CHAPTER II
LITERATURE REVIEWS

Infertility

Infertility is defined as an inability to conceive a child after one year of regular unprotected intercourse. It affects 15% of couples who try to conceive. Infertility is either primary, where no pregnancy has ever occurred, or secondary, where there has been a pregnancy regardless of the outcome [36].

Male Infertility

Male factor infertility represents about 25-40% of infertility cases [37, 38] and it has been associated with several genetic and non-genetic conditions. In clinical practice, a search for non-genetic causes, such as seminal infection, anatomical malformation and chemical insult, should precede any genetic analysis. However, genetic evaluation is also recommended even when infertility is apparently related to other obvious causes, as both conditions may coexist. From a genetic point of view, infertility patients are a high-risk group. The prevalence of numerical and structural chromosomal abnormalities in patients undergoing assisted reproduction is around 10% and 1% respectively, compared with 0.85% and 0.1% in the general population[29]. Genetic mutations are clinically relevant as a cause of male infertility and as a risk factor for transmission to the next generation.

Semen analysis is routinely performed to evaluate male infertility. Men, who have no spermatozoa in their ejaculates, after microscopic examination of the centrifuged specimen, are diagnosed as “azoospermic”. Testicular biopsy may provide further information. Those who have normal sperm production inside the testes are affected by obstructive azoospermia. This condition may be congenital or acquired[39]. In non-obstructive or secretory azoospermia, there is defective spermatogenesis that may be due to hypothalamic-pituitary insufficiency or primary testicular failure. Alternatively, azoospermia can be classified as pre-testicular (hypothalamic-pituitary insufficiency), testicular (seminiferous failure) or post-testicular (obstructive azoospermia) [40].

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Causes of male infertility

Male infertility may be due to congenital malformation or acquired pathology. In approximately 40-75% of cases, no pathological cause can be identified. This condition is called idiopathic male infertility [6, 41, 42].

1. Congenital bilateral absence of the vas deferens (CBAVD)

This is the most frequent abnormality of the extra-testicular duct system. It occurs in 1% to 2% of infertile men [43]. Without a vas deferens, the sperm cannot pass from the testis into the ejaculate. In some cases, CBAVD is a part of the phenotypic spectrum of cystic fibrosis, which is an autosomal recessive disorder with an incidence of 1 in 1,600 to 2,500 live births. Cystic fibrosis is caused by a mutation in CF transmembrane conductance regulator (CFTR) gene. This gene is responsible for transportation of chloride ions material across many surfaces of the body. More than 500 such mutations have been identified to date. Men born with CBAVD have several parts of their reproductive tract missing, including the vas deferens.

2. Abnormalities of testosterone (androgen) receptor gene

Androgens play a crucial role in the development of male reproductive organs and in male pubertal development. In adults, androgens are necessary for male fertility and male sexual function. Testosterone selectively binds to the androgen receptor to initiate and maintain spermatogenic processes and inhibit germ cell apoptosis. Mutations in the androgen receptor (AR) gene result in a condition known as androgen insensitivity syndrome (AIS). In this rare condition, tissues in the body cannot respond to circulating testosterone. In its severe form, known as complete AIS, males express a female phenotype, with breast development at puberty. In subtle forms, individuals may have ambiguous genitalia or normal male development with poor sperm production and infertility [44, 45].

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3. Infection and sperm production

Infection of the testis is known as orchitis. Mump orchitis is caused by a virus and it is perhaps the best known testicular infection. Mumps orchitis does not always accompany mumps. If it occurs in pubertal males, it can result in significant swelling

and pain, and may lead to total destruction of testicular sperm production. Less severe episodes of mump orchitis may result in cessation of sperm production for up to six months. As no effective treatment exists, it is important to prevent the infection by vaccinating children against mumps [41].

4. Sperm antibodies

Antibodies are substances that the body produces in response to an invasion by foreign organisms. The body's ability to tell what is self and what is foreign is usually 'programmed' before birth. As spermatozoa are not produced before puberty, the body recognizes them as foreign and, therefore, can produce antibodies against them. Spermatogenesis normally occurs inside the seminiferous tubules, which are excluded from the immune system by a "blood-testis barrier"[46].

Approximately 5% to 7% of men with infertility produce antibodies to their own sperm. This may occur as a result of testicular surgery or trauma, or following vasectomy. These antibodies may impair fertility by coating the sperm and interfering with their movement along the female reproductive tract. The sperm antibodies may also prevent egg and sperm interaction during fertilization. In some cases, sperm antibodies may directly damage the testis and impair spermatogenesis [47].

5. Varicocele

Valves inside testicular veins help in the drainage of blood against gravity from the testes back to the heart. If they are not functioning properly, blood will pool in the veins and this will result in venous dilation around the testis, forming a varicocele. This condition occurs more often on the left side of the body because the left testicular vein empties high into the left renal vein, while the right testicular vein drains directly into the nearby vena cava. To detect the presence of a varicocele, it is necessary to examine the patient standing-up.

Varicoceles are present in 15% of men in the general population, but they are more common in those with poor semen quality. Studies comparing men with and without varicoceles show that men with varicoceles have a lower average sperm count, decreased sperm motility and an increase in the number of abnormal spermatozoa.

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Even so, some men with varicoceles have normal sperm counts and many of them can father children [48].

6. Cryptorchidism or maldescended testes

Cryptorchidism is a condition where one or both testes fail to descend into the scrotum. It is the most frequent congenital abnormality of the male reproductive system, affecting approximately 3% of full-term and 33% of preterm male infants. At 1 year of age, the prevalence drops to about 1%, and remains the same into adulthood. Cryptorchidism poses an increased risk of developing testicular cancer. It also decreases sperm count and leads to male infertility. The incidence of infertility is about 32% and 59% in men with unilateral and bilateral cryptorchidism respectively[49]. In infertile couples, a medical history of cryptorchidism was found in 8% of males [50]. The cause of cryptorchidism is multifactorial and largely unknown. Possible causes and risk factors include endocrine disorders, anatomical abnormalities, and environmental and genetic factors [51].

Kunej provided evidence that Y chromosome microdeletions are not causally related to cryptorchidism. However, both conditions may coexist in the same patients[50]. Therefore, it is reasonable to include these patients in Y chromosome microdeletion screening, as the deletion may be iatrogenically passed on to their male offspring because of the increasing use of intracytoplasmic sperm injection [32].

7. Radiation damage

Radiation therapy is designed to attack and destroy rapidly dividing cells. As spermatogenic cells are actively dividing, they are very vulnerable. Attempts should be made to shield the testes during radiation therapy, but this is not always possible. Radiation can cause permanent damage to the testes and result in sterility. In some cases, the damage may be temporary and recovery is possible. Radiation also has the potential to cause genetic mutations in females. Female patients are, therefore, recommended to wait at least two years after radiation therapy before they attempt a pregnancy. Sperm storage should be considered if a man is about to undergo any medical treatment that could permanently impair his sperm production [52].

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8. Drugs and other substances causing infertility

Many common drugs and chemicals have a negative effect on sperm production and/or function.

8.1 Medication for cancer treatment

Anti-cancer drugs are designed to attack cancer cells. However, other dividing cells in the body can be affected. Spermatogenic cells in the testis are continually growing and are open to damage by cancer treatment. Many young men, who were treated with chemotherapy or radiotherapy in the past, may have low or absent sperm production and need infertility treatment. In some cases, spermatogonia have been totally destroyed and no treatment is possible. Others, who have a low level of sperm production, may be successfully treated with assisted reproductive techniques (ART)(52).

8.2 Environmental and chemical agents

Some environmental and chemical agents may cause infertility. One striking example is the pesticide called dibromochloropropane that is used to kill worms in pineapple plantations. Men exposed to this pesticide were found to have zero sperm count (53).

There is much debate about the possible long-term effects of pesticides and other industrial agents, such as heavy metals and glycol ethers, on male infertility. Further researches is urgently needed. However, studies are hampered by the fact that it may take some 25 years for an exposure to result in an infertility disorder later in life. Common sense would suggest caution in the use of such agents (54).

8.3 Anabolic steroids

Anabolic steroids were found to stop pituitary hormone stimulation of the testis, which results in reduced testicular size and low sperm count. Torres-Calleja reported that men using anabolic steroids have a decrease in count, motility, and percentage of spermatozoa with normal morphology. The levels of LH, FSH, and testosterone decreased during the intake of these steroids. Based on changes in hormonal profile and semen characteristics, they concluded that anabolic steroids alter male reproductive function (55).

8.4 Reactive oxygen species (ROS)

Increased levels of ROS can cause damage to the sperm membrane. Substances such as peroxides and hydrogen peroxide can be released by abnormal spermatozoa and

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white blood cells. ROS-mediated damage of sperm membranes has been reported to be responsible for impaired sperm motility [56]. Subsequently, spermatozoa lose their capability to undergo the acrosome reaction and to penetrate the oocytes [57].

9. Genetic abnormalities

Severe oligospermia and azoospermia may be associated with genetic abnormalities such as constitutive chromosome abnormalities and microdeletions of the Y chromosome [58]. In a survey of pooled data from 11 publications, involving 9,766 infertile men, the incidence of chromosome abnormalities was 5.7% [59]. Of these, sex chromosome abnormalities accounted for 4.2% and autosomal abnormalities for 1.5%. In comparison, the incidence of chromosome abnormalities in pooled data from three studies with a total of 94,465 newborn male infants, was 0.39%, of which 0.14% were sex chromosome abnormalities and 0.25% were autosomal abnormalities [60].

Large deletions of the Y chromosome were first demonstrated in azoospermic and oligospermic men in 1976 [13]. The deletions caused spermatogenetic failure because of the loss of genes controlling spermatogenesis. Such deletions are unidentifiable by conventional karyotyping and have to be specially tested for, using molecular techniques. As such, they are called "Y chromosome microdeletions [14]. The frequency of microdeletion is highest in azoospermic or severely oligospermic men.

The endocrine control of spermatogenesis

1. Regulation of the testes

The testes have two distinct compartments. The first comprises the seminiferous tubules, which contain germ cells called spermatogonia and Sertoli cells. Tight junctions between Sertoli cells form a diffusion barrier known as the blood-testis barrier that protects the germ cells from antibodies and environmental toxins. Spermatogenesis occurs inside the tubules, which are essentially avascular. This process takes approximately 70 days and is directed by genes located on the Y chromosome. Another 12-21 days are required for sperm transportation from the testis to the epididymis and the ejaculatory duct. The second compartment comprises the

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Leydig cells which produce testosterone. These cells are located in the connective tissues between the seminiferous tubules.

Normal testicular function requires the action of both follicle-stimulating hormone (FSH) and luteinizing hormone (LH). LH stimulates Leydig cells to synthesize and secrete testosterone. The action of LH is indirectly supported by FSH, which induces the appearance of LH receptors on Leydig cells. Testosterone is secreted both into the circulation and into the lumen of the seminiferous tubules, where it is concentrated to a level 50-100 times higher than in the blood. FSH binds to Sertoli cells and stimulates the production of androgen-binding protein (ABP), which serves to maintain the high local concentration of testosterone required for spermatogenesis. However, FSH levels do not provide an accurate prediction of the status of spermatogenesis on an individual basis [37]. One report found that variations in plasma FSH concentration can arise for reasons unrelated to spermatogenesis [61] and many patients with maturation arrest have normal plasma FSH concentrations.

2. Endocrine disorders

Male infertility is rarely caused by hormone deficiency (less than 1% of cases) [20]. Any condition that lowers FSH and LH levels, such as pituitary destruction by a tumor, can result in low or no sperm production and low blood testosterone levels [21-23]. On the other hand, when there is destruction of seminiferous tubules, spermatogenic cells will be absent or markedly diminished. In such cases, there will be low or no sperm production despite the very high FSH levels [62-64].

The Y chromosome and Y chromosome microdeletions related to spermatogenic failure

The Y chromosome

The human Y chromosome is approximately 60 Mb in length. It is the smallest chromosome and represents only 2% of the human genome. More than 30 genes and gene families that are involved in spematogenesis have been identified thus far on the human Y chromosome [65]. Some of these genes are located along the long arm in a region called Azoospermia factor (AZF) [66-68]. The AZF region on Yq11 has been subdivided into three spermatogenetic loci, by molecular deletion mapping: AZFa, AZFb and AZFc (Figure 1.1). These regions are believed to contain multiple genes

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required for different stages of spermatogenesis (Figure 2.1). Deletion in any or all of the three AZF regions will disrupt spermatogenesis[69]. On the other hand, Yq microdeletion was tested in 392 normospermic men and no cases of deletion were detected [70]. This supports the specific role of AZF microdeletion in spermatogenic failure.

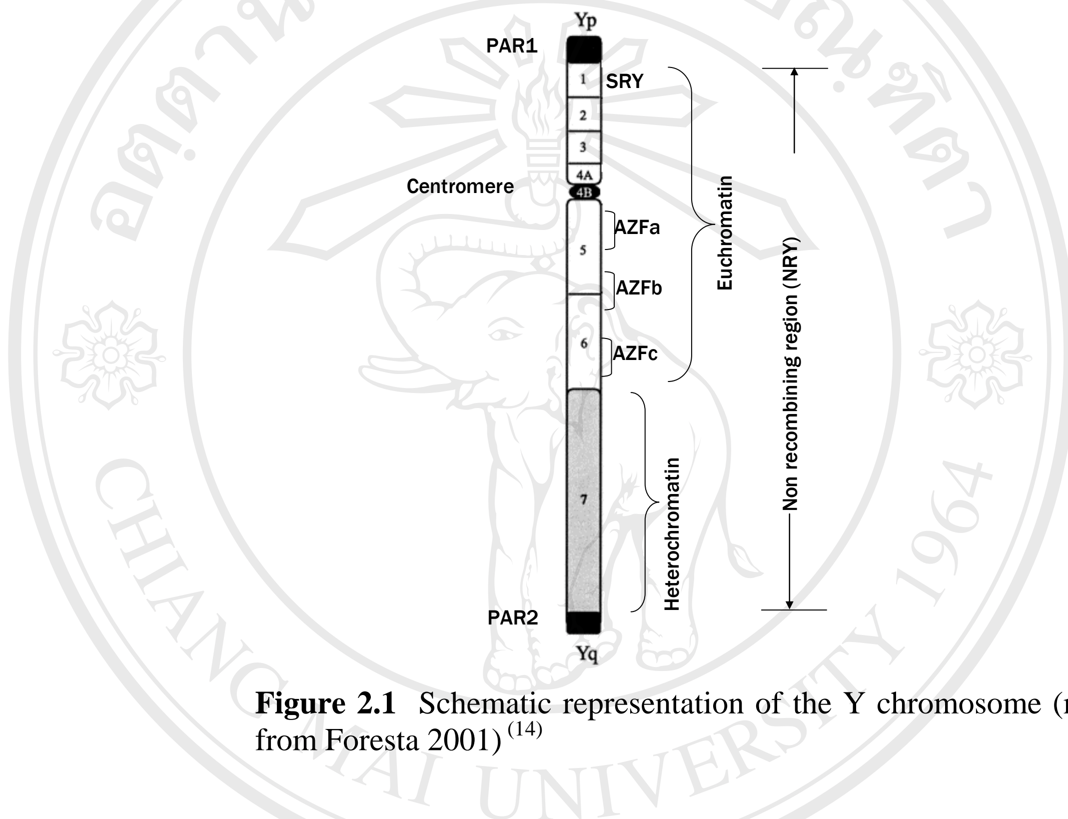

Figure 2.1 Schematic representation of the Y chromosome (modified from Foresta 2001) [14]

The majority of testis-specific genes are represented by multiple copies for example: one (*TGIF2LY*), two (*VCK, XKRY, HSFY, PRY*), three (*BPY2*), four (*CDY, DAZ*), six (*RBMY*) and approximately 35 (*TSPY*) copies on the Y chromosome [71].

The AZFa region is approximately 1100 kb in length and contains the single copy genes *DFFRY* (or *USP9Y*), *UTY* and *DBY*. AZFb overlaps with the proximal part of AZFc and includes the genes *RBMY*, *EIF1AY, SMCY, PRY* and *TTY2* [66, 71]. The complete deletion of AZFb removes 6.2 Mb (including 32 copies of genes and transcription units). The AZFc region includes 12 genes and transcription units, each present in a variable number of copies making a total of 32 copies [72]. The classical complete deletion of AZFc, the most frequent pattern among men with deletions of the

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Y chromosome, removes 3.5 Mb. Genes in this region that are known to be involved in spermatogenesis are *DAZ, CDY1, BPY2* [73].

In additional, AZFd region has also been proposed by Kent-First *et al.*[16]. Deletion of this region in patients with intact AZFa, AZFb and AZFc also results in oligospermia or azoospermia. AZFd has been localized between AZFb and AZFc, but a candidate gene has not yet been identified [16].

Y chromosome microdeletions

Microdeletions of the Y chromosome are the second most frequent genetic cause of spermatogenetic failure in infertile men after Klinefelter's syndrome [25].

1. Mechanism of Y chromosome microdeletions

It is currently believed that deletions almost invariably arise through homologous recombination between identical repeated sequence blocks located within Yq11, with loss of the genetic material between them [69, 71, 74, 75]. Intrachromosomal recombination events between repetitive specific HERV (Human Endogenous Retroviral) sequence blocks are now known to cause complete AZFa deletions [74, 76, 77]. The extent of extension for complete AZFa deletions has been estimated to be 782 kb [78]. Similarly, recombinations between large homologous sequence blocks composed of different repetitive sequence families are the major cause of complete AZFb and AZFc deletions and their sequence length is estimated to be 6.2 Mb (AZFb) and 3.5 Mb (AZFc), respectively [71, 75] as shown in Figure 2.2.

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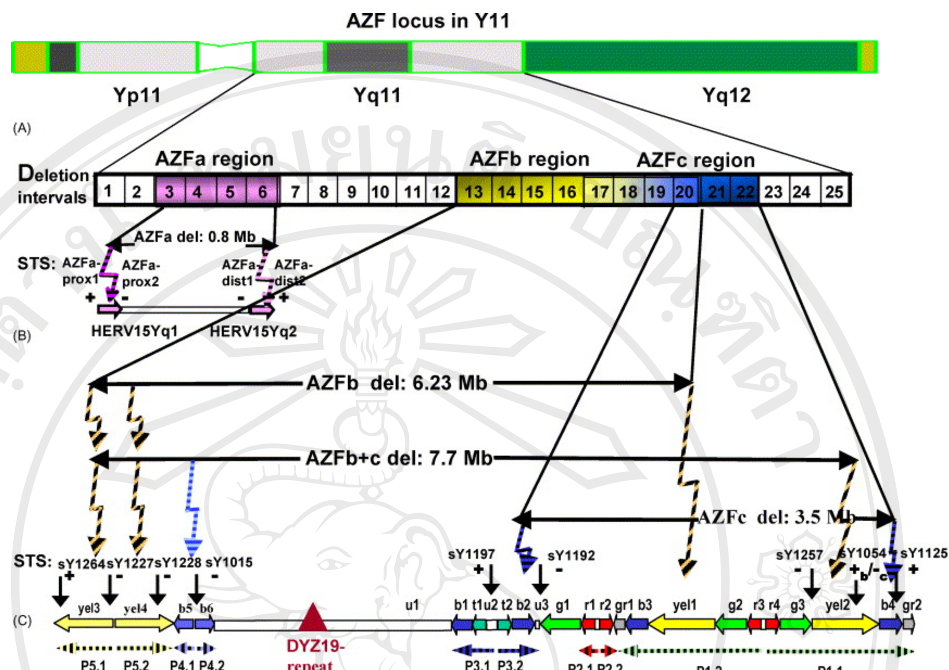

Figure 2.2 Schematic representation of the AZF locus in Yq11. (A) The AZF locus was subdivided by molecular deletion analyses into three regions by Vogt [79] who divided Yq11 into 25 intervals (D1–D25). (B) Complete AZFa deletions are always associated with SCO syndrome. The deletions are caused by recombination of two homologous HERV15Yq1/q2 blocks, located in the proximal region of Yq11. Complete AZFb deletions are always associated with meiotic arrest during spermatogenesis. Overlap of AZFb and AZFc are shown. (C) AZFb deletions are caused by a recombination between large homologous repetitive sequence blocks (or amplicons). They are designated as P1.1 and P1.2 (yel1 and yel2) in the P1 palindrome and P5.1 and P5.2 (yel3 and yel4) in the P5 palindrome, respectively. Similarly, AZFc deletions are caused by recombination between the blue amplicons b2 and b4. AZFc deletion ahow a mixed atrophy of germ cells patient testicular tubules and hypospermatogenesis. In patients with an AZF(b + c) deletion, the distal breakpoint was found to be not in the b4 amplicon but in the yellow amplicon yel2. The STSs which can be used to diagnose complete AZFa, AZFb, or AZFc deletions with simple duplex PCR experiments are given above each amplicon. The five possible palindromic structures P1–P5 and the DYZ19 repeat in AZFb are marked with the extensions of both arms below the amplicon structure in a similar colour code as that used for the corresponding amplicons [79].

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2. Prevalence of Y chromosome microdeletions

Many studies show that male infertility is associated with a high incidence of Y chromosome microdeletions, ranging from 4.3%-23.0% in azoospermic men and 0.1%-9.0% in oligospermic men (Table 2.1). The reason for such a wide difference is not clear, however, possible causes include: patient selection criteria, experimental design, environmental influences and ethnic or population variations [31, 33, 80-82]. Currently, it is difficult to make direct comparisons among reported results because there is no standardized methodology [18]. The frequency of Y chromosome microdeletion seems to increase with the severity of spermatogenic defect. Many genes may be present in several copies and thus their differential deletion may lead to dosage effects [67, 83].

Maurer reported 34 patients with Y chromosome mirodeletions. They found that deletions of the AZFc region were the most common (79%), followed by AZFb (9%), AZFbc (6%), AZFa (3%) and AZFabc (3%) [84]. Therefore, deletions in the AZFa interval occur at a much lower frequency than AZFb and AZFc [85].

To date, most studies have used sequence-tagged site [29] primers and reported a very wide variation in the incidence of Y microdeletions among infertile men [25], from 1% to 55% [19, 86]. Most of the STS primers used thus far amplify anonymous sequences, which do not belong to specific genes [10, 30, 87, 88]. However, the microdeletion of an STS could represent a clinically irrelevant polymorphism rather than the cause of spermatogenic failure [12, 31-34].

A review of articles published between 1996 and 1999 revealed that more than 150 markers have been used. Some of these markers may give inaccurate results because they are polymorphic or one of the repetitive sequences dispersed throughout the Y chromosome [89]. Because anonymous STSs were used, individual gene-deletion status was unavailable in most patients and genotype-phenotype correlation was not possible.

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Table 2.1 The prevalence of Y chromosome microdeletions in different parts of the world

Locations	**Prevalences**			**References**
	Azoospermia	Oligospermia	Idiopathic cause	
Korea	20%			Kim SW, *et al.* (1999) [6]
USA	20%			Silber SJ, *et al.* (1998) [90]
Israel	6.7%	3.6%		Kleiman SE, *et al.* (1999) [12]
Hong Kong (Chinese)		9% (AZFc)		Tse JY, *et al.* (2000) [82]
Hong Kong (Chinese)	8.5%	8.5%		Tse JY, *et al.* (2002) [22]
Spain	14%		7%	Martinez MC, *et al.* (2000)[91]
Germany			1.3%	Maurer B, *et al.* (2000) [92]
Finland	9%	0.1%		Aho M, *et al.* (2001) [93]
Greek (Cypriot)	12.5%		5.9%	Ioulianos A, *et al.* (2002) [94]
Japan	11.7%	7.5%		Nakashima M, *et al.* (2002) [95]
Japan			7.6% (12/157)	Sawai H, *et al.* (2002) [96]
The Netherlands			9.3% (ICSI)	Dohle GR, *et al.* (2002) [97]
Slovenia	8.6% (8/92)	1.5% (2/134)		Peterlin B, *et al.* (2002) [98]
Taiwan	10.6%			Lin YM, *et al.* (2002) [99]
Taiwan	23%	5%		Chen SU, *et al.* (2003) [100]
India (infertile men)			9.63%	Dada R, *et al.* (2003) [101]
Romania (infertile men)			10%	Raicu F, *et al.* (2003) [102]
Brazil			6.7%	SaoPedro SL, *et al.* (2003) [103]
Turkey	4.25%		3.3%	Sargin CF, *et al.* (2004) [8]
Turkey	14.3%	2.2%		Vicdan A, *et al.* (2004) [104]
Egypt	12%			El Awady MK, *et al.* (2004) [105]
Average	**12.5%**	**4.7%**	**6.74%**	

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3. Y chromosome microdeletions and infertility

Although Y chromosome microdeletions represent the most frequent molecular genetic cause of infertile in men, as many as 85% of azoospermic and 90% of severe oligospermic men do not have a deletion. Other Y chromosome related factors, such as variation in repeat sequences in multicopy gene families, mutation polymorphism in Y-specific genes or rearrangements such as duplications, could contribute to the infertile phenotype.

Intracytoplasmic sperm injection (ICSI) involves the direct injection of a single spermatozoon or a spermatid into the cytoplasm of an oocyte. In this technique, the natural selection of the spermatozoa is bypassed. Ejaculated, epididymal or testicular spermatozoa can be used, either fresh or freeze–thawed. The presence of a Y microdeletion does not seem to alter the fertilization of oocytes or the development of the resulting embryos [106]. The ICSI technique provides opportunities, hitherto not possible, for such men to be genetic fathers [107], however, it has inevitably increased the risk of transmitting male infertility from father to son. Kent-First [4] found that 9% of sons born after ICSI had Y chromosome microdeletions.

Natural transmission of deletions involving the entire AZFc region has been reported [15, 32, 108, 109] but sperm analysis was only available in two cases. Two fathers were able to father only one child whereas the father of four infertile sons was azoospermic many years after the natural conception of his sons. Consistent with this report, a progressive decrease in sperm number over time has also been reported in infertile men with an AZFc deletion [11, 31, 109]. Environmental effects or different genetic backgrounds may also account for the variable phenotypes observed.

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4. Genotype/phenotype correlation

Y microdeletions are specific for spermatogenetic failure as no deletions have been reported in a large number of normospermic men [110]. It is more appropriate to consider Y deletions as a cause of oligospermia or azoospermia rather than the cause of infertility because natural fertilization may occur even with relatively low sperm counts depending on the female partner's fertility status [25, 70].

Deletion of the entire AZFa region invariably results in complete Sertoli cell only syndrome (SCO) and azoospermia. Deletions of isolated genes of the AZFa

region, involving only the *USP9Y* gene or the *DBY* gene are related to a variable testicular phenotype [111, 112]. A complete deletion of the AZFa region implies that it will be virtually impossible to retrieve testicular sperm for ICSI.

Complete deletions of AZFb and AZF(b+c) are characterized histologically by SCO or spermatogenetic arrest resulting in azoospermia. Similar to the complete deletion of the AZFa region, several reports have shown that no spermatozoa are found at testicular sperm extraction (TESE) in these patients [7, 113]. ICSI should, therefore, not be recommended in cases of complete deletion of AZFa or complete deletion of AZFb or AZF(b+c) as they are incompatible with sperm retrieval.

Deletions of the AZFc region are associated with a variable clinical and histological phenotype [114-116]. AZFc deletions can be found in men with azoospermia or severe oligospermia and, in rare cases, can even be transmitted naturally to the male offspring [117]. There is a fairly good chance of retrieving sperm at TESE from a man with azoospemia and an AZFc deletion [4, 24, 98, 116, 118, 119]. Hypospermatogenesis is, therefore, the primary result of an AZFc deletion [120] and it seems to have a specific impact on the spermatogenetic process but not on fertility. The sons of these patients will be AZFc-deleted and will have an infertility problem when they reach reproductive age. Most AZFc deletions are found only in the patient's Y chromosome, but not in those of other family members, suggesting these are "*de novo*" mutations[121].

Partial AZF deletions have been identified repeatedly in different infertility clinics (AZFa [111], AZFb [7], AZFc [122]). However, they do not follow the strict genotype–phenotype correlation that has been observed for the complete deletion of the AZFa and AZFb regions [78]. This indicates the importance of genetic background and eventually of environmental factors in the expression of certain gene defects. Further study, that focuses on male fertility genes outside the Y chromosome, is needed.

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5. Clinical significance of Y chromosome microdeletions

The identification of Y deletions has diagnostic, prognostic and preventative treatment value. In azoospermic men, the presence of a complete AZFa or AZFb deletion has a

negative prognostic value for testicular sperm retrieval [7, 123]. All male offspring, who were conceived through ICSI using spermatozoa from patients with an AZFc deletion, have been reported to inherit this same deletion from their fathers [4, 116, 118]. Although no genital or other somatic cell defects have been reported in these ICSI-AZF offspring so far [121], it is good clinical practice to consider Y chromosome deletion analysis prior to ICSI treatment.

The number of spermatozoa in some men with Y chromosome microdeletions has been observed to deteriorate. Some genes, which are present in multiple copies (e.g. *DAZ* and *RBMY*), may be depleted over time resulting in progressively worse oligospermia and eventually azoospermia. Early detection of Y chromosome microdeletions in men and their ICSI offspring can improve clinical management. For example, sperm cryopreservation and testicular biopsy may be considered when there is still a good chance of success [124]. Cryoconservation of spermatozoa may avoid future invasive techniques such as TESE/ICSI [5].

Krausz *et al*. [110] concluded that:

1. Y microdeletions have been found almost exclusively in patients with <1 million spermatozoa/ml
2. Deletions are extremely rare in patients with a sperm concentration >5 million spermatozoa/ml (approximately 0.7%)
3. The most frequent deletions occur in the AZFc region (approximately 60%), followed by AZFb, AZF(b+c) or AZF(a+b+c) regions (35%), whereas deletions of the AZFa region are extremely rare (5%)
4. Isolated gene-specific deletions must be extremely rare and are found only for AZFa genes so far
5. Deletions may be found to be independent of other pathological conditions such as: varicocele, cryptorchidism, hypogonadotrophic hypogonadism, obstructive azoospermia.

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Methods of Y chromosome microdeletions detection

Initial discoveries of Y chromosome deletions in infertile men were made by karyotype analysis, where deletions of Yq11 were observed [13]. However, Y

chromosome deletions are often too small to be detected by standard karyotyping methods. Polymerase chain reaction (PCR), using sequence tagged sites (STSs) or gene-based markers and standard gel electrophoresis is now widely used to identify these microdeletions [92]. A commercial multiplex PCR primer-mix kit has also been developed (Promega, MI, USA). However, analysis of multiple PCR products on a gel, depending on their molecular sizes and band intensities, is sometimes complicated and dependent on the individual researcher's experience [27]. Recently, other research groups have developed new molecular techniques to scan for AZF microdeletions such as: Primed *in situ* labeling (PRINS) [26], DNA chip technology [27] and real-time PCR [28]. These protocols are interesting but they are also expensive, complex and require a certain level of expertise.

Multiplex PCR

Multiplex polymerase chain reaction (PCR) is defined as the simultaneous amplification of multiple regions of a DNA template by adding more than one primer pair to the amplification reaction mixture. It is often desirable to amplify several sequences of interest simultaneously in a multiplex reaction instead of performing many individual PCRs. Multiplex PCR systems uses primers for different loci, which amplify fragments whose product lengths do not overlap when analyzed on agarose or polyacrylamide gels. Multiplex PCR also offers a significant time and cost saving. Furthermore, reducing the number of pipetting steps minimizes the possibility of contamination and sample mix-up [125].

1. Multiplex PCR primer design

Multiplex PCR requires that all primer pairs in a reaction amplify their unique targets under a defined set of conditions. Most multiplex PCR reactions are restricted to amplification of three to ten targets. One reason is that a degree of flexibility is lost with each additional primer set included in the reaction. Increased numbers of primers also increase the probability of primer-dimer formation and non-specific amplification. The development of an efficient multiplex PCR requires strategic planning and often multiple attempts to optimize reaction conditions. Ideally, all primers in a multiplex reaction should amplify their individual target sequence with equal efficiency. In

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practice, it is difficult to predict the efficiency of a primer pair under these conditions. The primers used need to have similar characteristics, such as melting temperature (T_m), and should not exhibit significant interactions with each other or with unwanted regions of the template DNA. Oligonucleotides with near-identical annealing temperatures should work well under similar conditions [126].

2. General rules for multiplex primer design and optimization

Generally, all primers in a multiplex reaction should be matched for T_m. Care should be taken to avoid primers with complementary 3′ nucleotides. Each primer should be tested separately to determine optimal conditions. Once a panel of primer pairs has been generated, they need to be mixed sequentially and conditions optimized:

1. The length of individual primers should be 18-24 bases. Longer primers are more likely to result in formation of primer-dimers
2. The annealing temperature should be kept as high as possible. It is important to identify the annealing temperature for each primer pair and use the highest temperature in the multiplex reaction. Similarly, the minimum number of cycles should be used. Annealing temperature and cycle number are critical to the success of multiplex PCR.
3. Since multiple PCR products are simultaneously amplified, the pool of enzyme and nucleotides in a multiplex PCR can be a limiting factor, and more time is required for complete synthesis of all products. It is important to optimize reagent concentrations and extension times for each reaction. Longer extension times are required when compose to a single-target PCR[125].

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3. Primer design software (Primer Premier 5)

Primer Premier 5 software (PREMIER Biosoft International) integrates multiple-sequence alignment with primer design to facilitate the design of cross-species primers. The program uses a proprietary algorithm to calculate a minority consensus and designs primers to highly conserved regions of the sequence.

Primer Premier 5 provides comprehensive primer-design features that allow primers to be designed automatically or manually. Multiplex primer design ensures

that there are no cross-homologies. A search facility can be built into the database, and a synthesis order form is provided to process ordering.

4. Selection of primers

The most useful markers for PCR-based deletion analysis are those that are single copy or confined to a small region of the chromosome. Repetitive markers or genes that span large regions of the chromosome will rarely be informative [35]. Multiple, discontinuous deletions along a chromosome are rare and should be verified. Some markers will be naturally missing or polymorphic.

Development of multiplex PCR

Multiplex PCR primer design and optimization is a greater challenge than designing single PCR primer pairs, because multiple primer annealing events need to occur under the same conditions without interfering with one another [126]. Conditions for the amplification of multiple DNA targets in one tube differs from a simple merging of individual PCR reaction conditions [125] because PCR reaction would competes for the limited amount of resources available and each primer pair can interfere with the other. These competitions and interferences can be either diminished or magnified by subtle changes in PCR conditions. The PCR amplification of genomic DNA for clinical diagnosis requires strict compliance with good laboratory practice and basic principles of quality control to avoid artifact and misdiagnosis.

1. Specimens

In principle PCR analysis can be applied to a wide variety of tissues, including: whole blood or bone marrow treated with anticoagulants such as EDTA or citrate, serum or plasma, dried blood, buffy coat, bronchial lavage, cerebrospinal fluid, urine, stool, biopsy and cell culture material. As heparin inhibits PCR reactions, special precautions need to be considered when the use of heparinized material is intended.

With the multiplex PCR method, the source of genomic DNA for the detection of Y chromosome microdeletion is usually peripheral blood lymphocytes. Aknin-Seife. reported in 30 patients that multiplex PCR amplification for Y chromosome microdeletion using either buccal cells or leukocytes gave the same results [127]. Similarly, Katagiri found that DNA extraction and Y deletion assessments of

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spermatozoa and buccal cell provided a non-invasive approach with comparable results to that of leukocyte DNA [39]. Analysis for microdeletions of the Y chromosome in a single spermatozoon from a man with severe oligospermia indicated that spermatozoon (germ cells) have the same Y chromosome microdeletion as peripheral blood lymphocytes (somatic cells) [128].

2. Primer specificity and efficiency testing

All primer pairs should be tested under standard amplification conditions using the same DNA template concentration. Determination of the optimal conditions for each primer pair should be noted. A specific PCR product and a pre-determined amount of amplicon should be observed before combining primer into a multiplex PCR set. Female DNA was used as negative control to ensure that the primer pair did not amplify any non-Y-chromosome regions. Primer pairs that failed to amplify male sample or amplified female sample were eliminated.

3. Optimization of multiplex PCR conditions and components

The optimization of multiplex PCR reactions can pose several difficulties, including poor sensitivity or specificity and/or preferential amplification of certain specific targets. The presence of more than one primer pair in the multiplex PCR increases the chance of obtaining spurious amplification products, primarily because of the formation of primer dimers. Thus, the optimization of 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. However, special attention to primer design parameters such as homology of primers with their target nucleic acid sequences, their length, the GC content, and their concentration have to be considered. Ideally, all the primer pairs in a multiplex PCR should exhibit similar amplification efficiencies for their respective targets. This may be achieved through the utilization of primers with nearly identical optimum annealing temperatures [129].

3.1 $MgCl_2$ concentration

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The Mg^{++} ion concentration may affect all of the following steps: primer annealing, strand dissociation temperatures of both template and PCR product, formation of primer-dimer artifacts and enzyme activity and fidelity. High $MgCl_2$ concentration increases the yield of non-specific products and lowers the fidelity of synthesis. Meanwhile, a low concentration decreases the yield of PCR product. The Mg^{++} ions form complexes with dNTPs, primer and DNA templates. Thus, the optimal concentration of $MgCl_2$ should be selected for each PCR reaction experiment. When the DNA sample contains EDTA or other chelators, the $MgCl_2$ concentration should be raised proportionally in the reaction mixture.

3.2 dNTPs concentration

The dNTPs concentration has been reported to be very important in multiplex PCR. The concentration of each dNTP in the reaction mixture is usually 200 μM. When maximum fidelity of the PCR process is crucial, the final dNTP concentration should be 10-50 μM, since the fidelity of DNA synthesis is maximal in this concentration range. Lower dNTP concentration allows PCR amplification but lowers amount of product. For long PCR amplifications, increasing the concentration of each dNTP in the reaction mixture up to 350-500 μM is recommended.

One report [130] found that dNTPs stocks are sensitive to freezing/thawing cycles. The multiplex PCR did not work well when used freezed/thawed dNTP more than four cycles.

dNTPs/$MgCl_2$ balance

To work properly, *Taq* DNA polymerase required free magnesium (beside the magnesium bound by the dNTPs and the DNA). This is probably why increases in the dNTPs concentration can rapidly inhibit the PCR, whereas increases in magnesium concentration often have positive effects. By combining various amounts of dNTPs and $MgCl_2$, it was found that 200 μM each dNTPs work well in 1.5-2.0 mM $MgCl_2$.

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3.3 PCR buffer concentration

Standrad PCR protocols recommend a buffer of 10 mM Tris-HCl (pH 8.3-8.4) for both *Tag* DNA polymerase and recombinant DNA polymerase. This common Tris-HCl buffer system was intended to represent a starting point from which reaction

condition could be optimized for specific primer-template systems. However, it dose not represent the optimal buffer system for long distance PCR. Usually, primer pairs with longer amplification products work better at lower salt concentrations, whereas primer pairs with short amplification products work better at higher salt concentrations.

3.4 Amount of template DNA and *Taq* DNA polymerase

The concentration of enzyme used in a reaction has an impact on numerous factors of multiplex performance including yield and locus-to-locus signal balance. When the amount of template DNA is very low, efficient and specific amplification can be obtained by further lower the annealing temperature. Higher *Taq* DNA polymerase concentrations may cause synthesis of nonspecific products. The most efficient enzyme concentrations seem to be around 2.5 U/50 µl reaction volumes.

The use of HotStarTaq DNA polymerase can reduce or eliminate the generation of nonspecific PCR products that can result from mispriming and primer oligomerization. When using several primers, suboptimal conditions and reactions were set up at ambient temperature, particularly in multiplex PCR. The undesired PCR products compete with target sequence for dNTPs and primers, as a result, reduce the yield of some amplification products.

Comparison of specificity and efficiency of the multiplex PCR when using HotStarTaq and standard *Taq* DNA polymerase was determined. A significant enhancement in the yield of PCR products was obtained when using HotStarTaq. Furthermore, nonspecific PCR products were eliminated in all multiplex sets with HotStarTaq reactions.

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3.5 Use of adjuvant

The most difficult multiplex PCR reactions can be significantly improved by using a PCR additive, such as DMSO, glycerol or Q solution (Qiagen), which relaxes DNA and making template denaturation easier. Therefore, the usefulness of this adjuvant needs to be tested in each case.

3.6 Thermal cycling parameters

Pre-PCR activation of enzymes

The complete denaturation of the DNA template at the start of the PCR reaction is an importance key. Incomplete denaturation of DNA results in the inefficient utilization of template in the first amplification cycle and produces a poor yield of PCR product. Generally, the pre-PCR activation step is performed at 95°C for 12-15 min. This temperature should be used for activation of the enzyme even when a different temperature is used for denaturation during the PCR cycles. At 95°C, maximum enzyme activation is achieved within 15 min.

Denaturing time

Denaturing time of 30-60 seconds was sufficient to achieve good PCR products. If the amplified DNA has a very high GC content, denaturation time may be increased up to 3-4 min. However, too long a denaturing time, will increase the time that *Taq* polymerase is subjected to high temperatures, and then decreases the activity of *Taq* polymerase.

Annealing time

An annealing time of 30-45 seconds is commonly used in PCR reactions. Increase in annealing time up to 2-3 minutes did not appreciably influence the outcome of the PCR reactions. However, as the polymerase has some reduced activity between 45 and 65° C (interval in which most annealing temperature are chosen), longer annealing times may increase the likelihood of unspecific amplification products**.**

Annealing temperature

Annealing temperature is one of the most important parameters that need adjustment in the PCR reaction. Moreover, the flexibility of this parameter allows optimization of the reaction in the presence of variable amounts of other ingredients (especially template DNA). It was observed that the specific product can be detected, even in the presence of very low DNA template concentrations, if the annealing temperature is also decreased. However, if the same reaction is performed in the presence of a higher amount of DNA template, the low annealing temperature results in the appearance of many unspecific secondary products. Thus, it appears that by decreasing the amount of DNA template, the number of potentially unspecific sites is also decreased, making possible the drop in annealing temperature.

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Gradient PCR

Many thermal cyclers have a temperature-gradient function. Using this function, it is possible to easily determine optimal annealing temperatures by generating a temperature gradient across the heating block for the annealing step. In order to determine optimal annealing conditions, prepare some (at least 3) identical reactions and place in the block positions that most closely correspond to annealing temperatures.

Extension time

In multiplex PCR, as more loci are simultaneously amplified, the pool of enzyme and nucleotides becomes a limiting factor and more time is necessary for the polymerase molecules to complete synthesis of all the products. Extension time will play an important role in adjusting the outcome of the PCR reaction. Higher yields of PCR products were obtained when the longer extension time was used. Optimal amplification of all loci will require further adjustments in other factors influencing the reaction (buffer concentration, amount of individual primers).

Extension temperature

Usually, the extending step is performed at 70-75ºC. The DNA synthesis rate of *Taq* DNA polymerase is highest at this range of temperature (2-4 kb/min) and 1 min extending time is sufficient for the synthesis of PCR fragment of 2 kb. When large PCR fragment is amplified, the extending time is usually increase by 1 min for each 1,000 bp. If the prime annealing temperature is more than 55ºC, it is possible to perform annealing and extending at the same temperature because the activity of *Taq* DNA polymerase at this temperature is sufficient for the synthesis of PCR product up to 1-1.5 kb.

Final Extention

After the last cycle, the samples are usually incubated at 72°C for 5-15 min to fill-in the protruding ends of newly synthesized PCR products. Also, during this step, the terminal transferase activity of *Taq* DNA polymerase adds extra A nucleotides to the 3'-ends of PCR products.

Number of cycles

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The number of cycles is dependent on the amount of template DNA and the required sensitivity of the detection method. A cycling program usually consists of 25-45 cycles, depending on the number of copies of the starting template and the sensitivity of the detection system used. Increasing the number of cycles does not necessarily lead to a higher yield of multiplex PCR product; instead it may increase nonspecific background and may lead to artifacts.

Good laboratory practice (GLP)

Internal controls are particularly important in cases where the presence or absence of an amplification product is diagnostically relevant, such as gene deletions. A negative result must be clearly distinguishable from a technical failure of the assay. A primer specific for a gene which will be always present and amplified should be used as an internal control and added to all PCR reactions.

External positive and negative controls must be run in parallel with each multiplex (i.e. with each set of primers). A DNA sample from a fertile man without a Y chromosome microdeletion should be used as a 'no deletion' control. A female DNA sample should be used as a negative control to prove the specificity of Y chromosome specific primers and lack of amplicon contamination. In addition, a water sample must be run with each set of primers. The water sample is used to determine if there is reagent contamination [89]. For deletion detection PCR assays, a DNA sample is considered positive for a given marker when a PCR product of the expected size is present and considered negative if a product of the expected size is not obtained after three individual PCR attempts.

For contamination control, preventing the contamination of the reaction reagents is essential. In a PCR laboratory, aerosol-resistant pipette tips and clean gloves must always be used to avoid carryover and contamination of stock solutions. Reagents that are used for amplification reaction should be maintained and used separately from completed PCR amplification reactions. The best contamination control for PCR work is to use separate pre-amplification and post-amplification areas. A Laminar Flow Cabinet equipped with a UV lamp is also recommended for preparing the reaction mixture.

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