Quantitative PCR technique for the identification of microrearrangements of the AZFc region

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Abstract *Purpose:* The AZFc region spans about 3.5 Mb and contains many amplicons causing recombination events. Several papers have reported the occurrence of AZFc partial deletions resulting from non allelic homologous recombination (NAHR) (“gr-gr”, “b1-b3” or “b2-b3” deletions), particularly in infertile patients. *DAZ* genes are present in 4 copies and rearrangements involve a modification of the number of *DAZ* genes.

Methods: In addition to STS plus/minus PCR, we developed a quantitative technique using real time PCR (Q-PCR) to determine the number of *DAZ* genes. Fourteen DNA controls were selected to validate the use of Q-PCR to detect AZFc microrearrangements, and sperm DNA samples from 30 fertile men were studied.

Results: Rearrangements of 14 controls were well identified with Q-PCR, and 2 AZFc partial deletions were detected in fertile men (1 “gr-gr” and 1 “b2-b3”).

Conclusion: Q-PCR represents a well-adapted method to detect microrearrangements of the Y-chromosome, complementary to STS analysis.

Keywords AZFc · *DAZ* · Male infertility · Partial deletions · Q-PCR

Introduction

The human Y chromosome, which contains long, Y-specific repeats, is particularly susceptible to rearrangements such as deletions or duplication events generated by homologous recombination [1–5]. The interest of detecting Y chromosome rearrangements is becoming more and more important since the correlation between male infertility and Y chromosome microdeletions has been proved. Indeed, microdeletions of the Y chromosome long arm in the AZF (Azoospermia Factor) region may be responsible for up to 20% of cases of severe idiopathic male infertility [6]. Four types of deletions of the AZF region are identified: AZFa, P5-proximal P1 (AZFb), P5-distal P1 (AZFb + c) and AZFc [4, 7]. The most frequent findings are AZFc deletions, which represent at least 65% of all identified microdeletions and whose incidence could affect 1 in 4000 males in the overall population [8]. These deletions have only been detected in infertile populations, even if some rare cases of spontaneous transmission have been reported [8]. AZFc region spans about 3.5 Mb of the Y chromosome euchromatic long arm, and contains many genes and transcripts. The most studied so far are *DAZ* (*Deleted in AZoospermia*) genes, which are present in four nearly identical copies arranged in two clusters of two copies in head to head orientation (Fig. 1) [9]. AZFc microdeletions have been shown to be caused by unequal recombination between two homologous sequences (b2 and b4 fragments) removing the 4 *DAZ* genes (Fig. 1) [1]. “Complete” AZFb deletions were found to extend from palindrome P5 to the proximal arm of palindrome P1, 1.5 Mb within AZFc, then eliminating the first cluster of *DAZ* genes (*DAZ1* - *DAZ2*) (Fig. 1) [4].

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Fig. 1 (a) Localisation of the different markers present in the AZFc region used for Q-PCR and STS plus/minus PCR. (b) Localisation of *DAZ* genes. (c) Schematic view of the repetitive amplicon structure and palindromes of AZFc according to the Kuroda-Kawaguchi code (2001). (d) Schematic view of deletion type; extent of the deletion is shown by solid lines, whereas sequences which recombine losing only one of the two repeat copies are indicated by dotted lines

Recently, partial deletions affecting the AZFc region were identified and involve homologous recombination between “gr-gr” fragments, “b2-b3” fragments (or g1-g3) or “b1-b3” fragments (Fig. 1) [3, 5, 10]. Contrary to “complete” AZF deletions, the clinical relevance of partial deletion is still discussed. Indeed, several studies have shown the existence of partial AZFc deletions responsible for the loss of one cluster of the *DAZ* genes in fertile men [10–12]. In addition to deletion, duplication events and deletion followed by duplication events were identified, but their consequence in male infertility are unknown [3, 5, 11, 13–15].

Previous papers reporting the detection of AZFc partial deletions used several different techniques, namely FISH (fluorescence *in situ* hybridisation) on leukocytes or spermatozoa, Southern blotting or similar quantitative gene copy deletion assays associated with SNP (single nucleotide polymorphism) or SFV (sequence family variant) analyses [3, 11, 13, 16–18]. However, a major drawback of the PCR digest assays is that the variants studied appear to be polymorphic, making interpretation of the results difficult, and STS analysis alone does not allow the detection of duplications. Therefore, we set up a Q-PCR method for a quantitative gene dosage, using specific STS of *DAZ*. We combined STS plus/minus PCR screening, which indicates the presence and mechanism of deletion, and Q-PCR to study selected controls and a fertile population of 30 patients. The aims of this work were: (a) to prove the efficacy of Q-PCR in detecting rearrangements affecting *DAZ* gene copy number; (b) and to propose a new quantitative method, easy to adjust and faster than techniques used up to now. It would then be possible to screen a large population of fertile and infertile men, in combination with STS plus/minus PCR.

Materials and methods

Patients

Fourteen controls were selected according to their molecular and cytogenetic characteristics: -DNA from a patient with a complete AZFb deletion (P5 – proximal P1; AZFb –del) as a partial deletion control (lack of DAZ1-DAZ2 cluster) [4, 16] - DNA from a man carrying a *t*(X;Y)(qter->p22::q11->qter) as a duplication control [19] – DNA from a 47,XYY male was also included to validate the Q-PCR technique - DNA from 8 patients previously studied and characterized with FISH and quantitative techniques: 2 “b2-b3” partial deletion, 1 “gr-gr” partial deletion, 2 deletions followed by duplication and 3 with no AZFc rearrangements [11]. DNA from a man with proven fertility was chosen as a positive control and as a reference for Q-PCR, and female DNA as well as DNA from a man with an AZFc microdeletion (AZFc-del) were used as a negative control.

Thirty fertile men with normal semen analysis (sperm concentration $\gg 20.10^6/\text{ml}$) who consulted for couple infertility and for whom the cause of infertility was on the female side, were investigated in this study. The hospital ethics committee approved this study and informed consents were obtained from each patient included.

DNA extraction and STS plus/minus PCR

For the 30 fertile men, genomic DNA was extracted from 500 μl of sperm samples collected after masturbation and remaining after examination. DNA was extracted using the NucleoSpin Tissue kit (Macherey-Nagel, Germany) according to the manufacturer’s instructions. For the controls,

genomic DNA was extracted from blood peripheral leukocytes using the Nucleon Bacc3 kit (Amersham biosciences, UK).

STS sY1192 (Genbank accession number: G67166) and STS sY1197 (G67168) were tested in duplex PCR and amplifications were carried out as already described [1]. STS sY1291 (G72340) amplification was performed (annealing temperature 65°C) in duplex with the *SRY* gene STS (G38356) as control PCR amplification. PCR amplifications were carried out as previously described [3]. Control samples were included in all cases. Where there was no amplification of the STS sY1291 but both STS sY1192 and sY1197 were amplified, this was indicative of a “gr-gr” deletion. The absence of STS sY1192 amplification with both STS sY1291 and sY1197 amplified was indicative of a “b2-b3” deletion. Finally, when these 3 STS were absent, this was indicative of a “b1-b3” deletion (Fig. 1).

Quantitative real-time-PCR

Q-PCR was used to determine *DAZ* gene copy number. We used the Lightcycler apparatus and Faststart DNA Master SYBR Green I kitTM (Roche Molecular Diagnostics, Mannheim, Germany). Two sets of primers specific to *DAZ* genes, sY255 (G65827) and sY586 (G63907), were chosen. STS sY255 is located in the 10.8 kb fragment of the *DAZ* genes, which is present in 7 copies in the AZFc region depending on the *DAZ* gene number: 3 copies in *DAZ1*, 1 in *DAZ2* and *DAZ3*, and 2 in *DAZ4* [1, 9] (Fig. 1). STS sY586 is located in intron 6 of the *DAZ* gene, and the total number of this STS is expected to be four as there is one copy of it in each *DAZ* gene (Fig. 1) [1, 9]. The single copy STS sY1064 (G64723), located in the AZFa region, was used as a reference [20].

DNA samples were quantified and diluted to a final concentration of 1 ng/ μ l, and control DNA of a fertile male diluted to final concentrations of 10, 1 and 0.1 ng/ μ l. PCR were performed in 10 μ l volume in capillaries containing 1 μ l mix Faststart DNA Master SYBR GreenTM, MgCl₂ 4 mM, primers 0.4 nM each and 2 μ l of genomic DNA. Water control was systematically included to rule out possible contamination. After initial denaturation and *Taq* activation at 95°C for 8 min, samples were subjected to PCR amplification using 40 cycles of 95°C for 5 s, 58°C for 5 s (sY255 and sY1064) or 60°C for 8 s (sY586), and 72°C for 5 s (sY255 and sY1064) or 12 s (sY586). The melting curve consists of a progressive denaturation of PCR products (+0.05°/s) from 63°C (sY255 and sY1064) or 65°C (sY586) to 95°C, and a final cooling of capillaries at 37°C for 15 s.

Several technical parameters are essential to ensure the accuracy of the dosage: the calibration curve slope calculated from reference DNA samples (20, 2 and 0.2 ng) must be close to -3.32, corresponding to a 100% PCR efficacy, and error rate must be less than 10%. The melting curve analysis makes

it possible to ensure the specificity of the PCR products. An example of the Q-PCR results is presented in Fig. 2.

Values were assigned to each reference DNA sample (fertile male), according to the copy number of the marker used (7 for sY255, 4 for sY586 and 1 for sY1064) and the DNA quantity of the reference samples (ranging from 20 to 0.2 ng). Using these data, the quantity of target DNA in each sample was calculated, depending on the threshold cycle (Ct). The ratios sY255/sY1064 and sY586/sY1064 represent the number of sY255 and sY586 copies in each DNA sample tested. Gene dosage using Q-PCR was repeated at least three times for each DNA sample. Average dosage and average Ct values for each patient and for each STS were calculated. Table 1 shows results for reference DNA (fertile man). The cut-off value was determined following the ratio average for all the patients tested plus or minus standard deviation $6.47 \pm 1.38 = [5.09-7.85]$ for sY255 and $3.42 \pm 1.05 = [2.37-4.47]$ for sY586). Patients with dosage values in these ranges were considered as having 4 copies of *DAZ* genes.

Results

STS plus/minus PCR analysis

As expected, female and AZFb-del control was negative for STS sY1192, STS sY1197 and STS sY1291 whereas AZFc-del control was positive for STS sY1197 and negative for STS sY1192 and STS sY1291. The patient with 47,XXY and the patient with *t*(X;Y) were positive for all STS tested. A negative signal for STS sY1192 was observed in the 2 patients with “b2-b3” deletion and in 1 patient with a deletion followed by duplication from the Machev study. A negative signal for STS sY1291 was observed in the patient with “gr-gr” deletion and in the other patient with a deletion followed by a duplication (Table 2).

For the fertile population, 30 were positive for STS sY1197 whereas one was negative for STS sY1192 (F6) and another was negative for STS sY1291 (F29), corresponding to 1 “b2-b3” deletion (1/30; 3.3%) and 1 “gr-gr” deletion (1/30; 3.3%). No “b1-b3” deletion was observed in this study (Table 2).

Q-PCR results

One DNA sample, from a fertile male, was used as a reference DNA. Average dosage results were 4.14 ± 0.27 for STS sY586 (sY586/sY1064) and 7.24 ± 0.47 for STS sY255 (sY255/sY1064). Similar results were obtained for 6/14 controls used: 3 patients without rearrangements (M1, M2, M7), 2 with deletion followed by duplication (M6, M8) and the 47, XYY patient. For the last case, average dosage ratios were in

Fig. 2 Example of Q-PCR results. (a) 1- Melting curve obtained after sY255 and sY1064 PCR: the first peak (78.01°C) corresponds to sY1064 and the second peak (85.25°C) to sY255 PCR reaction. 2-melting curve after sY586 PCR with a peak (79.12°C) corresponding to sY586 PCR reaction. (b) Curve of fluorescence versus cycle number obtained for the reference range and 1: 12 samples tested with sY255, 2: 12 samples tested with sY1064 and 3: 10 samples tested with sY586. Threshold cycle (C_t) and curve for each sample are always included

the expected range (3.98 for sY586 and 6.95 for sY255), nevertheless C_t values were lower than reference values (fertile male) presented in Table I (26.99 versus 30.69 for sY1064, 20.66 versus 24.18 for sY255 and 24.61 versus

between the extreme curves of the reference range (20 and 0.2 ng). The samples curves are close to the 2 ng reference range. This parameter proves the quality of sample dosage and dilution. (c) The calibration curve (cycle number versus log concentration) obtained with the different dilutions of the reference sample (20 to 0.2 ng): 1: sY255 efficiency (slope) is -3.292 and error rate is 3.17%, 2: sY1064 efficiency = -3.393 and error rate = 3.34%, 3: sY586 efficiency = -3.783 and error rate = 2.78%

27.40 for sY586). Decreased average dosages were obtained for 4/14 controls: AZFb-del, 2 “b2-b3” del (M3, M5) and “gr-gr” del control (M4). Average dosages were increased for the $t(X;Y)(qter \rightarrow p22::q11 \rightarrow qter)$ duplication control

Table 1 Specific spectrofluorimetric values obtained for each STS tested in real time PCR with DNA of reference

STS	Tm (mean $\pm$ SD)	Tm (min-max)	Ct (mean $\pm$ SD)	Ct (min-max)
sY1064	77.82 $\pm$ 0.21	77.61–78.03	30.69 $\pm$ 0.56	30.13–31.25
sY586	79.19 $\pm$ 0.30	78.89–79.49	27.4 $\pm$ 0.74	26.66–28.14
sY255	85.17 $\pm$ 0.25	84.92–85.42	24.18 $\pm$ 0.37	23.81–24.55

Note: Ct: threshold cycle, Tm: melting temperature and SD: standard deviation.

(Fig. 3, Table 2). No amplification was observed for negative control AZFc-del and the female sample (data not shown).

2 fertile males (F6 and F29) out of the 30 tested presented decreased average dosages (6.6%) (Fig. 3, Table 2).

Discussion

Choice of the Q-PCR technique

Several techniques have been published allowing the detection of AZFc rearrangements primarily based on the number of *DAZ* gene copies. Polymorphisms (SFVs) specific to the different copies of the *DAZ* gene in the AZFc region were initially described by Saxena *et al.* (2000) when reporting the map of this region of the Y chromosome. These SNVs were proposed by de Vries *et al.* and Fernandes *et al.* to detect partial AZFc deletions. The latter reported new polymorphisms of this particular region (DAZ-SNVs). Interpreting results raised some problems, showing i) a high frequency of restriction profile anomalies similar in populations of men with or without spermatogenesis disorders [14, 16, 17], and ii) notably a high frequency of *DAZ2* polymorphism loss. Results obtained with the PCR assay method were often discordant with those obtained by other techniques; frequent gene conversion events rather than deletions may explain

these variabilities [21, 22]. The limits of this technique have thus been demonstrated, although there may still be some qualitative interest [11]. Moreover, some of these SNVs (sY581 for example) proved to be useful polymorphisms for population genetic studies [23, 24].

Accordingly, another technique must be used in order to detect partial deletions. De Vries *et al.* (2002a,b) used fiber-FISH to visualize the loss of one *DAZ* gene cluster: FISH is time consuming compared to Q-PCR and is not applicable if only DNA is available. Fernandes *et al.* (2002) used Southern blotting to complement SNV analysis; this technique is difficult to set up, requires a lot of time and an important quantity of genetic material and relies once more upon restriction polymorphism. More recently, the loss of specific AZFc STS was described, permitting the rapid and simple detection of AZFc partial deletion [3, 5]. Several studies using this technique had previously been published [12, 25–28]. Nevertheless, duplication or deletion followed by duplication, as already described [5], cannot be detected by this technique used alone. Only a quantitative technique could allow the detection of duplication [5, 11, 13, 15].

We decided to use real time PCR to quantify the number of *DAZ* genes. Q PCR is now widely used in diagnostic procedures in many molecular biology and genetics labs. Real time PCR is easy to adjust, highly reproducible, and can be used on large samples. It presents several other

Table 2 Summary of the results obtained from Q-PCR and STS analysis for the controls and the 2 fertile patients with identified partial deletions

Patient	Average dosage ratio sY586	Average dosage ratio sY255	STS 1291	STS 1192	STS 1197	Type rearrangement
fertile	4.14	7.24	+	+	+	—
AZFb-del	1.76	3.57	—	—	—	AZFb-del
47,XYY	3.98	6.95	+	+	+	—
T(X;Y)	7.86	11.80	+	+	+	t(X;Y)(qter->p22::q11->qter)
M1	3.74	7.04	+	+	+	—
M2	3.08	6.88	+	+	+	—
M3	1.62	4.62	+	—	+	b2-b3 del
M4	1.58	4.49	—	+	+	gr-gr del
M5	1.82	4.97	+	—	+	b2-b3 del
M6	3.63	7.28	+	—	+	b2-b3 del followed by duplication
M7	3.5	7.51	+	+	+	—
M8	3.63	6.91	—	+	+	gr-gr del followed by duplication
F6	1.63	3.41	+	—	+	b2-b3 del
F29	1.13	2.88	—	+	+	gr-gr del

Note: Q-PCR results consist of the average dosage ratios for each marker tested. Results obtained with STS sY1291, sY1192 and sY1197 are indicated (+ or - for presence or absence of this marker).

Fig. 3 Q-PCR results: dosage ratios obtained for all the patients tested (plot sY586 versus sY255). Most patients are close to normal values ($N=4$ for sY586 and $N=7$ for sY255). Surrounded points are the control sample presenting a complete AZFb deletion and the $t(X;Y)$ male

advantages compared to conventional methods. The technique is quick, does not require post PCR handling, which may cause contaminations, and uses only a very small amount of genetic material. Taking all these conditions into account, Q-PCR appears to be more profitable at a lower cost. Two specific markers were chosen in order to accurately dose the copy number of *DAZ*, according to the data available on the composition of *DAZ* genes: sY586 and sY255 (Fig. 1) [1].

Validation of Q-PCR

Our technique was validated by the results obtained for the controls used: the 3 patients without AZFc rearrangements (M1, M2, M7) were correctly identified: normal STS amplification and normal *DAZ* gene dosage. For the AZFb-del patient, dosage values for sY586 and sY255 were lower (1.76 and 3.57 respectively, instead of 4 and 7). These data were consistent with the STS PCR results. Similar results were obtained for “b2-b3” (M3, M5) and “gr-gr” deletion control (M4), with no STS sY1192 and STS sY1291 amplification respectively and decreased *DAZ* gene dosage. For the $t(X;Y)$ male, the translocation breakpoint was located between the AZFa and AZFb regions. This patient had 2 AZFc loci with twice as many *DAZ* gene copies. Due to the fact that the reference marker sY1064 was located in the AZFa region, dosage values were increased for both sY255 and sY586. For the 47,XXY male, dosage ratios were in the normal range with Ct values lower than the reference, as expected (Table 2). It is important to note that using the Y-chromosome reference makes it possible to avoid dosage interpretation problems relative to Y chromosome loss mosaicism, which is a common finding in infertile male population [29]. For the 2 patients with deletion followed by duplication (M6, M8), we obtained normal dosage using Q-PCR but with a negative signal in PCR plus/minus STS (Table 2). These results indi-

cate that PCR STS analysis used alone is not enough to detect deletion followed by duplication or duplication. A quantitative technique such as Q-PCR is needed to identify them.

Identification and characterization of partial AZFc deletions in the fertile population

Partial deletions were identified in 2/30 [6.6%] fertile males tested; one with a “gr-gr” deletion (F29) (3.3%) and one with a “b2-b3” deletion (F6) (3.3%). Results with Q-PCR showed a marked decrease dosage in only these 2 patients (Fig. 3 and Table 2). However, Q-PCR did not enable us to understand the molecular mechanisms leading to the deletion nor to determine which copies of *DAZ* genes were removed. Moreover, the number of *DAZ* genes could be polymorph in human populations. Therefore, the use of both techniques proved to be useful.

The effect of AZFc microrearrangements on male fertility, in particular partial deletions, is still under discussion. Indeed, partial deletions have mainly been detected in infertile men, but several papers indicated that they were also found in fertile control men [5, 10–12, 26–28]. Moreover, differences have been made between the incidence of “gr-gr” deletion and “b2-b3” deletion. Several studies have showed that only “gr-gr” deletion represents a risk factor for male infertility (25–28). Indeed, “gr-gr” deletion frequency was significantly higher in the infertile group than in the control. Studies have presented “b2-b3” deletion as a polymorphism not associated with a spermatogenesis failure [10]. Only 1 “gr-gr” deletion was found in our fertile population [3.3%], and this finding is in agreement with recent studies.

Further studies on larger fertile and infertile populations could easily be performed using Q-PCR and could allow a better understanding of the relation between AZFc rearrangements and male infertility and the potential function of *DAZ* and other genes of the AZFc region. Moreover, Q-PCR is equally adapted to the detection of duplications of the AZFc region as shown by controls $t(X;Y)$. These complementary methods offer promising future prospects concerning “b2-b4” duplications, for example, or deletions followed by duplication.

Conclusion

We report the validation of Q-PCR in the detection of microrearrangements of the AZFc region, useful in addition to STS and/or SNV analysis. This simple and rapid screening technique enabled us to identify 2 partial deletions, including 1 “gr-gr” and 1 “b2-b3” deletion. Moreover, Q-PCR allows the identification of duplication events. As already suggested, these rearrangements appear to be quite frequent, and involve multiple recombination mechanisms within the palindromes

identified in this Y chromosome region. These mechanisms and partial deletion boundaries are becoming better characterized; they appear heterogeneous as compared to those identified in complete AZFc deletions. Further studies in this field on larger populations should enable us to specify the different mechanisms and to better define the nature of genotype-phenotype correlations.

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