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Detection of the Most Common Genetic Causes of Male Infertility by Quantitative Fluorescent (QF)-PCR Analysis

Dijana Plaseska-Karanfilska[1], Predrag Noveski[1] and Toso Plaseski[2]
[1]*Macedonian Academy of Sciences and Arts, Research Center for Genetic Engineering and Biotechnology "Georgi D. Efremov", Skopje*
[2]*Clinic of Endocrinology and Metabolic Disorders, Faculty of Medicine, Skopje*
Republic of Macedonia

1. Introduction

Infertility is a major health problem today, affecting about 15% of couples trying to have a child. Impaired fertility of the male factor is causative in 20% of infertile couples and contributory in up to another 30-40%. Infertility already affects about 5-7% of the general male population and may further increase in the future, considering the apparent trend of declining sperm count in industrialized countries. Despite enormous progress in the understanding of human reproductive physiology, the underlying cause of male infertility remains undefined in about 50% of cases, which are referred to as idiopathic infertility (*Ferlin et al., 2006*). Most of the idiopathic cases are likely to be of genetic origin because the number of genes involved in human spermatogenesis is probably over thousands. At present, only few of the genes implicated in the processes of testis determination, testis descent and spermatogenesis have routine clinical importance. These include the cystic fibrosis transmembrane conductance regulator (CFTR) gene, whose mutations cause cystic fibrosis and absence of vas deferens and the androgen receptor (AR) gene, whose mutations cause the androgen insensitivity syndrome and spermatogenic damage.

1.1 Common genetic causes of male infertility

Chromosomal anomalies and microdeletions of the azoospermia factor (AZF) regions of the Y chromosome are the only common known genetic causes of spermatogenic failure. The frequency of these two genetic anomalies increases with the severity of the spermatogenic defect, reaching to an overall 30% (15% karyotype abnormalities and 15% of AZF microdeletions) in azoospermic men.

1.1.1 Sex chromosome aneuploidies

Sex chromosome aneuploidies, such as 47,XXY (Klinefelter's syndrome), 47,XYY and 46,XX males are the most common chromosome anomalies occurring at birth and in the population of infertile males (*Hetch & Hetch, 1987; Gekas et al., 2001*).

Klinefelter's syndrome (KS) is a form of primary testicular failure with testicular hypotrophy and elevated gonadotropin plasma levels, and it represents the most common

form of male hypogonadism. The prevalence of KS among infertile men is very high, up to 5% in severe oligozoospermia and 10% in azoospermia (*De Braekeleer & Dao, 1991*). The syndrome usually causes the arrest of spermatogenesis at the primary spermatocyte stage, but occasionally later stages of sperm development are observed. There are two forms of Klinefelter syndrome: nonmosaic, 47,XXY; and mosaic, 47, XXY/ 46, XY. Although previously believed to be sterile, it has been estimated that 25% of nonmosaic Klinefelter syndrome patients have sperm in their ejaculate (*Ferlin et al., 2007*). Men with the mosaic form of the disease may have residual spermatogenesis in their seminiferous tubules (*Foresta et al., 2005*). Klinefelter syndrome patients may try to achieve pregnancy using ICSI, but they risk producing offspring with chromosomal abnormalities (*Reubinoff et al., 1998*).
The karyotype 47,XYY is the second most frequent full aneuploidy of sex chromosomes. The spermatogenesis in XYY individuals range from severe oligozoospermia to normozoospermia (*Skakkebaek et al., 1973; Sharara et al., 1999*).
46,XX chromosomal abnormality is observed mainly in azoospermic males, with frequency of 0.9% (*Mau-Holzmann, 2005*). The phenotype is similar to Klinefelter syndrome, but with normal height and unimpaired intelligence. The *SRY* gene is present in most of the cases (*SRY*+ XX males); in these cases males are invariably infertile, and azoospermia results from testicular atrophy. The other category are *SRY*− XXmales, which assumes a mutation in an autosomal or X-linked gene involved in the sex determining cascade which should substitute the SRY, permitting testicular determination in absence of SRY.

1.1.2 Y chromosome microdeletions

1.1.2.1 Deletions of AZFa, AZFb and AZFc regions

Y chromosome microdeletions represent the etiological factor of 10-15% of idiopathic azoospermia and severe oligozoospermia (*Foresta et al., 2000; Ma et al., 2000*). In 1976, Tiepolo and Zuffardi provided the first evidence that the long arm of the Y chromosome is required for fertility in men, when they karyotyped 1170 men and found that six azoospermic men were missing most of the long arm of Y chromosome (*Tiepolo & Zuffardi, 1976*). Subsequently, this cluster on Yq11 became known as the azoospermia factor or AZF. The use of polymerase chain reaction (PCR) of sequence tagged sites (STS) has made possible the detection of small, interstitial deletions invisible by karyotyping (*Vollrath et al., 1992*). In 1996, the AZF region was subdivided into 25 deletion intervals (D1-D25) and the existence of three non-overlapping subregions, designated AZFa, AZFb and AZFc (*Figure 1A*), was proposed (*Vogt, 1996*). Subsequent DNA sequencing approaches revealed eight large palindromic regions containing an array of different ampliconic sequences (*Kuroda-Kawaguchi et al., 2001*) and demonstrated that these regions harbour a total of 12 different genes/gene families, most of which are exclusively expressed in testises (*Kuroda-Kawaguchi et al., 2001; Tilford et al., 2001, Scaletsky et al., 2003*). An overlap of 1.5Mb between distal AZFb and proximal AZFc was also demonstrated (*Repping et al., 2002*). Ampliconic sequences make up almost all of the AZFc sequence and 50% of the AZFb sequence (*Figure 1B*).
The frequency of AZF deletions in infertile men ranges in worldwide surveys from 5 to 20% (*Vogt, 1998; Krausz et al., 2003*). Y microdeletions are found almost exclusively in patients with azoospermia or severe oligozoospermia (*Simoni et al., 1998*). The prevalence of Y microdeletions among the infertile males from the Republic of Macedonia is 6.4%, among patients with azoospermia 16.7% and among those with severe oligozoospermia 2.8% (*Plaseski et al., 2003*). Deletions most frequently involve AZFc region, less frequently the

AZFb region, and only rarely the AZFa region. The most frequent deletions among Macedonian males are AZFc deletions, while AZFa deletions have not been detected (*Plaseski et al., 2006; Plaseski et al., 2008*).

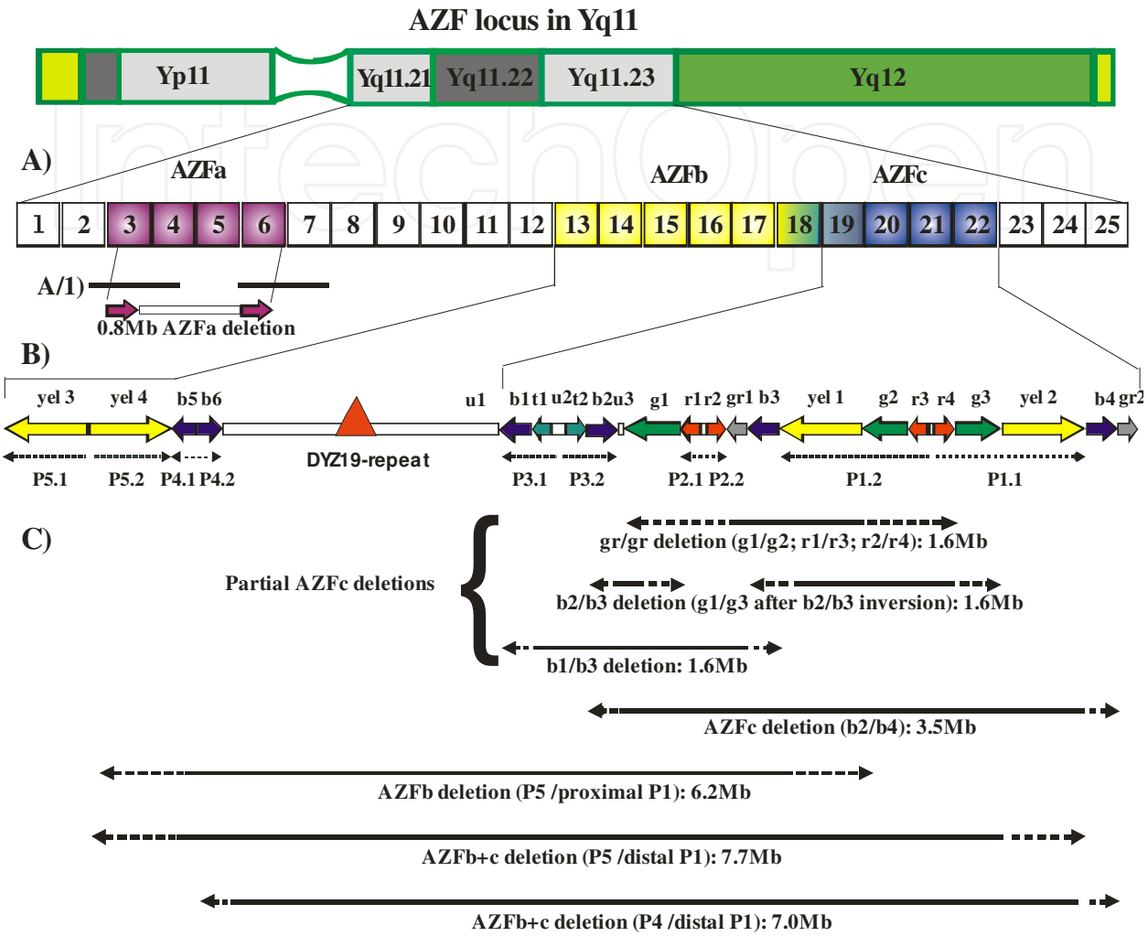


Fig. 1. Schematic view of the AZF locus in Yq1. **A)** Deletion map of AZF locus: 25 intervals (D1-D25) and three AZF regions (AZFa, AZFb and AZFc). **A/1)** Complete AZFa deletion, caused by recombination of two homologous HERV 15Yq1/q2 blocks; **B)** Structural organization of the different amplicons in the AZFb and AZFc regions belonging to five palindromic structures (P1-P5); **C)** Partial and complete AZFc, AZFb and AZFb+c deletions caused by recombination between different amplicons.

Distant homologous recombination between specific palindromic sequences is believed to be the mechanism for majority Yq deletions (Figure 1C) (*Kamp et al., 2000; Repping et al., 2002; Repping et al., 2003*), although deletions based on mechanism of nonhomologous recombination were also identified (*Costa et al., 2008*). The AZFa deletions are located in proximal Yq and are caused by recombination that take place between retroviral homologous sequences. These deletions account for less then 1% of all microdeletions of the Y chromosome reported to be associated with spermatogenic failure. Clinically, AZFa deletions are associated with complete absence of germ cells in the testes (*Vogt, 2005*). Complete deletions of AZFb have a size of 6.23 Mb and extend within a 1.5 Mb of the

proximal portion of AZFc. Deletions removing simultaneously part of the AZFb and AZFc regions result from homologous recombination, in which the proximal breakpoints are located in the P5 palindrome and the distal breakpoints mapped in either proximal P1 or distal P1 (*Repping et al., 2002*). Clinically, complete AZFb deletions are associated with meiotic arrest or Sertoly cell-only syndrome (*Ferlin et al., 2003*).

The most common AZFc deletion (b2/b4 deletion) eliminates a 3.5 Mb segment that contains 21 genes and is present in about 1 in 4.000 men worldwide (*Kuroda-Kawaguchi et al., 2001*). Deletions involving the AZFc region account for up to 90% of all Yq deletions with phenotypes varying from azoospermia to severe oligozoospermia (*Reijo et al., 1995, Simoni et al., 1997, Najmabadi et al., 1996)* and occasionally to milder oligozoospermia (*Oliva et al., 1998*). Although natural transmission of Y microdeletions has been reported, majority of the cases arise as a *de novo* event (*Edwards & Bishop , 1997*).

1.1.2.2 Partial AZFc deletions

Partial deletions within the AZFc region (gr/gr and b2/b3) that remove smaller portions of the AZFc region (1.6 and 1.8 Mb) are much more common and are present at various frequencies in different Y haplogroups (*Repping et al., 2003; Vogt, 2005*). Partial and polymorphic AZF deletions have been also reported in the AZFa (*Kamp et al., 2000*) and AZFb regions (*Ferlin et al., 2003*).

While the association of the complete AZFc deletion with spermatogenic failure is well established, the role of partial AZFc deletions and duplications on spermatogenesis and male infertility is still controversial. With the exception of one study among Han-Chinese population (*Wu et al., 2007*), all other studies reported no association between the b2/b3 deletion and impaired spermatogenesis. The results of the gr/gr deletion are more inconsistent; it is considered a new genetic risk factor by a number of research groups (*Kuroda-Kawaguchi et al., 2001; de Llanos et al., 2005; Ferlin et al., 2005; Gianchini et al., 2005, Gianchini et al., 2008*), but not by the others (*Machev et al., 2004; Hucklenbroich et al., 2005; Ravel et al., 2006; Carvalho et al., 2006; Lardone et al., 2007a; Lin et al., 2007*). These contradictory results may in part be due to the methodological differences and differences in the controls (fertile controls, general population, or normozoospermic men).

1.1.2.3 AZFc duplications

In addition to deletions, different duplications at the AZFc region have been reported. Duplications can occur on a chromosome with partial AZFc deletion and generate a chromosome with four DAZ genes, but lacking some STS markers (*Repping S et al., 2003; Repping S et al., 2004*). Recently, AZFc partial duplication has been shown to be a risk factor for male infertility in Taiwan (*Lin et al., 2007*). A higher incidence of increased number of DAZ genes was demonstrated in azoospermic and oligozoospermic men in Slovenia (*Writzl et al., 2005*). Additional studies are needed to determine the role of AZFc partial deletions and duplications in spermatogenesis and male infertility.

1.1.2.4 AZF candidate genes

The AZF regions include genes that are expressed during spermatogenesis and encode proteins necessary for specific stages of spermatogenesis as well as for maintaining the general housekeeping functions of the cells involved (*Lahn & Page, 1997*). The Dead box Y (DBY, recently renamed DDX3Y) encodes a putative RNA helicase. The ubiquitin-specific protease 9Y gene (USP9Y, previously known as DFFRY) encodes a protease with activity specific to ubiquitin that is involved in the regulation of protein metabolism (protein turn-over). Both

genes are located at the AZFa region and have homologous genes on the X chromosome. The exact role of the candidate genes in the AZFa region are largely unknown, owing to the extreme rarity of naturally occurring, single-gene-specific mutations. Complete deletions of AZFa region is rare, but is well documented and always associated with Sertoli-cell-only syndrome and consequently azoospermia (*Ferlin et al., 2007*). The translation Initiation Factor 1A Y isoform gene (EIF1AY) and the RNA binding motif (RBM) family are found on AZFb region. EIF1AY encodes an essential translation factor. The PTP-BL-related Y (PRY) family of genes is mapped to AZFb and AZFc regions and encodes proteins proposed to be involved in apoptosis. RBM and deleted-in-azoospermia (DAZ) genes encode RNA-binding proteins that are exclusively expressed in germ cells. In addition to DAZ, chromodomain Y genes (CDY1) are found on the AZFc region and encode a protein involved in DNA remodeling that can acetylate histone H4 in vitro. Among other Y chromosome genes, likely implicated in spermatogenesis but not related to microdeletions, TSPY is a candidate oncogene that, due to its limited expression pattern in germ cells, is thought to function as a proliferation factor during spermatogenesis. The quantities of AZF gene transcripts in testicular tissues of patients with different spermatogenic impairment have been recently examined and an important role of DDX3Y was suggested (*Kleiman et al., 2007; Lardone et al., 2007*).

1.1.3 Androgen receptor CAG repeats

Androgens are essential for male sexual development and for fertility. They act through the AR, which is a transcriptional factor that contains functional domains for DNA binding, ligand binding and transcriptional regulation. The 5' end of exon 1 of the AR gene includes a polymorphic CAG triplet repeat that codes for a polyglutamine tract. The number of CAG repeats in the normal population varies between 10 and 36. Expansion of the polyglutamine tract to >38 repeats in males leads to Kennedy disease [spinal bulbar muscular atrophy (SBMA)] (*LaSpada et al., 1991)*. In addition to neurological symptoms, SBMA patients show signs of hypogonadism, such as gynecomastia, impotence, testicular atrophy and reduced fertility.

In vitro studies have demonstrated a negative correlation between CAG repeat size and AR function (*Chamberlain et al., 1994*). The possible association of a long CAG repeat with male infertility in Asian populations was suggested because of a four-fold increase in the risk of impaired spermatogenesis in males who had >28 CAG repeats (*Tut et al., 1997*). Since then, the association of the long CAG repeat number in the AR gene and male infertility has been controversial.

We have also studied the possible effect of long CAG repeat tracts in the AR on infertility among Macedonian men (*Plaseski et al., 2007*). Our results showed that the mean CAG length dos not differ significantly between males with azoospermia, mild oligozoospermia, severe oligozoospermia, normozoospermia, or known causes of infertility and fertile controls. However, we found a significantly higher percentage of CAG repeats >26 (p = 0.022), >27 (p = 0.018) and >28 (p = 0.009) in males with mild oligozoospermia. Thus, our initial results indicated a possible association between CAG repeat length and mild oligozoospermia.

2. Screening for the presence of the most common genetic causes by quantitative fluorescent (QF)-PCR

Screening for chromosomal abnormalities is usually done by cytogenetic analysis and for AZF deletions by PCR analysis of several sequence tagged sites (STSs) in the three AZF

regions. Recently, we have described a multiplex QF-PCR method that allows simultaneous detection of the most common genetic causes of male infertility, i.e. sex chromosomal aneuploidies and AZFc and AZFb deletions, and some potential risk factors such as partial AZFc deletions/duplications and AR CAG repeats (*Plaseski et al., 2008*). This 11-plex QF-PCR analysis was shown as a rapid, simple, reliable and inexpensive method that can be used as a first-step genetic analysis in infertile patients. Here, we present a modified system, where we have included additional markers in the AZFa and AZFb region, as well as a marker for determination of the X/ chromosome 3 ratio.

2.1 QF-PCR method

The quantitative fluorescent (QF) polymerase chain reaction (PCR) included 13 markers: amelogenin gene which is present on X and Y chromosomes and allows for the determination of the Y/X ratio (AMEL marker), TAF9B gene that is present on chromosomes X and 3 and permits the determination of x/chr 3 ratio, four polymorphic X-specific short tandem repeat (STR) markers (XHPRT, DXS6803; DXS981 and exon 1 of the AR gene), three non-polymorphic Y-specific markers (SRY gene, sY86 in AZFa and sY134 in AZFb region), polymorphic Y-specific STR marker (DYS448), and co-amplification of DAZ/DAZL, MYPT2Y/MYPT2 and CDY2/CDY1 fragments that permit determination of the DAZ, MYPT2Y, CDY1 and CDY2 gene copy number. The details of the primers used in the 13-plex QF-PCR are given in Table 1, while the location of the markers on the Y chromosome is given in Figure 2.

The AMEL marker exploits the 6bp deletion on the X chromosome sequence, enabling amplification of specific X-chromosome (106 bp) and Y-chromosome sequences (112 bp). The TAF9B marker co-amplifies a fragment of the TAF9B gene on X chromosome (144bp) and the one on chromosome 3 (140 bp). The DAZ gene copy number was quantified using primers that co-amplify a fragment of intron 10 from DAZ gene (208 bp) and from the homologous autosomal locus DAZL on chromosome 3 (211 bp or 251 bp). The two MYPT2Y copies in the AZFc region were co-amplified with the MYPT2 gene on chromosome 1, giving fragments of 181 bp and 176 bp respectively. The relative ratio of the two CDY1 genes in the AZFc region and two CDY2 genes in the AZFb region, which share 98% nucleotide identity was scored by two PCR sets which amplify a 6bp nucleotide difference in the 5′ region, producing fragments of 200 bp for CDY1 and 194 bp for CDY2.

One primer in each set was labeled with 6-FAM or HEX fluorescent dye, which allowed the determination of the length of the different STR and STS alleles and for quantification of the relative Amel Y/Amel X, TAF9B-X/TAF9B-chr. 3, DAZ/DAZL, MYPT2Y/MYPT2 and CDY2/CDY1 ratios on ABIPrism 3130 Genetic Analyzer using a GeneMapper Software v.4.0 (Applied Biosystems, Foster City, CA, USA).

The PCR reaction mixture contained PCR buffer (Applied Biosystems), 50-100 ng genomic DNA, 200 μM each of the four dNTP's (dATP, dCTP, dGTP and dTTP), 2-8 pmol each of the primers, and 1.5U TaqGold polymerase (Applied Biosystems) in a total volume of 15μl. The PCR was performed under the following conditions: initial denaturation step at 95^0C for 5 minutes, followed by 28 cycles of 1 minute denaturation at 95^0C, 1 minute annealing at 58^0C and 1.5 minutes elongation at 72^0C; and final elongation at 72^0C for 30 minutes.

2.2 QF-PCR results

The normal results of the 13 markers included in the QF-PCR analysis in males without sex chromosome aneuploidies and AZF rearrangements are shown in Table 2 and Figure 3. The

Marker	Chromosome location		Sequence of the primers (5'-3')	Label	Repeat element	Allele size range in bp (expected)	Allele size range in bp (obtained)	Concen-tration (pmol)	Reference
AMEL	Xp22.1-p22.31 Yp11.2-p22.1	(F) (R)	CCC TGG GCT CTG TAA AGA ATA GTG ATC AGA GCT TAA ACT GGG AAG CTG	6-FAM	/	106 112	103 109	2	*Sullivan et al., 1993*
DXS6803	X chr	(F) (R)	GAA ATG TGC TTT GAC AGG AA CAA AAA GGG ACA TAT GCT ACT T	HEX	tetra	/	105-125	6	*Cirigliano et al, 2001*
TAF9B	TAF9B - X chr TAF9B - chr 3	(F) (R)	TTT GAC AGG TAG TTT TGG GTC A TGG TTT TGC CTA GGT CCA GT	6-FAM	/	144 140	147 143	4	*this study*
MYPT2	MYPT2Y - Y (AZFc) MYPT2 - chr 1	(F) (R)	CTC ACT ACA TGA CAT TCA GG GTT TCT TCC CAG TAT CTA GTA CAG TGC	6-FAM	/	181 176	180 175	2	*Machev et al., 2004*
CDY	CDY 1 - Y (AZFc) CDY 2 - Y (AZFb)	(F) (R)	GTT TCT TCC ACT GTA GAA ATT CAC CTC C GAA GTT TGC ATA GTG GAC AGC	6-FAM	/	200 194	203 197	4	*Machev et al., 2004*
DAZ	DAZ - Y (AZFc) DAZL - chr 3	(F) (R)	TTA AGT ACT ACT GTA GAC AC GTT TCT TGT ATA ATG TAG AAG AGT AGA GC	HEX	/	208 211/251*	211 214/254*	6	*Machev et al., 2004*
SRY	Yp11.2-p22.1	(F) (R)	AGT AAA GGC AAC GTC CAG GAT TTC CGA CGA GGT CGA TAC TTA	HEX	/	248	243	4	*Ogilvie et al., 2005*
DXS981	Xq13.1	(F) (R)	CTC CTT GTG GCC TTC CTT AAA TG TTC TCT CCA CTT TTC AGA GTC A	6-FAM	tetra	/	233-257	4	*Edwards et al., 1991*
XHPRT	Xq26.1	(F) (R)	ATG CCA CAG ATA ATA CAC ATC CCC CTC TCC AGA ATA GTT AGA TGT ACG	6-FAM	tetra	/	268-292	4	*Edwards et al., 1992*
AR	Xq11.12	(F) (R)	TCC AGA ATC TGT TCC AGA GCG TGC GCT GTG AAG GTT GCT GTT CCT CA	HEX	tri	/	247-309	8	*Plaseski et al., 2007*
sY134	Y (AZFb)	(F) (R)	GTC TGC CTC ACC ATA AAA CG ACC ACT GCC AAA ACT TTC AA	HEX	/	301	303	4	*Simoni et al., 1999*
sY86	Y (AZFa)	(F) (R)	GTG ACA CAC AGA CTA TGC TTC ACA CAC AGA GGG ACA ACC CT	6-FAM	/	326	317	4	*Simoni et al., 1999*
DYS448	Yq11.2	(F) (R)	CAA GGA TCC AAA TAA AGA ACA GAG A GGT TAT TTC TTG ATT CCC TGT G	6-FAM	hexa	/	346-376	6	*Ogilvie et al., 2005*

* in app. 25% of individuals a 40bp insertion polymorphism in DAZL intron 10 is present (*Machev et al., 2004*)

Table 1. Details of the primers used in the QF-PCR for the detection of the most common causes of male infertility

Notes

[1]NCBI MSY assembly based on Scaletsky et al, 2003

[2]Multicopy markers are designated with *

[3]For each deletion, black boxes indicate presence of STS, gray boxes indicate breakpoint intervals, and white boxes in

[4]The b2/b3 deletion is contiguous; it appears as shown because the deletion arises on Y chromosome with an inversion

Fig. 2. The location of Y chromosome markers included in the 13-plex QF-PCR system and schematic presentation of AZF deletions detected among Macedonian males

Marker	Normal result	Sex chromosomal aneuploidies			AZF deletions					Partial AZFc deletions			AZFc duplications	
		XXY	XX	XYY	b2/b4	AZFc-1	AZFc-2	AZFb	AZFb+c	AZFa+b+c	gr/gr	b2/b3	gr/gr + b2/b4dup	gr/gr & b2/b4	b2/b3
AMEL Y/X ratio	1 (1/1)	0,5	no Y	2	normal	normal	normal	normal	<1	<1	normal	normal	normal	normal	normal
DAZ/DAZL (Y/chr 3) ratio	2 (4/2)	normal	no DAZ	4 (8/2)	no DAZ	no DAZ	no DAZ	no DAZ	no DAZ	no DAZ	1 (2/2)	1 (2/2)	2 (4/2)	>3 (>6/2)	≥3 (>6/2)
MYPT2Y/MYPT2 (Y/chr 1) ratio	1 (2/2)	normal	no MYPT2Y	2 (4/2)	0,5 (1/2)	0,5 (1/2)	no MYPT2Y	normal	no MYPT2Y	no MYPT2Y	0,5 (1/2)	normal	0,5 (1/2)	>1,5 (>3/2)	normal
TAF9B (X/chr 3) ratio	0,5 (1/2)	1 (2/2)	1 (2/2)	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
CDY2/CDY1 - II (AZFb/AZFc) ratio	1 (2/2)	normal	no CDY1 no CDY2	normal	no CDY1	no CDY1	no CDY1	0,5 (1/2)	no CDY1 no CDY2	no CDY1 no CDY2	2 (2/1)	2 (2/1)	1 (2/2)	<0,5 (2/>4)	<0,5 (2/>4)
DXS6803/DXS 981/ XHPRT/ AR	1 allele of each STR marker	1 or 2 alleles[2]	1 or 2 alleles[2]	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
SRY	248 bp fragment	normal	normal[3]	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal	normal
sY134	303 bp fragment	normal	no fragment	normal	normal	normal	normal	no fragment	no fragment	no fragment	normal	normal	normal	normal	normal
sY86	317 bp fragment	normal	no fragment	normal	normal	normal	normal	normal	normal	no fragment	normal	normal	normal	normal	normal
DYS 448	1 allele	normal	no fragment	normal	normal	no fragment	no fragment	no fragment	no fragment	no fragment	normal	normal	normal	normal	normal

[1] the ratio is slightly higher in heterozygotes and homozygotes for DAZL polymorphism
[2] one allele in homozygotes and two alleles in heterozygotes for particular marker
[3] normal in SRY+ XX males; no fragment in SRY- XX males

Table 2. Results of the 11-plex QF-PCR in normal males and males with sex chromosomal aneuploidies, AZF deletions, partial AZFc deletions and b2/b4 duplication

normal results in a male DNA samples are presented by a Amel Y/X ratio of 1, due to the presence of one X and one Y chromosome, DAZ/DAZL ratio of 2, due to the presence of 4 DAZ genes in the AZFc region of Y chromosome and two DAZL genes, one on each chromosome 3. Around 30% of the samples showed the presence of a 40bp insertion polymorphism in the DAZL gene in a heterozygous or homozygous state. The DAZ/DAZL ratio was higher in the heterozygotes and homozygotes than in the individuals without this polymorphism due to the area of the 254bp peak being smaller than the 214bp peak. The normal MYPT2/MYPT2Y ratio is around 1 due to the presence of two copies of the gene in the AZFc region of the Y chromosome and one copy on each of the chromosomes 1, while the normal TAF9B-X/TAF9B-chr 3 ratio is 0,5 due to the presence of two copies of the gene on the chromosomes 3 and one copy on the chromosome X in males.

The four STR markers on chromosome X, as well as the one in the AZFb region on chromosome Y generate one PCR fragment due to the presence of one allele of each of the investigated markers. The non-plymorphic markers on the Y chromosome: SRY, sY134 (in the AZFb region) and sY86 (in the AZFa region) gave PCR fragments of 248 bp, 303 bp and 317 bp in males without chromosome aneuploidies and/or AZF rearrangements.

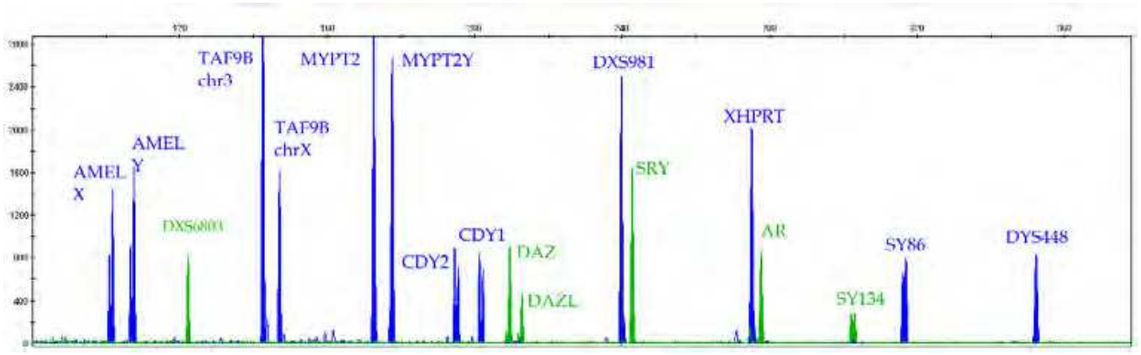


Fig. 3. Electrophoretogram of the 13-plex QF-PCR analysis in a blood sample of normal male.

2.2.1 QF-PCR results in sex chromosome aneuploidies

Among the studied males we detected four different chromosome aneuploidies: XXY or Klinefelter's syndrome (n=12), XX males (n=2), XYY males (n=2) and XY,XO mosaic male (n=1). All XXY and XX males, as well as one of the two XYY men were azoospermic, while the second XYY male and the XY,XO mosaic male presented with severe oligozoospermia. The electrophoreograms of the individuals with sex chromosome aneuploidies are shown in Figures 4-7 and the Y/X, DAZ/DAZL, MYPT2Y/MYPT2 and CDY2/CDY1 ratios are given in Table 2. All detected cases of chromosome aneuploidies were confirmed by cytogenetic analysis.

Klinefelter's syndrome (XXY) was detected by an abnormal Y/X ratio (~0.5), TAF9B-X/TAF9B-chr 3 ratio of ~1 and presence of two alleles from some of the STR markers on the X chromosome in a ratio of approximately 1:1 (Figure 4).

The two XX males were characterized by the absence of the Y fragment from the AMEL Y/X marker, DAZ, MYPT2Y, CDY1 and CDY2 fragments and TAF9B-X/TAF9B-chr 3 ratio of ~1 (Figure 5). In both XX males the SRY fragment was present, sY134, sY86 and DYS 448 fragments were absent and at least one of the four STR markers on the X chromosome

showed two alleles. Rarely, in XX males the SRY gene is not present in which case the PCR fragment from the SRY gene would be missing and 11-plex QF-PCR pattern would be same as in DNA from normal females.

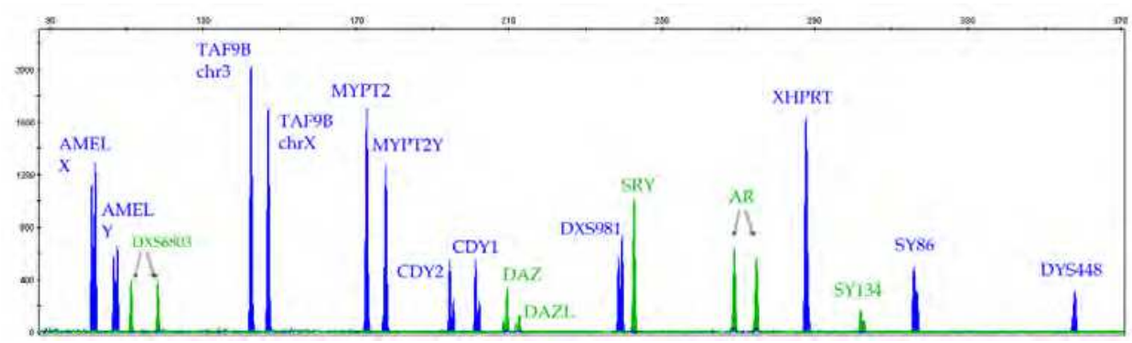


Fig. 4. Electrophoretogram of the 13-plex QF-PCR analysis in a men with Klinefelter's syndrome (XXY).

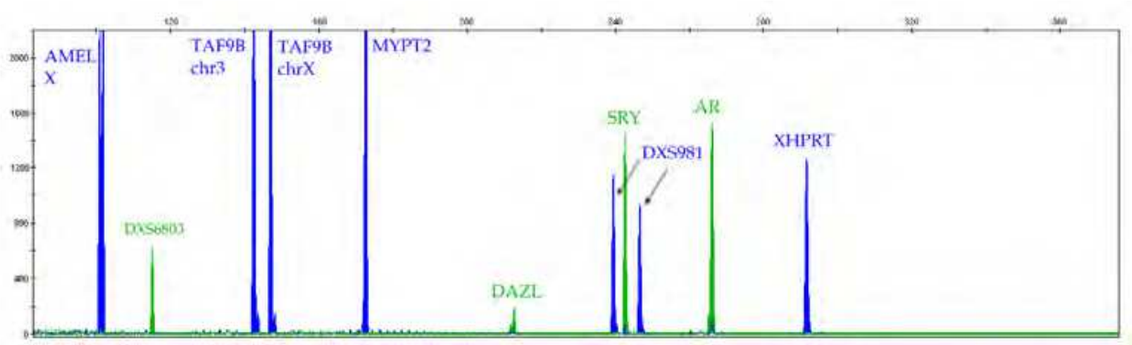


Fig. 5. Electrophoretogram of the 13-plex QF-PCR analysis in a men with XX male syndrome.

The XYY individuals showed also a specific pattern, characterized by abnormal Y/X (~2), DAZ/DAZL (~4) and MYPT2Y/MYPT2 (~2) ratios, while the TAF9B-X/TAF9B-chr 3 and CDY2/CDY1 ratios were within the normal range (Figure 6).

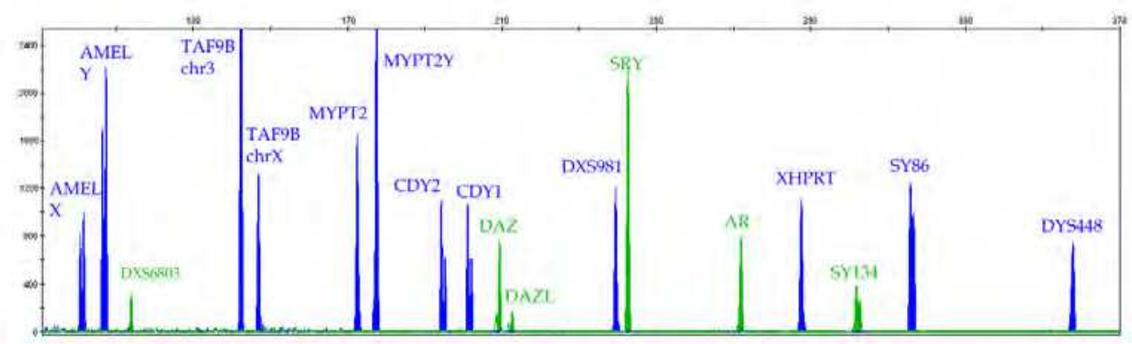


Fig. 6. Electrophoretogram of the 13-plex QF-PCR analysis in a men with XYY syndrome.

The 13-plex QF-PCR of XY,XO mosaic male showed abnormal Y/X (0,35) and MYPT2Y/MYPT2 (0,25) and DAZ/DAZL (0,59) ratios, while the CDY2/CDY1 and TAF9B-X/TAF9B-chr 3 ratios were normal (Figure 7). This result suggested that the Y chromosome is lost in approximately half of the white blood cells in this patient. The result was confirmed on a DNA isolated from a fresh blood sample and by cytogenetic analysis as well. Analysis of DNA extracted from the buccal swab of this patient showed a normal result. Unfortunately we were not able to analyze DNA isolated from the spermatozoa of this patient.

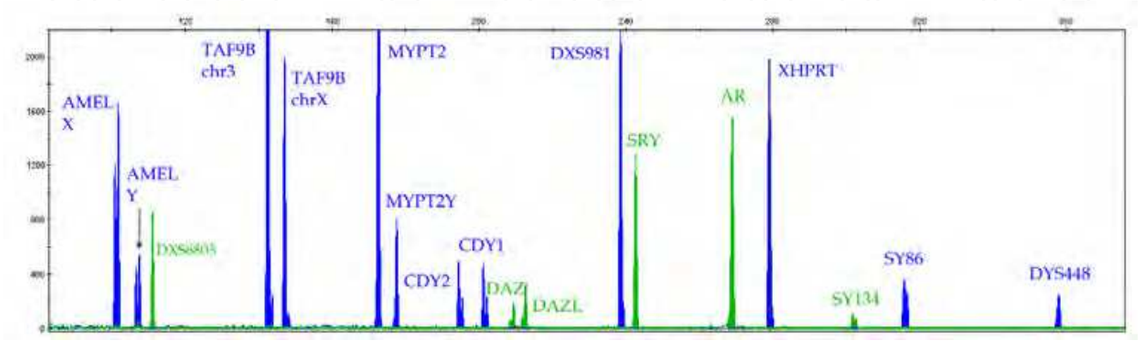


Fig. 7. Electrophoretogram of the 13-plex QF-PCR analysis in a XY/X0 mosaic male.

2.2.2 QF-PCR results in complete AZF deletions

During our previous work we have detected eight different AZF deletions, including the two partial AZFc deletions, gr/gr and b2/b3 deletions (*Plaseski et al., 2006, Plaseski et al., 2008*). Schematic presentation of the deletions is shown in Figure 2.

The 13-plex QF-PCR patterns obtained in the patients with different AZF deletions are given in Table 2. We were able to distinguish all six types of AZF deletions that we detected previously amongst infertile patients (*Plaseski et al., 2006*). In the six patients with b2/b4 deletions the DAZ fragment and the CDY1 fragments were missing, while the MYPT2Y/MYPT2 ratio was decreased suggesting the absence of one of the two MYPT2Y copies on the Y chromosome. All other fragments showed a normal pattern.

The initial screening for AZF deletions following the guidelines for the detection of Y microdeletions showed presence of AZFc deletions in two other patients (*Plaseski et al., 2006*). The analysis with additional STS markers showed that in these two patients the 5′ border of the deletion is identical and lies between sY 134 and sY 142 markers. The 3′ border differs and is identical to the 3′ border of the b2/b4 deletion in one of the two patients, while in the other it extends distal from the AZFc region (Figure 2). These two patients showed an identical pattern for all markers except for MYPT2Y/MYPT2 (Table 2). In one the MYPT2Y fragment was missing (Figure 8), while in the other it was present, but the ratio of MYPT2Y/MYPT2 was decreased to about half suggesting deletion of one MYPT2Y copy. In both patients markers sY134 in the AZFb region and sY86 in AZFa region were present, while the DYS 448 marker was absent.

During our routine screening for Y microdeletions we detected one patient with AZFb deletion. The additional Y STS markers showed that the 5′ border of this deletion is between sY 1228 and sY 1015, while the 3′ border extends in the 5′ part of the AZFc region between sY1291 and sY1191 (Figure 2). This deletion also gave a specific pattern with the 13-plex QF

PCR characterized by the absence of the DYS 448 and sY134 fragments and ratios of DAZ/DAZL and CDY2/CDY1 markers reduced to half (Table 2), suggesting that two of the four DAZ genes in the AZFc regions and one of the two CDY2 genes in the AZFb region were missing.

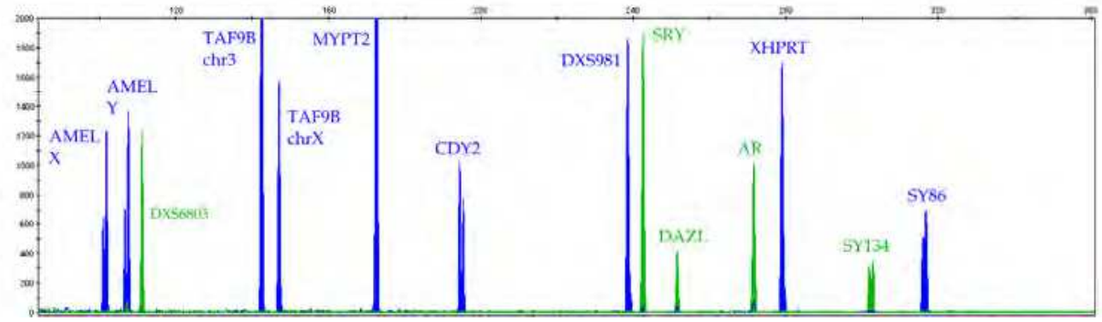


Fig. 8. Electrophoretogram of the 13-plex QF-PCR analysis in a men with AZFc-2 deletion.

The patient with AZFb+c deletion also showed a specific pattern with the 13-plex QF-PCR, which was the same as AZFc-1 deletion in all but sY134 marker that was missing and Y/X AMEL marker which showed a reduced ratio of 0.23, due to the presence of XY/X0 mosaicism (Table 2 and Figure 9). The XY/XO mosaicism was confirmed by cytogenetic analysis.
The QF-PCR result of the AZFa+b+c deletion showed absence of all markers in the AZF a, b and c regions (DAZ, MYPT2Y, CDY1, CDY2, sY134, sY86 and DYS448) (Figure 10). The patient with AZFa+b+c deletion also showed an abnormal Y/X ratio (0,42), suggesting that the Y chromosome was lost in half of the cells in this patient.

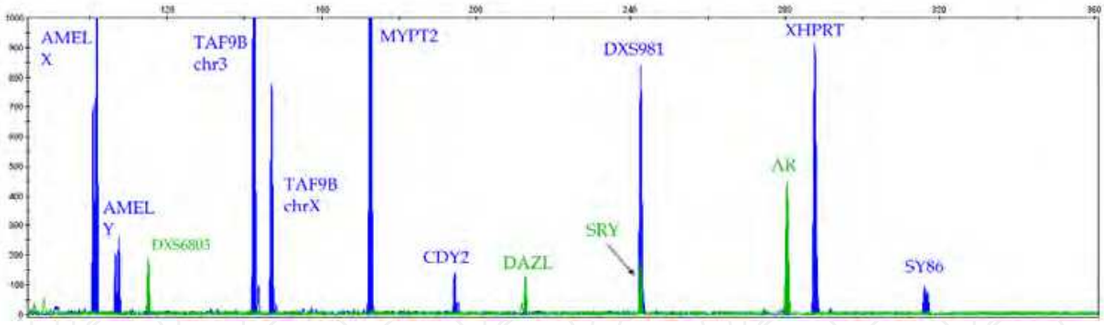


Fig. 9. Electrophoretogram of the 13-plex QF-PCR analysis in a men with AZFb+c deletion.

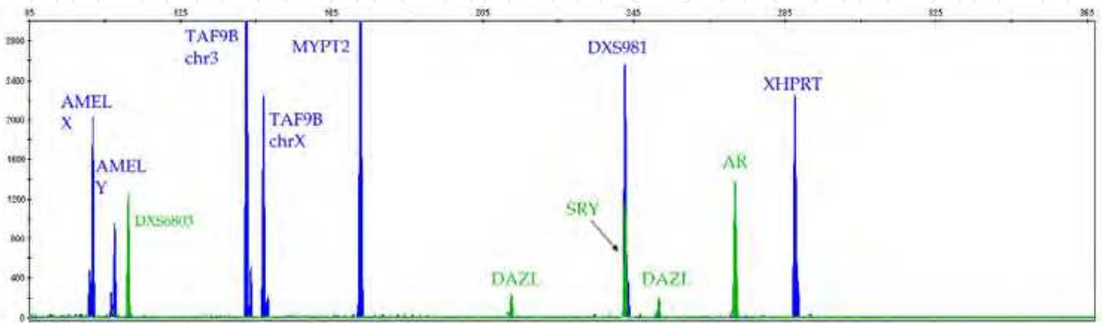


Fig. 10. Electrophoretogram of the 13-plex QF-PCR analysis in a men with AZFa+b+c deletion.

2.2.3 QF-PCR results in partial AZFc deletions

The 13-plex QF-PCR permitted detection of partial AZFc deletions and duplications (Table 2). Both partial AZFc deletions (gr/gr and b2/b3) showed abnormal DAZ/DAZL (~1) and CDY2/CDY1 ratios (~2), but differ in the MYPT2Y/MYPT2 ratios which are within the normal range in b2/b3 deletion and reduced in gr/gr deletion (~0.5). The QF-PCR analysis in men with gr/gr deletion is shown in Figure 11.

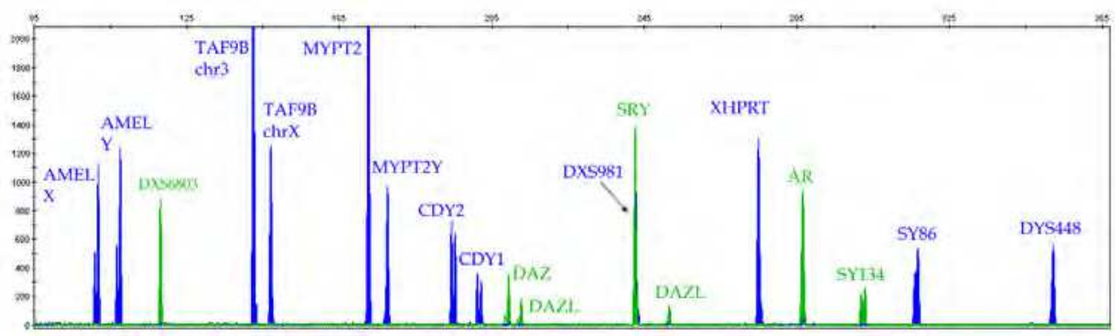


Fig. 11. Electrophoretogram of the 13-plex QF-PCR analysis in a men with gr/gr deletion.

Three of the previously detected patients with gr/gr deletions, showed normal or increased DAZ/DAZL levels, normal, increased or decreased CDY2/CDY1 levels, while the MYPT2Y/MYPT2 ratio was reduced to half, similar to that in the other gr/gr deletions (Figure 12). This rearrangement has probably arisen from gr/gr deletion followed by duplication. All three males with both b2/b4 duplication and gr/gr deletion differ from normal individuals in the MYPT2Y/MYPT2 ratio which is reduced to half.

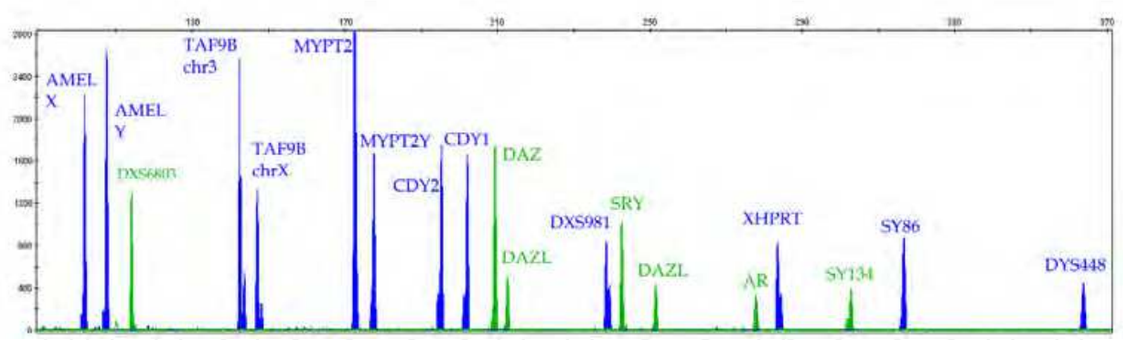


Fig. 12. Electrophoretogram of the 13-plex QF-PCR analysis in a men with b2/b4 duplication and gr/gr deletion.

2.2.4 QF-PCR results in AZFc duplications

The 13-plex QF-PCR detected also the duplications mediated by the amplicons in the AZFc region. Samples with gr/gr or b2/b4 duplications were characterized by increased DAZ/DAZL (>3) and MYPT2Y/MYPT2 (>1.5) ratios and decreased CDY2/CDY1 ratios (<0.5) (Table 2). Samples with partial AZFc duplication on chromosomes with b2/b3 inversion (b2/b3 duplications), showed increased DAZ/DAZL (>3), normal MYPT2Y/MYPT2 and decreased CDY2/CDY1 ratios (<0.5) (Figure 13).

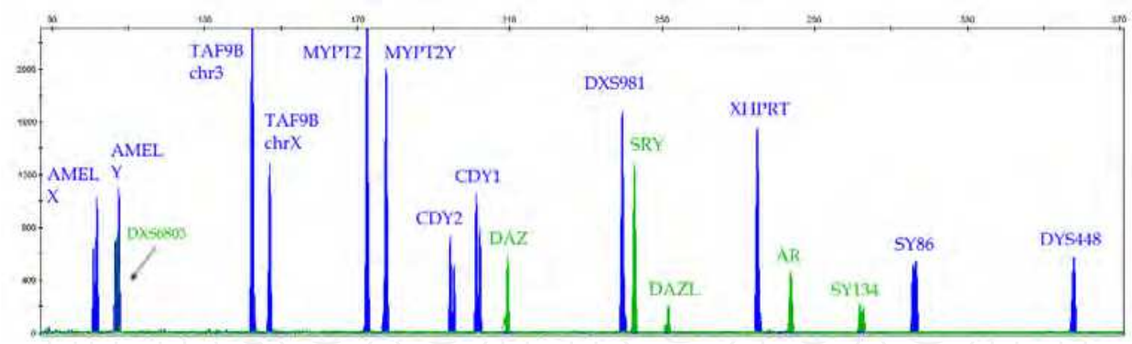


Fig. 12. Electrophoretogram of the 13-plex QF-PCR analysis in a men with b2/b3 duplication.

2.2.5 Detection of the number of AR CAG repeats

An additional advantage of our QF-PCR system is that it can also assess the number of the AR CAG repeats, since one of the STR markers on the X chromosome in the 13-plex QF-PCR involves the CAG repeats in the exon 1 of the AR gene. The PCR amplification using oligonucleotide primers surrounding the CAG repeat region in the exon 1 of the AR gene generated fragments with a size ranging from 247 to 309 bp, corresponding to the 13 to 34 CAG tandem repeats. The number of CAG repeats predicted by the GeneMapper software v.4.0. (Applied BioSystems) was previously compared with the actual CAG repeats determined by direct dideoxy terminator cycle sequencing using the BigDye Terminator Sequencing Kit v1.0 (Applied BioSystems) in several male DNA samples with 14, 19, 21, 25 and 29 CAG repeats.

3. Conclusion

In conclusion, we have developed a rapid, simple, reliable and inexpensive multiplex QF-PCR method, that can be used as a first-step genetic analysis in infertile/subfertile men to detect the most common genetic causes of male infertility (sex chromosomal aneuploidies and AZF deletions) and to study some potential risk factors (AZFc partial deletions and duplications and AR CAG repeats).

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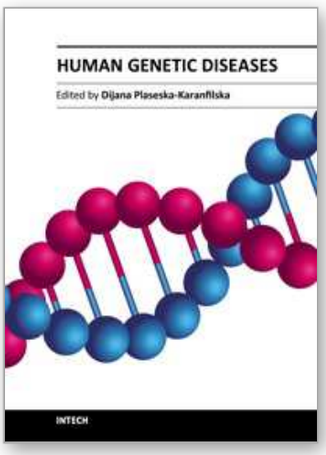
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The genetics science is less than 150 years old, but its accomplishments have been astonishing. Genetics has become an indispensable component of almost all research in modern biology and medicine. Human genetic variation is associated with many, if not all, human diseases and disabilities. Nowadays, studies investigating any biological process, from the molecular level to the population level, use the â€œgenetic approachâ€ to gain understanding of that process. This book contains many diverse chapters, dealing with human genetic diseases, methods to diagnose them, novel approaches to treat them and molecular approaches and concepts to understand them. Although this book does not give a comprehensive overview of human genetic diseases, I believe that the sixteen book chapters will be a valuable resource for researchers and students in different life and medical sciences.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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