Investigation on AZF-loci among infertile Sudanese patients

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DEDICATION

I dedicate this work to:

My great parents who have given me faith, love and support through the years.

My brothers and sisters.

My friends.
ABSTRACT

Infertility is an extraordinary public health problem, especially in our Arab world, affecting about 15% of couples seeking children. However, in 50% of these the male partner is responsible for infertility. Y chromosome classical microdeletions in the azoospermia factor (AZF) regions are known to be associated with spermatogenic failure. In the current study, twelve patients with primary male infertility were studied in order to investigate the molecular background of male infertility in Sudanese population using polymerase chain reaction technique.

Samples collected from Al-Sir AbuAlhassan centre, DNA extracted from Polymerase chain reaction (PCR) was performed on three loci spanning the AZFb subregions of the Y chromosome. The PCR was carried out using four primers.

Y chromosome classical microdeletions were detected in eight study participants (out of twelve).

Diagnostic bands of AZF b microdeletions (sY128, sY134) were detected in four study participants (out of twelve) 33.3%.

Diagnostic bands of AZFb (sY127) microdeletions were detected in two study participants 16.7% (out of twelve).

In conclusion the present study shows that the incidence of Y chromosome microdeletions is rare in Sudanese population and suggesting that other genetic, epigenetic, nutritional and local factors may be responsible for idiopathic azoos/oligozoospermic in our population.
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Chapter One: Introduction and Literature Review

1.1. Infertility:

Infertility is a real extraordinary problem worldwide. Infertility has been defined by the world health organization (WHO) as the inability to conceive naturally after at least one year of unprotected intercourse (Dohle et al., 2004). It is estimated that as many as 15% of couples worldwide who seeks children suffer from infertility (Pryor et al., 1997; Tse et al., 2000; Dada et al., 2003; Hellani et al., 2006).

Male causes for infertility are found in about 50% of infertile couples (Pryor et al., 1997; Ambasudhan et al., 2003). Genetic abnormalities contribute to a fair enough percentage of the idiopathic causes of male infertility (Vogt, 2004).

1.2. Semen analysis:

A semen analysis is a laboratory test to check a man’s semen and sperm. Semen contains sperm, which are the male sex cells that fertilize the female egg leading to pregnancy. The most common reasons for laboratory semen analysis in humans are as part of a couple’s infertility investigation. The characteristics measured by semen analysis are only some of the factors in semen quality. One source states that 30% of men with a normal semen analysis actually have abnormal sperm function. Parameters measured in a semen analysis are: sperm count, motility, morphology, thickness and thinness of semen, numbers of live sperm and the shapes of sperm.
A considerable and substantial attention has been focused on the role of genetic factors in spermatogenesis failure. It has been estimated that over 4000 genes are involved in the genetic control of human spermatogenesis (Granotten et al., 2004). The interstitial deletions of the Y chromosome are the most common structural abnormalities causing male infertility. It can be detected only by molecular techniques, such as polymerase chain reaction (PCR).

The human Y chromosome (fig 1.1) consists of long arm (q) and short arm (p). Y chromosome plays a central role in human biology. The presence or absence of this chromosome determines gonadal sex. The Responsible for the male phenotype is the testis-determining SRY gene (Sex-determining region Y) (Sinclair, 1990), which remains the most distinguishing characteristic of this chromosome. In addition to SRY, Y chromosome contains other genes with important functions in germ cell development, maintenance and then, related with male fertility. (Lahn, 1997).

![Figure 1.1: Human Y chromosome](image-url)
During the extensive search in the literature concerning Y chromosome microdeletions, we found only five Arab studies:
The first one was conducted in Egypt (El Awady et al., 2004), the second study was conducted among Saudis in Saudi Arabia (Hellani et al., 2006), the third study was Tunisian (Hadj-Kacem et al., 2006), the fourth study was conducted among Moroccan infertile men (Imken et al., 2007). The fifth study was a novel study that was conducted among Kuwaitis in Kuwait (Mohammed et al., 2007) where the authors reported a low AZF microdeletion prevalence of 2.4% (7/266).

1.3. The role of human Y-chromosome in male infertility:
The Y chromosome main task is to ensure that men can make and deliver sperm for the continuity of human species. In addition, the male sex determination role of the Y chromosome has been known for a long time, since it carries the sex determining region Y (SRY) gene, which switches the development of the indifferent gonad from the default female pathway to the male pathway, which results in the development of the testis (Goodfellow and Lovell-Badge, 1993; Yen, 2001).

The role of Y chromosome in male infertility was first elucidated in 1976 by Tiepolo and Zuffardi when they have proposed the existence of genes responsible for spermatogenesis on the Y chromosome long arm (Yq11), which was called the azoospermia factor (AZF). Micro deletions of the Y chromosome are the second most frequent genetic cause of spermatogenetic failure in infertile men.

Y chromosome micro(YCM) deletions are most commonly detected in men with azoospermia. Azoospermia is defined as absence of sperm, not having any measurable level of sperm in the male semen. It is associated with very low levels of fertility or even sterility) (Willott, 1982); It can also detect in severe
oligospermia. Oligospermia defined as less number of sperm in the ejaculate of the male or less than 20 million sperm per millilitre. Less commonly, men with sperm counts between 1–5 million sperm/ml semen will carry a microdeletion (Willott, 1982).

YCM is a family of genetic disorders caused by missing genes in the Y chromosome. Microdeletions in Yq11 overlapping three distinct 'azoospermia factors' (AZFa, AZFb and AZFc) represent the etiological factor of 10–15% of idiopathic azoospermia and severe oligozoospermia. (Ferlin et al., d. 1999).

This substantially proved the close association between Y chromosome deletions and male infertility. Men with YCM exhibit no symptoms and lead normal lives.

1.3.1. Y-chromosome long arm (q) deletions and male infertility:

Human chromosome deletions in Yq11 seem to occur frequently as de novo mutation events in men with idiopathic azoospermia or severe oligozoospermia. However, the molecular extensions of these deletions are variable.

At least three different intervals (i.e., the AZFa, AZFb, and AZFc) on the Y chromosome long arm (Yq11) (figure 1.2) are critical for male germ cell differentiation. They encode specific genes essential for the normal complex process of spermatogenesis. These intervals vary in length as they also vary in the encoded genes.

Structural abnormalities in these intervals, notably microdeletion events, were found to be the most prominent in azoospermic and oligozoospermic men with a normal karyotype (46, XY) (Reijo et al., 1995).

These microdeletions are only detectable by molecular techniques (such as PCR), because the deletion length is less than to be visualized or discerned by routine cytogenetic methods, and this is why they are called microdeletions.
Patients can show different deletion patterns, one or more AZF loci could be deleted (e.g. AZFb and AZFc), or even portions of variable lengths of one AZF locus may be deleted. Large scale of studies showed that the most frequently deleted region is AZFc, followed by deletions of the AZFb and AZF b-c or AZF a-b-c regions whereas deletions of the AZFa region are extremely rare (Saxena et al., 2000; Martinez et al., 2000; Tse et al., 2000; Foresta et al., 2001; Akbari Asbagh et al., 2003; Krausz et al., 2003; Ambasudhan et al., 2003; Yu et al., 2004; Carvalho and Santos, 2003; Fernandes et al., 2006; Omrani et al., 2006).

1.3.2. AZF loci deletions causing male infertility:

The azoospermia factor locus (AZF) is assumed to contain the genes responsible for human spermatogenesis. Three major deletion intervals have been defined and termed as AZFa, AZFb and AZFc. Point and/or major deletions of these genes loci on chromosome Y are significant causes of spermatogenic failure. Such mutations in these genes are thought to be pathologically involved in some cases of male infertility associated with azoospermia or oligozoospermia. Deletions in AZF genes intervals are thought to be pathogenetically involved in some cases of male infertility.

1.3.2.1. The AZFb region:

The AZFb region is located approximately between the subintervals 5M to 6B (Vogt et al. 1996). The region harbors about 3.2Mb sequences and encompasses several genes essential for normal spermatogenesis (Ferlin et al. 2003). (Figure 1.1).
AZFb Deletions:
The patients with AZFb microdeletions were found to have spermatogonia and primary spermatocyte normal in all the tubules observed (Vogt et al., 1996). Relative prevalence of deletions in AZFb region in infertile men is 16%, Azoospermia has Spermatogenic arrest. Micro deletions can remove AZFb alone or parts of AZFb. Several genes located in AZF regions are expressed in the testis and could therefore be viewed as “AZF candidate genes.” However, based on studies of infertile patients, only a few genes can actually be considered responsible for the AZF phenotype (Foresta et al, 2001).

![Figure 1.2 AZF region on Y chromosome. Three Main intervals AZFa, AZFb and AZFc.](image)

The universal manual of WHO clarify qualitative and quantitative criteria for man to be defined as fertile or infertile according to seminal profile in terms of azoospermia and oligozoospermia (Cooper et al. (2010)).
1.4. Objectives of the study:

To study the prevalence and molecular characteristics of Y chromosome microdeletions in AZF sub regions (AZF b) in Sudanese azoospermic and oligozoospermic infertile men using PCR.
Chapter Two: Materials and Methods

2.1. Study area.

This is a clinical-based study in Khartoum state the capital of Sudan. Study participants were enrolled in this study from Alsir Abu Alhassan Fertility Centre. The centre is one out of thirteen IVF centres in Khartoum. The centre established in 1998 to diagnose and treat infertility cases. Twelve males who approached the centre seeking investigation about their cases were agreed to participate in this study. They have been oriented about the aim of the study.

2.2. Sample collection:

Approximately 5 ml blood was collected from each patient in EDTA tubes, and centrifuged 4000 rpm for 5 min to separate the plasma from the blood and stored at -20°C to be transferred to further DNA extraction.

2.3. Semen analysis:

Study participants were subjected to semen analysis by the lab staff at Dr. Alsir Abu Alhassan Fertility Centre. Semen analysis performed according to world health organization (WHO) criteria 2010 (Cooper et al., 2010).

2.4. DNA EXTRACTION:

The blood sample was added to a falcon tube (15 ml). RBCs Lysis buffer (1:3) was added and mixed well and centrifuged at 4000rpm for 5 minutes. The supernatant was discharged; the process was repeated 5 times. WBCs was added and incubated at 37°C over night. NaCl was added and mixed well. Chloroform was added, mixed and then centrifuged at 4000 rpm for 5 min. The supernatant was transferred to a new falcon tube. Cold absolute ethanol (2:1) was added and mixed well, vortexed and kept at -20°C over night. The sample was then centrifuged at 13000 rpm for 15 min. The supernatant was removed by
pipetting; the pellet was washed with 70% ethanol twice and allowed to air dry. Double distilled water was added and the sample was kept overnight at 4°C and then stored at -20°C.

2.5. Primer selection:

Three sequence tagged sites (primer) at of AZFb region on chromosome Y at q arm (long arm) were selected. Internal control known as sex-determining region on Y chromosome (SRY) at p arm (short arm) were also selected according to Vogt et al. (1996). Shown in (table 2.1).

<table>
<thead>
<tr>
<th>STS</th>
<th>REGIONS</th>
<th>SEQUENCE</th>
<th>PRODUCT SIZE</th>
</tr>
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<tr>
<td>sY 14</td>
<td>SRY</td>
<td>F-5’GAATATTTCCCGCTCTCCGGA3’ R-5’GCTGCTGCTCCATATTCTTTGAG3’</td>
<td>472 bp</td>
</tr>
<tr>
<td>sY127</td>
<td>AZFb</td>
<td>F-5’GGCTGAGCTGAGCATGTACTTTTCGAGGA3’ R-5’GCA GCA GTA ATA AGG GA3’</td>
<td>274 bp</td>
</tr>
<tr>
<td>sY128</td>
<td></td>
<td>F-5’GGATGAGACATTTTCTGAGG3’ R-5’GCCCAGATGTAAACTGGACA3’</td>
<td>228 bp</td>
</tr>
<tr>
<td>sY134</td>
<td></td>
<td>F-5’TGC TGC TGC ACC ATA AAA CG3’ R-5’ACC ACT GCC AAA ACT TTC AA3’</td>
<td>301 bp</td>
</tr>
</tbody>
</table>

2.6. Polymerase Chain Reaction (PCR):

Polymerase chain reaction was carried out using four primers. The master mix was prepared to total volume of 25 µl. Buffer (10x) was added to a PCR tube, Mg++ and dNTPs are also added to the tube. Then the four primers were added. Tag (10 µ/l) polymerase was added. Then Double Distilled water was added. A number of PCR tubes were marked and DNA from each sample was added. Then the master mix was added to the PCR tubes. Finally the PCR tubes was inter the PCR machine. The PCR initiated with 94° C for 5 minutes followed by 45 cycle characterized by 94° C denaturation temperature for 45 seconds, 72° C
extension temperature for 1 minute, and 72° C annealing temperature for 45 seconds. Then the final extension temperature was 72° C for 5 minutes. PCR performed in 0.2 mL PCR sterile tubes for each individual sample. The PCR product was prepared to analyze by the Gel electrophoresis technique.

2.7. Gel electrophoresis:
The gel was prepared using agarose gel (2%), TBE buffer (10%) and distilled water, mixed well and put in the water bath for 10 minutes. Ethidium Bromide was added, and then the mixture allowed being cool at the room temperature. The DNA and Bromophenol Blue was mixed well and loaded in to the well. The gel was placed on the electrophoresis tank to be submerged by the running buffer then the tank was connected to power supply for 30 min.
Chapter Three: Results

4.1. Semen analysis results:

Twelve study participants' males agreed to participate in this study. Three study participants have sperm count of zero sperms/mL, so diagnosed as azoospermic. Nine study participants have sperm count between 1< and 20×10^6 sperms/mL, so diagnosed as oligozoospermic. table2.2.

<table>
<thead>
<tr>
<th>Site</th>
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<tr>
<td>Alsir Abu Alhassan Fertility Center</td>
<td>1</td>
<td>Azoospermic</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Azoospermic</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Azoospermic</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Oligozoospermic</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Oligozoospermic</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Oligozoospermic</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Oligozoospermic</td>
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<td></td>
<td>8</td>
<td>Oligozoospermic</td>
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<tr>
<td></td>
<td>9</td>
<td>Oligozoospermic</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Oligozoospermic</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Oligozoospermic</td>
</tr>
</tbody>
</table>
4.2. AZF subregions microdeletion results:

Diagnostic band of SRY gene (internal control) was detected in six study participants (out of twelve) 50% at 472 bp.
AZF b microdeletions (sY128, sY134) were detected in four study participants (out of twelve)33.3%
AZFb (sY127) microdeletions were detected in two study participants 16.7% (out of twelve).

Figure (2.3): Ethidium bromide stained agarose gels for detecting classical AZFb microdeletions. {(1-12): samples, 13: +ve control, 14: -ve control}. 
Chapter four: Discussion and Conclusion

In the present study, semen analysis has revealed that 25% were azoospermic and 75% were oligospermic.

In the present study, four STSs were used and microdeletions of AZFb subregion were detected in eight study participants (out of twelve) 66.7%. A prevalence in different geographical regions of 0.75% of Y chromosome microdeletion in 400 infertile men has been shown before, Medica et al. (2005) found Y chromosome microdeletions in 0.95% of 105 infertile males and van der Ven et al. (1997). Bor et al. (2002) found Y chromosome microdeletion in 0.98% of 204 infertile.

More intriguing, Kihalile et al. (2005) studied the occurrence of Y chromosomal microdeletions in two different populations, Japanese and Africans. They found Y chromosome microdeletion prevalence of 6.2% in Japanese, but absence of Y chromosome microdeletions in Africans. This may highlight the association of Y chromosomal microdeletions with certain Y chromosome haplogroups. Some Y haplogroups are more susceptible to deletions than others, while other Y chromosome haplogroups may confer protection against microdeletions.

In previous studies although the phenotypes associated with deletions are variable, and in general there is no clear correlation between the localization of the deletions (AZFa, b, or c) and the clinical phenotype (Forresta et al., 2001), the frequency of the Y chromosome deletion increases with the severity of the spermatogonic defect (Krausz et al., 2001). All the microdeletions likely to be related to azoospermia and oligozoospermia phenotypes, as Vogl, (2005a) and Behulova et al. (2011) did not record any microdeletion in the group of normospermic men.
The present study showed that, investigations on AZFb revealed that, microdeletion was found in azoospermic and oligozoospermic Sudanese subjects. Many studies agreed with these results (Behulova et al., 2011, Vogt et al., 1996, Reiho et al., 1995, Ferlin et al., 1999, Najmabadi et al., 1996, Pryor et al., 1997, Krausz et al., 1999, Girardi et al., 1997, Stuppia et al., 1997, Kim et al., 1999, Alkhalaif and Al-Shoumer, 2010).

To summarize, we suggest that the low prevalence of AZF classical deletions is influenced by ethnic factors, genetic background and Y chromosome haplogroups. Therefore, we strongly recommend conducting studies to analyze and then determine the distribution of Y-chromosome haplogroups in Sudan population. Moreover, other genetic, epigenetic, nutritional and environmental factors may be responsible for the oligozoospermia and azoospermia cases observed in Sudanese infertile males.

It is very important to increase the sample size of the researches on AZF loci microdeletion to confirm the association of the deletion with semen analysis. It is a need to identify the age, ethnic groups, nature of male work, family history of the males to detect if there is any cause of infertility other than Y microdeletion. Further investigation and evaluation of Endocrinological to find the effect of hormone levels in male infertility.

Another important test is Conventional cytogenetics (karyotyping) to describe the chromosomal constitution and give a comprehensive overview of genetic alterations of infertile male to identify the morphology of Y chromosome.
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