Guidelines for Y-chromosome microdeletions

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Note
These guidelines were written by Prof. Manuela Simoni and co-authors (see below) and were published in the International Journal of Andrology, 22: 292-299 (1999). They are not the outcome of a EMQN sponsored best practice meeting. EMQN is sponsored a Y-chromosome microdeletion best practice meeting which was organised by Prof. Simoni and held in L'Aquila, Italy on the 27/28 th May 2000.

Introduction
In addition to the "testis determining factor" (SRY), the Y chromosome harbours genes important for spermatogenesis. The „azoospermia factor“ (AZF) on the long arm of the Y chromosome, originally defined by Tiepolo and Zuffardi (1976), is now progressively being dissected into discrete genes (Lahn and Page, 1997; Vogt et al., 1997). The deletion of the AZF is thought to be pathogenetically involved in some cases of male infertility associated with azoospermia or severe oligozoospermia (Vogt, 1998). While until few years ago analysis of the AZF region was performed only for experimental purposes, the discovery of the DAZ gene (Reijo et al., 1995), the description of three discrete AZF regions (Vogt et al., 1996) and the diffusion of the PCR technology has rendered the molecular diagnosis of Y-chromosomal microdeletions popular and affordable (for review see Kostiner et al., 1998 and Vogt, 1998). As a result, PCR analysis of the Y chromosome is now routinely performed in the workup of the infertile male. However, there has been no consensus on how the diagnosis should be carried out and which DNA loci on the Y chromosome should be analysed.

The heterogeneity of the protocols employed and of the results published, together with the increasing diffusion of this diagnostic procedure suggested the necessity of a quality control programme (Simoni et al., 1996). Such an international programme started in 1997 as an official activity of the European Academy of Andrology. After completion of three trials the participants in the quality control programme and some experts in diagnostics of genetic diseases and in genetics of the Y chromosome were invited to evaluate the results and to contribute to the generation of the present guidelines for the molecular diagnosis of Y-chromosomal microdeletions.

Frequency and clinical relevance of microdeletions of the Y chromosome
Existing evidence suggests that genes located on the euchromatic region of the long arm of the Y chromosome play an essential role in spermatogenesis (Edwards & Bishop, 1997; Vogt, 1998). In fact, microdeletions are found in infertile men with azoospermia or severe oligozoospermia, while they are not detected in control groups of proven fathers. Moreover, deletions in infertile men are usually de novo events, probably originating in the germ line of the patient’s father (Edwards & Bishop, 1997). For practical purposes, the regions of the Y chromosome that contain genes probably relevant to male infertility are indicated as AZFa, AZFb and AZFc, respectively (Vogt et al., 1996).

At the present time, the literature published after the description of the DAZ gene (Reijo et al., 1995) reports data from more than 3000 infertile men and 1300 proven fathers (Nakahori et al., 1996; Qureshi et al., 1996; Stuppia et al., 1996; Reijo et al., 1996; Vogt et al., 1996; Najmabadi et al., 1996; Kent-First et al., 1996; Pryor et al., 1996; Foresta et al., 1997; Vereb et al., 1997; Kremer et al., 1997; Mulhall et al., 1997; Simoni et al., 1997; van der Ven et al., 1997; Stuppia et al., 1997; Girardi et al., 1997; Foresta et al., 1998; Liow et al., 1998; Stuppia et al., 1998; Brandell et al., 1998; Silber et al., 1998; Grimaldi et al., 1998; Kleimann et al., 1998; Selva et al., 1997; Kent-First et al., 1997; Seifer et al., 1999). Summarizing these published papers, microdeletions were found in about 7.3 % of the infertile men. The
vast majority of deletions were found in azoospermic men (66%), less so in men with sperm concentrations < 5x10^6/ml (28%) and sporadically in men with sperm concentrations >5x10^6/ml (6%). These data suggest that the expected frequency of microdeletions in "selected" infertile men should be around 7%. However, in the literature the frequency varies between 1% (van der Ven et al., 1998) and 55.5% (Foresta et al., 1998). This variation is probably mainly due to the selection criteria of the patients. However, other factors should be considered, such as accuracy of the PCR results and relevance of the deletion detected, especially in case of discontinuous deletions or deletions of isolated STS loci. Reviews of the literature have been published recently (Simoni et al., 1998; Vogt, 1998).

Although the role of Y-chromosomal deletions in male infertility appears obvious, formal proof of the involvement of discrete genes in male fertility is still lacking. This would require the discovery of cases of infertility due to point mutations and/or the demonstration in vitro of clear loss of function induced by site-directed mutagenesis. The function of these genes, however, is still unknown. Moreover, the nature of many Y-chromosomal genes, most of which are either multicopy or expressed in tissues other than the testis, renders the experimental analysis and the demonstration of a causal role in infertility extremely difficult. It is conceivable that several genes of the Y chromosome, which are specifically expressed in the testis and deleted in human male infertility, contribute to normal spermatogenesis (Vogt, 1998). What they exactly do, is basically unknown.

In summary, diagnosis of Y-chromosomal microdeletions as it is currently performed does not prove that the deletion caused the patient’s infertility. However, such a causal relationship is highly probable, provided that the loci have been shown and confirmed to be deleted only in infertile males and not in proven fathers in well controlled studies.

Indications for diagnosis
Given these premises, there are no absolute criteria or guidelines indicating which patients are candidates for molecular analysis. Patients undergoing intracytoplasmatic sperm injection (ICSI) may represent an obvious population for deletion screening due to the fact that the microdeletions are transmitted to the male offspring if assisted reproduction is performed (Kent-First et al., 1996, Kamischke et al., 1999, Page et al., 1999). In this case, the diagnosis of a deletion can influence the therapeutic option. Genetic counselling is then mandatory in order to provide information about the risk of producing an infertile son, although there is no proof yet that the son will indeed be infertile (Meschede et al., 1995; Meschede et al., 1997).

Therefore, for the time being indicators for molecular diagnosis of Y-chromosomal microdeletions remain open and clinicians should decide in which patients they regard this diagnosis as worthwhile and meaningful. It should be kept in mind that this is still a very experimental area of reproductive medicine and genetics.

Results from the first three trials of the External Quality Control Assessment Programme
Six DNA samples were sent to the participating laboratories subdivided into three trials. Participants (see Appendix) were requested to perform analysis according to their usual protocol. Twenty, 25 and 29 laboratories participated in the consecutive trials, respectively. In summary, the results have shown that:

1. Both simplex and multiplex PCR are used.
2. There is a wide inter-lab heterogeneity in the choice of primers used. Remarkably, none of the primers was common to all participants. Overall, 90 different primers were employed in trial 1 (9 of which amplified STSs in the AZF region, 23 in AZFb, 36 in AZFc and 22 other STSs, respectively), 77 primers in trial 2 (8 in AZFb, 24 in AZFb, 30 in AZFc and 15 in other regions, respectively) and 80 primers in trial 3 (9 in AZFb, 24 in AZFb, 36 in AZFc and 11 in other regions, respectively). The reason for the choice of primers was not indicated.
3. The rate of misdiagnosis was 5% (2/40 diagnoses) for trial 1, 4% for trial 2 (2/50 diagnoses) and 5.2% (3/58 diagnoses) for trial 3. Both false positive (i.e. failure to detect a deletion) and false negative (failure to amplify normal DNA) results were obtained. In general, the diagnosis of deletions involving the DAZ gene was accurate (no false positives), but false negative results leading to the wrong diagnosis of AZFc deletions were observed. False positive results were obtained in a sample with an AZFb deletion. Remarkably, both false positive and false negative results were obtained even when using more that one STS primer for each AZF interval. Finally, some laboratories produced "uncertain" results for some STSs, suggesting that the quality control samples might have been handled differently from the patient samples.
4. No data concerning internal quality control were given.

Guidelines for diagnostic testing
The diagnostic testing of deletions is performed by PCR amplification of selected regions of the Y chromosome.
Most of the STS primers used so far amplify anonymous sequences of the chromosome, which have been more or less precisely mapped but are not yet known to belong to specific genes. Although discrete genes are progressively being identified, there is no proof of their causal role in infertility, not even in the case of DAZ and RBM (Ruggiu & Cooke, 1999). Moreover, using STS primers specific for discrete genes does not increase the detection rate of microdeletions in DNA samples from ICSI candidates (Krausz et al., 1999). Therefore, for the time being, it is basically unimportant whether the STS primers used amplify anonymous regions or AZF candidate genes. What is important for the diagnosis is that the panel of STS primers are derived from regions of the Y chromosome which are unique and not polymorph in the Caucasian population and are well known to be deleted specifically in infertile men. These regions correspond to the AZFa, AZFb and AZFc regions according to Vogt et al. (1996). A putative fourth AZF region has been recently postulated by Kent-First et al. (1999).

According to the authors, a subgroup of patients with idiopathic infertility and variable sperm count would be included in the first choice set are: sY14 (SRY), sY84, sY86, sY127, sY134, sY254, sY255 (both in the AZFb region). The female DNA sample controls for the specificity and external positive and negative controls must be run in parallel with each multiplex, i.e. with each set of primers. Appropriate positive and negative controls are a DNA sample from a fertile man and from a women, respectively. The DNA sample from a fertile man controls for the sensitivity and specificity of the assay. The female DNA sample controls for the specificity and for contamination. In addition, a water sample, which contains all reaction components but water instead of DNA, must be run with each set of primers. The water sample controls for reagent contamination.

In summary, the diagnostics of Y-chromosomal microdeletions should be performed by multiplex (at least duplex) PCR amplification of genomic DNA, using the ZFX/ZFY as internal PCR control. A DNA sample from a fertile male and from a woman and a blank (water) control should be run in parallel with each multiplex.

For a more detailed description of internal quality control please consult Neumaier et al. (1998).

b. First choice (minimal set) of STS primers

In principle, the analysis of only one non-polymorphic STS locus in each AZF region is sufficient to determine whether any STS deletion is present in AZFa, AZFb or AZFc. However, analysing two STS loci in each region reinforces diagnostic accuracy, since deletions usually involve more than one STS locus. Therefore, it was agreed that at least two STS loci in each AZF region should be analysed. Based on the experience of the first three trials of external quality control and considering the multiplex PCR format, the STS primers included in the first choice set are:

- For AZFa: sY84, sY86
- For AZFb: sY127, sY134
- For AZFc: sY254, sY255 (both in the DAZ gene)

These STS primers have been shown to give reproducible results in multiplex PCR reactions by several laboratories and are those most frequently used among the participants in the quality control assessment scheme. Moreover, the SRY gene should be included in the analysis as a control for the testis determining factor on the short arm of the Y chromosome and for the presence of Y-specific sequences when the ZFY gene is absent (e.g. in XX males).

In summary, the set of PCR primers which should be used in multiplex PCR reactions as first choice for the diagnosis of microdeletion of the AZFa, AZFb and AZFc region includes: sY14 (SRY), ZFX/ZFY, sY84, sY86, sY127, sY134, sY254, sY255.

The sequence of the primers and the suggested PCR conditions are reported in the
Appendix. The use of this primer set will enable the detection of over 90% of the deletions in the three AZF regions. This is the first choice set for diagnosis and it should be kept in mind that the extent of the deletion is not assessed by this method and genotype/phenotype correlations are not possible. Furthermore, deletions outside the AZFa, AZFb and AZFc regions will not be detected. Nevertheless, the adoption of this minimal set of primers by all laboratories is judged to be sufficient, at the moment, for routine diagnostics. It will also allow a minimal standardization and a better comparison of laboratory performance and inter-laboratory variability.

c. Second choice of STS primers

Once a deletion is detected, if one wishes to determine whether the extent of the deletion is similar to that of the regions designated as AZFa, AZFb and AZFc, the analysis can be extended to STS loci known to cross the proximal and the distal borderlines of each AZF region. This analysis could include:

- AZFa: sY82(+), sY83(-) (proximal border), // (distal border) sY87(-), sY88(+)
- AZFb: sY135(+), sY114(-) (proximal border), // (distal border) sY143(-), sY152(+)
- AZFc: sY143(+), sY152(-) (proximal border), // (distal border) sY157(-), sY158(+)
- Yq12 (distal heterochromatin, positive control): sY160/DYZ1.

(+/-) indicates that the locus should be present in the corresponding patient group
(-/-) indicates that the locus should be absent in the corresponding patient group

This choice might be useful to establish genotype/phenotype correlation. Even in this case, deletions outside the AZFa, AZFb and AZFc regions will not be detected.

In summary: in any case the routine diagnostics of deletions of the Y chromosome should include the set of primers indicated under 5b. If a more extensive analysis is desired, the set of primers indicated under 5c might be used. However, the choice of the primers for the extended analysis is left to the individual laboratories. The extended analysis should be performed in multiplex PCR reactions using the internal and external controls indicated above.

d. STSs primers which should not be used

A number of STS primers currently used by some participants in the quality control assessment scheme should no longer be used anymore for the following reasons:

- sY16: is redundant when using sY14
- sY55: this STS locus is repetitive and dispersed throughout the Y chromosome (intervals 3C, 6A)
- sY75: this STS locus is repetitive and dispersed throughout the Y chromosome (intervals 4A, 6)
- sY109: this STS locus is repetitive and dispersed throughout the Y chromosome (intervals D11, D23)
- sY112: this STS locus is repetitive and dispersed throughout the Y chromosome (D12, D14, D22).
- sY135 is recommended instead of sY112 for the analysis of interval D12.
- sY132: this STS locus is repetitive and dispersed throughout the Y chromosome (intervals 3 and 6)
- sY164: this STS locus is repetitive
- sY138: this STS locus is repetitive
- sY155: this STS locus is polymorphic
- sY272: this STS locus is polymorphic

Moreover: sY156 and Y6HP52 analyse the same locus in interval D20. Therefore only one of them should be used.

In summary: the use of the following primers should be abandoned: sY16, sY55, sY75, sY109, sY112, sY132, sY164, sY138, sY153, sY155, sY272.

e. Control and repetition of the test

If the results of a multiplex PCR indicate a deletion, the whole set of primers should be repeated in simplex PCR, since there is no reason to repeat the test in the same manner. It is known that simplex PCR is less subject to amplification failure. If the result is ambiguous and/or a technical failure must be suspected, the multiplex reaction should be repeated. If the multiplex does not work for a specific DNA sample, the primer set may be run in simplex reactions. There is no general advice as to the number of repetitions. The test should be repeated until the results are clear and reproducible (good laboratory practice).

Reporting

Reports should be written in a standardized format and should be clear to the non-specialist. Guidelines on how to write reports on the outcome of molecular genetics investigations on a patient can be found at the internet site:

http://www.leeds.ac.uk/medgs/guidelines/report-writing

According to those guidelines, reports must be clear, concise, accurate, fully interpretative, credible and authoritative. Reports should be typed, word-processed or created by computer. Hand-written reports are not
acceptable. Reports must include the following information:

- clear identification of the laboratory.
- date of referral and reporting.
- patient identification: full name, date of birth and unique laboratory accession/identification number.
- restatement in some form of the clinical question being asked (e.g. diagnosis of microdeletion of the Y chromosome), and the indication (e.g. azoospermia, ICSI, etc.).
- tissue studied (e.g. blood, buccal smear, etc).
- method used (e.g. multiplex PCR amplification).
- outcome of the analysis: a tabular form of the various STS loci analysed is preferred. Avoid the use of + and -, which can be misinterpreted. Use words instead (e.g.: present/absent, or similar).
- a written interpretation understandable by the non-specialist.
- signature of at least one person responsible for the results. Preferably, all laboratory data are reviewed and all outgoing reports are signed by two independent assessors.

**Participation in External Quality Control Assessment Programme**

The laboratories performing AZF diagnostics should join an external quality assessment (EQA) scheme. It is fundamental that the DNA samples received from the organizers of the EQA programme are processed exactly in the same way as patients’ samples are handled, including reporting. Results should be returned by mail to the organizers as patient (i.e. DNA sample) reports written in English. E-mail or fax can be used for quick communication, but the final evaluation of the results will be performed exclusively on reports received by mail.

The assessment of the performance of the laboratories participating in the international quality control assessment scheme organized by the European Academy of Andrology will include both a general report and individual reports and recommendations to the participants. The results will be assessed by independent reviewers.

The present guidelines represent a first effort towards standardization and improvement of the quality of the molecular diagnosis of Y chromosomal microdeletions. They have been compiled with the active participation of many colleagues working in this field (see Appendix) who have expressed consensus on the issues described here. These guidelines derive from the state-of-the-art knowledge on this rapidly evolving, still experimental field and will be updated as soon as new data from the published literature suggest its necessity. In any case, a formal review of these guidelines will be made one year from the date of issue.

**References**

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Appendix

a. Participants in the 1st meeting

The first meeting to evaluate the results of the first three trials and generate guidelines for the diagnosis of Y-chromosomal microdeletions was held in Noordwijkerhout, The Netherlands, on May 13, 1999 during the first conference of the centers of the European Academy of Andrology. The following persons participated in the meeting and/or contributed to the guidelines:

Organiser:
- Prof. Dr. M. Simoni, Institute of Reproductive Medicine of the University, Domagkstr.11, D-48129 Münster, GERMANY

Participants in the meeting:
- Dr. E. Baldi, Unità di Andrologia, Dipartimento di Fisiopatologia Clinica, Viale Pircisini 6, I50139 Firenze, Italy
- Dr. E. Bakker, Department of Laboratory of the Clinical Genetics, Wassenaarseweg 72, NL-2333 Leiden, The Netherlands
- PD Dr. J. Gromoll, Institute of Reproductive Medicine of the University, Domagkstr. 11, D-48129 Münster, Germany
b. Suggested PCR protocol for the first choice testing

Two multiplex reactions were designed for the analysis of the three AZF deletion regions on the Y chromosome. Both multiplexes contain 5 fragments, i.e. the three AZF loci and the two control fragments SRY and ZFY. Reaction mixes (kits) are prepared in batches sufficient for e.g. 200 reactions, tested and filled out in smaller size aliquots (sufficient for 10 or 20 reactions) for storage at –70°C.

For a deletion test 200 ng of genomic DNA of the patient and 2 U of AmpliTaq are added to 20.6 µl of the prepared mix, giving a total reaction mix with an end volume of 25 µl. The mix then contains 0.75-6.1 pmol of each primer, multiplex buffer (16.6mM (NH₄)₂SO₄, 66.7 mM Tris-HCl pH 8.8, 6.7 mM MgCl₂, 6.8 µM EDTA and 5 mM Beta-mercapto-ethanol) 10% DMSO, 1.5 mM dNTP's, 160 ng/µl BSA, 2U AmpliTaq (Perkin-Elmer), and 200ng DNA.

Amplification conditions (as tested using a Perkin-Elmer 9700/9600 or 2400 PCR machine) start with an initial denaturation step of 5 minutes, followed by 25 cycles of 30 seconds denaturation (94°C), 30 seconds annealing (55°C) and 4 minutes elongation (65°C), ended by a last elongation step of 5 minutes and cooling to 4°C.

Reaction products (10 µl) are separated on a 2% NuSieve (FMC BioProducts, Rockland, ME) plus 0.5% MP (Boehringer) agarose gels in 1 x TBE for 480 Vhr. An example of both multiplexes is given in figure 1.

Legend to Figure 1:
Examples of both Multiplex PCR. Multiplex A: lane 1 phi X-Healil size marker, lane 2 water , lane 3 female DNA, lane 4 DNA of normal male, lane 5 DNA of AZFb deleted patient, lane 6 DNA of AZFc deleted patient.

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