Cytogenetic and AZF microdeletions on the Y chromosome of infertile men

K. Ben Makhlouf1,2,3, I. Samri2, L. Bouguenouch2, N. Boudra3, N. Elghachtouli1, K. Ouldim2

1Laboratory of biotechnology, Faculty of Sciences and Techniques, PO Box : 2202, Road og Immouzer, Fez, Morocco
2Unit of Medical Genetics and Oncogenetics, Hospital University Center, Fez, Morocco
3Assisted reproductive technologies center, Fez, Morocco
*Corresponding author : kaoutar.benmakhlouf@yahoo.com

Abstract
Intervals V and VI of Yq11.23 regions contain responsible genes for spermatogenesis, and are named as “azoospermia factor locus” (AZF). Deletions of these genes are thought to be pathogenetically involved in some cases of male infertility associated with azoospermia or oligozoospermia. The aim of this study was to develop a method for the detection of the AZF region of the human Y chromosome in the Unit of Medical Genetics and Oncogenetics UGMO at the Hospital University Center Hassan II-FEZ. We applied multiplex polymerase chain reaction (PCR) using several sequence-tagged site (STS) primer sets, in order to determine microdeletions in the Y chromosome. Our findings suggest that the technique is validated, so genetic screening should be advised to infertile men before starting assisted reproductive treatments.

Key words: Male infertility, Y chromosome microdeletion, AZF regions.

Introduction
Infertility affects approximately 9% of couples attempting pregnancy, and it is estimated that a male factor is involved in approximately half of cases [1]. Evidence for direct involvement of the long arm of the Y chromosome in male infertility was first established by cytogenetic analysis [2]. The presence of the azoospermia factor (AZF) locus, containing one or more genes necessary for normal spermatogenesis, was then proposed as important for fertility and mapped to deletion Yq-interval VI. Finally, polymerase chain reaction (PCR) and recommended sequence-tagged sites (STS) allowed the definition of Y microdeletions as three close and nonoverlapping subregions: AZFa, AZFb, and AZFc. Genotype and phenotype correlations have been described within the AZF region. Complete or incomplete deletions of the AZFa region were observed only in azoospermic patients, in association with Sertoli cell–only syndrome (SCO) [3]. Phenotype of men with a complete AZFb deletion corresponded to azoospermia with spermatogenetic arrest at the pachytene stage [3]. In contrast, approximately half of men with incomplete AZFb deletion had spermatozoa in the ejaculate [4-6]. Patient phenotypes with AZFc deletions ranged from moderate oligozoospermia to azoospermia, with various histologic aspects. Finally, no testicular spermatozoa could be retrieved in cases of large deletions including and extending beyond the AZFc region (AZFbc or AZFac) [7-9].

Recently, infertility treatment has been performed by intracytoplasmic sperm injection (ICSI) and in vitro fertilization (IVF) techniques. However, deletions on the Y chromosome might be spread to the male offspring, causing the persistence of infertility problem over the next generations [10].

In this study, we develop a method to demonstrate the presence or absence of microdeletions on infertile cases with azoospermia and oligozoospermia before
ICSI/IVF, by using multiplex polymerase chain reaction (PCR) method.

Materiel and methods

Patients
Deletion testing was performed in men with a sperm concentration <5.11/mL (azoospermia or severe oligozoospermia).

Extraction of genomic DNA from peripheral blood lymphocytes was performed using a standard technique.

Sequence-Tagged Sites and Polymerase Chain Reaction
The screening method for microdeletions was consisted of 20 primer pairs that are homologous to previously identified and mapped sequence-tagged sites STS [11-17]. These primers will amplify nonpolymorphic short DNA segments from the Y chromosome when used in PCR [16, 18]. Y chromosome deletions in the regions that are amplified by these primer sets have been associated with male infertility [12, 16, 19-27]. The primers have been combined into five Multiplex Master Mix sets for use in multiplex PCR. This makes it possible to determine the presence or absence of all 20 STS by performing five concurrent PCR amplifications (Figure 1). Adjacent regions of the Y chromosome do not generally appear as sequential amplification products within a single multiplex amplification reaction. Exceptions are SY242 and SY208 of the DAZ locus, which are represented as sequential amplification products using Multiplex B Master Mix reactions, and SY84 and SY86 of the DYS273 and DYS148 loci, which are represented as sequential amplification products in Multiplex E Master Mix reactions.

Figure 1. Example of amplification using male genomic DNA as template. Amplification of male genomic DNA (MD115A) (lanes 1, 3, 5, 7, 9), as well as a negative DNA control (lanes 2, 4, 6, 8, 10), for each of the five Multiplex Master Mixes.

Multiplex Master Mixes A–D contain a control primer pair that amplifies fragments of the X-linked SMCX locus. The fifth Multiplex Master Mix, Multiplex E, contains a control primer pair that amplifies a unique region in both male and female DNA (ZFX/ZFY). These control primer pairs are internal controls for the multiplex amplification reactions and test the integrity of the genomic DNA sample. Finally, Multiplex E Master Mix also includes a primer pair that amplifies a region of the SRY gene, acting as control amplification for the testis-determining factor on the short arm of the human Y chromosome.

Results
To detect microdeletions in the Y chromosome following multiplex PCR, the worksheets presented in Table 1 are used to analyze the PCR reactions (presence or absence of the expected PCR products). Therefore, If there are any PCR products absent from the reactions, they can be mapped using the Y Chromosome Map Worksheet (Table 2). All deletions should be contiguous. If multiple amplification
bands are missing for a sample, and those bands do not map to adjacent regions of the Y chromosome (Table 2), they represent dropout bands and are not deletions. In this case, the test is repeated. A single missing amplification band in a sample may also represent a dropout band and thus the test is repeated to confirm that the locus is not amplified. A locus that is present in a sample may not be amplified if there is a mutation in one of the primer binding sites for the locus. Diagnostic results obtained with this method should only be interpreted in conjunction with other clinical or laboratory data. Figure 2 shows an example of gel analysis of sample DNA.

We have observed some nonspecific bands that appear above or below the expected amplification product on the agarose gel. These nonspecific bands represent different conformations of the same amplification product. This phenomenon is sometimes seen in gel electrophoresis because of the pH. In addition, no PCR amplification products were detected with the negative controls (no DNA template). Accordingly, these nonspecific bands have no effect on the analysis.

### Discussion

Recent advances in molecular biology suggested that microdeletions of the Y chromosome represent an important cause of male infertility and the most frequent cause of severe testiculopathy [28]. Y chromosome was thought to be poor in terms of gene content as its q arm constitutes mostly of heterochromatic region. But recently, it came into consideration due to the discovery of genetic complexity of AZF region which is divided into three nonoverlapping loci: AZFa, AZFb, AZFc spanning through intervals V and VI. Molecular mapping analysis has shown that these loci harbor not all but at least some of the genes responsible for spermatogenesis [29, 30].

The negative effects of abnormal semen characteristics and sperm quality on fertility can be overcome with in vitro fertilization (IVF) using conventional methods of fertilization, or with intracytoplasmic sperm injection (ICSI). The use of ICSI provides an effective treatment for severe male factor infertility. Indeed, ICSI is recommended following failure of fertilization using conventional methods of IVF because the technique bypasses the zona pellucida and oolemma.

---

**Table 1.** PCR Amplification Product Profile for Test Sample. Worksheets: We record the presence (+) or absence (−) of the PCR amplification products for each sample.

<table>
<thead>
<tr>
<th>STS</th>
<th>Locus</th>
<th>Product Size (bp)</th>
<th>Map Position</th>
<th>Sample (+/−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SY254</td>
<td>DAZ</td>
<td>380</td>
<td>18</td>
<td>+</td>
</tr>
<tr>
<td>SY157</td>
<td>DYS240</td>
<td>290</td>
<td>20</td>
<td>+</td>
</tr>
<tr>
<td>SY81</td>
<td>DYS271</td>
<td>209</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>SY130</td>
<td>DYS221</td>
<td>173</td>
<td>11</td>
<td>+</td>
</tr>
<tr>
<td>SY182</td>
<td>KAL_Y</td>
<td>125</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>SMCX</td>
<td></td>
<td>83</td>
<td>Control</td>
<td>+</td>
</tr>
<tr>
<td>SYPR3</td>
<td>SMCY</td>
<td>362</td>
<td>7</td>
<td>+</td>
</tr>
<tr>
<td>SY127</td>
<td>DYS218</td>
<td>274</td>
<td>9</td>
<td>+</td>
</tr>
<tr>
<td>SY242</td>
<td>DAZ</td>
<td>233</td>
<td>16</td>
<td>+</td>
</tr>
<tr>
<td>SY208</td>
<td>DAZ</td>
<td>140</td>
<td>17</td>
<td>+</td>
</tr>
<tr>
<td>SMCX</td>
<td></td>
<td>83</td>
<td>Control</td>
<td>+</td>
</tr>
<tr>
<td>SY128</td>
<td>DYS219</td>
<td>228</td>
<td>10</td>
<td>+</td>
</tr>
<tr>
<td>SY121</td>
<td>DYS212</td>
<td>190</td>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>SY145</td>
<td>DYSF51S1</td>
<td>143</td>
<td>14</td>
<td>+</td>
</tr>
<tr>
<td>SY255</td>
<td>DAZ</td>
<td>124</td>
<td>19</td>
<td>+</td>
</tr>
<tr>
<td>SMCX</td>
<td></td>
<td>83</td>
<td>Control</td>
<td>+</td>
</tr>
<tr>
<td>SY133</td>
<td>DYS223</td>
<td>12</td>
<td>SY133</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>177</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SY152</td>
<td>DYS236</td>
<td>15</td>
<td>SY152</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SY124</td>
<td>DYS215</td>
<td>8</td>
<td>SY124</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>109</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMCX</td>
<td></td>
<td>83</td>
<td>Control</td>
<td>+</td>
</tr>
<tr>
<td>SY133</td>
<td>ZFX/ZFY</td>
<td>496</td>
<td>Control</td>
<td>+</td>
</tr>
<tr>
<td>SY14</td>
<td>SRY</td>
<td>400</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>SY134</td>
<td>DYS224</td>
<td>303</td>
<td>13</td>
<td>+</td>
</tr>
<tr>
<td>SY86</td>
<td>DYS148</td>
<td>232</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>SY84</td>
<td>DYS273</td>
<td>177</td>
<td>4</td>
<td>+</td>
</tr>
</tbody>
</table>
**Figure 2.** Example of Y chromosome deletion gel analysis. The amplification products from Multiplex A–E Master Mix reactions are shown. Each Multiplex Master Mix is used for amplification of Male Genomic DNA (MD115A) samples (lanes 1, 3, 5, 8, 10) and a representative sample containing (lanes 2, 4, 7, 9, 11). Bands resulting from the amplification of the positive Male Genomic DNA control are compared to those bands resulting from the amplification of the test genomic DNA. The marker (lanes M) is the 50bp DNA Step Ladder. Negative control (no DNA template) (lane 6).

**Table 2.** Y Chromosome Map Worksheet. Palindromes 8, 7, 6, 5 and 4 map in the proximal direction of SY121 and in the distal direction of SY182 (1). SY121 is located at the distal boundary of P4 (8). AZFb extends from P5 to proximal P1 (8). AZFc includes P1 and P2 (8). At least one copy of SY157 maps outside the AZFc boundary.

To deliver the male chromosomes directly into the ooplasm. However before performing such treatment for male infertility, genetic counseling is recommended. Indeed, if the infertility is due to a genetic defect such as microdeletions of the Y chromosome, this defect will be transmitted to the male offspring. Therefore, the Y chromosome screening is worthwhile especially when the sperm count is inferior to 5 million /ml. Here, we have shown a method based on multiplex PCR that can be used in genetic counseling before treatment for male infertility with IVF or ICSI.
Reference


