Quadruplex real-time polymerase chain reaction assay for molecular diagnosis of Y-chromosomal microdeletions

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Objective: To develop a rapid and reliable method for molecular diagnosis of Y-chromosomal microdeletions.
Design: Study of diagnostic accuracy.
Setting: Molecular diagnostics laboratories in three hospitals.
Patient(s): A total of 701 men with nonobstructive azoospermia or oligozoospermia from three hospitals.
Intervention(s): We developed a quadruplex real-time polymerase chain reaction (PCR) assay and evaluated its performance in molecular diagnosis of Y-chromosomal microdeletions.
Main Outcome Measure(s): Analytic sensitivity, analytic specificity, clinical sensitivity, and clinical specificity.
Result(s): The limit of detection of quadruplex real-time PCR assay was 100 pg genomic DNA. The method attained 100% analytic specificity, 100% clinical sensitivity, and 100% clinical specificity.
Conclusion(s): We have successfully upgraded the diagnostic method published by the European Academy of Andrology and the European Molecular Genetics Quality Network. Our method was validated to be fast, simple, contamination free, of high analytic sensitivity and specificity. Therefore, it is strongly suggested that such quadruplex real-time PCR assay can be readily applied as clinical routine in the near future. (Fertil Steril® 2012;97:864–9. ©2012 by American Society for Reproductive Medicine.)
Key Words: Y-Chromosomal microdeletions, real-time PCR, EAA/EMQN guidelines, HANDS, azoospermia, oligozoospermia

Y-Chromosomal microdeletions (YCMD) are the second most frequent genetic cause of spermatogenetic failure in infertile men after Klinefelter syndrome (1). Nowadays, the molecular mechanism resulting in such deletions had been clarified that it is due to the homologous recombination between identical repeated sequences in the male-specific region of the Y chromosome (MSY) which was classically subdivided into three regions: *AZF*a, *AZF*b and *AZF*c, respectively (2, 3). Deletions in the entire *AZF*a region invariably result in complete Sertoli cell–only (SCO) syndrome and azoospermia (4, 5), which implies the virtual impossibility to retrieve testicular sperm for intracytoplasmic sperm injection (ICSI). Similarly, complete deletions of *AZF*b and *AZF*b+c result in azoospermia such that no sperm can be obtained by testicular sperm extraction (TESE) (5, 6). *AZF*c deletions can be found in men with azoospermia or severe oligozoospermia and, rarely, can even be transmitted naturally to the male offspring (7–9), which means that such deletions are compatible with residue spermatogenesis. In general, TESE and ICSI should not be recommended to those patients who carried microdeletions covering the entire *AZF*a or *AZF*b regions. In contrast, in men with azoospermia and *AZF*c deletion, there is a fairly good chance of retrieving sperm from TESE and that children can be conceived by ICSI (7, 8, 10, 11).

Diagnosis of YCMD may clarify the cause of nonobstructive azoospermia or oligozoospermia, may have prognostic value, and may influence therapeutic options (12–14). Because there are no clinical parameters beyond azoospermia or severe oligozoospermia that can be

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used to predict its occurrence, YCMD only can be diagnosed by molecular methods. Currently, molecular testing of such deletions is mainly based on polymerase chain reaction (PCR) amplification of selected sequence-tagged sites (STSs) within specific *AZF* regions. Considering that different diagnostic protocols might result in inaccurate or wrong diagnoses, guidelines were published by the European Academy of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN) to provide standardization for molecular diagnosis of such deletions, and it is well recognized so far (15). The method in those guidelines performed two quintuplex PCR reactions to detect six STS regions: sY84 and sY86 for *AZF*a, sY127 and sY134 for *AZF*b, and sY254 and sY255 for *AZF*c. *SRY* and *ZFX/Y* regions are also involved as internal controls. The primer sets in the guidelines, enabling the detection of almost all of the clinically relevant deletions and >95% of the deletions reported in the literature in the three *AZF* regions (15), thus meet the demand of routine diagnosis. However, some technical issues remain to be solved in the method proposed by the guidelines. First, multiplex primer pairs in one reaction facilitate the formation and accumulation of nonspecific PCR products, which would lower the analytic sensitivity and specificity. Moreover, relative long products amplification followed by electrophoresis makes the method time consuming and labor intensive. Finally, and the most important, the use of electrophoresis for product analysis makes the method vulnerable to carryover contamination which can be fatal to diagnosis.

To address these problems, we developed a quadruplex real-time PCR assay for molecular diagnosis of YCMD based on the EAA/EMQN guidelines. In this assay, the real-time PCR platform allows detection of amplification through fluorescence intensity accumulation in a closed-tube setting, thus eliminating the carryover contaminations and reducing the time consumption and labor intensity. For alleviating primer dimer formation in the quadruplex reaction, a homotag-assisted nondimer system (HANDS) (16) was introduced in the assay.

MATERIALS AND METHODS

Samples

Peripheral blood samples from 701 men with nonobstructive azoospermia or oligozoospermia were obtained from Xiamen Maternal and Child Health Hospital (n = 451), Fuzhou General Hospital (n = 72), and Fujian Provincial Maternal and Child Health Hospital (n = 178). The Research Ethics Committees of the three hospitals approved the study protocols. All samples were coded, and the code numbers were known only to the technician who collected and coded the samples. Genomic DNA were extracted from 200-μL blood samples with the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. The concentration of extracted genomic DNA was determined by measuring the UV absorbance at 260 nm with the Nanovue plus spectrophotometer (GE Healthcare).

Primers and Probes

According to the EAA/EMQN guidelines, six STS regions, sY84, sY86, sY127, sY134, sY254 and sY255, were chosen as targets. *SRY* and *ZFX/Y* regions were also involved as

TABLE 1

Information of primers and probes.

Name	Sequence (5′ to 3′)	Concentration (nmol/L)
Reaction A		
SRY-F	GCAAGCCCTCACGTAGCGAATAGAGAATCCCAGAATGCGAAA	200
SRY-R	GCAAGCCCTCACGTAGCGAACTGTGCCTCCTGGAAGAAT	200
SRY-P	FAM-AAGCAGCTGGGATACCAGTGGAAAATGCT-BHQ1	200
sY86-F	GCAAGCCCTCACGTAGCGAACTCACAGTCCTTGAGGCTA	40
sY86-R	GCAAGCCCTCACGTAGCGAAAAGACAGCATCTACAACCCA	40
sY86-P	HEX-ATCAAGCTATGGCCAGGGCTGGTTCC-BHQ1	200
sY127-F	GCAAGCCCTCACGTAGCGAAGGCTCACAAACGAAAAGAAA	400
sY127-R	GCAAGCCCTCACGTAGCGAACTTTTGTATAATTAGCATCTCATGAA	400
sY127-P	ROX-ACTGGAATCTACCAAAGCCCACTGTGTTCATG-BHQ2	200
sY254-F	GCAAGCCCTCACGTAGCGAAGGGTGTTACCAGAAGGCAA	40
sY254-R	GCAAGCCCTCACGTAGCGAAGTACGAATACAATACCCTAGCA	40
sY254-P	CY5-TCGTGCCAAACACTGTTTTTGTTGGTGGAA-BHQ2	200
Reaction B		
ZFX/Y-F	GCAAGCCCTCACGTAGCGAACCACCTGGAGAGCCACAA	200
ZFX/Y-R	GCAAGCCCTCACGTAGCGAAACAAAGCCCCTGCATGAGA	200
ZFX/Y-P	FAM-ACCAGCAAGGCAGAGAAGGCCATTGA-BHQ1	200
sY84-F	GCAAGCCCTCACGTAGCGAAGATTCAGTGGGACCCTTTCTT	200
sY84-R	GCAAGCCCTCACGTAGCGAAGGAGGCTTCATCAGCAAGA	200
sY84-P	HEX-AAGCTGGCTAACTCCTTTCAAAAGGTTTTGTCTT-BHQ1	200
sY134-F	GCAAGCCCTCACGTAGCGAAGAGGAATAGTACAGGTCAAAGGAA	200
sY134-R	GCAAGCCCTCACGTAGCGAATCTTTCAGTCACAGAACGCTT	200
sY134-P	ROX-ATAGATGGGGTTGATACTAAAGTTTAAAACATCTGGAACATTCTACT-BHQ2	200
sY255-F	GCAAGCCCTCACGTAGCGAAGTTACAGGATTCGGCGTGAT	40
sY255-R	GCAAGCCCTCACGTAGCGAACTCGTCATGTGCAGCCAC	40
sY255-P	CY5-AGGTAGGTTTCAGTGTTTGGATTCCGCA-BHQ2	200
Universal primer	GCAAGCCCTCACGTAGCGAA	1,600

Guo. Diagnosis of Y-chromosomal microdeletions. Fertil Steril 2012.