The prevalence of Y chromosome microdeletions among spontaneous recurrent pregnancy loss and infertile males in the Sri Lankan Sinhalese population

By

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# TABLE OF CONTENTS

ACKNOWLEDGEMENT ..............................................................................................v

DECLARATION ...........................................................................................................vi

ABSTRACT ..................................................................................................................vii

LIST OF TABLES .........................................................................................................ix

LIST OF FIGURES .......................................................................................................x

1. INTRODUCTION .....................................................................................................1

1.1. General overview .................................................................................................1

1.2. Spermatogenesis .................................................................................................6

1.2.1 Genetic Control of Spermatogenesis .................................................................8

1.3. Human Male Infertility .....................................................................................8

1.3.1. Immunological factors ....................................................................................9

1.3.2 Idiopathic male infertility ...............................................................................11

1.3.2.1. Genetic Causes of Male Infertility .............................................................11

1.3.3.1. Molecular causes of male infertility and RPL ........................................12

1.3.3.2. Chromosomal abnormalities .................................................................15

1.3.3.3. Sperm chromosomal abnormalities .........................................................15

1.3.4. Klinefelter's syndrome and variants ...............................................................16

1.3.5. Y chromosomal translocations ....................................................................18

1.3.6. Autosomal translocations .........................................................................18

1.3.7. Androgen Insensitivity Syndromes ............................................................19

1.4. The human Y chromosome ..........................................................................20

1.4.1. Evolution of the human Y chromosome .......................................................20

1.4.2. Structure of the human Y chromosome ....................................................23
1.4.3. Gene content of the human Y chromosome ................................................................. 24
1.4.4. Molecular mapping of the human Y chromosome .......................................................... 27
1.4.4.1. Non-coding and repetitive DNA .............................................................................. 29
1.4.5. Azoospermic factors and the Y chromosome ............................................................... 30
1.4.6. Other spermatogenesis genes at AZFc region ............................................................. 40
1.5. Mechanism of microdeletion ............................................................................................ 41
1.6. AZF genes as candidates for Azoospermia Factor ............................................................... 42
1.7. Assisted reproduction Techniques and male Infertility ....................................................... 42
1.7.1. The concern of assisted reproduction techniques ........................................................ 42
1.8. The diagnosis of Y chromosome micro deletion ................................................................. 43
1.9. Objectives ......................................................................................................................... 47

2. MATERIALS AND METHODS .......................................................................................... 48

2.1. Ethical concerns ............................................................................................................... 48
2.2. DNA resource .................................................................................................................. 49
2.3. Research participants and Inclusion criteria .................................................................... 49
2.4.1. Extraction using QIAamp® DNA Blood Mini kit ....................................................... 53
2.4.2. Quantification of extracted DNA .................................................................................. 54
2.5. Y chromosome micro deletion analysis by multiplex PCR .................................................. 54
2.5.1. Selection and designing of Primers .............................................................................. 54
2.5.2. PCR optimization ......................................................................................................... 55
2.5.2.1. Multiplex PCR analysis .......................................................................................... 55
2.5.2.2. Monoplex PCR analysis ......................................................................................... 58
2.5.3. Polymerase Chain Reaction components and conditions ............................................ 60
2.6. Agarose gel electrophoresis ............................................................................................. 63
2.6.1. Preparation of agarose gel ......................................................................................... 63
2.6.2. Detection of PCR amplified product ............................................................................ 64
2.7. Further classification of a microdeletion ................................................................. 65

2.8. Polymorphism analysis of sY156 region .................................................................... 67

2.8.1. Primer design ........................................................................................................ 67

2.8.2. DNA sequencing ................................................................................................... 67

2.8.3. Incorporation of additional STS loci to deleniate the deletion of sY156; sY 1086 and BPY2 .. 68

2.9. Measures of quality assuarance ................................................................................ 71

2.10. Software tools and electronic database information .............................................. 72

2.11. Statistical analysis .................................................................................................. 72

3. RESULTS ..................................................................................................................... 73

3.1. PCR optimization and Multiplex PCR ...................................................................... 73

3.2. Prevalence of microdeletions among Sinhalese population ..................................... 75

3.3. Follow up analysis of deletion at sY156 ................................................................... 75

3.3.1. Verfication of absence of a PCR amplified product as a true deltion or a polymorphism ............ 76

3.3.1.2. Amplification of sY156 with extended primers .................................................. 76

3.4. Patient data analysis .................................................................................................. 77

3.5. Summary of Y chromosome microdeletion analysis ................................................ 78

4. DISCUSSION ............................................................................................................... 79

4.1. Multiplex PCR Optimization .................................................................................. 80

4.2. Polymorphisms in genetic markers ........................................................................... 80

4.3. Importance of extensive Y microdeletion analysis .................................................. 81

4.3.1. Flanking STS loci analysis & sequencing ............................................................... 81

4.4. Effectiveness of Y chromosome microdeletion analysis .......................................... 81

4.4.1. Implications of microdeletion analysis on RPL males ........................................... 82

4.4.1.2. Implications on azoospermic men ..................................................................... 83

4.4.1.3. Other parameters effecting micodeletion detection rate ......................................... 84

4.4.1.4. Novel techniques beyond traditional PCR for microdeletion analysis ..................... 86
4.4.1.5. Implecation of scrutinised STS markers ................................................................. 87
4.4.1.6. Impact on homogenous sample population ............................................................ 88
5. CONCLUSION .................................................................................................................... 90
REFERENCES ....................................................................................................................... 92
APPENDIX 1: GENES AND ITS FUNCTIONS ....................................................................... 102
APPENDIX 2: LIST OF ABBREVIATIONS .......................................................................... 104
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DECLARATION

The work provided in this thesis, unless otherwise referenced, is the researcher’s own work.
ABSTRACT
The pathogenic relationship between the presence of Y chromosomal microdeletions and male infertility is unclear. Nevertheless, a causal relationship is thought to be probable when loci are shown to be deleted in infertile males but are present in fertile males. The successful mapping of the entire Y chromosome had resulted in the availability of a method of identifying and locating genes based on PCR analysis of specific sequence-tagged sites (STS).

Researchers in Sri Lanka have attempted to examine the association of Y chromosome microdeletions with infertility and recurrent pregnancy loss in Sri Lankan men using the multiplex PCR amplification system suggested in the state of the art recommendations made by the European Academy of Andrology/European Molecular Genetics Quality network (EAA/EMQN) guidelines. The patient population consisted of male partners of women experiencing RPL, oligozoospermic men and azoospermic men. None of the men in the RPL group had any microdeletions in the AZF regions or partial deletions in the AZFc region. In the azoospermic group one had a complete AZFc deletion and 2 others had a deletion at sY254, a sequence tagged site (STS) in the AZFc region. None of the men in the oligozoospermic group had deletions. The detection rate of deletions in the Sri Lanka population using the European recommendations appeared to be very low. Similar findings have been reported in India with the European recommendation. This has been addressed in a study done in India where Indian men with azoospermia were tested for Y chromosome microdeletions using a panel of novel STS markers. In this study, among 340 azoospermic men 82.8% had deletions in the AZFc region, followed by 55.2% in the AZFb region, and 24.1% in the AZFa region. In the light of the above
findings, in the current study, the above mentioned Sri Lankan samples were tested using the Indian panel of STS markers reported by Thangaraj and colleagues. Of the 50 RPL males and 50 oligozoospermic men neither of the groups had Y microdeletions. Among azoospermic men 80% had deletions at sY156 which belongs to AZFc region which was recommended by the Indian study. Complete or partial deletions were present among azoospermic men in the sY156 region. Follow-up analysis on patients absent for deletion in the sY156 loci showed that proximal and distal markers within the same region (BPY2 and sY1086) were also present. Sequencing the region flanking and including the sY156 did not revealed polymorphisms. Y specific genes found within the AZF locus such as DFFRY and BPY2 remained intact in all patients.

In conclusion none of the STS markers in the panel were deleted in men whose partners experienced RPL, however, the marker at sY156 was deleted in 80% of infertile men with azoospermia. This suggest that adding the sY156 marker to the EAA/EMQN panel would increase the diagnostic yield for Y chromosome microdeletions in men with azoospermia in Sri Lanka.

**Keywords:** azoospermia, microdeletions, male factor infertility, multiplex polymerase chain reaction, recurrent pregnancy loss, sequence tag sites (STS), Y chromosome
LIST OF TABLES

Table 1.1. Factors associated with male infertility along with their distribution .................. 10
Table 1.2. A summary of recommended tests for indications associated with infertility.......13
Table 1.3. Human Y genes with known functions mapped in AZF deletion interval ..........102
Table 2.1. lower reference limits for semen characteristics according to WHO guidelines....51
Table 2.2. General guidelines for optimization of a multiplex PCR ................................57
Table 2.3. Multiplexes analysed and their relative locations.............................................59
Table 2.4. Sequence tagged sites used for microdeletion analysis ..................................62
Table 2.5. AZFc partial deletion patterns and their inference ..........................................66
LIST OF FIGURES

Figure 1.1. Spermatogenesis ................................................................. 7
Figure 1.2. Genetic etiologies of human male infertility ......................... 14
Figure 1.3. A proposed path of the evolution of human Y chromosome .......... 22
Figure 1.4. Schematic representation of important regions in the Y chromosome .......... 23
Figure 1.5. Active genes on the human Y chromosome .......................... 26
Figure 1.6. The cytogenetic bands and deletion interval map ...................... 28
Figure 1.7. Schematic representation of human Y chromosome AZFa region .......... 33
Figure 1.8. Schematic representation of human Y chromosome AZFb region .......... 34
Figure 1.9. Schematic view of AZFb and AZFc deletion intervals .................. 39
Figure 1.10. Schematic representation of human Y chromosome AZFb region .......... 40
Figure 1.11. Detailed analysis of an STS loci ........................................ 46
Figure 2.1. Detailed map of Y chromosome ........................................... 59
Figure 2.2. Physical position of the STS loci used to detect the partial AZFc deletion ...... 66
Figure 2.3. A screen shot of the USCS genome browser ........................... 69
Figure 2.4. A screen shot from MSY Breakpoint Mapper ............................ 70
Figure 3.1. Agarose gel photograph of electrophoresed PCR multiplex products ........ 74
1. INTRODUCTION

1.1. General overview

According to World Health Organizations (WHO) definition health does not merely mean the absence of disease or infirmity, it’s a state of complete physical, mental and social well being. In this context reproductive health addresses the reproductive processes, functions and system at all stages of life. Reproductive health is a universal concern for both men and women. Because reproductive health is such an important component of general health it’s a prerequisite for social, economic and human development. As healthy and active population in contrary to sick and tired people becomes a prerequisite of social and economic development it is important to address the issues curtailing for a healthy population. Infertility and Recurrent Pregnancy Loss (RPL) among married couples are such issues with a worldwide occurrence (Fernandez Pelegrina et al. 1991).

Sri Lanka’s fertility levels began to decline even before policy decisions were made to introduce family planning at the national level during early 1960s (Sri Lanka Health and Demographic survey,2000). During next four decades a steady decline was observed in crude birth rate (CBR) and total fertility rate (TFR) in the country. The CBR declined from 36.6 per thousand of the population in 1960 to 18.4 in 2004. Similar trends were seen in total fertility rates. Four factors have been identified as the most important determinants of Sri Lanka’s falling rate, which include proportion married, contraception, post partum infecundability, and induced abortions. There are likely to be substantial long term implications associated with declining fertility. One of the major consequences of low fertility is changes in the age structure of the population, which will undoubtedly have an impact on Sri Lanka’s socio-economic and development activities. According to the standard projections made by De
Silva et al. regarding the future course of fertility, it is likely that Sri Lanka’s TFR will continue to fall about 1.49 children per women by 2030. In due course even though some of the traditional family values have changed, for the average Sri Lankan couple having a child is still a very important necessity. The pressure applied by the family, further increases this need. Thus, inability to do so therefore leads to great personal as well as social trauma. The long standing myths that are present in our society such as the female partner is always the cause of infertility and its association with general misfortune deepens the tragedy.

There is limited information on the problems of infertility due to the paucity of will conducted studies. The only community based study aimed at studying the prevalence and risk factors for primary and secondary infertility was carried out in 1994 in the district of Colombo. This study used WHO definitions and according to these criteria, prevalence of primary infertility was found to be 4.1 percent with secondary infertility being 16.1 percent. According to the perceptions of the care providers, there has been a definite increase in the number seeking treatment for this problem in the last few decades. In the community based study referred to, the significant risk factors for primary infertility among women seem to be similar to those reported from other countries. These factors include woman’s current age being high, younger age at menarche, women being employed, absence of dysmenorrhoea and irregular menstrual cycles. Care providers perceive that there is an increase in the incidence of problems among males specially those related to quantity and quality of sperms. In this study other factors suggested as linked to male infertility (genetic etc.) had not been investigated. There is a dearth of information, literature refering genetic contributions to male infertility and RPL cases in Sri lanka. The present study is to investigate the relationship between male factor infertility and recurrent pregnancy loss in relation with microdeletions in the Y chromosome.
Among married couples not all are fortunate for a conception or a conception that do not result in a delivery of a baby. Probably no aspect of infertility is more traumatic than becoming pregnant and then losing the baby to miscarriage. Worldwide, couples view infertility as a tragedy which carries social, economic and psychological consequences. About 15 percent of all couples attempting pregnancy are unable to conceive naturally (Fernandez Pelegrina et al. 1991). The WHO has defined infertility as a period of one year without conception. Infertility is either primary, when no pregnancy has ever occurred, or secondary where there has been a pregnancy regardless of the outcome. Infertility can be permanent (irreversible) or sub fertility which means the probability of spontaneous conception may be decreased. All men who are sterile would be considered infertile, but not all men who are infertile are sterile, because an infertile man can father a child with medical help or with simple change in his life style (Winston 1991). Male factor infertility accounts for approximately 50 percent of all infertile cases (Meschede et al. 2000). Most cases of male infertility can be attributed to sperm production resulting in low sperm count and/or poor motility. These can caused by varicocele, obstruction of the spermatic ducts, agglutination of sperm, high semen viscosity, necropermia, low volume of ejaculation, impotency and high sperm density (Greenberg et al. 1978). When no cause is known the man is described as having idiopathic infertility. Consequently the estimated proportion of men with idiopathic infertility comprises 15 to 24 percent (Ferlin et al. 2007). 20 to 25 percent of cases of infertility among men are due to variation on semen profile (McLachlan et al. 1998). According to the semen profile there are four (4) major categories namely oligozoospermia (low sperm count), asthenozoospermia (low motility), teratozoospermia (abnormal shape and size) and azoospermia (complete absence of sperm). Male infertility is manifested as quantitative abnormality (azoospermia, cryptozoospermia and oligozoospermia), or as qualitative abnormality (asthenospermia, teratozoospermia and necropermia) or both.
(Hargreaves 2000; Ferlin et al. 2007). There are numerous known causes of male infertility which can be grouped into several major categories. The etiologies of male infertility can be associated with several genetic and non genetic conditions. Genetic causes comprises of microdeletion of the Y chromosome, Mitochondrial DNA mutations, monogenic disorders and multifactorial disorders. Hormonal causes, Hypogonadotrophic hypogonadism, Impotency, Previous scrotal or inguinal surgery, Varicocele, Exposure to chemicals, Environmental factors and Immunological factors comprises non genetic conditions. Genetic factors have been found to play a role in about 10 percent of male infertility (Vogt et al. 1996).

Recurrent Pregnancy loss (RPL) is another aspect of infertility. It is estimated that 1 to 3 percent of pregnancies terminate by recurrent miscarriages (Dhont 2003). Pregnancy loss is common and in most cases it can be considered as natures method to select for a genetically normal offspring. RPL only adds more questions and concerns for couples battling infertility and trying to give birth to a healthy baby. The aetiology of spontaneous RPL is multifactorial. The male factor i.e. sperm quality, chromosomal anomalies and paternal age, is rarely discussed and has been poorly evaluated in RPL. A parental carrier of a structural chromosome rearrangement i.e. reciprocal or robertsonian translocation is associated with a history of RPL in approximately 3.5 percent of couples (Chandley et al. 1975; Elliott et al. 1997; Stephenson et al. 2002). Recurrent pregnancy loss (RPL) is distinct from infertility, defined by 2 or more failed pregnancies (Coulam 1992). Seminal studies by Boue et al. and Hossold et al. has postulated that 50 percent of clinical abortions result from chromosomal abnormalities. When the cause is unknown, each pregnancy loss merits careful review to determine whether specific evaluation may be appropriate. The incidence of pregnancy loss among all women is about 20 percent. Statistics show that if the first pregnancy ended in a miscarriage the second has similar possibility of the same outcome, after 2 miscarriages, however the risk rises to 25
to 30 percent, so only about 4 percent of women will experience 3 or more consecutive miscarriages (Empson et al. 2002). For this reason most experts recommend that a woman see a fertility specialist if she has experienced two miscarriages in a row. Most clinicians focus their evaluation of RPL on the female, without much consideration of the male. Recurrent literature demonstrates that the male contributes to RPL due to genetic factors, semen factors or due to other factors such as age (Homonnai et al. 1980; Hill et al. 1994; Hill et al. 1994; Sbracia et al. 1996). A study by Saxena et al. showed reduction in test scores of sperm function such as hypooosmotic swelling (HOS), acrosomal status (AS) and nuclear chromatin decondensation (NCD) in male partners of couples with idiopathic RPL suggests that sperms with altered or lowered competencies, upon fertilization may lead to development of an unstable embryo resulting in early pregnancy loss. Genetic causes of recurrent miscarriage are rare but identification of a genetic cause may have important risk implications for future children, parents and other relatives. In a pilot study to evaluate the association between Y chromosome microdeletions and RPL, reported a significant proportion (82%) of 17 male partners of women with RPL had Y chromosomal microdeletions primarily in the AZFc region. This may have been the reason for the RPL experienced by the male partners. The above study was criticized because of several shortcomings identified in the experimental design and interpretation of the results. A similar study reported 16% of men with RPL had microdeletions in the AZF region of the Y chromosome. In contrary a similar study done in the Finnish population had concluded that Y chromosome microdeletions were not associated with RPL in the population. The above study was conducted according to guidelines provided by the European Academy of Andrology (EAA) / European Molecular Genetics Quality Network (EMQN) for diagnostic laboratories, with the use of six sequence tagged sites (STSs) which has a probability to detect up to 95% of all reported Y microdeletions in the AZF region. A recent study carried
out by Sri lankan scientists on Y chromosome microdeletions on male partners of women experiencing RPL found that none of the men in the population had microdeletions (Wettasinghe et al. 2010). The study used the multiplex PCR amplification system suggested in the state of art recommendations made by the EAA/EMQN (Simoni et al. 2004).

1.2. Spermatogenesis

Spermatogenesis is a long and complex process requiring about 70 days and involving a series of continuous cellular changes of distinct cell types generated by mitotic and meiotic cell divisions. The process of spermatogenesis can be divided into three major stages 1) mitotic proliferation of spermatogonia 2) meiosis and 3) spermiogenesis (Figure 1.1) (Clermont 1966; Clermont et al. 1970). In the initial stages, spermatogonia divide via mitoses, giving rise to primary spermatocytes, which in turn undergo the first meiotic division leading to secondary spermatocytes. Through the second meiosis these cells produce haploid cells (round spermatids) which elongate during the spermiogenesis process (elongated spermatids) and finally differentiate into mature spermatozoa, by condensation of the chromatin, substitution of histones with protamines and formation of the acrosome and the other sperm components. However our knowledge of the mechanisms regulating spermatogenesis is still poor, and only recently has research focused on the identification of genes specifically involved in its regulation.
Figure 1.1. Spermatogenesis
1.2.1 Genetic Control of Spermatogenesis

Although the developmental process of spermatogenesis has been extensively studied and clearly described our knowledge of the genetic control of spermatogenesis remains very limited. From the genetic point of view the process of spermatogenesis can be assumed to be regulated by orchestrated co-ordinated expression of many genes. For instance in mice there are many genes involved in spermatogenesis (Matzuk et al. 2002). Mutation of the genes involved in male reproduction affects specific testicular cell types and reproductive function resulting in male infertility or subfertility in the mouse. In man, both structural and numerical chromosomal abnormalities and mutations of many genes can result in the disruption of spermatogenesis. This happens especially in the testis specific genes found along the human Y chromosome.

1.3. Human Male Infertility

Human infertility can result from a variety of factors. In 1987 the WHO established a Task Force on the Diagnosis and Treatment of infertility with the objective of creating a standard protocol for the investigation of infertile couples. Normal semen was classified as containing a sperm concentration of at least $20 \times 10^6$ spermatoza/ml of which more than 40% are progressively motile more than 60% are alive and over 50% show normal morphology. In addition the semen should not contain more than $1 \times 10^8$/ml of white blood cells (Comhaire et al. 1987). Human infertility is a major health problem affecting about 15% of couples seeking to have children and a male factor can be identified in about half of these cases. A
significant proportion of infertile men are affected either by oligozoospermia (reduced sperm production) or azoospermia (lack of any sperm in the ejaculate). About 1 out of 1000 human males is infertile owing to spermatogenic failure (Hull 1985). The cause of infertility in these men may be related to different underlying testicular histological pathologies ranging from the complete absence of germ cells (Sertoli cell-only syndrome) hypospermatogenesis and maturation arrest. The spermatogenic failure can be the consequence of systemic diseases, endocrinological disorders, obstruction or absence of seminal pathways, or infections. Nevertheless up to 50% of the cause of male infertility is idiopathic (Foresta et al. 2005) and in some individuals are due to genetic defects. In 50% of involuntarily childless couples, a male infertility associated factor is found together with abnormal semen parameters. A fertile partner may compensate for the fertility problem of the man and thus infertility usually becomes manifest if both partners have reduced fertility. Male fertility can be reduced as a result of congenital or acquired urogenital abnormalities, urogenital tract infections, increased scrotal temperature (e.g. as a consequence of varicocele), endocrine disturbances and genetic abnormalities.

1.3.1. Immunological factors

In 30-40% of cases, no male infertility associated factor is found (idiopathic male infertility). These men present with no previous history of fertility problems and have normal findings on physical examination and endocrine laboratory testiong. However semen analysis reveals a decreased number of spermatozoa (oligozoospermia), decreased sperm motility (asthenozoospermia) and many abnormal forms of sperm (teratozoospermia).
These sperm abnormalities usually occur together and are called oligo-asthenoteratozoospermia (OAT) syndrome. Table 1.1 summarises the main male infertility-associated factors. Idiopathic male infertility may be explained by several factors, including endocrine disruption as a result of environmental pollution, reactive oxygen species or genetic abnormalities.

<table>
<thead>
<tr>
<th>Male infertility associated factor</th>
<th>Distribution%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idiopathic male infertility</td>
<td>31</td>
</tr>
<tr>
<td>Maldescended testes</td>
<td>7.8</td>
</tr>
<tr>
<td>Urogenital infection</td>
<td>8.0</td>
</tr>
<tr>
<td>Disturbances of semen deposition and sexual factors</td>
<td>5.9</td>
</tr>
<tr>
<td>General and systemic disease</td>
<td>3.1</td>
</tr>
<tr>
<td>Varicocele</td>
<td>15.6</td>
</tr>
<tr>
<td>(Endocrine) Hypogonadism</td>
<td>8.9</td>
</tr>
<tr>
<td>Immunological factors</td>
<td>4.5</td>
</tr>
<tr>
<td>Obstructions</td>
<td>1.7</td>
</tr>
<tr>
<td>Other abnormalities</td>
<td>5.5</td>
</tr>
</tbody>
</table>

**Table 1.1.** Factors associated with male infertility along with their distribution
1.3.2 Idiopathic male infertility

Idiopathic male infertility is often associated with genetic and epigenetic abnormalities. The possibility that many cases of idiopathic male infertility might have genetic background was bolstered by the failure of most clinical therapies to correct deficient spermatogenesis. Genetic causes account for 10-15% of severe male infertility cases. Such abnormalities include chromosome translocations and aneuploidies, Y chromosome microdeletions and androgen receptor gene mutations. Numerous other genes are likely to be associated with male infertility. Initial reports from human studies have identified several candidate genes, including Protamine-1 and Protamine-2 genes, DAZL1, SPO11, EIF5A2, USP26 and others. The genetic causes are either detected at the cytogenetic and/or at the molecular levels. In addition to gene mutations and polymorphisms, damage to the chromatin resulting in single and double strand DNA breaks affects male fertility. Epigenetic abnormalities such as gene imprinting may also contribute to male infertility.

1.3.2.1 Genetic Causes of Male Infertility

Now a days many couples seek fertility treatment. Having a good understanding of genetic abnormalities in infertility will help the practitioner as well as the counsellor to provide correct advice to such couples. Men with very low sperm counts can be given a reasonable chance of paternity, using in vitro fertilisation (IVF), intracytoplasmic sperm injection (ICSI) and sperm harvesting from the epididymis or the testis in case of azoospermia. However the sperm of infertile men show an increase in aneuploidy, other genetic abnormalities and DNA damage and carry the risk of passing genetic abnormalities to the next generation. Although there are
prospects for screening of sperm (Griffin et al. 2005; Carrell 2008), current routine clinical practice is based on screening peripheral blood samples.

Human male infertility can be caused by:

1) Chromosomal aneuploidies and rearrangements between sex chromosomes, between sex chromosome and an autosome and between autosomes

2) Androgen insensitivity syndromes

3) Submicroscopic microdeletions of spermatogenes inYq11

1.3.3.1. Molecular causes of male infertility and RPL

The advancement of assisted reproductive techniques (ART) has emphasized the necessity for clinicians to recognize the role that genetics play in male factor infertility cases as because new technologies such as intracytoplasmic sperm injection (ICSI), allow men with suboptimal sperm quality to overcome natural selection mechanisms and produce a viable zygote (Ferlin et al. 2007). Some andrologists have voiced concern about concealing reproductive defects through ART that might have negative consequences at the epigenetic level (De Rycke et al. 2002; Thompson et al. 2002). Thus development of techniques to test for genetic abnormalities or unfavourable polymorphisms before performing ART is critical. Consequently it is necessary to determine the underlying genetic basis of male factor infertility to develop appropriate screens for abnormal phenotypes and to discover more effective solutions for infertile couple’s problems. Abnormalities in the genetic message passed on through ART can have serious implications for the developing zygote (Carrell 2008). On the other hand social factors such as late marriage and late child bearing have resulted both women and men recognizing their infertility and recurrent pregnancy loss in their
late 30s. Therefore considering the current social situation it is necessary to have a pre-evaluation on one's own genetic constituent in advance.

Genetic abnormalities have been identified in men with unexplained oligozoospermia and azoospermia including numerical and structural chromosomal abnormalities. Genetic factors involved in male infertility manifest as chromosomal disorders, mitochondrial DNA (mtDNA) mutations, monogenic disorders, multifactorial disorders and endocrine disorders of genetic origin. Table 1.2 summarises the indications and recommended tests for genetic evaluations (Esteves et al. 2011)

<table>
<thead>
<tr>
<th>Indications</th>
<th>Recommended tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men with infertility of unknown etiology and sperm concentration &lt;10 million.ml who are candidates for ART</td>
<td>Y chromosomemicrodeletion and G-band karyotype</td>
</tr>
<tr>
<td>Non-obstructive azoospermia in a male considering testicular sperm retrieval for ART</td>
<td>Y chromosome microdeletions and G-band kartotyping</td>
</tr>
<tr>
<td>Azoospermic or oligozoospermic men with the absence of at least one vas deferens at physical examination</td>
<td>CFTR gene mutation analysis</td>
</tr>
<tr>
<td>Azoospermic men with signs of normal spermatogenesis (e.g., obstructive azoospermia of unknown origin)</td>
<td>CFTR gene mutation analysis</td>
</tr>
<tr>
<td>History of recurrent miscarriage or personal/familiar history of genetic syndromes</td>
<td>G-band kartotyping</td>
</tr>
</tbody>
</table>

Table 1.2. A summary of recommended tests for indications associated with infertility
Figure 1.2. Genetic etiologies of human male infertility. Developmental disorders causing human male infertility result from failure of gonadal development of testis determination, endocrinopathies, well known genetic syndromes, and numerical and structural chromosomal abnormalities.
1.3.3.2. Chromosomal abnormalities

The association between chromosomal abnormalities and male infertility was first reported by Chandely. Chromosomal abnormalities can be numerical (e.g., trisomy) or structural (e.g., inversions or translocations). The incidences of sex chromosomal and autosomal abnormalities in infertile men were observed to be 15~ and 6~ fold greater respectively than those found in the general population. In a survey of pooled data from 11 publications reporting on 9766 infertile men seeking fertility treatment there was an incidence of chromosomal abnormalities of 5.8%, the sex chromosomal abnormalities accounted for 4.2% and autosomal abnormalities account for 1.3% (Johnson 1998).

Based on the frequencies of chromosomal aberrations in patients with different sperm concentration, karyotype analysis is indicated in azoospermic men and in oligozoospermic men with <5 million spermatozoa/ml (Vincent et al. 2002). If there is a family history of recurrent abortions, malformations or mental retardation, karyotype analysis should be requested, regardless of the sperm concentration.

1.3.3.3. Sperm chromosomal abnormalities

Sperm can be examined for chromosomal normality using multicolour fluorescent in situ hybridisation (FISH). Aneuploidy in sperm, particularly sex chromosome aneuploidy, is associated with severe damage to spermatogenesis (Sbracia et al. 1996; Van Assche et al. 1996; Machev et al. 2005) and is also seen in men with translocations. FISH analysis of spermatozoa needs techniques to separate populations of genetically abnormal sperm from normal sperm or to safely screen individual spermatozoa before IVF and ICSI. It should be
used to assess spermatozoa from men with defined andrological conditions (Tempest et al. 2009).

### 1.3.4. Klinefelter’s syndrome and variants

Klinefelter’s syndrome represents one of the most common cause of azoospermia (De Braekeleer et al. 1991; Van Assche et al. 1996; Okada et al. 1999). In the majority of cases the syndrome is characterized by a 47,XXY chromosome complement. Variants such as 47,XXY/46,XY mosaicism; 48,XXXY; 48,XXYY; and 49,XXXXY have been reported to manifest Klinefelter’s symptoms (Bielanska et al. 2000). Klinefelter patients with increasing number of chromosomes in their karyotype shift their sexual phenotype to the female site. This suggests an X chromosomal dosage effect on the male’s gonad development (Vogt 2004). In male infertility the 47 XXY karyotype is found in 11% of azoospermic and 0.7% of oligozoospermic men. Men with Klinefelter mosaicism 46,XY/47,XXY have severely reduced levels of germ cells in their testes. The production of 24,XY sperm has been reported in 0.9% to 2.1% of men with Klinefelters mosaicism and in 1.4 to 25% of men with somatic karyotype 47,XXY. However rare cases of fertility and proven paternity have also been reported amongst oligozoospermic Klinefelter patients.

**47,XXY**

Men with 47,XXXY karyotype occur frequently (1:750) but their fertility status is variable. If infertile they are usually oligozoospermic. Although the normal formation of the X-Y pairing complex in the germ cells is inhibited resulting in infertility, the majority of spermatozoa are normal.
45,X/46,XY mosaic or mixed dysgenesis

Human male infertility is often related to chromosome abnormalities. Klinefelter syndrome (XXY) and specific translocations are well established causes of male infertility (Foresta et al. 2005). Two important gene defects conclusively associated with spermatogenic failure are point mutations in the androgen receptor and the cystic fibrosis transmembraneconductance regulator (CFTR) gene commonly associated with congenital vas deference abnormalities. The Y chromosome is an obvious area of interest in the study of male factor infertility because it contains many of the genes that are critical for spermatogenesis and the development of male gonads (Reynolds et al. 2005). Apart from some nuclear genes mitochondria have their own genome capable of producing many essential components of the respiration chain that have a profound impact on sperm motility. The quality and quantity of sperm production may be affected greatly by both environmental and genetic factors. Sperm mitochondria play an important role in spermatozoa because of the high adenosine triphosphate (ATP) demand of these cells. There are over 50 monogenic disorders associated with male infertility. Monogenic genetic disorders occur as a direct consequence of a single gene being defective. Such disorders are inherited in a Mendelian pattern.
1.3.5. Y chromosomal translocations

Translocation of terminal portions of the short arms of X and Y chromosomes result in an X chromosome with translocated SRY. This result in a 46,XX male, occurring in 1:20,000. In these men a 46,XX genotype is associated with male gonadal differentiation resulting in intersexual phenotypes i.e. hypospadias, micropenis and cryptorchidism. They will be sterile because of the loss of Yq that includes the spermatogenesis genes in the AZF locus (Vogt et al. 1996).

Y chromosome DNA can also be translocated onto autosomes. Autosomal translocation of Y chromosome are observed in balanced or nonbalanced karyotypes. These men have normal external genitals or hypogonadism. In most familial cases, the distal heterochromatin of Yq(Yq12) is translocated to the short arm of an acrocentric chromosome and the Ychromosome is broken in Yq11 where the spermatogenesis genes are localized (Smith et al. 1979) resulting in azoospermia. Translocations with the breakpoint in the Yq12 do not result frequently in severe spermatogenic failure. However improper pairing and segregation of the chromosomes during mitosis and meiosis may result in a mildly reduced spermatogenesis (Thielemans et al. 1998).

1.3.6. Autosomal translocations

Chromosomal aneuploides and translocations between autosomes (Robertsonian and reciprocal) were found less frequent than sex chromosomal abnormalities. Instead, structural aberrations (translocations and inversions) are found 10 times more frequently in infertile men than in fertile men(Vogt 2004). Pericentric inversions in chromosome 1,3,5,6 and 10 seem to interfere with meiosis leading to a reduced rate of post-meiotic sperm numbers or
even azoospermia. Acrocentric chromosomes 13 and 14 were involved in about two-thirds of the cases of Robertsonian translocation (Vogt 1995).

1.3.7. Androgen Insensitivity Syndromes

The androgen receptor (AR) is a ligand-activated, intracellular transcriptional regulator (Zhou et al. 1994) that belongs to the steroid receptor superfamily (Gronemeyer et al. 1995; Mangelsdorf et al. 1995). Androgens are cholesterol derivatives. Like other steroid hormones they serve as signaling molecules to coordinate the expression of complex gene programmes in higher eukaryotes. Steroid hormones work by binding to intracellular receptors which themselves orchestrate the transcriptional response. Failure of the AR to activate genes in the presence of androgen results in target organ resistance to androgen. This phenomenon known as androgen insensitivity syndrome (AIS) causes abnormal sexual development in 46,XY individuals.

The AR gene is located on the long arm of the X chromosome (Xq11-12) along 90Kb. Complete or partial deletions almost always seriously disrupt AR function, resulting in complete androgen insensitivity syndrome. Other aberrations involve insertions or deletions of several nucleotides in length. Complete AIS is inevitable if these mutations cause frame shifts, which results in premature translation termination. Point mutations such as missense, nonsense or splice mutations, can result in complete partial or minimal AIS. Specific point mutations associated with male infertility have been found in the transactivation domain (TAD) and androgen-binding domain (ABD) region of the AR gene (Ghadessy et al. 1999; Yong et al. 2000).
1.4. The human Y chromosome

1.4.1. Evolution of the human Y chromosome

The Y chromosome, one of the smallest human chromosome (approximately 60MB), is the male-specific chromosome that has a very important function in sex-determination. Genes found along the Y chromosome are required for sexual development and spermatogenesis. The human Y chromosome is mostly devoid of genes, while the X chromosome, its meiotic pairing partner contains several thousand genes (Graves 1995). The Y chromosome is probably the most bizarre part of the human genome, reflecting its unique status as a huge block of largely non-recombinating DNA, maintained in a permanently heterozygous state and transmitted solely through males (Brown et al. 1983). Comparative studies strongly suggest that the X and Y chromosomes in mammals are descended from a homologous pair of autosomes (Graves 1995; Higgs et al. 1996) and that the Y had lost most of the genes that it once contained, in response to its lack of recombinational exchange with the X and its permanent heterozygosity (Higgs et al. 1996; Rice et al. 1999). This hypothesis is further supported by the existence of regions of homology between the two sex chromosomes (Lahn et al. 1999).

The evolution of the human sex chromosomes from a pair of autosomes involves a series of steps (Lahn et al. 1999) (Figure1.3). Consider a sex determining locus, SRY, located on a proto-sex chromosome. Genes linked to the sex-determining locus may acquire sexually antagonistic functions. Selection will then favour a reduced recombination rate between the sex determining locus and sexually antagonistic genes. In the absence of recombination over most of the Y chromosome, mutations will accumulate rapidly and degeneration of the non-recombining region will occur fast (Charlesworth et al. 2000). Degradation of the Y
chromosome requires dosage compensation between the sexes to restore equality of the dosage of gene products from X-linked loci in males and females. This is because dosage differences may be deleterious for many genes (Charlesworth 1996). In humans and other eutherian mammals dosage compensation is achieved by the inactivation of one of the two X chromosomes in females. By comparing X-Y nucleotide divergence at 19 homologous genes located in the non-recombining region of the X and Y chromosomes, for ‘evolutionary strata’ were identified along the human X chromosome. Within each stratum, the number of copies of genes in X and Y chromosomes differ by almost an equal amount should indicate that recombination has ceased at the same time for all the genes in question. However the different groups of X and Y copies of genes indicated that recombination ceased at different time points. Large inversions which are known to suppress recombination could account for this step wise pattern (Lahn et al. 1999).
Figure 1.3. A proposed path of the evolution of the human Y chromosome (Lahn et al. 1999). During the evolution it was postulated that human Y chromosome underwent four inversions, which suppressed recombination between the ‘proto’ sexchromosomes. Each inversion (designated 1-4) is thought to have reduced the size of the pseudoautosomal region (white) and enlarged the non recombining portions of the X (yellow) and Y (blue) chromosomes. Time points at which the human X and Y may have diverged from the sex chromosomes of other mammals are indicated.
1.4.2. Structure of the human Y chromosome

The human Y chromosome is made up of a short and a long arm, denoted as Yp and Yq respectively. Both Yp and the proximal portion of Yq consist of euchromatin, whilst the distal segment of Yq is composed of heterochromatin. This latter region generally varies in length and normally comprise between one-half to two-thirds of Yq. Therefore, Yq is cytogenetically dichotomized into euchromatic proximal region (Yq11) and a heterochromatic distal region (Yq12). The euchromatic short arm is denoted Yp11.

Figure 1.4. Schematic representation of important regions in the Y chromosome
1.4.3. Gene content of the human Y chromosome

The human Y chromosome is unique in its bipartite structure and function. Towards the telomeres it presents the pseudoautosomal regions (PAR1 and PAR2) that undergoes meiotic recombination with the X chromosome and thus ensure the proper segregation of X and Y during male meiosis. These pseudoautosomal regions differ in their sequential composition from the much larger non-recombining region on the Y (NRY). Skaletsky *et al.* (2003) renamed NRY to MSY (male-specific region of the Y chromosome) because they observed that the designation NRY did not reflect the dynamic evolutionary events occurring in this part of the Y chromosome. The euchromatic portion of MSY only encompasses 23Mb that contains 156 transcription units including 78 protein-coding genes that collectively encode 27 distinct proteins. The MSY is made up of three main gene groups. The first group is composed of X-transposed genes represented by two coding units approximately 99% identity to the corresponding X chromosome. The second group consists of 16 coding genes that are generally single-copy and expressed biquitously. These genes are involved in housekeeping cellular activities and are probably involved in functions other than male reproduction. The third group is formed of ampliconic multicopy class genes that encompasses approximately 10.2Mb distributed in Yp and Yq, which consists of large regions where gene copies show near absolute sequence identity (~99.9%) they are exclusively testis specific and are implicated mainly in spermatogenesis and male fertility.

PAR1 is located at the terminal region of the short arm (Yp) and the PAR2 at the tip of the long arm (Yq). PAR1 and PAR2 cover approximately 2600 and 320-kb of DNA respectively. The pseudoautosomal regions and in particular PAR1 are where the Y chromosome pairs and exchanges genetic material with the pseudoautosomal region of the X chromosome during
male meiosis. Consequently genes located within the PAR are inherited in the same manner as autosomal genes. The euchromatic region is distal to the PAR1 and consists of the short arm paracentric region the centromere and the long arm paracentric region. The heterochromatic region comprises distal Yq corresponding to Yq12. This region is assumed to be genetically inert and polymorphic in length in different male populations since it is composed mainly of two highly repetitive sequences families DYZ1 and DYZ2 containing about 5000 and 2000 copies of each respectively.

Whereas PAR1 and PAR2 represent the 5% of the entire chromosome the majority of the length of the Y (95%) is made by male specific region (MSY). This includes the euchromatic and heterochromatic regions of the chromosome. Whereas the heterochromatic region is considered genetically inert the euchromatic region has numerous highly repeated sequences but also contains some genes responsible for important biological functions.
Figure 1.5. Active genes on the human Y chromosome. Genes named to the right of the chromosome have active X chromosome homologues. Genes named to the left of the chromosome lack known X homologues. Genes in red are widely expressed housekeeping genes; genes in black are expressed in the testis only; and genes in green are expressed neither widely, nor testis specifically (AMELY (amelogenin Y) is expressed in developing tooth buds, whereas PCDHY (protocadherin Y) is expressed in brain). With the exception of the SRY (sex-determining region Y) gene, all the testis-specific Y genes are multicopy. Some multicopy gene families form dense clusters, the consistent loci of which are indistinguishable at the resolution of this map. Three regions often found deleted in infertile men, AZFa, b, c (azoospermia factor region a, b, c), are indicated (Lahn et al. 2001).
1.4.4. Molecular mapping of the human Y chromosome

The first attempts at mapping the Y chromosome were based on cytogenetically detectable deletions on this chromosome by chromosome banding patterns. These preliminary studies led for the first time to the hypothesis that a gene or genes located on Yq were related to spermatogenic failure (Tiepolo et al. 1976). Similar studies defined also a region associated with sex determination (Jacobs et al. 1966; Fraccaro et al. 1982).

The first molecular map of the human Y chromosome was proposed by Vergnaud et al. (1986) by using different Y-specific probes on patients with microscopically detectable Y chromosomal anomalies. They subdivided the Y chromosome into 7 intervals corresponding with naturally occurring deletions of this chromosome. Six years later a more precise deletion map of the Y chromosome based on the detection of about 200 sequence-tagged sites (STS’s) was constructed by Vollrath et al. (1992). The presence or absence of these STS’s on a large set of patients with a wide range of Y chromosomal anomalies subdivided the euchromatic region into 43 ordered intervals all defined by naturally occurring chromosomal breakpoints. This collection of ordered STS’s along the Y chromosome have been extensively used in order to define shortest deleted regions associated with particular phenotypes and then in identifying Y chromosomal genes and exploring the origin of Y chromosome disorders.

Finally a complete physical map of the Y chromosome was generated with 196 overlapping DNA clones by a library of yeast artificial clones (YAC) from a human XYYYY male which covered 98 percent of the euchromatic region (Foote et al. 1992). These physical maps of the human Y chromosome have certainly accelerated the search for new genes and made it much easier to explore the biology of this chromosome.
Figure 1.6. The cytogenetic bands and deletion interval map of human Y chromosome (Foresta et al. 2001)
1.4.4.1. Non-coding and repetitive DNA

One of the greatest challenges for mapping the human Y chromosome was presented by, NRY-specific amplified regions which comprise about one-third of the euchromatic NRY (Tilford et al. 2001). These euchromatic amplified regions are diverse in composition, size copy number and orientation with some occurring in tandem repeats others as inverted repeats and still others dispersed throughout both arms of the Y chromosome (Tilford et al. 2001). Most Y-specific genes are found in these amplified regions (Tilford et al. 2001).

Much of the human Y chromosome is composed of repetitive satellite DNA, the bulk of which is located in the block of heterochromatin in the Yq (Figure1.6). This heterochromatic region mostly consists of the 3.4-kb DYZ1 and the 2.5-kb DYZ2 repeat families which together account for about 50-70% of the DNA content of the human Y chromosome (Cooke et al. 1983). The heterochromatin region of the human Y chromosome is assumed to have no genes. However in the heterochromatic region of the Y chromosome of the Drosophila, several genes have been identified (Carvalho et al. 2000). This finding suggests that there may be some genes to discover in the heterochromatin of the human Y chromosome. Unlike the genes found in human Y chromosome all known functional genes in D.melanogaster Y chromosome have essential male specific functions and all are believed to be single-copy (Carvalho et al. 2000).
1.4.5. Azoospermic factors and the Y chromosome

The role of Y chromosome in male infertility was first elucidated in 1976 when Tiepolo and Zuffardi proposed the existence of a key factor which controls spermatogenesis encoded by a gene that is localized within the euchromatic region of the Y chromosome long arm (Yq11) named as azoospermica factor (AZF) because the first six men observed with microscopic terminal deletions in Yq by routine karyotyping were azoospermic (Tiepolo et al. 1976). Of all the genetic factors, Y chromosome microdeletions are a frequent cause of infertility in males. A microdeletion is defined as a chromosomal deletion that spans several genes but not large enough to be detected using conventional cytogenetic methods. Studies have revealed that microdeletions are more prevalent in men who are azoospermic and severely oligozoospermic (Katagiri et al. 2004). This substantially proved the close association between Y chromosome deletions and male infertility. The prevalence of microdeletion in azoospermic men was found to range from 10 to 15 percent (Dohle et al. 2002; Foresta et al. 2005). In oligozoospermic men the prevalence of microdeletions was 5 to 10 percent (Foresta et al. 2005). It is essential to consider these deletions when discussing ART because microdeletions are always passed on to the male offspring and fertilization and pregnancy rates are not affected by microdeletions on the AZFc region when using ICSI (Peterlin et al. 2002). The presence of Yq microdeletion was historically detected on the long arm of the Y chromosome, Yq. A particular area of interest on Yq is the azoospermica factor region (AZF region). The region spans 10-Mb and frequently found deleted in men with unexplained spermatogenic failure. This region contains genes involved in the growth and development of sperm. The AZF region contains three subregions referred to as azoospermia factor AZFa, AZFb and AZFc. The most common aberrations that occur in the AZF region are multiple gene deletions in the AZFb and AZFc areas. Researchers are attempting to characterize
deletions in the AZF region so that they can be used to determine treatment for infertile males. Figure 1.6 displays the different AZF regions of the Y chromosome and the locations of genes associated with AZF region. Interstitial and terminal deletions in AZFa, or AZFb, or AZFc alone or in any combination of the Y chromosome long arm (Yq) are all associated with dramatic nonobstructive spermatogenic failure. Studies show that there is a clear cause-effect relationship between AZF loci deletion/s and male infertility. Among the three AZF regions, deletion of AZFc is more frequent (roughly 50%), followed by AZFb and AZFa (Layman 2002). The AZFc deletion is caused by recombination between two similar direct repeats (between amplicons b2 and b4). The absence of the AZFb/b-c regions are gross “microdeletions” which are associated with divergent testicular histological profiles, ranging from sertoli cell-only syndrome (SCOS) hypospermatogenesis (HS) to spermatogenic arrest (SGA) (Vogt et al. 1996). Apart from infertile men presenting with AZF microdeletions appear otherwise healthy. The microdeletions are usually detected by performing sequence tagged site (STS) based PCR techniques on patients DNA extracted from peripheral blood. Therefore in addition to GTG karyotyping Y chromosome deletion detection by PCR is also a mandatory test in the azoo/oligozoospermic patient workup. Worldwide Y chromosome microdeletion assay has become a routine test (Ferlin et al. 2007). Y chromosome microdeletion screening provides diagnostic prognostic and preventive impacts. In addition it can improve genetic counseling. Moreover, infertile men are known to be at increased risk of androgen deficiency and testicular neoplasia (Giannouli et al. 2004; Nathanson et al. 2005). Apart from complete AZF region deletions a new type of Yq deletion has recently attracted the attention. Partial deletion patterns within the AZFc region have been proposed and then described the spermatogenic effect of these deletions was found to vary among different populations.
The AZFa region has been estimated to span 800 kilobases (kb) and four candidate genes have been isolated. The two main genes located in the AZFa region are DFFRY (Drosophila fat facets related Y) later named USP9Y (Ubiquitin-specific protease 9) and DBY (dead box on the Y). USP9Y is located in interval 5C and expressed in a wide range of tissues. DFFRY and its X-homologue, DFFRX, might be involved in male germ cell development (Foresta et al. 2001). Deletion of DFFRY will cause reduction of germ cells rather than their complete absence (Brown et al. 1998; Foresta et al. 2001). Distal to DFFRY there are two genes, DBY (DEAD/H box polypeptide, Y chromosome) and UTY (ubiquitously transcribed tetratricopeptide repeat gene, Y chromosome). These genes were mapped to interval 5C/D (Lahn et al. 1997). Deletion of DBY alone or in combination with UTY has been observed in a subset of patients with spermatogenesis defects. Deletions in the AZFa region that remove both of these genes cause Sertoli cell-only syndrome, a condition characterized by the presence of Sertoli cells in the testes but a lack of spermatozoa in the ejaculate (Vogt 2005). DBY the major gene located in the AZFa region has a probable role in infertility because it is localized in the testis and is involved in the development of premeiotic germ cells. DBY consists of 17 exons and encodes for a putative ATP dependent RNA helicase as it belongs to the DEAD-box proteins. The USP9Y gene is also involved in spermatogenesis (Smith et al. 2008). Shortening or deletion of the USP9Y gene causes azoospermia (Galan et al. 2007), oligozoospermia or oligoasthenozoospermia (Ehrmann et al. 1998). Recently an anonymous expressed sequence tag (AZFaT1) has been mapped proximal to DFFRY.
AZFb; AZFb spans from the distal portion of deletion interval 5 to the proximal end of deletion interval 6 and it spans around 1-3Mb of DNA. Deletion of the region cause arrest of spermatogenesis at the primary spermatocyte stage, indicating that the region is essential for fertility. The main gene in the AZFb region is RBMY (RNA binding motif on the Y) and there are six copies of the gene located on the Y chromosome. RBMY1 codes for an RNA binding protein which is a testis-specific splicing factor expressed in the nuclei of spermatogonia, spermatocytes and round spermatids. In a study by Lavery et al., RBMY1 expression was reduced in azoospermic men. A family of PRY genes are involved in the regulation of apoptosis, an essential process that removes abnormal sperm from the population of spermatozoa. In cases in which all the genes in the AZFb region except RBMY and PRY are deleted, patients present with hypospermatogenesis. However if both the RBMY and PRY genes are removed spermatogenesis is arrested completely, indicating that RBMY and PRY are the major genes involved in fertility in the AZFb region.
AZFc; Deletions in the AZFc region produce a wide range of phenotypes, many of which are associated with low sperm concentration due to reduced spermatogenesis. AZFc deletions cause approximately 12% of nonobstructive azoospermia and 6% of severe oligozoospermia (Kuroda-Kawaguchi et al. 2001). In many cases, men can still achieve fertilization with the assistance of ART (Vogt 2005). Studies demonstrate that only the AZFa and AZFb regions are needed to initiate spermatogenesis but that without the AZFc region, spermatogenesis will not be completely normal. Complete deletions of the AZFc region may occur in two different ways: either as a result of a previous deletion within the AZFc or spontaneously from a normal AZFc region. A study by Zhang et al. found that there were more complete deletions of the AZFc region in groups with existing partial deletions in that area of the Y chromosome (Zhang et al. 2007). This result was replicated in a study of Italian men with a high frequency of partial deletions in the AZFc region (Arredi et al. 2007). A deletion of the AZFc region may also predispose men to Y chromosome loss, leading to sexual reversal. Several studies have found this deletion to be a premutation for 45,X (Siffroi et al. 2000) and for the mosaic phenotype 45,X/46,XY (49).
The AZFc region is prone to many smaller subdeletions that are thought to be caused by intrachromosomal recombinations. These partial deletions produce a wide array of phenotypes, ranging from normospermic to azoospermic, due to many factors, including the interaction of the environment and the genetic background. Genetic studies of ethnic groups produce diverse results because of the variations in their genomes that have evolved over generations to cope with environmental pressures specific to their region. Kuroda-Kawaguchi together with his colleagues constructed a meaningful map of the AZFc region after sequencing the entire AZFc region. They found that AZFc consisted of three palindromes with six distinct ampliconic families. These amplicons have been given the names: turquoise(t), gray(gr), green(g), yellow(y), blue(b) and red(r) Figure 1.9. As mentioned earlier, the AZFc region is delimited by two 229 Kb amplicons, proximal and distal, termed b2 and b4 respectively. Most of the AZFc deletions were between these two amplicons as witnessed by Kuroda-kawaguchi et al. which spans approximately 3.5 Mb and is mapped to the deletion intervals 6D-6F, which removes all of AZFc locus. The association of b2/b4 complete AZFc deletions (also called classical AZFc deletion) with spermatogenic failure is well established as the observed phenotype range from azoospermic to severe oligozoospermia. It was reported that patients with AZFc deletions and oligozoospermia will be exposed to a progressive decrease in their sperm counts overtime, as a result of spontanious progressive regression of the germinal epithelium. This is why it is recommended for these patients to cryopreserve their semen for latter use when diagnosed avoiding the risk of becoming completely azoospermic or to avoid future more invasive techniques such as TESE/ICSI (Girardi et al. 1997; Simoni et al. 1997; Saut et al. 2000; Calogero et al. 2001; Dada et al. 2003). Almost all b2/b4 AZFc deletions are essentially identical in molecular extention, however, individuals carrying these microdeletions show variable grades of testicular failure, ranging from severe hypospermatogenesis, to
spermatoenic arrest (SGA) to Sertolicell only syndrome (SCOS) although the former is the usual finding in testicular biopsies (Vogt 2005). It is of importance to know that mature spermatozoa (residual spermatogenesis) can generally be recovered from testicular tissue biopsies of azoospermic patients with AZFc deletions, whereas this usually is not the case in patients with complete AZFa or AZFb deletions (Saut et al. 2000; Vogt 2005). Consequently, TESE for ICSI treatment is not recommended for patients with complete AZFa or AZFb deletions, because usually they are unsuccessful (Krausz et al. 2000; Choi et al. 2004).

Genes within the AZFc region are present in multiple copies. The region includes 12 genes and transcription units each present in a variable number of copies making a total of 32 copies (Simoni et al. 2004). Candidate genes within the AZFc region include 4 copies of the *DAZ*, 3 copies of *BPY2* (Basic Protein on Y chromosome, 2) and 2 copies of *CDY1* (*CDY1a* and *CDY1b*; Chromodomain protein, Y chromosome1) (Reijo et al. 1995; Yen 1998; McElreavey et al. 2000; Kuroda-Kawaguchi et al. 2001). The strongest candidate among them, in which its deletion is responsible for the AZFc phenotype, is the *DAZ* gene family which encode putative RNA-binding proteins, that are exclusively expressed in the male germ cells, specifically in the spermatogonia and early primary spermatocyte (Menke et al. 1997; Moro et al. 2000; Saxena et al. 2000; Kuroda-Kawaguchi et al. 2001; de Vries et al. 2002; Oates et al. 2002; Repping et al. 2002). Noteworthy, DAZ gene family have no X chromosome homolog, but surprisingly have two autosomal homologs, *DAZL1* (DAZ like autosomal 1) located on chromosome 3 short arm (3p24) and BOULE located on chromosome 2 long arm (2q33). DAZL is expressed exclusively in the germ cells where it encodes for a RNA binding protein (Foresta et al. 2005; Ferlin et al. 2007). Therefore, it is suggested by Foresta et al. (2001) that *DAZL1* may synergistically act in combination with DAZ during spermatogenesis. DAZL1 allows a small degree of spermatogenesis to survive in the majority of AZFc deleted men (Silber et al. 1998) which may explain the residual spermatogenesis in
many AZFc deleted men. BOULE is also exclusively expressed in the testis, where it regulates meiosis through controlling the translation of CDC25 transcript (Luetjens et al. 2004; Krausz et al. 2006; Kostova et al. 2007).

There was an argument about how many copies of DAZ gene are present within the AZFc region. Reijo et al. (1995) claimed that there is only one copy of DAZ, while several others suggested that there are seven copies (Glaser et al. 1998; Yen 1998; Moro et al. 2000). However, Saxsena et al. (2000) demonstrated that there are at least four DAZ copies (DAZ1-DAZ4) found in two clusters. The proximal DAZ1 and DAZ2 (situated in P2 palindrome) in one cluster, and the distal DAZ3 and DAZ4 (situated in the large P1 palindrome) in a second cluster, where the two genes in each cluster are arranged in two head-to-head clusters in amplicons r1-r4. This model was widely accepted and can explain why there is no point mutation events were detected within the DAZ genes, because if one is mutated the others will, of course, compensate (Fernandez Pelegrina et al. 1991; Ferlin et al. 2007).

Recently, a substantial attention was given to the CDY1 gene as being the second candidate gene within the AZFc region, which encodes a histone acetyltransferase (Vogt 2005). This gene is specifically expressed in the testis, which may signify its importance in spermatogenesis. There is no Y chromosome homologue in any nonprimate or even in prosimians, suggesting that it originated in primate Y chromosome recently. There is a homologue, CDYL on chromosome 6 that is transcribed ubiquitously. CDY1 is present in two copies, CDY1a and CDY1b, within the AZFc region, one being embedded within the DAZ cluster, and the other is found within the distal AZFc region (Yen 1998; Machev et al. 2004; Foresta et al. 2005). Likewise DAZ, CDY1 has an autosomal homolog on human chromosome 13, called CDYL in contrast to the DAZ autosomal homologs, this gene is expressed
ubiquitously (Foresta et al. 2005; Vogt 2005; Krausz et al. 2006). CDY1 genes are mapped to intervals 6F of the Y chromosome.

Therefore, studies of the partial deletions of the AZFc region have produced conflicting results that relate to the genetic makeup of the haplogroups studied. The complex interaction of genes and environment makes it difficult to replicate the results of studies and definitively associate subdeletions with infertile phenotypes. The AZFc region also contains genes involved in spermatogenesis. The DAZ gene has four copies on the Y chromosome. DAZ genes are thought to serve a variety of roles throughout the spermatogenic process because they are expressed in all stages of germ cell development. They regulate translation, code for germ cell–specific RNA binding proteins, and are involved in the control of meiosis and maintenance of the primordial germ cell population. Deletions of the DAZ genes can cause a spectrum of phenotypes ranging from oligozoospermia to azoospermia additionally, DAZ gene expression was reduced in azoospermic patients, and partial deletions of DAZ genes seem to be related to oligozoospermia (Writzl et al. 2005).
**Figure 1.9.** Schematic view of AZFb and AZFc deletion intervals in distal Yq11.

Comparative view on the schematic AZFb and AZFc deletion intervals with their underlying genomic sequence structure (Kuroda-Kawaguchi *et al.* 2001; Vogt 2004). Molecular extensions of the estimates AZFb, AZFc and AZFc plus AZFc deletions are given in Mb. The five possible palindromic structures P1-P5 in this Yq11 region are marked each with the extensions of both arms below the amplicon structure. Only the large p1 palindrome and the P3 palindrome include more than one amplicon type. The AZFb deletion overlaps with the AZFc deletion as indicated.
1.4.6. Other spermatogenesis genes at AZFc region

Other genes mapped to the AZFc region of the Yq include \( BPY2 \) (basic proteinY2), \( PRY \) (PTA-BL related Y) and \( TTY2 \) (testis transcript Y20). Although the functions of these genes have yet to be determined they do share common characteristics such as existing in multiple copies on the Y chromosome; they are expressed uniquely in the testis and are Y-specific (Lahn et al. 1997). Restriction mapping has also identified three \( PRY \) and \( TTY2 \) genes in the proximal portion of AZFc, which are thus unlikely to be involved in the spermatogenic disruption experienced by patients with deletions restricted to DAZ (Yen 1998).

Figure 1.10. Schematic representation of human Y chromosome AZFc region along with different regions
1.5. Mechanism of microdeletion

AZF deletions are caused by homologous recombination of specific repetitive sequence blocks. Microdeletions on autosomes are caused by local recombinations between repetitive homologous sequence blocks. AZF microdeletions on the human Y chromosome are also postulated to occur in similar ways (Vogt 2004). It has now been revealed that the Yq11 is largely composed of five Y-specific repetitive sequence blocks or palindrome structures especially in the AZFc deletion interval (Kuroda-Kawaguchi et al. 2001; Skaletsky et al. 2003).

Homologous recombination between specific palindromic sequences (also known as amplicons) is believed to be the mechanism for the formation of various Yq deletions (Mannan et al. 2000; Repping et al. 2002). For instance, generation of AZFc deletions is caused by recombination involving b1 and b4 amplicons (Kuroda-Kawaguchi et al. 2001) and removes all four copies of the DAZ gene and three copies of the BPY2 gene. DNA sequence alignments within AZFc also revealed possibility for the formation of smaller subdeletions. Detailed analysis of the AZFc region using new molecular markers has conformed the existence of three such deletions; namely, gr/gr, b1/b3 and b2/b3 (also known as g1/g3) (Fernandez Pelegrina et al. 1991; Repping et al. 2002). The most prevalent deletion, gr/gr (Repping et al. 2002) is caused by recombination between amplicons g and r, resulting in the loss of two of the four copies of the DAZ gene and one of the three copies of the BPY2 gene. The outcome of b1/b3 and b2/b3 deletions is similar leading to the retention of two DAZ gene copies and one or two BPY2 gene copies. These deletions have resulted in spermatogenic failures.
1.6. AZF genes as candidates for Azoospermia Factor

Many of the AZF genes have been postulated as candidate genes involved in human male fertility. The genes have been proposed based on their expression profiles and targeted disruption of homologues genes in mice which ultimately result in sterile phenotype. These genes are designated as “spermatogenesis phase marker” or “male fertility index” genes, because they are useful tools for diagnosing patient’s spermatogenesis disruption phase and for predicting the presence and quality of his mature sperms (Vogt 2004). Nevertheless scientist have not been able to elucidate a direct link between a Y chromosome gene and male infertility. Table 1.3 at appendix 1 summarizes genes with known function within the AZF locus.

1.7. Assisted reproduction Techniques and male Infertility

1.7.1. The concern of assisted reproduction techniques

Intracytoplasmic sperm injection (ICSI) is the direct introduction of a spermatozoon into an oocyte to achieve fertilization and pregnancy when the number of spermatozoa in the ejaculate is very low or even absent. In the latter case ICSI can be performed using spermatozoa obtained from the epididymis or directly extracted from testicular tissue. Furthermore techniques of spermatid injection into oocytes may be performed and first term pregnancies have been achieved in humans (Tesarik et al. 1995). Despite the worldwide diffusion of ICSI in recent years, the possible risks that might ensue from its indiscriminate use have been considered only recently. These concerns arose especially with the recent advances in genetically determined male infertility (Foresta et al. 2001). ICSI arouses more
fears of the transmission of genetic abnormalities to offspring than other forms of assisted reproduction techniques because it bypasses all the physiological mechanisms related to fertilization which need an active motile spermatozoon to undergo normal capacitation and acrosome reaction and to start all mechanisms required to penetrate the oocyte. By bypassing these steps ICSI allows an altered spermatozoon to fertilize an oocyte. This consequently increases the risk of transmitting genetic defects to the offspring. In other words a genetic defect giving rise to abnormal spermatogenesis that can be surmounted by ICSI could be transmitted to the children produced. The concerns are most remarkable for male infertility related to Y chromosome microdeletions, since Y-deleted patients are strong candidates for ICSI as in most cases spermatozoa or spermatids suitable for the procedure can be recovered from semen or the testis but all male offspring will invariably inherit the deleted Y chromosome from the father (Kent-First et al. 1996).

1.8. The diagnosis of Y chromosome micro deletion

The Y chromosome though representing only 2-3 percent of the haploid genome harbours about 107 genes and pseudogenes. Many of these are responsible for spermatogenesis and other male related functions, and deletion of any of these can result in infertility. Y-chromosome microdeletions are common in about 10-15 percent of men with azoospermia or severe oligozoospermia. These microdeletions are too small to be detected by karyotyping, they can be easily identified using polymerase chain reaction (PCR). Cytogenetic studied demonstrated that deletion of the distal euchromatin of the Y chromosome was associated with infertility. More recently the Y chromosome was mapped with high resolution into a series of seven deletions (Kuroda-Kawaguchi et al. 2001). The genetic complexity of AZF
locus located in the Yq could be determined by the use of sequence-tag sites (STS). Sequence-tagged site is a short region along the genome whose exact sequence is found nowhere else in the genome. The uniqueness of the sequence is established by demonstrating that it can be uniquely amplified by the PCR. The DNA sequence of an STS may contain repetitive elements, sequences that appear elsewhere in the genome, but as long as the sequences at both ends of the site are unique, we can synthesize unique DNA primers complementary to those ends, amplify the region using the PCR, and demonstrate the specificity of the reaction by gel electrophoresis of the amplified product. The STS markers of the AZF region have been useful to identifying microdeletions in DNA from blood and sperm (Hellani et al. 2005) of infertile men. It is critical that azoospermic and severely oligozoospermic men be tested for microdeletions both for accurate diagnosis and genetic counseling before performing ART (Cram et al. 2000; Sadeghi-Nejad et al. 2007). However, the lack of association between testicular phenotype and genotype in affected men forces clinicians to employ costly methods, such as polymerase chain reaction (PCR), to determine diagnosis. Sprinkled throughout the AZF region is a myriad of transcriptional units with possible critical roles in the human male (Lahn et al. 1997; Kuroda-Kawaguchi et al. 2001). The initial analysis of Y-chromosome microdeletions were performed with uncertified and heterogeneous markers (sequence-tagged sites[STS] ) that were frequently uninformative or unreliable. The precise mapping of the Y-chromosome allowed the use of specific STSs of high quality (Foresta et al. 2001; Skaletsky et al. 2003). Introducing guidelines for molecular diagnosis of Yq microdeletions (Simoni et al. 2004) enabled the Yq microdeletion test to become more homogeneous and reliable. The Y chromosome contains 300 sequence tagged sites (STS), which correspond to the AZF regions and could be exploited for easier characterization of microdeletions (Foresta et al. 2001). Screening protocols have ranged from the use of a single STS in the DAZ region to the use of 131 STSs that cover the Y
chromosome. The microdeletion detection rates also vary widely from 1 to 58.3 percent in these studies. It is still a question how many and which loci should be included in the screening of Y chromosome microdeletion. It has been suggested that the use of a larger number of STSs in a screening protocol does not increase the frequency of microdeletions detected. However it is also true that that a larger number of STSs can protect against inaccuracy and also lead to the detection of clinically irrelevent polymorphic variants. With the help of European Academy of Andrology (EAA) guidelines (Simoni et al. 2004) and EAA/EMQN (Eueopean Molecular Genetics Quality Network) external quality control programme (http://www.emqn.org/emqn/), Yq testing has become more homogeneous and reliable in different routine genetic laboratories. Furthermore Mitra et al. demonstrated the utility of this strategy by developing a targeted multiplex PCR using STS specific to the Indian population. This type of procedure could be used as an initial screen for Y chromosome microdeletions before employing more expensive and technically challenging testing methods. However, to be effective, specific STS would need to be defined for different ethnic populations.

Other researchers in our Unit have attempted to examine the association of Y chromosome microdeletions with infertility and recurrent pregnancy loss in Sri Lankan men using the multiplex PCR amplification system suggested in the state of the art recommendations made by the European Academy of Andrology/European Molecular Genetics Quality network (EAA/EMQN) guidelines (Wettasinghe et al. 2010). The patient population consisted of male partners of women experiencing RPL, oligozoospermic men and azoospermic men. None of the men in the RPL group had any microdeletions in the AZF regions or partial deletions in the AZFc region. Of the 153 men in the azoospermic group one had a complete AZFc deletion and 2 others had a deletion at sY254, a sequence tagged site (STS) in the AZFc region. None of the men in the oligozoospermic group had deletions. The detection rate of
deletions in the Sri Lanka population using the European recommendations appeared to be very low in the Sri Lankan population (Simoni et al. 2004). Similar findings have been reported in India with the European recommendation. This has been addressed in a study done in India where Indian men with azoospermia were tested for Y chromosome microdeletions using a panel of novel STS markers (Thangaraj et al. 2003). In this study, among 340 azoospermic men 82.8% had deletions in the AZFc region, followed by 55.2% in the AZFb region, and 24.1% in the AZFa region. In the light of the above findings, in the current study, the above mentioned Sri Lankan samples were tested using the Indian panel of STS markers reported by Thangaraj and colleagues.

**Figure 1.11.** Detailed analysis of an STS loci with its corresponding amplified product size along with forward and reverse primers in block letters where as the unique sequence in simple letters

>chrY:12942302+12942517 216bp TTGACTGCTTATTCTACACAAGGC
CAGGGGAAATTGGTGTTT
TTGACTGCTTATTCTACACAAGGCaatttcacattaattaatgtatgtaa
tcctcatagcttctctgttaaagtagacactggtttttcctatatttcaca
tgaagaaccaatcaggaacatctccatgtatattggaacaggaattgaaagaatttacagtgtgtaagcaaaaactcagttttagAA
AACCCAATTTCCCCTG
1.9. Objectives

1. To determine the association of Y chromosome microdeletions with male partners of women experiencing RPL, with the STS markers used in the Indian study.

2. To determine the prevalence and patterns of Y microdeletions in oligozoospermic and azoospermic men using STS markers used in Indian study.
2. MATERIALS AND METHODS

This chapter highlights all the methods used in this investigation. An ethical approval to conduct the study was the first issue to be considered though research participants were not recruited due to the existence of in house blood/DNA resources of RPL and infertile male Sri-Lankans. Thus this chapter begins with ethical concerns. A description of pre-existing blood/DNA resource in terms of phenotypic characteristics of recruited research participants, their inclusion criteria is provided in the second part of the chapter. The next portion of this chapter illustrates the DNA extraction methods employed to fill up DNA samples which were low in volume during the study. The selected sequence tag sites (STS) and further analysis methods described in the fourth and fifth parts of this chapter respectively. This is followed by a description of agarose gel electrophoresis, sequencing and the measures taken to assure the quality of genotyping, and a summary of sources of reagents used in the assays. The final two portions of the chapter express the statistical methods employed to analyse the microdeletion analysis data and the electronic databases and software tools used in the study.

2.1. Ethical concerns

The study was conducted with the approval of the Ethical Review Committee of the Faculty of Medicine, University of Colombo. The investigation protocol of the study has been designed and implemented in accordance with the Declaration of Helsinki (2008) of the World Medical Association and other applicable research ethics guidelines. As already existing biological materials (blood/DNA) in the laboratory was used, no volunteers were recruited in the investigation. The recruitment of research participants for the collection of the above mentioned blood / DNA samples were performed for the previous studies and all
volunteers were recruited after obtaining written informed consent using formal documentation, in conjunction with a verbal discussion, in the language of their choice (either Sinhala or English) as only the Sinhalese population in Sri Lanka was selected for the study.

2.2. DNA resource

The blood / DNA samples, which have been collected according to the protocol approved by the Ethics Review Committee of the Faculty of Medicine, Colombo for the studies of this nature, was available in the Human Genetics Unit, Faculty of Medicine, Colombo. This unique DNA sample pool utilized for the genetic analysis consists of a population based DNA collection from 250 Sinhalese RPL and infertile (oligozoospermic and azoospermic) men. A total of 50 DNA samples from each category was randomly selected for this study.

2.3. Research participants and inclusion criteria

Two hundred couples with three or more spontaneous RPLs were recruited for a case-control genetic association study of genetic thrombophilic polymorphisms with RPL at the Human Genetics Unit, Faculty of Medicine, University of Colombo according to a protocol approved by the Ethics Review Committee of the faculty. The couples had also undergone karyotyping prior to recruitment and those with chromosomal abnormalities had been excluded. The RPL partners were negative for genetic thrombophilic polymorphisms and invited the male partners to participate in the current study. Seventy six male partners gave written informed
consent and participated in the study. Among the men recruited for the study a seminal fluid analysis (SFA) report was available for 50, of which 5 (10%) men were oligozoospermic, 2 (4%) men were asthenozoospermic, 2 (4%) men were asthenozoospermic/oligozoospermic and 41 (82%) men were normospermic. Fifty (50) infertile men with oligozoospermia and fifty (50) nonobstructive azoospermia who were seeking andrologic investigation for male-factor infertility from a private reproductive biology laboratory. The patients were counseled and their written consent was obtained before the study. A complete history and physical examination was performed. Physical examination included assessment of secondary sexual characteristics, testis size and consistency. Abnormalities in semen analysis when compared with reference values (Table 2.1) indicates a comprehensive andrological examination. As important treatment decisions are based on the result of semen analysis it is essential that the complete laboratory work-up is standardised (WHO Laboratory Manual for the Examination and Processing of Human semen). WHO has standardised ejaculate analysis by distributing and publication of a standardised manual. According to the manuals guidelines the parameters were as follows (Table 2.1).
### Table 2.1. Lower reference limits (5th centiles and their 95% confidence intervals) for semen characteristics according to WHO guidelines

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lower reference limit(range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semen volume(mL)</td>
<td>1.5 (1.4-1.7)</td>
</tr>
<tr>
<td>Total sperm number($10^6$ per ejaculate)</td>
<td>39 (33-46)</td>
</tr>
<tr>
<td>Sperm concentration($10^6$ per mL)</td>
<td>15 (12-16)</td>
</tr>
<tr>
<td>Total motility(% motile)</td>
<td>40 (38-42)</td>
</tr>
<tr>
<td>Progressive motility(PR,%)</td>
<td>32 (31-34)</td>
</tr>
<tr>
<td>Vitality (live spermatozoa,%)</td>
<td>58 (55-63)</td>
</tr>
<tr>
<td>Sperm morphology(normal forms,%)</td>
<td>4 (3.0-4.0)</td>
</tr>
</tbody>
</table>

**Other consensus threshold values**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>&gt;7.2</td>
</tr>
<tr>
<td>Peroxidase-positive leukocytes($10^6$ per mL)</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>MAR test(motile spermatozoa with bound particles,%)</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Immunobead test(motile spermatozoa with bound particles,%)</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Seminal zinc(umol/ejaculate)</td>
<td>&gt; 2.4</td>
</tr>
<tr>
<td>Seminal fructose(mol/ejaculate)</td>
<td>&gt;13</td>
</tr>
<tr>
<td>Seminal neutral glucosidase(mU/ejaculate)</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

*PR=progressive; NP=non progressive; MAR=Mixed antiglobulin reaction.*
The patients were categorized according to their mean sperm concentrations: azoospermia (no sperm in the ejaculate); severe oligozoospermia (<5x10⁶ /ml); oligozoospermia (5-20x10⁶/ml) and cases with normal sperm counts (>20x10⁶/ml) and cases with normal sperm counts (>20x10⁶/ml). Healthy women were used as negative controls during optimization of multiplex polymerase chain reactions (PCR).

2.4. Extraction of DNA

The investigation was commenced with the existing anonymous DNA sample collection available at Human Genetics Unit, Faculty of Medicine, University of Colombo acquired from RPL and infertile males. However, along with the progression of the study, the DNA samples which were low in volume were filled up by further DNA extraction. The DNA extraction had been performed using QIAamp® DNA Blood Mini kit (Qiagen Ltd., UK) with existing venous blood which had been collected (into EDTA containing tubes) and stored at −80°C. The manufacturer’s protocol was exploited as guidance for the DNA extraction. Adequate precautions were taken to avoid any possible contaminations.
2.4.1. Extraction using QIAamp® DNA Blood Mini kit

This kit is intended for fast and efficient purification of total DNA. The concept of the DNA extraction and purification procedures is composed of lysis of leukocytes, loading lysate onto the QIAamp® spin column where DNA binds to the QIAamp® membrane and purging of impurities effectively (proteins and other possible contaminants) via two centrifugation or vacuum steps, and elusion of the immaculate DNA from the QIAamp® spin column.

Prior to the DNA extraction procedures the samples were equilibrated to room temperature (RT) (15°C - 25°C) and buffer AW1, buffer AW2 and QIAGEN Protease were prepared according to the instructions. Whole blood (or Phosphate Buffer Saline mixed blood) 200 µl was added into a 1.5ml centrifuge tube containing 20µl of QIAGEN Protease. Subsequent to a brief mixing, 200µl buffer AL was added and mixed thoroughly (to ensure adequate lysis) by inversion of tube (15 times), followed by vigorous shaking by pulse vortex for at least 1 minute. Then the reaction mixture was incubated at 70°C for 10 minutes. Subsequently 200µl of ethanol (96 -100%) was added to the reaction mixture and mixed by inverting the tube (10 times) which was followed by additional vigorous shaking (to ensure efficient binding). Then one half of the reaction mixture was carefully transferred to a QIAamp® Mini column placed in a 1.5ml centrifuge tube and centrifuged at 1850 × g (3000 rpm) for 3 minutes. Acquired filtrate was discarded and the ‘column’ was placed back in to the tube and the same procedure repeated with the remaining half of the reaction mixture. Later, 500µl of buffer AW1 was added carefully in to the ‘column’ kept in the tube and centrifuged at 4500 × g (5000 rpm) for 1 minute. Subsequently 500µl of buffer AW2 was added to the ‘column’ and centrifuged at same speed for 15 minutes. Then the ‘column’ was placed in a clean 15ml centrifuge tube and filtrate was discarded. Later 300µl of buffer AE, equilibrated to RT (15-25°C), was pipette directly on to the membrane of ‘column’ and incubated at RT for 5
minutes and centrifuged at 4500 × g (5000 rpm) for 2 minutes. Finally in order to obtain a maximum concentration, elute containing DNA was reloaded, re-incubated and re-centrifuged as described earlier. The eluted DNA samples were labeled and stored at -20°C.

2.4.2. Quantification of extracted DNA

Prior to any genetic investigation, the freshly extracted DNA was quantified using a spectrophotometer (GeneQuant pro, Korea) at genetic laboratory of the Asiri Surgical Hospital, Colombo to confirm the presence of sufficient concentration of DNA. The procedure involves the following; Using blank (distilled water) to neutralize the measurement of the spectrophotometer, testing a 100µl of mix (5 µl of extracted DNA samples diluted with 95µl of distilled water), and reading the spectrophotometer measurements.

2.5. Y chromosome micro deletion analysis by multiplex PCR

2.5.1. Selection and designing of Primers

The study was conducted by analysing 2 genes (DFFRY, BPY2) and 23 sequence tagged site (STS) markers of the Y chromosome. Primers of known sequences for STS loci were searched against NCBI Uni STS database. The primer designing programs used include NCBI Primer BLAST (Johnson et al. 2008), and Primer3 version 0.3 (http://frodo.wi.mit.edu/primer3/) over the World Wide Web (Rozen et al. 2000). To check the specificity of the designed primers, In-silico PCR analysis in USCS genome browser was used. In-Silico PCR searches a sequence database with a pair of PCR primers, using an
indexing strategy for fast performance. When search is done it returns a sequence output file in fasta format containing all sequence in the database that lie between and include the primer pair. The fasta header describes the region in the database and the primers.

All primers were supplied in a dry form and were reconstituted with freshly prepared 1x Tris-EDTA (TE) buffer (at pH 8.0) at the commencement of the assay. Pre-calculated volume of 1x TE was added to make the final concentration of primers as 50µM. The primers used in PCR amplification are listed in Table 2.4. The dNTPs were supplied as four separate vials containing one base each and the reaction dNTPs solution (for 1000µl) was prepared by adding 20µl of each dNTP (total of 80µl) to 920µl of distilled dH₂O.

2.5.2. PCR optimization

Multiplex PCR is a demanding PCR technique used to amplify several products in a single reaction using multiple sets of oligonucleotide primers (Chamberlain et al. 1988). This technique often requires extensive optimization because primer-dimers and other nonspecific products may interfere with the amplification of specific products. Amplification specificity is also influenced by factors such as the PCR buffer and primer concentration.

2.5.2.1. Multiplex PCR analysis

The loci used in the construction of the each multiplex were determined from an extensive review of literature. Selected loci should have an annealing temperature within ±5°C. Also
the loci tested should have gel separable amplification product. The size ranges for each loci were determined using NCBI UniSTS data base. To test for possible repetitive sequences primers used were aligned with the sequence database at the National Centre for Biotechnology Information (NCBI) using the Basic local Alignment Search Tool (BLAST). BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search results list the accession numbers that contained sequences producing significant alignment to the query sequences. The accession numbers are listed in order of their alignment score. The alignment score is defined as the degree of similarity between the query sequence primer and the sequence being compared (Karlin et al. 1993). The sequences with the higher the score, the greater extent the nucleotide sequences are related. If a query sequence primer returned multiple GenBank accession numbers with a maximum score the most recent entry and or sequence with a larger flanking region was used. Multiple sequences of the flanking region of STS loci can exist due to the frequency of sequence variation inherited in human populations. Each multiplex PCR encompassed control SRY when ever appropriate. If amplification of a particular locus was poor the primer concentration for that locus was increased. For the initial construction of the multiplex primer set all of the primer pairs were combined yielding a final concentration of 1.0 µM. This concentration value was chosen based on a review and comparison of previous Y STS multiplex studies of markers that are also used in the present study. Unlike previous published multiplex PCR primer mixture design protocols (Henegariu et al. 1997) in which many other experimental conditions, such as MgCl₂ concentration and buffer concentration were modified to achieve better amplification, only the primer concentrations, annealing temperature and annealing time were adjusted and sticked to the inhouse PCR protocol. If one PCR product was higher in intensity relative to the other amplicons in the multiplex the appropriate primer pair concentration were lowered to generate a more balanced multiplex.
<table>
<thead>
<tr>
<th>Step 1</th>
<th>Optimize cycling conditions for individual primer pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prepare 25ul containing only one primer pair.</td>
</tr>
<tr>
<td></td>
<td>Adjust temperature and duration of annealing and</td>
</tr>
<tr>
<td></td>
<td>extension steps until similar product yields are</td>
</tr>
<tr>
<td></td>
<td>obtained with all primer pairs.</td>
</tr>
<tr>
<td>Step 2</td>
<td>Perform multiplex PCR</td>
</tr>
<tr>
<td></td>
<td>Use equimolar concentrations of all primer pairs and</td>
</tr>
<tr>
<td></td>
<td>the optimized cycling conditions from step 1.</td>
</tr>
<tr>
<td>Step 3</td>
<td>Optimize multiplex conditions</td>
</tr>
<tr>
<td></td>
<td>Adjust following parameters according to the guidelines</td>
</tr>
<tr>
<td></td>
<td>below until the desired yield is obtained:</td>
</tr>
<tr>
<td></td>
<td>✓ Primer concentration in steps of 0.1-0.2µM</td>
</tr>
<tr>
<td></td>
<td>✓ Annealing temperature in steps of 1°C</td>
</tr>
<tr>
<td></td>
<td>✓ Extension time in steps of 30 sec</td>
</tr>
<tr>
<td></td>
<td>No product detected:</td>
</tr>
<tr>
<td></td>
<td>✓ Increase primer concentration</td>
</tr>
<tr>
<td></td>
<td>✓ Decrease annealing temperature</td>
</tr>
<tr>
<td></td>
<td>Low product yield:</td>
</tr>
<tr>
<td></td>
<td>✓ Increase primer concentration</td>
</tr>
<tr>
<td></td>
<td>✓ For short products decrease extension time</td>
</tr>
<tr>
<td></td>
<td>✓ For long products increase extension time</td>
</tr>
<tr>
<td>Step 4</td>
<td>If necessary, perform further optimization</td>
</tr>
<tr>
<td></td>
<td>(to be used if steps 1-3 do not produce desired</td>
</tr>
<tr>
<td></td>
<td>results)</td>
</tr>
<tr>
<td></td>
<td>DMSO</td>
</tr>
<tr>
<td></td>
<td>Betane</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
</tr>
</tbody>
</table>

Table 2.2. General guidelines for optimization of a multiplex PCR- modified (Henegariu et al. 1997)
2.5.2.2. Monoplex PCR analysis

Each primer pair was initially tested in a PCR singleplex reaction on three different DNA samples, two from male donors and one from a female. During multiplex PCR if any of the STS loci failed to amplify a singleplex analysis was performed. Two males were chosen in case amplification of one of the male templates failed. The female was chosen as a negative control to ensure that the primer pair does not amplify any non-Y-chromosome regions.

Each patient genomic DNA was tested for a total of twenty three previously published sequence tagged sites (STS) that are mapped at intervals 5 and 6 of AZF region located at long arm of Y chromosome (Thangaraj et al. 2003). Primer details of the STS loci of interest with four multiplex PCR sets, each containing 3-4 primer pairs (Table.2.3). Specific primers used for each multiplex and the expected PCR product sizes are listed in Table 2.4. Rest of the primers were amplified as singleplexes. The internal control used was sex determining region (SRY) gene.
**Figure 2.1.** Detailed map of Y chromosome

<table>
<thead>
<tr>
<th>Multiplex</th>
<th>STS markers</th>
<th>Loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mix A</td>
<td>sY83+sY581</td>
<td>AZFa+AZFc</td>
</tr>
<tr>
<td>Mix B</td>
<td>sY741+sY615+sY158</td>
<td>AZFa+AZFc</td>
</tr>
<tr>
<td>Mix C</td>
<td>sY152+sY148+sY156</td>
<td>AZFc</td>
</tr>
<tr>
<td>Mix D</td>
<td>sY110+sY118+sY124+sY143</td>
<td>AZFa+AZFb</td>
</tr>
<tr>
<td>Mix E</td>
<td>sY2320+sY100+sY121+sY98</td>
<td>AZFa+AZFb+AZFc</td>
</tr>
</tbody>
</table>

**Table 2.3.** Multiplexes analysed and their relative locations
2.5.3 Polymerase Chain Reaction components and conditions

PCR was carried out in 25µl reaction volume containing 2µl template DNA (100-150ng), 5xGoTaq Green master mix (Promega, Madison, WI, USA), 1.5mM MgCl₂, 2.5mM dNTP’s mix (2.5mM each of dTTP, dCTP, dGTP, dATP), oligonucleotide primers (0.1 to 2µmol/L each of the forward and reverse primers (Table 2.3), 1U of Taq DNA polymerase and nuclease free sterile distilled water. PCR was carried out in 0.2ml PCR Microfuge tubes. The amplification reaction was performed in a programmable thermal cycler. PCR conditions used for STS markers were as follows: initial denaturation (94°C for 5 minutes) and subsequent denaturations (94°C for 4 seconds) was same for all the samples. Extension was 72°C for 2 minutes. Different annealing temperatures that were used for different STS markers were as follows: 60°C for 15 seconds for sY83, sY254 and sY581; 60°C for 1 minute for sY740, sY746, sY741, DFFRY, sY742, sY615 and sY743; 55°C for 30 seconds for sY86; 53°C for 30 seconds for sY84 and sY158; 57.4°C for 1 minute for sY2320, sY113, sY100, sY98 and sY121; 58°C for 30 seconds for sY127, sY255; and 55°C for 1 minute for sY110, sY143, sY124, sY156, sY148, sY247, sY118s and Y152. The amplified products were resolved on appropriate agarose gels. Positive and negative controls were run concurrently with each patient sample. A positive control (fertile male DNA) and a negative control (female DNA) were included with each set of reaction to ensure that all primers worked and that no contamination occurred during the study. Further distilled water instead of genomic DNA was used as blank to check for any DNA contamination.

With a PCR product of the expected size, the reaction was regarded as positive and indicative of absence of deletion for the studied STS loci. To detect the heterochromatic region, sY160 was used in all cases. The presence of deletion results in the absence of synthesis of the
desired product, therefore absence of band corresponding to any of the studied region is indicative of presence of the deletion of the particular region. Negative PCR products (no amplification) of the same STS in 3 consecutive independent attempts with the presence of internal control, testis determining gene (SRY) as well as positive control. Moreover the deletions with respect to the various STS markers were reconformed by performing temperature gradient PCR in absence or presence of 5 percent dimethyl sulphoxide (DMSO; Sigma Chemical Co;St.Louis,MO,USA) was interpreted as deletions for a particular STS. The absence of the products corresponding to control primer is indicative of a problem with that particular PCR amplification. If the control bands was present with the male genomic DNA control but not with the sample DNA, it suggests that there may be a problem with the genomic DNA used as template i.e; impurities, inaccurate, DNA quantitation or degraded DNA. Men deleted for any of these markers were further analyzed using markers contiguous to the deleted marker.
<table>
<thead>
<tr>
<th>STS</th>
<th>Locus</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sY83</td>
<td>AZFa</td>
<td>275</td>
</tr>
<tr>
<td>sY746</td>
<td></td>
<td>216</td>
</tr>
<tr>
<td>sY740</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>sY2320</td>
<td></td>
<td>82</td>
</tr>
<tr>
<td>sY741</td>
<td></td>
<td>97</td>
</tr>
<tr>
<td>DFFRY</td>
<td></td>
<td>111</td>
</tr>
<tr>
<td>sY742</td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>sY615</td>
<td></td>
<td>130</td>
</tr>
<tr>
<td>sY743</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>sY98</td>
<td>AZFb</td>
<td>266</td>
</tr>
<tr>
<td>sY100</td>
<td></td>
<td>111</td>
</tr>
<tr>
<td>sY110</td>
<td></td>
<td>179</td>
</tr>
<tr>
<td>sY113</td>
<td></td>
<td>304</td>
</tr>
<tr>
<td>sY118</td>
<td></td>
<td>218</td>
</tr>
<tr>
<td>sY124</td>
<td></td>
<td>109</td>
</tr>
<tr>
<td>sY121</td>
<td></td>
<td>190</td>
</tr>
<tr>
<td>sY143</td>
<td></td>
<td>311</td>
</tr>
<tr>
<td>sY152</td>
<td>AZFc</td>
<td>125</td>
</tr>
<tr>
<td>sY148</td>
<td></td>
<td>202</td>
</tr>
<tr>
<td>sY156</td>
<td></td>
<td>950</td>
</tr>
<tr>
<td>sY581</td>
<td></td>
<td>252</td>
</tr>
<tr>
<td>sY158</td>
<td></td>
<td>231</td>
</tr>
<tr>
<td>sY160</td>
<td>Heterochromatic region</td>
<td>236</td>
</tr>
</tbody>
</table>

**Table 2.4.** Sequence-tagged sites (STS) used for microdeletion analysis of human Y chromosome along with their location and amplification product sizes
2.6 Agarose gel electrophoresis

The most common and rapid way of analyzing PCR products is by standard agarose gel electrophoresis, a technique used to separate charged molecules by their size. The negatively charged DNA molecules are moved towards anode with an electric field through a porous matrix formed by agarose and ‘Tris/borate/EDTA’ (TBE) buffer. The advantages of this method include that the gel is easily poured and it does not denature the DNA samples, and the samples can be recovered if needed.

2.6.1. Preparation of agarose gel

Agarose is a chain of sugar molecules extracted from seaweed. Determination of the requisite strength (%) of an agarose agar gel (weight/volume) is depended on the expected DNA fragment size(s) (product size) because when the agarose concentration of a gel is increased it reduces the migration speed and permits separation of smaller DNA molecules and vice versa.

An agarose agar gel was made by dissolving appropriate (calculated) amount of agarose in a calculated volume of 1x TBE buffer, at high temperature by microwave heating. The solution was allowed to cool down (solution was stirred or swirled while cooling) to approximately 60°C. Ethidium bromide, an intercalating agent, which has the property to fluorescence when illuminated with Ultra Violet (UV) light was added to the solution to make a final concentration of 0.5μg/ml [5 μl ethidium bromide stock (10 mg/ml) per 100 ml gel solution]. The solution was again stirred to diffuse the ethidium bromide. Gels were poured into gel casting moulds (Consort, Belgium) which contains the well forming comb and allowed to set in the mould at RT. The fixed (solid) agarose agar gel was submerged into an electrophoresis
chamber (Consort, Belgium) filled with the running 1 x TBE buffer. When voltage of the electrophoresis increases the DNA fragments move faster than before. But utilization of high voltages is limited as it may melt the gel by heat and also may decrease the resolution (above 5 to 8 V/cm approximately) of the gel. If a DNA fragment has more base pairs (more length) the fluorescence will be high, in turn bands become brighter. DNA molecules of the same size move at the same speed. Since smaller molecules move faster than the larger molecules they separated in to different bands.

2.6.2. Detection of PCR amplified product

Detection of amplified region of the DNA (PCR product) was carried out by loading 10µl of loading mix (by adding 5µl of Promega loading buffer (15% (w/v) Ficol to 5µl of PCR product) into the wells formed by the comb on a prepared agarose gel. For all analysis electrophoresis was carried out at 55V (small chamber) or 90V (large chamber) for an average of ten minutes. The band(s) was visualised by illumination under UV light and documented using the UV transilluminator documentation system (Uvitec,UK). Detection of PCR product on prospective batches was carried out with a positive sample or with a DNA ladder.
2.7. Further classification of a microdeletion

In an event of detection of a microdeletion following procedure was followed to confirm the deletion. In this step we checked for AZFc partial deletions using sY1291 and sY1191 primer sets (Repping et al. 2003) which detect the unique fragments flanking the DAZ1/DAZ2 doublet at the u3 segment (proximal) and the P2/P1 palindrome junction (Figure 2.3). The physical position of the STS’s within the AZFc region is shown in Figure 2.2. The sequences of both primer pairs and their expected product sizes are also shown. In order to detect partial AZFc deletions we relied on sY1191 and sY1291 to discriminate the AZFc partial deletion patterns, by applying the results shown in Table 2.5.
**Fig 2.3.** Physical position of the STS loci used to detect the partial AZFc deletion. Each palindrome is marked by a specific STS loci as indicated.

<table>
<thead>
<tr>
<th>STS Loci</th>
<th>Deletion Patterns</th>
</tr>
</thead>
<tbody>
<tr>
<td>sY1291+</td>
<td>No deletion</td>
</tr>
<tr>
<td>sY1291-</td>
<td>Deletion gr/gr</td>
</tr>
<tr>
<td>sY1191+</td>
<td>No deletion b2/b3</td>
</tr>
<tr>
<td>sY1191-</td>
<td>Deletion b1/b3</td>
</tr>
</tbody>
</table>

**Table 2.5.** AZFc partial deletion patterns and their inference
2.8. Polymorphism analysis of sY156 region

2.8.1. Primer design

A BLAST search (http://blast.ncbi.nlm.nih.gov/) was used to obtain the DNA sequence of 200 bp proximal and distal to the sY156 primer binding region. Additional oligonucleotide primers within the flanking region of sY156 were designed as such the amplified product include the region of sY156 and its proximal and distal area. The flanking primers generated a DNA fragment with a size of 1130 bp, where as in the absence of deletion no corresponding band was generated.

2.8.2. DNA sequencing

The DNA sequencing was performed with the same primers as for PCR using the ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequencing fragments were purified using Centriflex® gel filtration cartridge Edge Biosystem (USA). Sequencing of purified extraction products were analyzed using an ABI PRISM 3130 Genetic Analyzer. This system works on the basis of the Sanger dideoxy chain termination principle. It contains four ddNTPs with different fluorescence labels and AmpliTaq® DNA polymerase. At the end of the reaction PCR products of varying sizes all terminating with a fluorescent-labelled dideoxy nucleotide are generated. The sequence is then read out automatically by capillary electrophoresis of cycle sequencing products through an automated sequencing machine. Sequence data was confirmed by sequencing both sense and antisense strands of DNA (bidirectional sequencing), on at least two independently
amplified PCR products for each sample. DNA sequencing analysis was done using BioEdit Sequence Alignment Editor, version 7.1.3.

2.8.3. Incorporation of additional STS loci to deleniate the deletion of sY156; sY 1086 and BPY2

Individuals who had deletions in any of these STS markers were further analysed by using the STS markers in the vicinity of the deleted loci. The STS which showed the deletion was identified using NCBI comprehensive database of sequence tagged sites (UniSTS). This site contain information on STSs along with their PCR primer pairs, genomic position, genes and sequences. The sequence of the STS loci was compared with similar sequences especially with human genome using basic local alignment search tool (BLAST). The genomic position was identified using map viewer. Further, by the help of UCSC PCR facility the position of the amplified region was identified. By clicking the dense STS marker and by scrolling the view, flanking STS markers were selected (Figure 2.2). Further flanking STS markers were selected using MSY Breakpoint Mapper (Figure 2.3) (http://breakpointmapper.wi.mit.edu/mapper.html).
Figure 2.4. A screen shot of UCSC genome bowser depicting the relative positions of STS loci of interest a. Y chromosomesmome idiogram depicting the relative position of the region along with flanking STS loci marked in red
The following STSs are between BY2-7 (chrY:23553339-23554203) and BY2-7 (chrY:25593331-25594095):

**Figure 2.5.** A screen shot from MSY Breakpoint mapper depicting the STS loci flanking sY156 and their relative positions
2.9. Measures of quality assurance

The PCR amplification of genomic DNA for Y chromosome microdeletion diagnosis requires strict compliance with good laboratory practice and basic principles of quality control.

Due to the enormous sensitivity of PCR, immense preventive measures were taken to preclude contamination. This includes physical separation of pre-PCR and post-PCR activities, products and apparatus including laboratory coats, pipettes etc. In addition, other routine precautionary methods such as usage of aliquots of reagents, negative control (reaction mixture containing all reagents except DNA) in each and every PCR batch, and modification of laboratory techniques etc. was also used. If any PCR amplified products were observed in the DNA free negative controls then the entire PCR batch was repeated. All in house PCR batches had either a DNA ladder (50p or 100bp).

Our study was conducted in compliance with the guidelines provided by EAA/EMQN scheme. In summary, the diagnostics of Y chromosomal micro-deletions should be performed by multiplex (at least duplex) PCR amplification of genomic DNA, using an internal control. A DNA sample from a fertile male and from a woman and a blank (water) control should be run in parallel with each multiplex.
2.10. Software tools and electronic database information

The software and electronic databases used in these investigations are as follows:

1. National Centre for Biotechnology information (NCBI), USA was searched to obtain DNA sequences and gene mapping

2. UCSC genome browser

3. MSY mapper used to explore flanking STS loci.

4. Primer 3 for designing primers

2.11. Statistical analysis

In general parametric statistics were used. Comparisons of qualitative data were carried out by means of Chi-square test ($\chi^2$) in 2x2 contingency tables. While quantitative data were handled by means of unpaired student’s t-test for independent samples. A P value of less than 0.05 was adopted to indicate statistical significance for each test.
3. RESULTS

3.1. PCR optimization and multiplex PCR

Genomic DNA from patients were examined using different sets of multiplex PCR primers that spanned across the entire AZF locus and the short arm of the human Y chromosome. Different concentrations of primers were tested. After several trials it was revealed that the optimal concentration of primer was 100µM. Although the primer concentration was similar at each case, annealing temperature was different for each multiplex primer mix. Mix A contained sY 81 and sY581 primers in a ratio of 1:1. Mix B contained sY 158, sY615 and sY741 primers in a ratio of 1:1:1. Mix C contained sY146, sY152 and sY156 primers in a ratio of 0.5:0.5:1. Mix D contained sY 110, sY118, sY124 and sY143 primers in a ratio of 0.5:0.2:1:1. Mix E contained sY 98, sY100, sY121 and sY2320 primers in a ratio of 1:1:1:1. Repeated amplification failure of any particular primer pair suggested a deletion. On several occasions, suspected deletions indicated by multiplex PCR were found to be false, when PCR reactions using a single primer pair showed the presence of the ‘missing’ DNA fragments.
Figure 3.1. PCR products from multiplex A, B, C, D, E separated on 3% agarose gel.

1. Multiplex A sY83 (275 bp) and sY581 (252bp)

2. Multiplex B sY158 (231bp), sY615 (130bp) and sY741 (97bp)

3. Multiplex C sY148 (202bp), sY152 (125bp) and sY156 (950bp)

4. Multiplex D sY110 (179bp), sY118 (218bp), sY124 (109bp), sY143 (311bp)

5. Multiplex E sY98 (266bp), sY100 (111bp), sY121 (190bp), sY2320 (82bp)

L is the 100bp ladder
3.2 Prevalence of microdeletions among Sinhalese population

Screening of 50 males with RPL with the STS markers specific to AZF region did not detected any deletions. A resent study which was conducted by Bellver et.al (Bellver et al. 2010) in a group of recurrent spontanious abortion (RSA) males, screening Yq microdeletions for 20 STS loci did not detect any deletions. Deletions were not observed for 50 oligozoospermic men as well. In another Sri lankan study, which incorporated STS loci recommended by EAA/EMQN, deletions were not observed among RPL males (Wettasinghe et al. 2010).

None of the azoospermic men had deletions at AZF region except at sY156 which represented AZFc region. Out of 50 azoospermic men 40 (80%) had deletion at sY156 locus. The rest (20%) were not detected with a deletion at sY156.

None of the patient groups showed deletion of the marker sY160. This suggests that the heterochromatic terminal region of the Y chromosome was intact in our study group. Neither groups did show any AZFc partial deletions.

3.3 Follow up analysis of deletion at sY156

For those who detected with deletion of sY156 were further subjected to analysis of secondary markers (sY1086 and BPY2). BPY2 and sY1086 flanks sY156 proximally and distally respectively. These STS loci will help to determine the boundaries of the deleted AZFc region. Upon analysis of 40 azoospermic men who had deletions at sY 156 loci, all were present with the BPY2 marker. In contrast the same group had deletions at sY1086
intermittently. Upon amplification we observed 13 out of 50 azospermic men were deleted for sY 1086.

3.3.1. Varification of absence of a PCR amplified product as a true deletion or a polymorphism

The observed high percentage of azospermic men with suspected sY156 deletion was an unexpected outcome. Thus it triggered the following questions.

1. Was sY156 polymorphic in the Sri lankan Sinhalese population?

2. Did the polymorphism contribute to the failure of PCR amplification of sY156 using the primer set or is it a true deletion?

3.3.1.2. Amplification of sY156 with extended primers

Using the extended primers it was possible to determine whether the deletion was a true one or whether amplification failure was caused by sequence variation under a primer. Flanking extended primers were designed based on the gene sequence from the GenBank database (Accession No. AC025735.4) flanking 500bp upstream and downstream of sY156 primer binding sites. If the sY156 fragment was intact, the expected size of amplified product would be about 1130 bp. There will be no PCR product or the amplified fragment would be shorter if the sY156 site was truly or partially deleted.

Out of 40 azospermic men 36 men had true deletions. The rest had partial deletions which correspond with amplified products ranging between 600bp to 700bp. These 4 men may be having partial deletions at sY156 loci. The observed amplifications may be caused by flanking regions, which represent 500bp up steam and downstream of sY156 primer binding.
sites. The above 4 subjects were subjected for sequencing to detect the deleted region within the extended sY156 primer binding site.

An equivocal amplification was observed in both RPL and oligozoospermic men for sY156, this infers absence of any polymorphism at sY156 loci. This result was further confirmed by alignment of sequence result with a reference genome with MEGA version 5 software.

3.4. Patient data analysis

The mean age of the azoospermic men was 33.8 years old, ranged between 25-40 years. The mean infertility duration was 5.4 years. The highest percentage of infertility (16.9%) was among men who are 35 years old, followed by men aged 34 years (12.3%). The mean volume of semen collected was 1.95 ml where as the maximum and minimum semen volumes collected ranges from 1.5ml to 3ml respectively. Majority of oligozoospermic men belong to 35 years of age. The rest of the parameters were more similar with that of azoospermic men. Testicular biopsy studies revealed that Most azoospermic men (47%) belong to spermatogenic arrest followed by Sertoli cell only syndrome.
3.5. Summary of Y chromosome microdeletion analysis.

A relationship could not be found among RPL males and recurrent pregnancy loss by the use of Indian panel of STS markers reported by Thangaraj and colleagues. Y chromosome microdeletions were not detected among Oligozoospermic men. Among azoospermic men microdeletion at sY156 was a common occurrence. The region covered by sY156 is probably devoid of any polymorphic genetic markers.
4. DISCUSSION

4.1. Multiplex PCR Optimization

Under optimal PCR conditions amplicons should give a single product of a particular base pair for a unique set of primers. The use of multiple primer pairs in the same reaction requires extensive optimization because amplicons are often amplified with different efficiencies (Polz et al. 1998). The presence of more than one primer pair in the multiplex PCR increases the chance of obtaining spurious amplification products, mainly due to the formation of primer dimers (Brownie et al. 1997). These nonspecific products may be amplified more efficiently than the desired target, consuming reaction components. Thus, the optimization of a multiplex PCR should aim to minimize or reduce such nonspecific interactions. Empirical testing and a trial and error approach may have to be used when testing several primer pairs, because there are no means to predict the performance characteristics of a selected primer pair even among those that satisfy the general parameters of primer design (Henegariu et al. 1997).

To optimize the PCR condition, two most important parameters were checked, the primer concentration and the annealing temperature. The first few rounds of thermal cycling have substantial effect on the overall sensitivity and specificity of PCR (Pryor et al. 1997). Success of specific amplification depends on the rate at which primers anneal to their target and the rate at which annealed primers are extended along the desired sequence during the early, middle and late cycles of the amplification. Annealing temperature is one of the factor which prevent optimal annealing rates. A gradient PCR machine would be ideal for the above task. In general primers should be present in excess. However, primer concentrations have to
be adjusted in a multiplex condition when the efficiency of amplification of one or more sets has to be compromised to ensure that sharpness of all bands in the multiplex PCR. Therefore several primer concentrations had been tested in this study. In general it was observed 1:1 ratio was the optimal primer concentration, except for sY156 which has got a higher molecular weight.

4.2. Polymorphisms in genetic markers

The sole deletion observed among azoospermic men in the study group at sY156 STS marker could be regarded as a rare variant or a true deletion. Nevertheless, no deletions at sY156 have so far been reported in men with proven fertility, and in our set of RPL men included. Literature review has elucidated, deletion in sY156 was observed among azoospermic men. It is therefore cannot exclude the possibility that the observed deletion might be related to infertility in our patient population as well. On the other hand there is a possibility to fail the PCR amplification, because a genetic marker in the Y chromosome may represent a normal polymorphism or a rare variant (Pryor et al. 1997; Vogt 1998). Genetic polymorphisms are differences in DNA sequences among individuals, groups or populations (Conn et al. 1940). The sources of polymorphisms include single nucleotide polymorphism (SNPs), sequence repeats, insertions, deletions and recombination. There are some instances where small deletions were found in fertile men, which in most cases due to polymorphisms in the genetic markers (Torroni et al. 1990; Vogt et al. 1996). It was recommended that STS loci which are polymorphic and amplify repetitive sequences on the Y chromosome should be abandoned (Maurer et al. 2000). The European Academy of Andrology (EAA) published guidelines for the molecular diagnosis of Y chromosomal deletions in 1999 and 2004. According to the
guidelines 12 STS markers had to be abandoned from Y chromosome microdeletion analysis due to polymorphisms and repetitive dispersion throughout the Y chromosome. For instance sY153 a sequence tagged site at AZFc region with polymorphism has been abandoned from Y chromosome microdeletion analysis. In contrary a study which was done to reappraise the use of sY 153 as a marker of Y chromosome microdeletion has shown real deletions and single nucleotide polymorphism (16173 C>G) in a Chinese population consisting of normospermic oligospermic and azoospermic (Unpublished data). The incidence of polymorphism varies between populations. In a nother instance a fertile Asian man had a SNP that led to false positive Y chromosome deletion result (Thornhill et al. 2002). The above observations supports the existence of differences in polymorphism between the Caucasian and non Caucasian population and selection of genetic markers should be based on population diversity.

4.3. Importance of extensive Y microdeletion analysis

4.3.1. Flanking STS loci analysis & sequencing

A fertile man who appered to be deleted for sY84 in the AZFa region was further subjected for analysis by amplifying the proximal and distal markers of sY84 which did not show any deletions (Thornhill et al. 2002). Sequencing the region flanking and including the sY84 revealed a single base alternation under the reverse primer, which probably caused the amplification failure. This observation underlines the importance of using at least two linked STS markers for the reliable diagnosis of Y chromosome microdeletions as proposed by the EAA guidelines. In accordance with that criteria sY1086 and BPY2 was selected as proximal and distal markers for sY156. Upon amplification it was observed 13 out of 50
azzospermic men were deleted for sY 1086. Sequencing the region flanking the deletion was an effective method to detect partial deletions or full deletions as was proven in the study.

4.4. Effectiveness of Y chromosome microdeletion analysis

4.4.1. Implications of microdeletion analysis on RPL males

One of the most significant findings of the work is that none of the male partners of women experiencing RPL had any microdeletions. Seminal fluid analysis reports indicated the majority of them to be normospermic. These results are in complete contrast to what was initially published by Dewan et al. In a pilot study Dewan et al. (Dewan et al. 2006) reported that 82% of male partners of women with RPL had Y chromosomal microdeletions, majority representing the AZFc region, which may have been the reason for the RPL experienced by their partners. This study has been criticized because of several pitfalls identified in the experimental design and interpretation of their results (Noordam et al. 2006). Kaare and colleagues (Kaare et al. 2008) conducted a similar study on 40 male partners had women with RPL using 37 STS loci spanning the whole Y chromosome and concluded that Y chromosome microdeletions were not associated with RPL in the Finnish population. Recently a Sri Lankan study conducted in our laboratory using STS loci recommended by EAA/EMQN concluded, that Y chromosome microdeletions were not found in RPL Sinhalese males. Thus in the Sri Lankan scenario Y chromosomal microdeletions do not appear to be important in the etiology of RPL and routine Y chromosome microdeletion testing is not necessary for male partners of women experiencing RPL.
4.4.2. Implications on azoospermic men

In the study microdeletions restricted to loci sY156 is somewhat a significant incidence. Microdeletions in sY156 have been detected in several Asian and European studies among azoospermic men. When compared with the literature sY156 deletion has been accompanied with several other linked STS loci and genetic markers. These markers are sY254, sY255, OX7, and Y6HP52. Simoni et. al (Simoni et al. 2004) recommended analysis of either sY156 or Y6HP52 has the same circumstances as both the markers analyze the same locus in interval D20 of the Y chromosome map. Majority of the azoospermic men showed deletions at sY156. This result was intriguing since pY6H sequences contain some homology to dhMiF1, a fertility gene sequence on the Y (Vogt et al. 1991) chromosome of Drosophila hydei (Vogt et al. 1991). A “de novo” microdeletion was detected in 2 out of 19 karyotipically normal sterile males. In both cases a probe of the pY6H sequence family was present. Since AZF, a human spermatogenesis gene has been mapped to Y interval 6, Vogt et al. postulated that the microdeletion may affected the functional DNA structure of the AZF gene. In primary spermatocytes, pY6H family loci contain RNA transcripts and associated proteins in the lamp brush loops. RB97D a protein which is essential for spermatogenesis and bound to a specific lamp brush loop, which contains two copies of a well characterized RNA binding domain, the RNA recognition motif, followed by a proline-glutamine rich domain and found in only spermatocytes nuclei. The protein and its loop binding function are evolutionarily conserved and thought to be essential for fertility (Heatwole et al. 1996).

Although BPY2 did not show any deletions among azoospermic men, there were several instances in families whose father and sons had an identical deletion involving AZFc with loss of the genes DAZ, BPY2, and CDY1, but showed different phenotypes (Chang et al. 2006).
1999; Saut et al. 2000). This confirms that AZFc microdeletions can be associated with features ranging from normal fertility, to mild oligozoospermia, to infertility characterized by severe oligozoospermia (Krausz et al. 2006) or azoospermia.

### 4.4.1.3. Other parameters effecting micodeletion detection rate

Microdeletion frequency in a sample of infertile men is not significantly related to the number of STS loci analysed (Simoni 1998). Kent-First and colleagues have analysed a large number of STSs in different Y chromosome regions. They have shown that each STS is statistically correlated with male infertility (Kent-First et al. 1999). It seems that patients’ selection criteria have a much more profound effect on the rate of detection of microdeletions that do the numbers of STSs analysed (Simoni et al. 1997). This variability in the detection of microdeletions between studies is probably explained by the different clinical selection criteria used by different research groups. Stringent selection of patients according to histologic, endocrinologic, and clinical criteria have been found to be associated with high deletion frequencies (Reijo et al. 1995; Stuppia et al. 1996; Foresta et al. 2001). For instance Foresta and colleagues have studied patients with idiopathic azoospermia and bilateral Sertoli cell only syndrome and found a very high number of Yq11 microdeletions (Kent-First et al. 1996; Foresta et al. 1998). In contrast, less stringent criteria for selection has been associated with low deletion frequencies in studies on a large number of men with oligospermia. Our study shows the influence of the selection criteria on the reported incidence of microdeletions; we had a low rate of Y chromosome deletions in our patient population. This may be probably due to following reasons.
Some infertile men may have cell populations in peripheral blood leukocytes that have a normal Y chromosome and yet have some sperm populations those with Y deletions. In a recent study, two infertile men who did not carry a deletion in peripheral blood cells conceived sons by assisted reproduction who had Yq microdeletions indicating that two fathers were mosaic for Y chromosome deletions (Jones et al. 1996). Y microdeletion analysis is usually performed on DNA extracted from peripheral blood lymphocytes (PBL) because it is easy to obtain. Although Y microdeletion analysis detects deletion of the Y genes at the stoma level it is generally assumed that the genomic DNA isolated from PBL is considered to be similar to that in germ cells such as spermatozoa and spermatids. However, PBL originate from the mesoderm. Thus, there is a possibility that the analysis of the Y chromosome for microdeletions from DNA samples isolated from PBL may not correspond with the results obtained at the germ cell level.

In several studies sperm DNA has been preferred over blood DNA to detect any germinal mosaicism and microdeletions (Hellani et al. 2005; Dada et al. 2007; Sakthivel et al. 2008). Sperm DNA might have higher rate of deletions and DNA damage as a result of oxidative stress. Wang et al. reported that reactive oxygen species levels are higher in the semen of infertile men (Wang et al. 2003). Multiple displacement amplification (MDA) is a technique used in the amplification of very low amounts of DNA and reported to yield large quantities of high-quality DNA (Hellani et al. 2004). Application of MDA into single cells made it possible to amplify DNA from a single sperm (Hellani et al. 2004; Hellani et al. 2005). MDA yields a consistent amplification of STS in the control sperm DNA.
4.4.1.4. Novel techniques beyond traditional PCR for microdeletion analysis

PCR analysis only indicates the presence or absence of a particular locus, other rearrangements such as duplications, triplications events and point mutations would not be detected. Both duplications and triplication events are relatively frequent in the Yq region and may result in deregulation of gene (Spurdle et al. 1993; Jobling et al. 1996; Santos et al. 1996). Quantitative PCR (qPCR), also known as real-time PCR, has become a powerful tool for the amplification, identification and quantification of nucleic acids. It’s ability to quantitatively and specifically detect genes has been invaluable for both diagnostic and research applications (Schweitzer et al. 2001). The diagnostic method for Y-chromosomal microdeletions in the EAA/EMQN guidelines has been successfully upgraded to a real-time polymerase chain reaction assay that can be readily applied as clinical routine. The hallmark of the real-time PCR, compared with conventional end-point methods, is the observation of the crossing point parameter that permits the detection of cross contamination during PCR start up or the presence of low quality DNA in the samples used. This information, provided by the technique, improves the safety because it reduces the risk of undetected false-negative results resulting from technical artifacts or PCR contamination. These methods were validated to be fast, simple, contamination free, high analytic sensitivity and specificity. In addition end point PCR techniques cannot detect mosaicism.

Comparative genome hybridization (CGH) has been identified as a potential methodology to improve aneuploidy analysis of gametes. In this process DNA from a test sample and normal reference sample are labeled differentially, using different fluorophores and hybridized to several thousand probes. The probes are derived from most of the known genes and non-coding regions of the genome, printed on a glass slide. The fluorescence intensity of the test
and of the reference DNA is then measured, to calculate the ratio between them. Modification of the technology to employ microarrays instead of metaphase chromosomes has facilitated the analysis of thousands of discreet loci. Literature data have showed that more than half of the variability between human genomes is due to submicroscopic copy number variations of DNA, and that these CNVs are responsible for some complex diseases, even more than single nucleotidetide polymorphisms (Freeman et al. 2006; McCarroll et al. 2007). There are several studies which has detected Y microaberrations, including microdeletions and microduplications (Pinkel et al. 1998; Fiegler et al. 2007). In a study which was carried by Ivanka et al. found Y microaberrations in 5 patients. The first patient was carrying a 16 Mbp deletion in Yq11.2 a region which involved many loci linked with non-obstructive spermatogenic failure where as others were detected with microduplications and polymorphism. In contrast to PCR array CGH could determine exactly the size and the boundaries of deletions on Y-chromosome. Thus array CGH analysis of spermatozoa of men with azoospermia could be used as a powerful method for high resolution detection of genomic imbalances across whole genome.

4.4.1.5. Implecation of scrutinised STS markers

The STS markers prescribed by EAA claim to detect over 90% of the microdeletions in the AZF loci. However, in several studies at home and in India, the deletion range was between 3-5% of subjects for the STS markers recommended by EAA. Subsequently by amalgamation of other STS loci the deletion detected rate has been increased (Saxena et al. 2000; Thangaraj et al. 2003; Sakthivel et al. 2008). It has been suggested that the inability to find Y chromosome deletions in certain populations may be due to the fewer number of STS markers, heterogeneity of markers used among different studies, and also ethnic variations. In
the study it was assumed that a panel of 23 STS markers which has been been validated and a deletion detecting range of 4-6% in Delhi and Chennai respectively in India which represent a similar popular genetic structure. Ironically it seems that a single STS loci was powerful enough to detect deletions among oligospermic and azoospermic population. Thus, it infers that another set of markers specialty in and around sY156 should be used for future studies along with a larger number of sample with homogenous phenotypes and using large number of STS markers which will eventually identify markers specific for Sri lankan population will increase the detection of Y chromosome microdeletions.

4.4.1.6. Impact on homogenous sample population

The frequency of Y chromosome microdeletions is related to sample size, differences in inclusion criteria for enrolling subjects into the study, and the number of markers tested. In a study conducted by Foresta et al. (Foresta et al. 1998), 18 azoospermic males with Sertoli cell only syndrome, a highly specific group, was screened and deletions found in 55.5% of the cases. In a follow up study of 130 infertile males, who were azoospermic/severely oligospermic (110) and moderately oligospermic (20), no deletions were found in moderately oligospermics males. The former study had a homogenous group of patients, and maybe the frequency of Y chromosome microdeletion is higher in such cases. In contrast, in the latter study, despite a larger sample size, the patients included belong to different groups - azoospermia, and moderate oligospermia. Y chromosome microdeletions were present in 28 cases (25.4%). In the present study the results were in accordance with the past research in which microdeletions were detected mostly in the AZFc region. Interestingly the microdeletions were limited to a single genetic marker (sY156) in majority (80%) of
azoospermic men. According to the testicular biopsy analysis the azoospermic men belong to Sertoli cell only, and spermatogenic arrest categories. Thus the azoospermic men were not a homogenous group in the study population. Absence of microdeletions on other STS loci (other than sY156) may be due to the lack of homogeneity among oligozoospermic and azoospermic participants.
5. CONCLUSION

In conclusion none of the STS markers in the Indian study panel were deleted in men whose partners experienced RPL, however, the marker at sY156 was deleted in 80% of infertile men with azoospermia. This suggest that adding the sY156 marker to the EAA/EMQN panel would increase the diagnostic yield for Y chromosome microdeletions in men with azoospermia in Sri Lanka.
REFERENCES


##APPENDIX 1: GENES AND ITS FUNCTIONS

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Number of copies and code</th>
<th>Protein homolog to</th>
<th>Tissue RNA expression</th>
<th>Copies to Yp interval</th>
<th>Yq11 interval</th>
<th>X chromosome homolog</th>
<th>Autosome homolog</th>
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103
# APPENDIX 2: LIST OF ABBREVIATIONS

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<td>ART</td>
<td>Assisted reproductive techniques</td>
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<tr>
<td>AS</td>
<td>Acrosomal stats</td>
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<tr>
<td>AZF</td>
<td>Azoospermic Factor</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CBR</td>
<td>Crude birth rate</td>
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<tr>
<td>CDY</td>
<td>Chromodomain Y gene</td>
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<td>DAZ</td>
<td>Deleted in Azoospermia Gene</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EAA</td>
<td>European Academy of Andrology</td>
</tr>
<tr>
<td>EMQN</td>
<td>European Molecular Genetics quality Network</td>
</tr>
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<td>EtBr</td>
<td>Ethidium Bromide</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle Stimulating Hormone</td>
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<td>H₂O</td>
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<td>ICSI</td>
<td>Intra-Cytoplasmic Sperm Injection</td>
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<td>IVF</td>
<td>In vitro fertilization</td>
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<td>MSY</td>
<td>Male-specific region of the Y chromosome</td>
</tr>
<tr>
<td>NRY</td>
<td>Non-recombining region of the Y chromosome</td>
</tr>
<tr>
<td>OAT</td>
<td>Oligo astheno teratozoospermic</td>
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<td>OD</td>
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<td>P</td>
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<td>PAR</td>
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<td>PCR</td>
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<td>Rpm</td>
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<td>RT-PCR</td>
<td>Real-time PCR</td>
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<td>SCO</td>
<td>Sertoli cell-only</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<td>SPSS</td>
<td>Statistical package for the social sciences</td>
</tr>
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<td>SRY</td>
<td>Sex determining region of Y gene</td>
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<tr>
<td>STS</td>
<td>Sequence tagged site</td>
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<td>TFR</td>
<td>Total fertility rate</td>
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**Table 1.3.** Human Y Genes with known Functions mapped in AZFa,AZFb and AZFc Deletion Interval