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SHORT COMMUNICATION

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A New Approach for Screening for Y Microdeletions: Capillary Electrophoresis Combined with Fluorescent Multiplex PCR

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***Purpose*:** To apply capillary electrophoresis for rapid screening for Y microdeletions.
***Methods*:** A set of 25 specific sequence tagged sites that cover the azoospermia factor a, b, and c regions of the Y chromosome was amplified in 5 fluorescent multiplex sets each including 5 primer pairs. One of the primers of each pair was labeled with a fluorescent tag attached to the 5′-end. After PCR amplification, analysis of the obtained PCR products was performed using capillary electrophoresis (ABI Prism 3100 Genetic Analyzer). The method was employed to determine Y microdeletions in azoospermic ($n = 49$) and severe oligozoospermic ($n = 149$) men.
***Results*:** The number of PCR cycle (from 45 to 30) and the amount of DNA template (20-fold) used in fluorescent multiplex PCR were reduced because of the high sensitivity of capillary electrophoresis. Approximately 1000 multiplex PCR sets from 198 patients were analyzed simultaneously within 50 h. Y microdeletions were found in 3 out of the 198 azoospermic or severe oligozoospermic men.
***Conclusions*:** Application of capillary electrophoresis for detection of PCR products provides a semiautomated, high throughput method for rapid screening for microdeletions on the Y chromosome.

KEY WORDS: Capillary electrophoresis; male infertility; multiplex PCR; oligo-azoospermia; Y-chromosome microdeletions.

INTRODUCTION

Y chromosomal microdeletions in the azoospermia factor region (AZF) have been implicated as one of the major causes of male infertility (1,2). The availability of intracytoplasmic sperm injection (ICSI) in the treatment of male infertility implies a risk of transmission of Y microdeletions from father to son (3–5). The frequency of Y microdeletions has been reported to range from 0 to 58% in infertile men (3–12). Therefore, screening for Y microdeletions with subsequent genetic counseling is recommended in many IVF centers before ICSI.

The development of a PCR-based sequence tagged sites (STSs) mapping strategy allows the rapid screening of a large number of infertile patients for Y microdeletions (6,13). However, PCR using a single STS primer pair is not practical especially when the number of STSs to be analyzed is increased. Thus, multiplex PCR has been suggested to be the choice for screening for microdeletions on the Y chromosome in infertile men. In previously developed PCR assays, amplified DNA fragments were separated by traditional agarose gel electrophoresis and visualized with ethidium bromide under UV illumination. However, this approach is labor-intensive with low sensitivity and resolution. An alternative way for analyzing PCR products is available through capillary electrophoresis (CE). In this paper, we have introduced CE (ABI Prism 3100 Genetic Analyzer, Applied Biosystems, United States of America) for the separation and detection of PCR products for rapid screening for Y microdeletions.

MATERIALS AND METHODS

Patients

Genomic DNA was extracted as described previously (14). Azoospermic ($n = 49$) and severe oligozoospermic ($n = 149$) men, who were investigated previously by a multiplex PCR method using traditional agarose gel electrophoresis for detection of PCR products (12), were reanalyzed by fluorescent multiplex PCR combined with CE. All participants gave written consent according to the study protocol, which was approved by the Regional Ethics Committee.

Fluorescent Multiplex PCR Amplification of STSs

Our previously described multiplex PCR protocol (14) was modified. Twenty-five nanograms of genomic DNA was added to a mixture of PCR buffer containing 1.5 mM $MgCl_2$ (Qiagen, Germany), 0.2 mM of each dNTP (Boehringer Mannheim, Germany), 2.5 IU of HotStarTaq DNA polymerase (Qiagen), different concentrations of the each primer (Table I) and adjusted with redistilled H_2O to a final volume of 25 μL. One of the primers of each pair was labeled

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Table I. STSs Employed for Screening for Y Microdeletions

Multiplex set	STSs	Labeling dye	Target size (bp)	Primer concent. (pmol)[a]
I	sY14	FAM	472	3.4
	sY132	FAM	143	3.4
	sY84	HEX	326	3.2
	sY152	HEX	125	2.4
	sY272	FAM	93	2.4
II	sY269	FAM	94	2.0
	sY139	HEX	120	1.3
	sY153	FAM	139	1.2
	sY155	FAM	349	1.8
	sY138	HEX	170	1.9
III	sY160	FAM	236	1.2
	sY143	FAM	311	1.4
	sY144	HEX	143	0.7
	sY255	FAM	126	0.8
	sY254	HEX	350	1.3
IV	sY243	HEX	118	2.1
	SPGY	FAM	400	2.6
	RBM1	HEX	800	2.8
	sY273	FAM	93	3.9
	sY164	HEX	590	4.3
V	sY117	HEX	262	1.1
	sY166	FAM	115	1.3
	sY150	HEX	158	1.4
	sY277	FAM	310	1.8
	sY158	FAM	231	1.3

Note. Nonstandard abbreviations used: HEX, 4, 7, 2′, 4′, 5′, 7′-Hexachloro-6-carboxyfluorescein; FAM, carboxyfluorescin; bp, base pair.
[a] Amount of the primer added to PCR.

with a fluorescent tag either FAM (carboxyfluorescin) or HEX (Hexachloro-6-carboxyfluorescein) to the 5′-end. The concentration of primers, PCR product size, and labeled dye for each STS are listed in Table I. Amplifications were carried out on a Thermocycler (Gene Amp 9700, Applied Biosystems) with the following program for the each of the multiplex set; initial denaturation at 95°C for 15 min followed by 30 of cycles at 95°C for 30 s (denaturation), 60°C for 30 s (annealing), 72°C for 2 min (extension). A final extension was carried out at 72°C for 7 min.

The Use of CE

The ABI Prism 3100 Genetic analyzer (Applied Biosystems) was used for CE. Samples were prepared by mixing 2 μL of PCR product with 10 μL formamide and 1 μL internal size standard, ROX-500 (Applied Biosystems), where after the samples were denaturated for 5 min 96°C, snap-cooled and injected at 15 kV for 5 s into a 36-cm capillary containing POP-4 polymer (Applied Biosystems). Electrophoresis was performed at 15 kV for 35 min at 60°C. Gene Scan Analyzer Buffer containing EDTA was used as running buffer. All fluorescent dyes were detected by filter set D. Data represented as peak heights were analyzed in the ABI Genescan analysis software (Applied Biosystems).

Internal quality control for the diagnosis of Y microdeletions was performed as described previously (14).

RESULTS

Our previously published multiplex PCR protocol was optimized for fluorescent detection of PCR products by CE. Because of the high sensitivity of CE, we decided initially to reduce the number of PCR cycles and tested 25, 30, 35, 40, and 45 cycles for the PCR amplification. The optimum results with strong signals and low background were obtained with 30 cycles, and this amplification was used in further experiments. Accordingly, primer concentrations used in the each multiplex assay were reduced and adjusted to generate PCR products of measurable intensity following 30 PCR cycles. The new primer concentrations used in each multiplex set are shown in Table I. The use of CE as detection method further made it possible to reduce the amount of DNA template used for PCR. In the new approach we used 20-fold lower DNA template concentration compared to the previous assay (14).

Additionally, in this study, we used the HotStarTaq DNA polymerase. This polymerase can be stored in the preprepared, ready-to-use mastermix at −20°C, which is practical in a routine setup. Mastermix, up to 1-year-old, containing HotStarTaq DNA polymerase was tested and found to be stable.

Screening 198 men having severe oligozoospermia or azoospermia for Y microdeletions with the new protocol, we identified the same three men with Y microdeletions previously found by a multiplex PCR method using traditional agarose gel electrophoresis for detection of PCR products. In Fig. 1, the results of screening for Y microdeletion by CE in two oligozoospermic men one without (Fig. 1(A)) and one with a Y microdeletion (Fig. 1(B)) are shown.

DISCUSSION

In this study, we modified and extended our previous methodology by introducing for the first time an automated CE for screening for Y microdeletions.

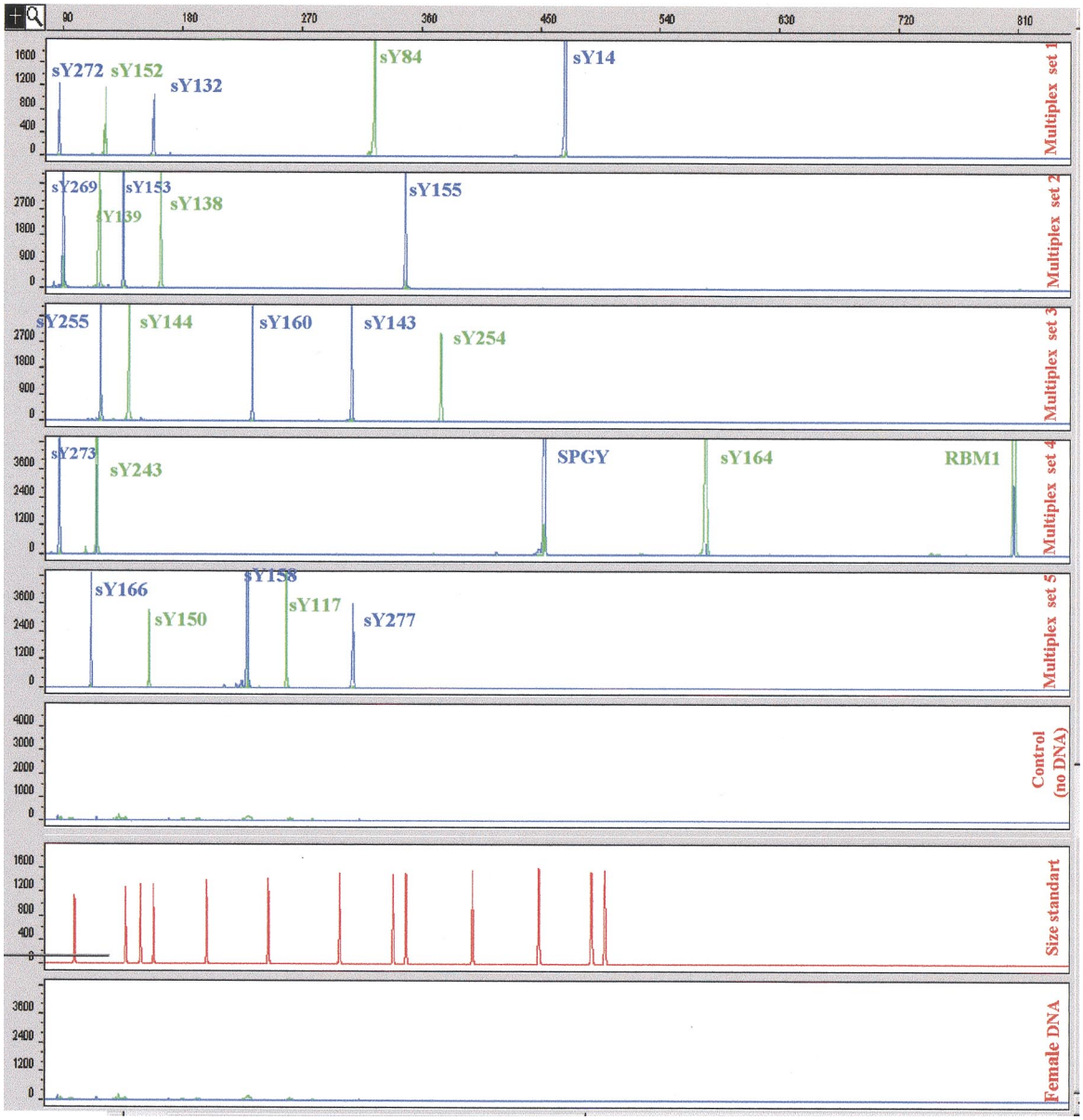

Fig. 1. (A) Electropherograms of five fluorescent multiplex PCR sets in an oligozoospermic man having no Y microdeletions. Twenty-five peaks representing 25 amplified DNA fragments from AZF region by the fluorescent multiplex PCR were observed (first five electropherograms). The PCR products labeled with FAM and HEX dye are represented as green and blue colored peaks respectively. Gene Scan-500 standard labeled with ROX (represented as red colored peaks) was used as a size marker. Female genomic DNA, which controls for specificity and for contamination, was used as a negative control. To control for reagent contamination, a sample with PCR reaction mixture without DNA added was used. The target size is displayed at the horizontal axis at the top of the electropherograms and the fluorescent intensity is displayed along the vertical axis. (B) Electropherograms of five fluorescent multiplex PCR sets in an oligozoospermic man having Y microdeletions. Deletion of 11 out of 25 amplified DNA fragments from AZF region in the five fluorescent multiplex PCR sets were found (represented by asterisk (*)). Deletions are in the multiplex set I; sY152, in the multiplex set II; sY269, sY153, sY155, in the multiplex set III; sY255, sY254, in the multiplex set IV; sY243, SPGY, in the multiplex set V; sY150, sY277, sY158.

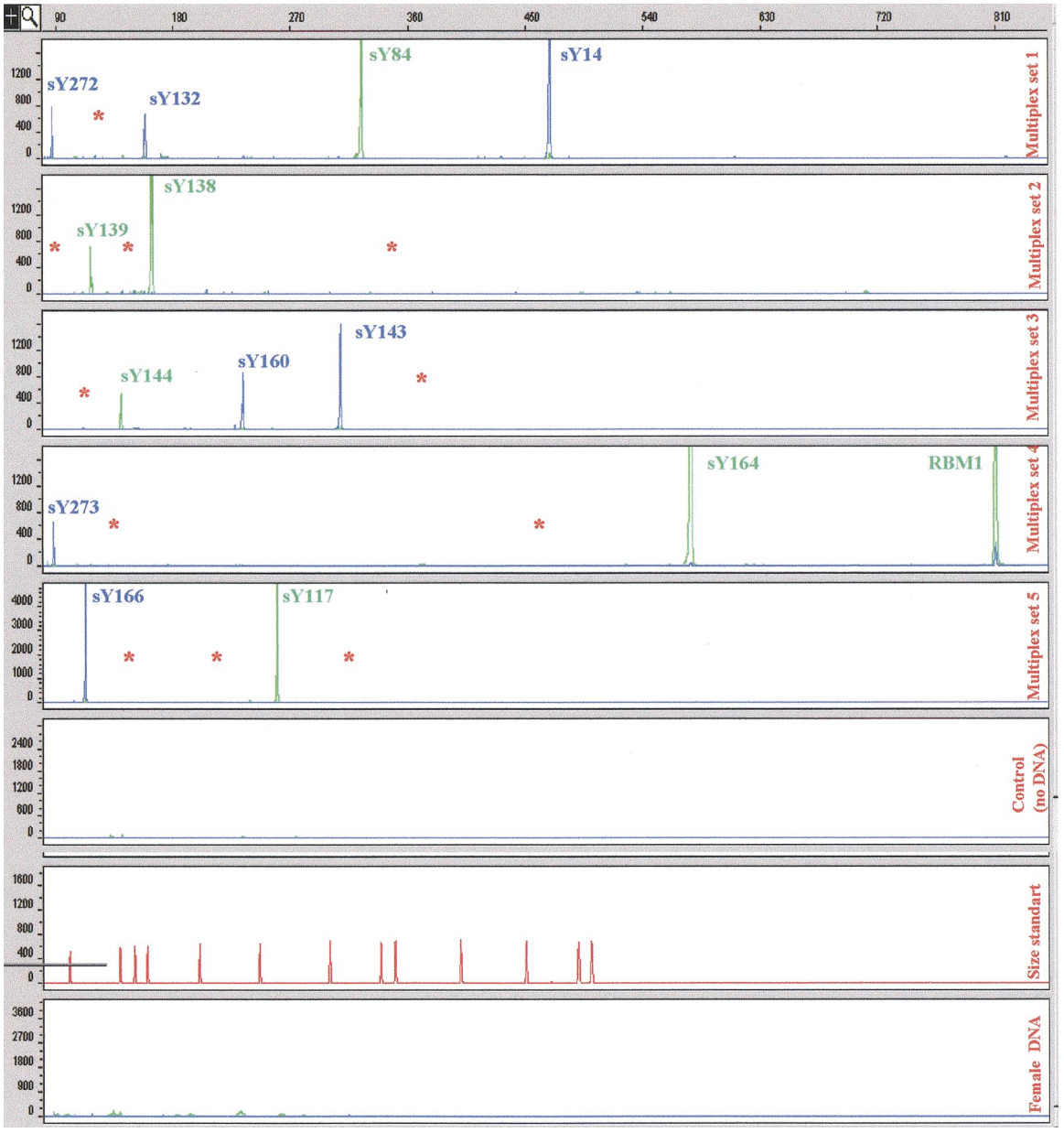

Fig. 1. (B) (*Continued*).

The major advantages of the CE for screeening for Y microdeletions are that minute amounts of DNA is required, high throughput, increased sensitivity of the analysis, automated electrophoresis, high resolution of DNA separation, and reduced risk of contamination due to minimal sample handling.

This method is based on separation of DNA molecules by size and dye color, which makes it possible to distinguish between multiple DNA fragments electrophoresed in a single capillary. With the CE, there is the potential to label primers with three different colored fluorochromes (TET, HEX, and FAM) and reserve the fourth color (ROX) for a size marker. This gives the CE added flexibility, since different but comigrating DNA fragments can still be distinguished on the basis of color. Thus, this technique is particularly suitable to the multiplex PCR approach used for detection of Y microdeletions. In the literature the number of STSs used for the determination of the integrity of Y chromosome have varied

from 1 to 131 (3–12). In our assay, we used 25 STSs that were amplified with 25 primers labeled with two fluorescent tags (FAM or HEX) producing PCR products ranging from 93 to 800 bp. These PCR products were separated by ABI Prism 3100 Genetic Analyzer that can analyze 16 multiplex PCR sets (80 PCR products) simultaneously within 45 min by using 16 capillaries.

The most commonly used method for separation of the PCR products is gel electrophoresis. However, a major disadvantage of the gel electrophoresis is that its sensitivity is considerably lower than that of CE. In this study, we confirmed this by lowering the concentration of DNA template 20-fold, reducing the primer concentrations and the number of PCR amplification from 45 to 30 cycles in our multiplex PCR, when it was combined with CE. The latter is particularly important for the reliable PCR, since the reduced number of amplification minimizes the risk of the contamination in PCR. Another problem of using agarose gel electrophoresis combined with ethidium bromide as a method for PCR product detection is the lack of standardization of electrophoretic conditions. This problem can be circumvented by using CE. Since the internal size standard labeled with ROX dye and the unknown fragments are subjected to exactly the same electrophoretic conditions, problems like lane-to-lane or run-to-run variation can be eliminated. A further drawback of the agarose gel electrophoresis is that it is time-consuming. It requires considerable time to prepare the gel, prepare and load the samples, and further postseparation analysis to visualize products. For example, in our previous multiplex PCR method combined with agarose gel electrophoresis (14) the total minimum working time was 3 h to obtain information from 14 samples. However, in CE all these steps are performed simultaneously reducing analysis time to 45 min for 16 samples. Even while samples are being analyzed, it is possible to review the results on the computer screen and reprogram for testing any results, which are unsatisfactory. Accordingly, CE allows rapid and semiautomated screening of a large number of samples.

In our previous multiplex protocol using agarose gel electrophoresis (14), primers were combined so that the amplified products differed in size from adjacent STSs by at least 10 bp, which was necessary for efficient separation. However, CE can separate DNA fragments with a difference of a single base pair, and potentially numerous PCR products can be analyzed in a single capillary.

A further important improvement in this study is the use of HotStarTaq DNA polymerase, which makes the multiplex approach more practical and suitable for routine screening since this polymerase can be included into a preprepared mastermix and stored at -20°C until used. Thus, working load and pipetting steps are minimized, which may reduce the possibility of errors and contamination.

A total of 198 men having severe oligozoospermia or azoospermia were investigated both by using previously described multiplex PCR protocol combined with agarose gel electrophoresis and by using the CE. Although both methods revealed that 3 of 198 men had a Y microdeletion, we have observed that the number of experimental repeats due to unsatisfactorily results and work load were dramatically minimized using CE.

In summary, we conclude that the fluorescent multiplex PCR approach using HotStarTaq DNA polymerase and combined with CE is a good candidate to be the gold standard method in the diagnosis of microdeletions on the Y chromosome.

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