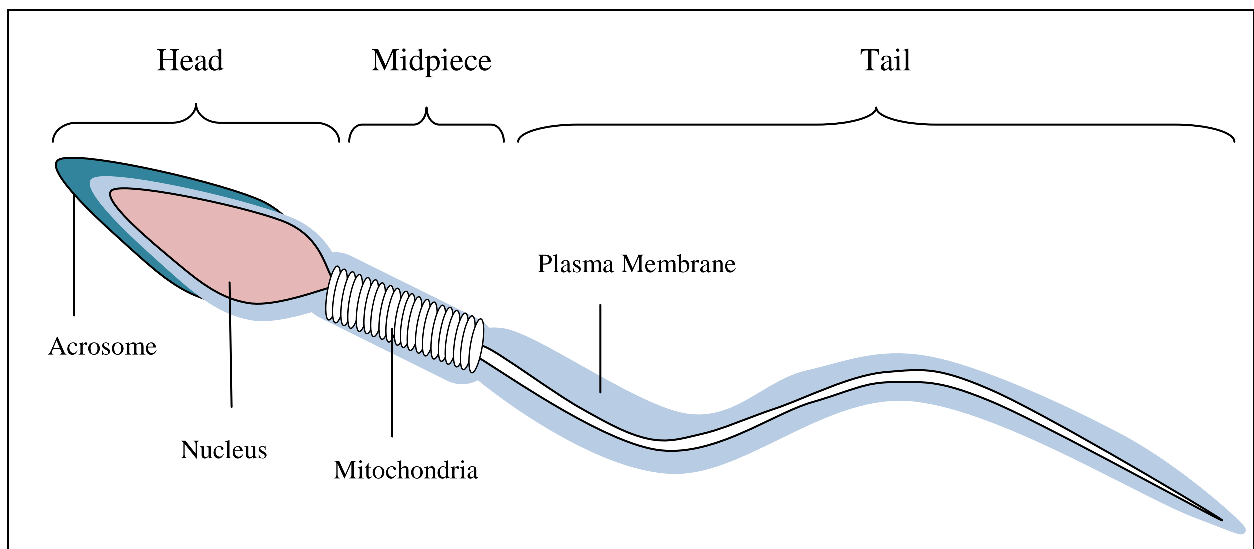


Figure 2: The structures found in a completely functional spermatozoon.

Mature, non-motile sperm are transported to the epididymis by peristalsis, where they gain motility. The complex process of spermatogenesis takes between 64 and 72 days to completely form each functional sperm (Holstein, Schulze, & Davidoff, 2003).

Spermatogenesis Defects: Non-Obstructive Azospermia (NOA)

There are two main causes of NOA: Sertoli cell-only syndrome (SCOS) and maturation arrest (Silber & Disteche, 2012). In cases of maturation arrest, there is a disruption of type-B spermatogonia that leads to an increase in the formation of type-A cells, without any further development or movement along Sertoli cells (Holstein, Schulze, & Davidoff, 2003). Early germ cells fail to develop further than meiosis I and therefore do not mature into functional sperm. Biopsy results of these cases present Sertoli cells and immature spermatogonia in the testes. However in 60% of NOA cases related to maturation arrest, very few spermatocytes are actually able to progress, but they remain in the tubules of the testis and must be retrieved surgically.

As suggested by its name, Sertoli cell-only syndrome is marked by the presence of Sertoli cells, without any germ cells in the seminiferous tubules. This occurs when both A and B-type spermatogonia are absent and no spermatogoniogenesis, the important first step of sperm production, takes place. SCOS may be present at birth or acquired by triggers such as x-radiation (Holstein, Schulze, & Davidoff, 2003). However, similar to maturation arrest cases, about 60% of SCOS cases show very minimal sperm production, though any mature sperm that may form still remain in the seminiferous tubules.

Diagnosis

Semen analysis

According to the WHO updated laboratory manual for semen analyses, samples should be collected into a sterile container after a minimum of two days and maximum of seven days of

abstinence. Semen must be kept at ambient temperature (20-37˚C) and analyzed within one hour of ejaculation. Samples are first macroscopically examined for appearance, liquefaction, viscosity, volume and pH. Microscopic examination determines important factors of sperm count and concentration, motility, vitality, and morphology (World Health Organization, 2010). Samples that are suspected to be azospermic require an additional centrifugation step and must be reexamined microscopically. Results that remain negative require a second semen analysis after 2-3 weeks, to confirm the diagnosis.

Physical exam, medical history and hormone profiles

A complete medical history is required to assess birth defects, exposures, chronic illnesses and medications that may have had a causative effect. Physical examinations of AZF-deleted azospermic males show normal findings, although small testes are often observed (Silber & Disteche, 2012). Hormone profiles include follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone level tests. When it comes to males with non-obstructive azospermia, there has not been a definitive correlation relating high or low levels of these hormones with the diagnosis of Y-microdeletions (Ambulkar, et al., 2014) (Kumar, Dada, Gupta, & Kucheria, 2006). However, a number of studies suggest that LH and FSH levels are abnormally high in azospermic AZF-deleted males (Wang, Zhang, Qi, Zhao, & Xu, 2011) (Zhang, et al., 2013).

Testicular biopsy

In order to confirm non-obstructive azospermia, testicular fine-needle aspiration (TEFNA) of the testes is required and often carried out in combination with testicular sperm extraction (TESE) in case sperm are found. TESE is done by opening the scrotum and removing large sections of testicular tissue from various regions of one or both testes, and examining seminiferous tubules

for sperm. TEFNA is an aspiration procedure in which a needle is placed into various regions of one or both testes, to extract fluid that is then examined for sperm. These procedures will show rare or complete absence of sperm, spermatids, and/or spermatocytes in non-obstructive azospermic males affected by Y-microdeletions (Krausz, Hoefsloot, Simoni, & Tuttelmann, 2014). Repeated TEFNA and TESE procedures are harmful to a male's reproductive health, physical condition, and testosterone regulation, and may cause irreversible damage to the testes. For this reason, surgical techniques to obtain sperm should be avoided if there is a low chance, or after repeat failures to find viable sperm.

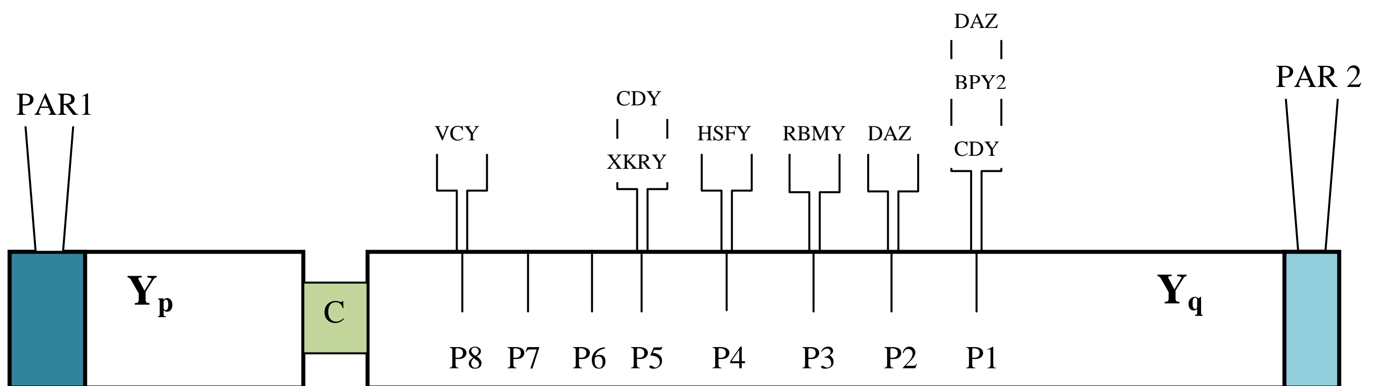
Molecular testing

The only way to definitively detect and diagnose an AZF-deleted male is by molecular genetic testing such as DNA sequencing, PCR using STS markers, or fluorescent in situ hybridization (FISH) techniques (Krausz, Hoefsloot, Simoni, & Tuttelmann, 2014) (Mozdarani & Ghoraeian, 2012). To date, these molecular methods are the only reliable way to test for Y-microdeletions. These techniques will show an absence of necessary functional genes to spermatogenesis in affected males.

Y Chromosome

The Y chromosome spans over 58 million base pairs and contains more than 200 genes, 72 of which are coding genes (Genetics Home Reference, 2016) (Ensembl, 2016). The Y chromosome contains a male-specific region (MSY) and pseudoautosomal regions termed PAR1 and PAR2 (Krausz, Hoefsloot, Simoni, & Tuttelmann, 2014). PAR regions share homology with the X-chromosome and therefore recombine by meiotic exchange, in an autosomal manner. MSY does not undergo recombination with the X chromosome. It consists of heterochromatic (densely

packed DNA) sequences and euchromatic (loosely packed) sequences. Three classes of euchromatin exist: X-transposed (highly identical regions to the X-chromosome), X-degenerate (X-linked homologous genes) and ampliconic sequences (Simoni, Bakker, & Krausz, 2004). The ampliconic sequences of the Y chromosome are composed of highly repetitive sequences that are organized into eight palindromes or inverted repeats, termed P1-8 as shown in Fig. 3 (Krausz, Hoefsloot, Simoni, & Tuttelmann, 2014). These palindromes make up 25% of the Y-chromosome and contain essential spermatogenesis genes. Palindromic sequences are unstable and susceptible to homologous recombination between identical repeats; the formation of hairpin loops in euchromatic regions allows for DNA cleavage, removal of gene(s) and re-annealing of remaining DNA—the mechanism that leads to Y microdeletion infertility (Noordam & Repping, 2006). However, deletions of the heterochromatic region of Yq do not have any effect on genital differentiation and development.



C: centromere
PAR: pseudoautosomal region
P1-8: palindromes
Yp: short arm of Y-chromosome
Yq: long arm of Y-chromosome

Figure 3: Y-Chromosomal map of pseudoautosomal regions and palindromes containing select genes.

Y-microdeletions

In 1976, deletions in the Yq11 location of the Y chromosome were found in 6/1170 azospermic males. These deletions suggested that the Yq11 region contains genes that control spermatogenesis; the locus was accordingly termed azospermia factor (AZF) region (Tiepolo & Zuffardi, 1976). Subsequent studies subgrouped the AZF region into AZFa, AZFb and AZFc regions based on the correspondence between the deleted region and the observed histological spermatogenesis defects (Vogt, et al., 1996). In other words, spermatogenesis disruption was shown at the same phase in all cases that were deleted in the same subregion; this was observed for three different phases in which spermatogenesis was interrupted, suggesting each locus is active at different points in sperm development and that deletions of different AZF regions cause spermatogenic failure at different phases (though all may result with essentially the same phenotype). It has been confirmed and is continuously being studied, that each AZF subregion contains genes that are essential for different parts of the spermatogenesis process (Silber & Disteche, 2012). Following the complete sequencing of MSY, Repping et al clarified a new AZF pattern in which AZFb and AZFc regions slightly overlap (Repping, et al., 2002).

Deletions in the AZF region are specific to varying degrees of spermatogenic failure (Krausz, et al., 2001). Though microdeletions in AZFc have been found in normospermic males, there has not been any report of significant numbers of fertile males with these mutations (Simoni, Tuttelmann, Gromoll, & Nieschlag, 2008) (Krausz, Forti, & McElreavey, 2008). The presence of deletions in males that still fathered children naturally is a reason why it is important to consider AZF deletions as a cause of azoospermia rather than a cause of infertility. The incidence of AZF deletions ranges from 1-55.5% in infertile men worldwide; the wide range may be due to inconsistent exclusion criteria, differences in demographics and varying study designs (Krausz,

Forti, & McElreavey, 2008) (Calogero, Garofalo, & D'Agata, 1999).

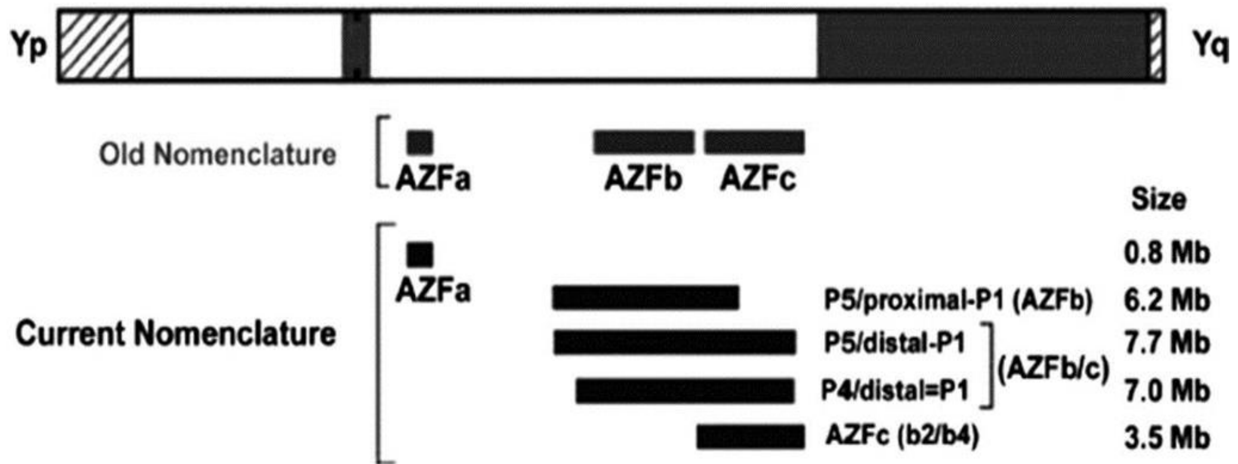


Figure 4: Map of the Y-chromosome and classic (complete) microdeletions of AZF subregions. Image adapted from (Wettasinghe, Jayasekara, & Dissanayake, 2012)**.**

AZFa

The AZFa region spans 1100 kb and contains two single copy genes, USP9Y (DFFRY) and DDX3Y (DBY). DDX3Y is thought to have an important role in ATP binding, RNA binding, hydrolysis and the formation of molecular interactions (OMIM, 2016). Disruption of these processes within the testes may be the cause of spermatogenic failure. USP9Y encodes a ubiquitin-specific protease that may function as a regulator of protein turnover, by removing conjugated ubiquitin in various cell types including the testes, heart and brain (Gene Cards, Human Gene Database, 2016). DDX3Y is expressed specifically in spermatogonia and has testes-specific transcripts, which suggests that this gene plays a more crucial role in spermatogenesis than USP9Y, which is ubiquitously expressed (Foresta, Ferlin, & Moro, 2000). Fig. 4 shows the complete deletion of AZFa, which removes 792 kb (approximately 0.8 Mb),

eliminating both USP9Y and DDX3Y (Krausz, Hoefsloot, Simoni, & Tuttelmann, 2014). This deletion results in azospermia due to Sertoli cell only syndrome (Vogt, et al., 1996). The diagnosis of males with complete AZFa deletions that show SCOS implies that the possibility of successful sperm extraction for ICSI is virtually zero. The frequency of such deletions is rare among all populations (Silber & Disteche, 2012).

Partial deletions of AZFa remove only one gene and may still cause damaging pathology. However, the frequency of these deletions is extremely low and has a very inconsistent impact on spermatogenesis (Kleiman, et al., 2012). Deletions that remove only the DDX3Y gene result in a significant reduction or the complete absence of germ cells (Foresta, Ferlin, & Moro, 2000). In other words, these partial deletions cause mild to severe Sertoli cell only syndrome. Carriers of the USP9Y only deletion have shown phenotypic expressions ranging from azoospermia to normal sperm concentration, indicating that the loss of this gene is not necessarily a cause of hypospermatogenesis and that its function could be considered as a “fine-tuner” (Luddi, et al., 2009). Because of inconsistencies in phenotypic expression and the rarity of partial AZFa microdeletions, screening for the loss of individual genes is not included in routine testing.

AZFb

The complete deletion of AZFb removes 6.2 Mb and all the genes within this region. Complete deletions of AZFb+c remove all of AZFb and a portion or the complete region of AZFc. This combined deletion spans 7 to 7.7 Mb (shown in Fig. 4) and removes up to 32 gene copies and transcription units (Silber & Disteche, 2012) (Simoni, Bakker, & Krausz, 2004). These major deletions are the result of a homologous recombination between palindromes P1 and P5, or P1 and P4. One particularly important candidate gene of AZFb is RBMY1, which is a testes-specific, multi-copy gene that may have a role in RNA processing and a regulatory function in

translation during the early stages of spermatogenesis (OMIM, 2016). Deletions that include the complete loss of AZFb are marked by Sertoli cell only syndrome or spermatogenesis arrest, both of which lead to non-obstructive azospermia (Krausz, Hoefsloot, Simoni, & Tuttelmann, 2014). The frequency of AZFb deletions is 1-2% (Oates, Silber, & Page, 2002) (Repping, et al., 2002). Similar to deletions of AZFa, no sperm is typically found in males carrying AZFb or AZFb+c-deletions during TESE, in which the chance of successful sperm retreival is practically zero (Kleiman, et al., 2011). However in one case, an oligospermic male was found to have a complete AZFb deletion (Soares, et al., 2012); while another case of a severely oligospermic male (less than one million sperm/ml) had a deletion of the entire AZFb+c region with spermatid arrest (Longepied, et al., 2010). This may indicate that such deletions may not be as damaging as originally understood; however these are extremely rare incidences. The uncommon phenotypes may be explained by different Y-haplotypes, differences in the magnitude of deletions or perhaps the presence of a polymorphism at the primer binding site rather than a deletion.

Smaller, partial deletions of AZFb remove the entire palindrome P4, causing a decrease in spermatocyte maturation but not complete arrest; this may be due to the retention of other AZFb genes such as XKRY, HSFY and CDY2 (Kichine, et al., 2012). Partial AZFb and AZFb+c deletions can be transmitted from father to son.

AZFc

Deletions of AZFc region present varying clinical pictures and histological characteristics (Oates, Silber, & Page, 2002). The complete deletion removes 3.5 Mb by homologous recombination between P3 and P1 (seen in Fig.4), and removes 21 copies of genes and transcriptional units (Kuroda-Kawaguchi, et al., 2001). These deletions represent 60-70% of all Y-microdeletions and account for 13% of mutations found in NOA males (Silber & Disteche,

2012). Generally AZFc deletions are associated with trace amounts of spermatogenesis and may thus be found in males with phenotypes of both azospermia and oligospermia (Krausz, Hoefsloot, Simoni, & Tuttelmann, 2014). In rare cases, AZFc microdeletions may be passed on naturally from an oligospermic father to son (Kuhnert, et al., 2004); otherwise these mutations are obligatorily transmitted by ICSI in azospermic men. Deletions that involve AZFb+c occur by two major mechanisms: the homologous recombination between P5 and P1, removing 42 gene copies and 7.7 Mb or between P4 and P1, removing 38 copies that span 7 Mb (Simoni, Bakker, & Krausz, 2004).

The major candidate gene cluster of AZFc is DAZ, which has four copies that code for RNA-binding proteins that play a critical role in the spermatogenic formation of haploid gametes. DAZ gene copies (DAZ1-4) exist as two clusters that are made up of inverted pairs. Another important gene of AZFc is CDY, which encodes proteins that are specifically expressed in mature spermatids and function as histone acetyltransferases (Lahn, et al., 2002).

Males with NOA and AZFc deletions may undergo sperm retrieval procedures TESE or micro-TESE and have an approximate 50% chance of successfully finding sperm for use in ICSI (Lo Giacco, et al., 2014) (Simoni, Tuttelmann, Gromoll, & Nieschlag, 2008). The success rates of these sperm recovery methods are highly dependent upon technique—where microdissection TESE is superior due to minimal testicular tissue excision and more sufficient sperm extraction (Weill Cornell Medical College, 2001) (Hopps, et al., 2003).

Males with partial deletions usually have compensatory gene copies to balance deletions (Noordam & Repping, 2006). Ferlin et al. found that deletions that remove DAZ1/DAZ2 are likely associated with spermatogenic failure, whereas DAZ3/DAZ4 deletions have little or no

effect on male infertility (Ferlin, et al., 2005). The partial deletion of CDY1 also showed no effect, as the gene was likely compensated for by the second copy of CDY (OMIM, 2016). Partial deletions may be considered as risk factors for male infertility. The partial gr/gr AZFc microdeletion is commonly observed in infertile males; however its significance is not yet clear because of the presence of such deletions in normospermic men (Giachini, et al., 2005).

Indications of Y Microdeletion Screening

Because clinical parameters such as testicular volume, hormone levels and the presence of testicular malformations are not predictive of Y microdeletions, genetic testing is required. Y chromosomal screening is recommended for males diagnosed with idiopathic causes of non-obstructive azospermia. Molecular screening is usually not recommended for males with chromosomal abnormalities, obstructive azospermia, hypogonadotropic hypogonadism, history of testicular tumors or severe injury or chemotherapy. However there have been examples of deletion carriers who show spermatogenic failure that is accompanied by a diagnosis in addition to azospermia. Therefore any diagnosis, as long as non-obstructive azospermia is present, is an indication for AZF deletion screening.

For example, some studies list varicocele as an exclusion criterion when gathering samples for research and doctors often do not test for AZF deletions before carrying out a varicocelectomy. However this is an erroneous practice in that deletion carriers may have varicocele and therefore screening for deletions beforehand could prevent an unnecessary procedure (Moro, Marin, Rossi, Garolla, & Ferlin, 2000). TESE or TFNA operations may also be avoided for those males who show clinically significant deletions in AZFa, AZFb or AZFb+c.

Azospermic patients that are candidates for TESE and ICSI should also complete deletion screening because TESE is ineffective and is not recommended for cases of AZFa complete deletions. Micro-TESE on the other hand, may be attempted in azospermic microdeletion carriers of the AZFb or AZFb+c regions—only in cases where the mutation breakpoint lies within the P4 palindrome. For most other cases, biopsies and surgical treatment may be attempted but the chances are low and the deletion and infertility phenotype will be passed on to all sons. Thus the diagnosis of deletions serves prognostic purposes and may influence therapeutic decisions.

A study by Sheikhha et. al found that 5/25 (20%) of azospermic males seeking ICSI were positive for microdeletions of multiple AZF regions (Sheikhha, et al., 2013). An additional 12 males showed microdeletions within one region, for a total of 17/25 significant results. This is alarming because of the exceptionally high rate of mutations in azospermic candidates attempting ICSI. The problem with assisted reproduction for these males is that they are highly increasing the risks of having a child with the same fertility issues, or ending a pregnancy in abortion or miscarriage due to an abnormal fetus. Presence of deletions may prevent ICSI from occurring, but in the event of a successful treatment, the mutations are always inherited and may even expand in the son (Krausz, Hoefsloot, Simoni, & Tuttelmann, 2014) (Dai, et al., 2012). Therefore AZF deletion screening is important and necessary to provide genetic counseling that would allow couples to understand the full consequences of passing on a deletion.

METHODOLOGY

Ethical Considerations

The present study was approved by the Ethics Committee of Birzeit University's Clinical Laboratory Science Program and carried out in compliance with general principles of the

Declaration of Helsinki (World Medical Association, 2013). All participants were informed about the present study's purpose, procedure, risks and benefits before voluntarily signing the consent form. No names or potential identifiers were used at any point in the study. Such confidential information was kept with medical personnel who assigned each participant with a new identification number to be used solely for the purpose of this study. This method provided strict confidentiality and gave participants confidence that their privacy was protected. There were no foreseeable risks involved.

Study Population

A total of 93 participants including 41 infertile, azospermic men, 50 normospermic men and two females were enrolled in this study. Cases were referred by and collected from the northern, central and southern regions of the West Bank from Razan IVF centers, Al-Hiba center and Palestine European Fertility Centers, between March 2016 and June 2016. Due to the sensitive nature of the study, questionnaires and sample collection were carried out through lab technicians in order to avoid discomfort, minimize refusals and allow patients to maintain their anonymity. Control samples were obtained similarly, to maintain consistency in the study. All participants signed an informed consent form, answered a questionnaire and gave two to three mL of blood.

Selection Criteria

Cases were referred to the study when at least two semen samples were analyzed in accordance to WHO guidelines and found to be absent of any sperm, even after centrifugation (World Health Organization, 2010). Negative TEFNA or TESE results were preferred as confirmation of the absence of sperm in the testes, but were not always available. Therefore, physical examination or ultrasonography was also accepted as a method to exclude obstructive azospermia in a few cases.

A short, detailed questionnaire was given to all male participants to determine their eligibility for the study. Infertile males with hypogonadotropic hypogonadism, endocrinopathies that improved with medication, a history of severe testicular trauma or infection, chronic disease, surgery or chemotherapy were excluded. Men younger than twenty or above fifty were also excluded from the study to exclude abnormalities that typically affect these ages. A normal karyotype is ideally required as an important criterion; however the lack of cytogenetic labs in Palestine and the high cost of karyotypes made it very difficult to include this test in the present study. Strict criteria are necessary for correct selection of non-obstructive azospermic cases.

Fertile male DNA was used as a positive control. Fertile males were considered as those with sperm concentrations of no less than 20 million/mL, normal hormone profiles and two or more children conceived naturally. Female DNA was used as a negative control.

MATERIALS AND METHODS

Genetic Analysis

Allele-specific polymerase chain reaction (PCR) was used to screen the Y chromosome for STS that are recommended by the European Academy of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN) (Krausz, Hoefsloot, Simoni, & Tuttelmann, 2014). A total of 19 forward and reverse primers (designed by Hylabs), shown in Table 1, were included in the study. The primers were chosen from non-polymorphic regions of AZF and are therefore reliable for diagnosis. Most of the STSs are single-copy except for sY254, sY255, sY153 and sY160 which are multi-copies chosen for their outer proximal or distal locations.

STS Primer Pairs			
STS	**Forward Primers**	**Reverse Primers**	**Size (bp)**
ZFX/Y	5′ACCRCTGTACTGACTGTGATTACAC-3′	5′GCACYTCTTTGGTATCYGAGAAAGT-3′	495
SRY	5′-GAA TAT TCC CGC TCT CCG GA-3′	5′-GCT GGT GCT CCA TTC TTG AG-3′	472
sY86	5′-GTG ACA CAC AGA CTA TGC TTC-3′	5′ - ACA CAC AGA GGG ACA ACC CT – 3′	318
sY84	5′-AGA AGG GTC CTG AAA GCA GGT-3′	5′-GCC TAC TAC CTG GAG GCT TC-3′	326
sY82	5′ATC CTG CCC TTC TGA ATC TC-3′	5′CAG TGT CCA CTG ATG GAT GA-3′	264
sY1064	5′-GGG TCG GTG CAC CTA AAT AA-3′	5’TGCACTAAAGAGTGATAATAAATTCTG-3′	110
sY1182	5′-ATG GCT TCA TCC CAA CTG AG-3′	5′-CAT TGG CCT CTC CTG AGA CT-3′	247
sY88	5′-TTG TAA TCC AAA TAC ATG GGC-3′	5′-CAC CCA GCC ATT TGT TTT AC-3′	123
sY127	5′-GGC TCA CAA ACG AAA AGA AA-3′	5′-CTG CAG GCA GTA ATA AGG GA-3′	274
sY134	5′-GTC TGC CTC ACC ATA AAA CG-3′	5′-ACC ACT GCC AAA ACT TTC AA-3′	301
sY105	5′-AAG GGC TTC TTC TCT TGC TT-3′	5′-AGG GAG CTT AAA CTC ACC GT-3′	301
sY1224	5′-GGC TTA AAC TTG GGA GGG TG-3′	5′-CAA AGA GCC TCC CAG ACC A-3′	640
sY143	5′-GCA GGA TGA GAA GCA GGT AG-3′	5′-CCG TGT GCT GGA GAC TAA TC-3′	311
sY153	5′-GCA TCC TCA TTT TAT GTC CA-3′	5′-CAA CCC AAA AGC ACT GAG TA-3′	139
sY254	5′-GGG TGT TAC CAG AAG GCA AA-3′	5′-GAA CCG TAT CTA CCA AAGCAG C-3′	380
sY255	5′-GTT ACA GGA TTC GGC GTG AT-3′	5′-CTC GTC ATG TGC AGC CAC-3′	123
sY1291	5′-TAA AAG GCA GAA CTG CCA GG-3′	5′-GGG AGA AAA GTT CTG CAA CG-3′	527
sY1191	5′-CCA GAC GTT CTA CCC TTT CG-3′	5′-GAG CCG AGA TCC AGT TAC CA-3′	385
sY160	5′-TAC GGG TCT CGA ATG GAA TA-3′	5′-TCA TTG CAT TCC TTT CCA TT-3′	236

Table 1: Primer pairs that amplify DNA within AZFa, b and c of the Y chromosome.

DNA Extraction

Whole blood was collected into EDTA tubes and frozen at -20C. Blood samples were thawed slowly at room temperature just before DNA extraction. DNA membrane elution kits (Qiagen DNEasy Blood and Tissue kit, #69504) were used to extract DNA from peripheral blood lymphocytes by following the included protocol. Qualitative analysis of the DNA was done by observing bands on 2% electrophoresis gels during optimization steps. Quantitative analysis

included the assessment of purity and concentration of DNA by measuring the optical density with a spectrophotometer (ThermoScientific Nanodrop LITE). The purity and concentration of all isolated DNA samples were found to be within normal ranges (purity: 1.7-1.85 and concentration: >30ng/µl) and stored at -20˚C until required for PCR.

PCR Protocol

PCR reactions were carried out with a total reaction volume of 25 µl. Each PCR tube aliquot contained: 12.5 µl of master mix (ThermoScientific ReddyMix, #AB-0575/DC/LD/A which includes loading dye, Taq polymerase, dNTPs, $MgCl_2$ and buffer), 0.6 µl each of forward and reverse primers and 8.3 µl of distilled water. 3 µl of DNA was then added for a total volume of 25 µl (1X). All reactions and components were kept on ice and immediately placed into the thermocycler after mixing and spinning the tubes.

Each primer was first tested at its expected, optimal annealing temperature to confirm the primers function properly and amplify the expected product. Temperatures were calculated based on the A, G, C and T content using the following equation:

$$[4(G+C) + 2(A+T)]-5$$

The annealing temperatures of all 19 primers fit within a 6 degree range. To save time and avoid confusion, one standard PCR program was created and optimized to work for all primers.

The initial program began with denaturation at 95˚C for 3 minutes, followed by 35 cycles of:

1. Denaturation for 30 seconds, 94˚C
2. Annealing for 30 seconds, 56˚C
3. Extension for 45 seconds, 72˚C

The final step was elongation for 7 minutes at 72˚C, followed by cooling to 4˚C.

Gel Electrophoresis and Visualization

Amplicons were analyzed on 2% agarose gels, which were prepared by combining 0.5g agarose, 25 mL of 1X tris acetate EDTA (TAE) buffer and 0.3 µl ethidium bromide. Electrophoresis was run for 30-45 min at 70V and the resulting bands were visualized under UV light. Suspected deletions were repeated three times, and additionally tested with newly extracted DNA in case of false negative results due to degradation.

Statistical Analysis

To compare the frequency of microdeletions in the Palestinian population to that of other populations, statistical analysis was carried out using the binomial distribution equation (1) and the z-test (2) to reject or accept the null hypothesis, as explained later:

$$\textbf{(1)}\quad b(x; n,P_0)= ({}_nC_x)(P^x)(1-P_0)^{n-x}$$

$$\textbf{(2)}\quad z=(p-P_0)/\sigma$$

RESULTS

From 41 infertile males analyzed for Y microdeletions in three AZF locations, four men (~9.8%) were found to have ten deletions in a total of seven different locations (See Table 2). All other azospermic and fertile male controls showed characteristic bands for all primers. Fig. 5 shows a normal PCR gel with no deletions. One case, P-10 had only one location deleted at sY1191. Two cases, P-03 and P-38 showed deletions in two locations each: sY1291 and sY160, and sY1191 and sY1224 respectively. Lastly, P-17 had deletions in 5 locations: sY254, sY255, sY1191, sY1291 and sY1224. Fig. 6 and Fig. 7 show a PCR gel image of one deletion from P-17, at location 254 and 255, respectively.

Region	STS	P-03	P-10	P-17	P-38	Female	Fertile
AZFb	sY1224				del	del	
	sY153			del		del	
AZFc	sY254			del		del	
	sY255			del		del	
	sY1191		del	del	del	del	
	sY1291	del		del		del	
Heterochromatin	sY160	del				del	

Table 2: AZF deletions found in four unrelated, infertile azospermic males P-03, P-10, P-17 and P-38. Negative (female) controls were deleted in all STS. Fertile males showed no microdeletions.

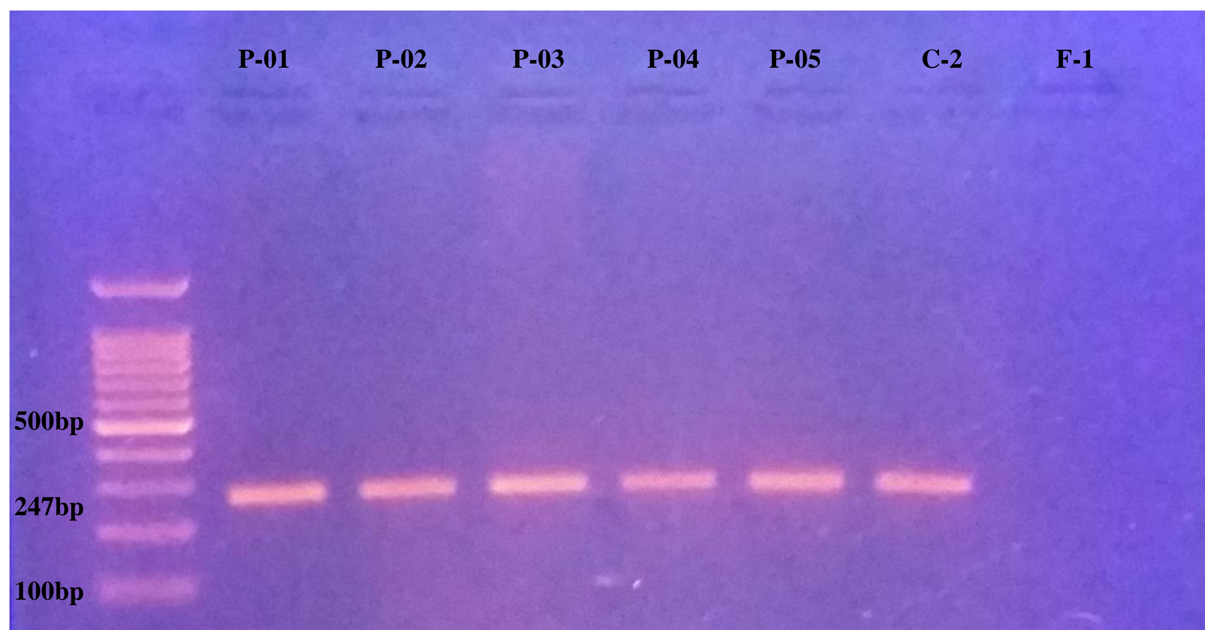


Figure 5: PCR gel visualized with UV light shows cases P1-5, positive male control C-2 and negative female control F-1. Amplified fragments of 274bp are shown, for STS marker sY127.

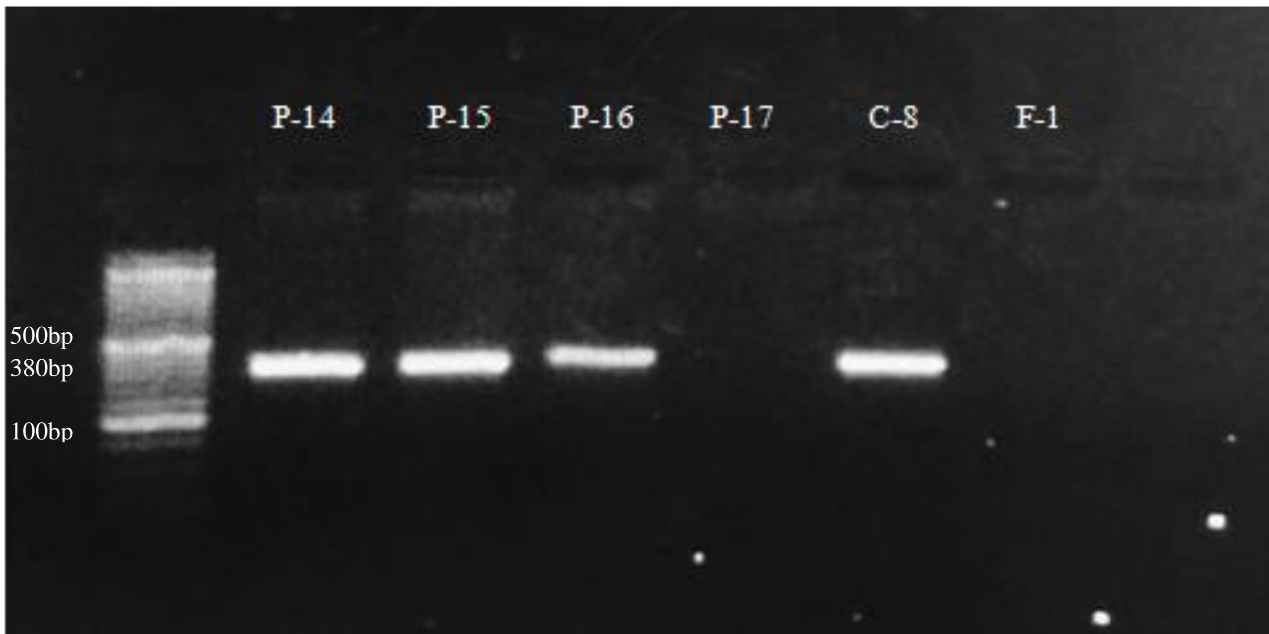


Figure 6: PCR results for four cases using STS primer sY254. Cases P14-16 and fertile male control (C-8) show the expected 380bp bands, indicative of a positive result for the normal presence of sY254. Female control (F-1) shows expected deletion, and case P17 also shows a deletion. The last well (far right) is blank.

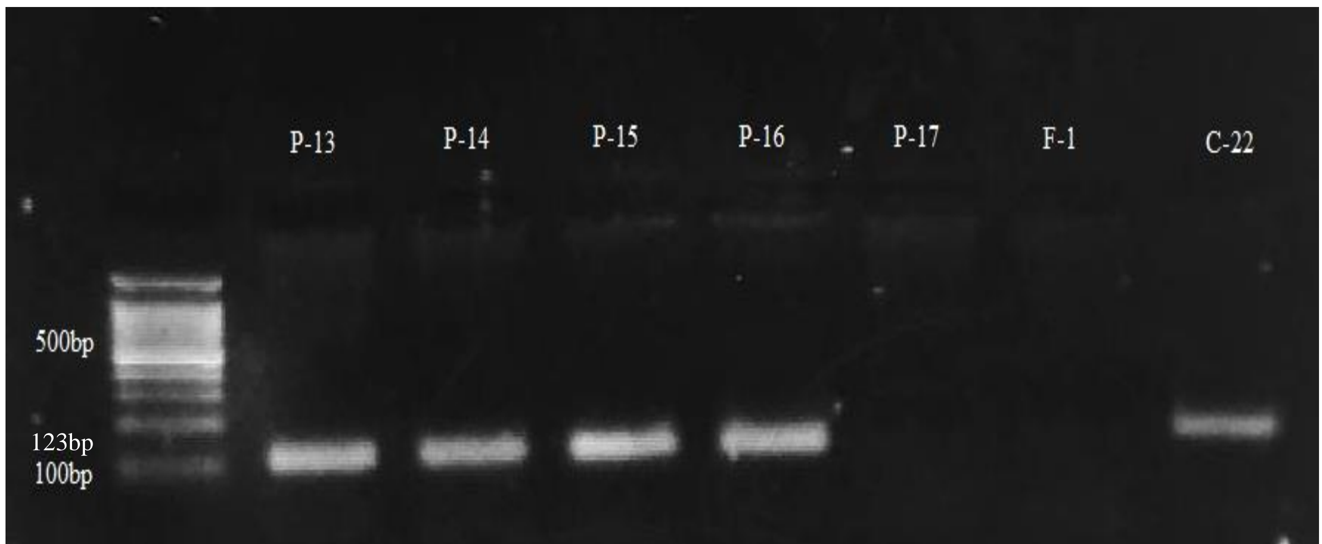


Figure 7: PCR results for four normal cases (P13-16) using STS primer sY255. Case P-17 and negative control (F-1) do not show a band, indicating deletion.
Fertile male control (C-22) shows the expected 123bp band.

All males showed negative results after at least one TEFNA or TESE procedure. The four affected patients were all under the age of 35 and showed primary infertility for at least two years. The questionnaires revealed that both cases and controls were generally healthy individuals, with no illnesses or known hazardous exposures. A few cases presented with prolonged exposure to pesticides, residency near Israeli settlements (that may leak toxic material into the surrounding environment), trauma or infection of the testes, however none of these showed deletions.

Complete AZFc Deletion in Case P-17

Azospermia case P-17 was found to have a complete deletion of AZFc and partial deletion in AZFb, that included loss of DNA in five different locations. This is the first reported case of a complete Y-microdeletion in Palestine. P-17 is a 34 year old male that has been married for 14 years and infertile for the entire duration. The couple suffers from male-factor only, primary infertility. He had no previous marriages and does not have children. ICSI was attempted four times to date, only to result in recurrent failure. Medical and family history is normal, with no current or previous exposures to toxic chemicals, radiation, extreme or prolonged time in heat, infections or other known infertility-inducing factors.

DISCUSSION

Y chromosome deletions are one of the most common genetic causes of male infertility. The prevalence of AZF microdeletions varies worldwide among both azospermic and oligospermic males; it generally ranges from 1-13% worldwide but may reach up to 55% (Suganthi, Vijesh, Jayachandran, & Benazir, 2013) (Silber & Disteche, 2012). The wide range of frequencies is a

reflection of several factors that include varying selection criteria, accuracy in testing and social restrictions that limit sample collection.

In this study, the AZF region of the Y chromosome was analyzed for microdeletions in 41 males, using 19 EAA/EMQN recommended STS primers (including two control locations). The results showed that 4 out of the 41 infertile azospermic cases had deletions in at least one AZF region, whereas no deletions were detected in the fertile controls. The frequency of microdeletions observed in the Palestinian population was 9.8%. Table 3 shows the incidence in Palestine among those of Middle Eastern and other world regions. Results of the current study were very similar to results of studies done in Jordan (8.3%) and the United States (10.4%). Very low deletion frequencies were reported in Algeria (2%) and Tunisia (1.3%), while a detailed study in Saudi Arabia found an exceptionally high incidence of 20.5%. The major variation between these regions is due to geographic and environmental differences, sample size, methods of deletion detection and inclusion criteria, among other factors.

Region	**Frequency**	**Reference**
Palestine	9.8%	This study
Jordan	8.3%	(Khabour, Fararjeh, & Alfaouri, 2014)
Saudi Arabia	20.5%	(Atia, Abbas, & Ahmed, 2015)
Turkey	1.3%	(Balkan, Tekes, & Gedik, 2008)
Morocco	14.2%	(Naasse, et al., 2015)
Tunisian	11.8%	(Rejeb, et al., 2008)
Indian	7.6%	(Mitra, et al., 2008)
Syria	6.8%	(Alachkar, Wafa, & Moassass, 2013)
Algeria	2%	(Chellat, et al., 2013)
Japan	11.7%	(Nakashima, Koh, Namiki, & Yoshida, 2002)
United States	10.4%	(Stahl, et al., 2010)

Table 3: Frequencies of AZF microdeletion in select populations.

Seven deletions were found in the AZFc region, one deletion was found distal to AZFc, in the heterochromatic region of the Y chromosome and two were found in AZFb. This data is consistent with most studies, which show that AZFc deletions are the most common (60-80%) among the three AZF regions (Atia, Abbas, & Ahmed, 2015) (Krausz, Hoefsloot, Simoni, & Tuttelmann, 2014) (Silber & Disteche, 2012). The high frequency of microdeletions in the AZFc region (7/10) may be due to the presence of highly repetitive gene sequences, which predispose the Y-chromosome to intrachromosomal breaks and recombination, and consequently result in deletions.

Two cases P-10 and P-03 showed partial deletions in AZFc, in which extensional STS were missing, rather than major markers. Case P-17 showed a complete deletion of the entire AZFc region in which major markers sY254 and sY255 were deleted, removing all copies of the DAZ gene. The extended analysis of this case revealed the loss of two other AZFc sites, sY1291 and sY1191, as well as STS sY153 located in AZFb, which indicated the broad extent of the deletion.

Although complete deletions of AZFc are detrimental to male fertility, the chance of successfully fathering a child via ICSI is still higher (50%) than the likelihood of an AZFb or AZFa-deleted male (~0%). However, to date, the affected males of this study have not yet had success after attempting intracytoplaspic sperm injection. In classic deletions of AZFb, the entire region is deleted. In molecular terms, this means that sY127 and sY134, major AZFb markers would have to be deleted, along with the extensional markers, sY1224, sY143, and sY153. (Krausz, Hoefsloot, Simoni, & Tuttelmann, 2014). Complete AZFb deletions imply that the likelihood of finding viable sperm for use in successful ICSI is virtually zero. Cases P-17 and P-38 each showed a microdeletion in the AZFb region, although the deletions were only partial. It may be suggested that these two males have a decreased chance of reproductive success, but it cannot be

assumed that the partial AZFb deletions rule out future success, even though their recurring failures seemingly point to that conclusion.

Since the first successful in vitro fertilization (IVF) in 1977, there have been advancements in assisted reproductive treatments and a surge in IVF centers worldwide to give infertile couples a chance of having their own child. Azospermic males often undergo numerous procedures to search for sperm and attempt sperm retrieval to be used for ICSI. The growing field of IVF has allowed doctors to bypass natural selection, in order to give couples a chance at having biological children. But without proper screening and counseling, IVF centers may be increasing infertility and genetic disorders in the world population. In addition to the risk that is placed on the population, the mental, physical and reproductive health of the couple enduring IVF may also be affected. Repeat TESE procedures in search of sperm may cause testis devascularization and fibrosis, damaging the tissues (Shah, 2011). Long-term testosterone deficiency also results often in males who have had numerous TESE procedures (Everaert, et al., 2006). In NOA males with successful TESE (sperm is found), only about 37% of cases result in one live birth after six different ICSI cycles (Vloeberghs, et al., 2015). Depression and anxiety from experiencing recurrent implantation failures or abortions may cause mental and physiological health problems that lead to hormonal abnormalities (Deka & Sarma, 2010) (Volgsten, Svanberg, Ekselius, Lundkvist, & Poromoaa, 2008).

The importance of genetic studies of male infertility in Palestine has been demonstrated by (Qumsiyeh, Borqan, & Obeid, 2014) and (Shaqalaih, 2007), whose data showed that a significant portion of idiopathic infertile males had genetic abnormalities—specifically chromosomal disorders such as Klinefelter's syndrome. Both studies reinforced the need for molecular screening of azospermic males before seeking assisted reproduction, as most of the affected

individuals either had no chance to father a child or had a very high chance of producing an abnormal fetus by ICSI. The results of this study have also shown that genetic testing is necessary for the infertile Palestinian male population. The presence of important deletions in this region highlights the importance of screening at least azospermic males before attempting sperm retrieval techniques and reproductive assistance.

Y-microdeletions are considered de novo events (Foresta, Moro, & Ferlin, 2001) since the fathers of affected individuals do not usually reveal any microdeletions. However in cases of azospermic males with known deletions, who are undergoing ICSI, the phenotype of the deletion may deteriorate in their future sons (Krausz, Hoefsloot, Simoni, & Tuttelmann, 2014). In other words, with the help of reproductive assistance, when the deletion is transmitted from father to son, the phenotype worsens with each successive generation. This may be due to the widening of the microdeletion, in which more DNA is lost through recombination (Stuppia, et al., 1996). Azospermic patients face more difficult hurdles when attempting assisted reproduction technologies such as ICSI, if of course, they first had success with TESE. Some may have one or a few viable sperm, some have few sperm that are all unviable, and others do not have any sperm or sperm precursors in the testes.

If males with Y microdeletions were diagnosed and informed of the consequences, they might choose to opt out of unnecessary, invasive, costly procedures and try an alternative option such as adoption. It would be unfair to the expecting couple, who after exhaustive testing, repetitive cycles of IVF, expensive treatment and medications, have a child (if male) with the same infertility condition as his father. Doctors should completely inform their patients of all risks and benefits related to IVF, including the dangers of repeat surgical procedures, ICSI and the future health of potential children, and then allow the parents to decide; the inclusion of genetic

screening in the infertile male workup is therefore important for providing a diagnosis, prognosis and for offering couples appropriate genetic counseling. It is also important for the health of potential pregnancies, and the psychological and emotional wellbeing of the couple.

Statistical Analysis

To compare the results of the Palestinian population to other populations, this study was considered as a binomial experiment where the distribution is the probability of a number of successes, from a fixed number of independent trials. Given that $\mathbf{P_0}$ is the probability of success (for statistical purposes, success equals an AZF-deleted male), **x** is the probability of a given number of successes and **n** is a fixed number of trials, binomial probability is equal to:

$$b(x;\, n, P_0) = ({}_nC_x)(P^x)(1-P_0)^{n-x}$$

The incidence of microdeletions in azospermic males ranges worldwide, but is generally between 8-12% (Jungwirth, et al., 2014). Because there is no data on the incidence of microdeletions in azospermic males of the Palestinian population, the prevalence that is common in most studies (10%) was used ($P_0 = 0.1$). In this study, the number of trials is n=41, the Palestinian population frequency is p=.098 and the number of successes x=4. The binomial probability P was calculated to be P(4)=0.21.

Given these variables, the hypothesis was tested using a one-tailed z-test for proportions, where **σ** is the standard deviation:

$$Z = (p - P_0)/\sigma$$

H_o: The proportion of microdeletions does not differ from that of most populations' proportions.

H: The proportion of microdeletions in this study differs from that of most populations.

The z-score of the binomial distribution was found to be -0.431, with the associated P value of 0.333, which was higher than the value at $\alpha=0.05$. This indicated that the result at $p<0.05$ was not significant and therefore the null hypothesis was accepted, that the deletion frequencies of this population do not differ from most other studies.

Essentially this means that the Palestinian population has a Y microdeletion frequency that is comparable to other world regions, and should thus be considered as a significant genetic condition that should be screened for during male infertility lab analyses.

Recommendations

To improve this study, semen samples may be used in place of blood, as the source of DNA. A study by Atia et al. showed that 2% more Y microdeletions were found when using sperm DNA rather than peripheral blood DNA, in the same males (Atia, Abbas, & Ahmed, 2015). Other improvements include the use of multiplex PCR to test internal control primers simultaneously with AZF primers for better quality assurance, as well as DNA sequencing of azospermic samples to pinpoint microdeletions and possible single nucleotide polymorphisms.

A follow-up study of couples that choose to continue attempting TESE and ICSI would be beneficial to confirm or refute the importance of AZF deletion screening in this population, by measuring the success rate versus failure to reach live birth. If ICSI is successful in any of the

microdeletion-positive cases, a study can be done on male offspring to test for genetic abnormalities and the inheritance of the father's infertility.

Because a significant percentage of microdeletions were found in this study, it is recommended that other Y chromosomal abnormalities such as androgen receptor mutations are investigated. It would also be interesting to study the link between genetic male infertility and environmental exposures such as toxic waste released from Israeli settlements or large industrial plants around Palestine.

CONCLUSION

The growing availability of IVF treatments over the last few decades has provided hope for couples struggling with severe infertility, particularly for non-obstructive azospermic males. However, in overcoming the barriers of natural fertilization there is concern that the frequency of genetically acquired infertility and other heritable conditions will increase and thus widen the reproductive failure of future generations. To prevent this from happening, genetic screening and counseling is important. The Palestinian population is affected by numerous genetic disorders, including Y-chromosome infertility. The deletion frequency found in this study strongly suggests the need for microdeletion screening of azospermic Palestinian males, to help in maintaining the health of future generations' natural reproduction.

جامعة بيرزيت
BIRZEIT UNIVERSITY
Master of Clinical Laboratory Science Program

Consent Form
موافقة على المشاركة في البحث

الباحثة: شادن طه **الهاتف:** 0599490489 **البريد الألكتروني:** **sataha@uh.edu**

عنوان الدراسة: Detection of Y-microdeletions in Azospermic Palestinian Men

سوف يتم عمل الدراسة على 150 من الرجال و 2 من السيدات (للتأكد من جودة الاختبار).

إذا كنت توافق على أن تكون في هذه الدراسة، سوف يطلب منك التوقيع بالموافقة وإعطاء عينة من الدم.

لا يوجد أي مخاطر متوقعة.

الخصوصية

سيتم الاحتفاظ بسجلات هذه الدراسة في سرية تامة. ولن توجد أي معلومات في أي تقرير قد يتم نشره من شأنه أن يجعل من الممكن التعرف على هوية الشخص المتطوع بحيث تكون جميع العينات مشفرة. ولن يتم استخدام اي أسماء.

لديك الحق في طرح الأسئلة حول هذه الدراسة والحصول على إجابات عنها في أي وقت. لا تتردد في الاتصال بالباحث عن طريق البريد الالكتروني أو رقم الجوال المذكورة أعلاه. واذا رغبت في الحصول على نتائج الأختبار يمكن ارسالها عن طريق البريد الالكتروني أو من خلال المختبر الذي تم اخذ عينة الدم فيه.

الموافقة

توقيعك يشير إلى أنك قررت التطوع كمشارك في البحث لهذه الدراسة وقد فهمت المعلومات التي تم قرائتها أو تفسيرها شفويا.

توقيع هذه الوثيقة يعني أن الدراسة البحثية ، بما في ذلك المعلومات الواردة أعلاه، تم فهمها بعد القراءة أو الشرح شفويا ، وأنك توافق طوعا على المشاركة.

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التاريخ:______________

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