A simple, low cost and non-invasive method for screening Y-chromosome microdeletions in infertile men

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BACKGROUND: Recent investigations emphasized a high prevalence of Y-chromosome microdeletions in men having severely impaired spermatogenesis. Screening of these men is recommended prior to assisted reproduction techniques. METHODS: The aim of this study was to define a reliable and efficient method to detect Y-chromosome deletions in infertile men. At first the feasibility of using a cytobrush to collect buccal cells as a source of DNA was tested. Then, a multiplex PCR in accordance with European recommendations (European Andrology Academia: EAA) was compared with a commercial kit. The test population consisted of 18 infertile male patients (with a known Y-deletion). Both buccal and blood cells were used for DNA extraction. A specific DNA extraction protocol was carried out on the buccal cells. RESULTS: Between 4±10 µg of DNA were retrieved per brush, allowing for several PCR attempts. The commercial kit failed to detect an AZFa deletion. Furthermore, markers sY130, sY133 and sY153, included in the kit, are not reliable. Both false negative and false positive results were generated by the commercial kit. CONCLUSION: A multiplex PCR performed pursuant to EAA recommendations is proposed. When the testing is conducted with DNA extracted from buccal cells, this protocol is simple, accurate and affordable.

Key words: male infertility/PCR/screening/Y chromosome

Introduction

Recent advances in assisted reproduction techniques (ART), especially ICSI, have altered the prognosis of male infertility. Some men, once regarded as sterile and previously relying on donation of sperm, may now father their own children. This raises the question of the transmission of genetic abnormalities, related to infertility, to offspring. The most frequent causes of male infertility are chromosomal abnormalities which have been found in karyotypes of 10–15% of men with azoospermia and 5% of men with oligospermia (De Braekeleer and Dao, 1991; Dohle et al., 2002). The French Guide for Good Clinical and Biological Practices in ART (1999) therefore recommends genetic screening for patients relying on ICSI, especially in the case of non-obstructive azoospermia or severe oligozoosperma.

As early as 1976, large Y-chromosomal (Yq) deletions had been detected by standard karyotype analysis in six azoospermic males leading to the description of an essential factor in spermatogenesis called the Azoospermia Factor (AZF) (Tiepolo and Zuffardi, 1976). The region surrounding AZF was subsequently involved in male infertility ascribed to azoospermia or severe oligozoosperma (Vogt et al., 1998). Anonymous markers, called Sequence Tagged Sites (STS), mapped along the entire length of the Y chromosome, allowed the detection, through PCR, of small size deletions within the AZF region, not identifiable with standard cytogenetic techniques (Vollrath et al., 1992). Analysis of these microdeletions resulted in the identification of three loci in Yq11 involved in the control of spermatogenesis, corresponding to three non-overlapping regions: AZFa, AZFb, AZFc (Ma et al., 1993; Reijo et al., 1995; Vogt et al., 1996). Some authors have recently proposed the existence of a fourth region, AZFd, in close proximity to AZFc (Kent-First et al., 1999).

Microdeletion detection should be readily available throughout the country since this test has been included in the recommended guidelines for male infertility (Kostiner et al., 1998; Krausz et al., 1999; Sharlip et al., 2002). Nevertheless, some French centres do not choose to perform Y-chromosomal microdeletion diagnosis. Such centres advance theoretical reasons: the test is not compulsory, there is doubt regarding its diagnostic efficiency, the detection has no consequence on the management of the infertility, and lack of information on genetic counselling. In addition, there are practical issues: absence in the French Social Security Nomenclature, no national quality control, blood samples are dispatched only after short delays, and finally there is no consensus on the appropriate operating mode, especially concerning STS choice for Y-chromosomal detection. The position of the STS on the Y

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The first European quality control confirmed the absence of a consensus protocol among the 29 participating centres (Simoni, 1998; Maurer et al., 2001; Simoni et al., 2001). During the first European meeting of Andrology (European Academy of Andrology: EAA), 16 European countries, including France, thus proposed ‘Laboratory guidelines for molecular diagnosis of Y-chromosomal microdeletions’ (Simoni et al., 1999). It is generally recommended that Yq microdeletion should only be offered to selected infertile patients with <5×10^6 sperm/ml. This study recommends ways to minimize some of the practical problems, therefore enabling wider use of the test. The usefulness of a specific method to sample that is associated with a fast DNA extraction technique is described. The study compares a technique recommended by the European guidelines with a commercially available kit.

### Materials and methods

#### Patients

The first group consisted of 18 patients with AZFa, AZFb or AZFc microdeletion detected previously in another centre, with a protocol using 15 STS. The second group consisted of 12 patients characterized by an isolated teratozoospermia at >90%.

#### Sampling

Each patient was submitted to (i) blood sampling through peripheral venous tapping into an EDTA-containing tube and (ii) collection of buccal cells by brushing.

The buccal cell sampling consisted of a careful brushing of the internal side of both cheeks, with two nylon endocervical type brushes (‘Hospital’s brush’, item 0134; GPS, Mozzo, Italy). After collection, the samples were stored in a freezer (−20°C) until DNA extraction.

#### DNA extraction

DNA was extracted from peripheral blood by the standard phenol-chloroform technique and resuspended in Tris EDTA (pH 8). DNA was extracted in parallel from buccal cells by Chelex resin extraction technique (Chelex® 100 resin; Bio-Rad) (Walsh et al., 1991). DNA concentration was measured with the standard spectrophotometric method and with a colorimetric method using strips (DNA DIPSTICK™; Invitrogen). DNAs were stored at 4°C.

#### PCR

A technique prepared following international recommendations (Simoni et al., 1999; Simoni, 2000) was compared with a commercial kit (Y-chromosome deletion detection system version 1.1, Promega). PCRs were performed on a PTC 200 thermocycler (MJ Research).

### Table I. Result of a PCR with the Promega kit (kit p) and our own PCR in five patients

<table>
<thead>
<tr>
<th>Zone</th>
<th>STS</th>
<th>GORkit p.</th>
<th>GOR</th>
<th>MENkit p.</th>
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<th>G2</th>
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</table>

(+) indicates that the locus is present.

(−) indicates that the locus is absent.

(+/−) indicates that the result is doubtful because of a weak band.

(NA) indicates not analysed.

Patient HUG presents an AZFb, d and c deletion.

Patient GOR presents an AZFc deletion.

The known AZFa deletion in patient MEN was not detected with the Promega kit.

Patient RIV presents a macrodeletion of the three regions AZFa, b and c.

Patient MEN presents an AZFa deletion, detected with our own PCR.

Patient G2 presents an AZFb deletion.

H is a control, fertile man.

The present results with our own PCR, for each patient, fit perfectly with the data obtained previously in a different laboratory, using a PCR protocol with 15 STS.

Reference:

Our own technique from international recommendations
For a first screening, eight STS were analysed in two multiplex PCR: sY84 and sY86 for AZFa, sY127 and sY134 for AZFb, sY254 and sY255 for AZFc.

The two multiplex PCR consisted of: Mix 1: SRY – ZFY – sY84 – sY134 – sY255 and Mix 2: SRY – ZFY – sY86 – sY127 – sY254. As internal controls, SRY (sY14) and ZFY were included in both PCR multiplexes. At the beginning of the study, the STS sY117 was included in Mix 1, but its use was discontinued.

Several controls were used for each PCR reaction: a blank without DNA; female DNA; male DNA with a known AZF deletion; and fertile male DNA. Internal controls were SRY and ZFY.

The experiments were made in triplicate. To decrease the risk of contamination, a female technician performed all experiments.

Promega kit: Y-chromosomal Deletion Detection System, version 1.1
This kit tests 18 STS (Foote et al., 1992; Vollrath et al., 1992; Reijo et al., 1995; Affara et al., 1996; Kent-First et al., 1996a; Vogt et al., 1997), mapped among AZF a, b, c and d regions. The kit includes several controls: a tube without DNA, a male DNA, and an internal control SMCX—checking the integrity of the tested DNA sample by amplifying a sequence in the X chromosome. Experiments were made in triplicate, according to the manufacturer’s recommendations, with some modifications mentioned in the Results section.

Results
DNA extraction from buccal cells
DNA extraction from buccal cells was possible for all patients. Cytobrushing proved to be simple, fast and painless and was well accepted by patients. The extraction step with Chelex resin also proved to be simple, fast (1 h 30 min) and did not require any organic solvents. Spectrophotometer and colorimetric strips gave similar results: for a final volume of 200 µl, DNA concentrations ranged from 20–50 ng/µl, with a quantity of DNA between 4–10 µg by cytobrush. Furthermore, it was possible to get DNA of similar quality and quantity even when brushes were kept at room temperature for 3 weeks, enabling the use of standard mail. Extracted DNA was stable at +4°C for up to 18 months since a PCR amplification after that period was successful (data not shown).

PCR
Blood versus cytobrush
In the 30 patients, it was possible to amplify DNA extracted from either buccal cells or leukocytes, with identical results.

Promega kit versus the study’s PCR protocol in accordance with European recommendations
Table I shows selected results obtained with standard PCR and the Promega kit. Since sY117 did not add any information, this marker was removed from the protocol.

PCR conditions supplied by Promega proved to be inadequate. First, the Taq polymerase supplied in the Promega kit was changed to another brand, Hot Star Taq (Qiagen®), leading to a cost increase. Second, it was necessary to adapt their programme to our thermocycler. Regarding the European recommendations about PCR, the elongation step was modified (45 s at 72°C versus 4 min at 65°C), the hybridization temperature was slightly increased (58°C versus 55°C) and the number of PCR cycles was increased (34 versus 25).

Some major discrepancies appeared between the standard technique and the Promega kit. An AZFa deletion (patient MEN) was not detected by the Promega kit—the deletion lies between sY81 and sY182. In contrast, following the STS recommended by the international guidelines, the deletion was detected, encompassing sY84 and sY86.

Furthermore, the kit is based on some inappropriate STS. For example, sY153 is present in multiple copies (Tilford et al., 2001); this can explain a weak amplification for some patients with an AZFc deletion. SY130 and sY133 also led to frequent false positive PCR results. A blast search with sY133 sequence retrieved three different clones giving rise to the presumption that this STS is not a single copy. By contrast, apart from sY254 and sY255 corresponding to DAZ, the four other STS included in the recommended PCR are present in a single copy.

In addition, PCR multiplex ‘C’ from the Promega kit, gave a non-specific fragment within the control female DNA and the internal control corresponds to the smallest piece of each multiplex, with a risk of preferential amplification.

We also analysed 12 patients selected because of their isolated teratozoospermia [>90%, according to David’s classification (modified)]. No deletion was detected in the DNA from these patients, which does not support the existence of a fourth AZF area, ‘AZFd’ (Kent-First et al., 1999).

Costs comparison between the two techniques
The respective costs for each technique were estimated, labour cost being excluded. The cost per patient for our standard PCR is about 6.1 Euros. The Promega kit is more than 10 times this price, around 88 Euros. Extraction costs with Chelex are reasonable, being 0.6 Euros per patient.

Discussion
Even if substantial evidence supports Y-chromosomal microdeletion testing, implementation of the testing in a laboratory poses practical problems that are only partially solved. Collecting buccal cells by cytobrush offers many benefits: low cost, painless and ability to mail samples. DNA extraction with Chelex resin is fast, simple and does not need phenol-chloroform. There is usually enough DNA to perform at least 200 PCR.

The Promega kit, mainly used in the USA, is seldom used in France, partly because of its high cost. It appears difficult to implement, especially with some of the thermocyclers (Perkin Elmer 9600) or some Taq Polymerases (H.Lejeune, unpublished data). About 1/20 DNA samples did not yield useful results. The study also highlighted some cautionary notes regarding the Promega kit YDDS 1.1.

The AZFa region is not accurately analysed. Our patient (MEN) azoospermia phenotype corresponds to a Sertoli cell only syndrome (SCOS) which is expected from a patient with AZFa deletion. This deletion was correctly identified using the European recommendations.

Promega has announced a new modified kit that can detect AZFa deletions. This new kit is similar to the old one, with an additional fifth mix including sY84, sY86, SRY, ZFY and sY134. This new kit is not yet available. According to Promega the new kit will be more expensive than version 1.1.
Internal control corresponds to the smallest piece of each multiplex, leading to a risk of preferential amplification, PCR multiplex C from the Promega kit gives a non specific fragment within the control female DNA.

Many non-specific bands are detected in multiplex D from the Promega kit, in deleted patients, possibly because certain STS, mainly sY130, sY133 and sY124 are not single copies.

The Promega kit includes the detection of a fourth region, the so-called AZFd, which is associated with a severe teratozoospermia phenotype (Kent-First et al., 1999). STS are questionable as sY152 corresponds to an intronic region of DAZ and sY153 seems to be polymorphic or in multiple copies. All the STS loci analysed for defining AZFd (i.e. sY145, sY153, sY152, sY220, sY150, sY232, sY262, and sY221) had been earlier mapped to the AZFc region (interval 6C-6E). (Reijo et al., 1995; Vogt et al., 1997). According a published study (Kuroda-Kawaguchi et al., 2001), sY152 and sY153 are located within AZFc proximal limits. The present study on a limited number of patients could not confirm the existence of a fourth region. European recommendations for Y-chromosome microdeletion detection address routine diagnosis and are strongly supported by scientific societies [American Urological Association (AUA), American Association of Bioanalysts (AAB), American Society for Reproductive Medicine (ASRM), Human Fertilisation and Embryo Authority (HFEA)], in order to standardize the analysis process. The results of the first European quality control study were very informative and resulted in modifications of the test. First, they allowed the withdrawal of non-informative STS (sY55, sY75, sY138, sY143, sY231, sY272 and sY167). Second, they showed conflicting results between laboratories for some STS: sY152, sY153, sY236, sY166 and sY147 (Vogt et al., 1997). Third, they pinpointed that the analysis of too many STS in the AZFc region may lead to false positive results. Finally, the requirement for negative controls was emphasized (female DNA, blank) and a positive control (fertile male DNA).

Other microdeletion detection protocols were recently proposed, such as the PRINS technique (Kadandale et al., 2002). These protocols are of interest, but they are time consuming, complex and seem to be quite difficult to perform in a routine diagnosis.

The French Society for Human Genetics (SFGH) also edited practical recommendations for Y-chromosome microdeletion detection, adapted from the recommendations of the EAA. Analysis using STS situated within candidate genes does not show any clear advantage compared with the screening using the STS currently recommended. The SFGH recommends a simple two-step screening. For detection—sY84 and sY86 (AZFa), sY127 and sY134 (AZFb), sY254 and sY255 (AZFc). The analysis of these markers is sufficient to detect >90% of the deletions. Additional markers to define the borders of the deletion—AZFa: sY82 (+), sY83 (–) proximal border, and sY87 (–), sY88 (+) distal border. AZFb: sY135 (+), sY114 (–) proximal border, and sY142 (–), sY152 (+) distal border. AZFc: sY142 (+), sY152 (–) proximal border, and sY157 (–), sY158 distal border. The (+) indicates that the locus should be present when the corresponding region is deleted, (–) indicates that the locus should be absent when the corresponding region is deleted. The SFGH confirms the inadequacy of sY153 because it corresponds to repeated sequences.

In conclusion, European and French recommendations for Y-chromosome microdeletion detection highlight the need for a routine diagnosis offered to selected infertile patients with <5×10⁶ sperm/ml. As such, the test should be accurate, reliable, easy to perform, reproducible, fast and inexpensive.

Along these lines, it is proposed that a simple Y-chromosome microdeletion detection protocol including (i) a simple and practical sampling mode—brushing of buccal cells, (ii) a simple extraction step—Chelex resins and (iii) a valid PCR protocol in accordance with the aforementioned recommendations. It consists of eight STS amplified in two multiplex PCR: sY84 and sY86 (AZFa), sY127 and sY134 (AZFb), sY254 and sY255 (AZFc), plus SRY and ZFY as internal controls. Nevertheless, the limits of this approach must be stressed, mainly because the deletion can be present in a mosaic state or consist of only a reduction of DAZ copy numbers. Furthermore, studies are still needed to confirm the possible correlation between phenotype and genotype.

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