Identification of X- and Y-chromosome-bearing human sperm separated by free-flow electrophoresis using Y-chromosome-specific polymerase chain reaction

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ABSTRACT

The effectiveness of separation of X- and Y-bearing sperm by free-flow electrophoresis was examined by use of the F-body test and polymerase chain reaction (PCR). For each ejaculate the ratio of X- and Y-bearing sperm was analyzed by determination of Y-chromosome-specific sequences (DYZ 1) before and after fractionation by free-flow electrophoresis. Fraction number 16, corresponding to the one peak near the cathode was more than 80% F-body positive, and the DYZ 1 in it was highly amplified; while fraction number 21, the peak near the anode, was F-body negative and also DYZ 1 negative. The data were consistent with our previous results; i.e., X-bearing sperm were more negatively charged than Y-bearing sperm.

Persistent efforts have been made to obtain male or female offspring by prenatal diagnosis and artificial insemination with X- or Y-bearing sperm. In human beings, over 200 X-linked recessive diseases, typically affecting only hemizygous males, have been identified. For the purpose of therapy, many scientists have developed methods of separating X- and Y-bearing sperm (4, 8).

Attempts on the separation of human sperm by means of galvanization have been made by various workers (5-7). The results obtained, however, were in disagreement even when investigators used the same method. The conflict occurred mainly because of the poor methods for detection of X- and Y-chromosomes in sperm. All of these workers used a fluorescence method, in which the F-body, corresponding to a repeating sequence in the long arm of the Y-chromosome, was stained with quina-craine mustard. However, the results obtained by this staining varied depending on the lot number of chemicals, samples, and observer's technique.

Recently, sex-chromosome-specific genes or probes have become available as a consequence of the remarkable developments in human gene map-

ping. A Y-chromosome-specific repeating DNA family, determination of Y-chromosome-specific sequences (DYZ 1), was first described by Cooke (2) as a 3.4 kb male-specific fragment in *Hae* III digests of genomic DNA. It is a major component of the heterochromatic region of the long arm of the Y chromosome. Nakagome *et al.* (9) selected useful segments with high male-specificity for polymerase chain reaction (PCR) and made new primers for amplification. In this paper, we applied the PCR to amplify this specific region of the Y-chromosome for evaluating the electrophoretically separated human sperm.

Human semen was collected from healthy men by masturbation after sexual abstinence for at least 4 days. Normospermic semen, which had a density of more than 5×10^7 sperm/ml and more than 80% motility, was used for the experiments.

In order to purify the sperm, the semen was processed by Percoll linear gradient centrifugation and the 'swim down' method previously described (5). The obtained sperm were washed twice with a buffer containing 0.226 M sucrose, 30 mM MgSO₄, 0.5 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES (N-2-

Table 1 *List of Primers Used for PCR*

Locus	Primer	Primer sequence	Location	Length (bp)
DYZ1 ^a				
A	Y1-3F	5'-TCGAGCCCTTTCAATTTGAGTCC-3'	247- 269	900
	Y1-6R	5'-AGAATGGACACGAATGCTCAAATT-3'	1146-1123	
B	Y1-6F	5'-AATTTGAGCATTTCGTGTCCATTCT-3'	1123-1146	1024
	Y1-4R	5'-AATGCCCTTGAATTAAATGGACT-3'	2146-2124	
C	Y1-4F	5'-AGTCCATTTAATTCAAGGGCATT-3'	2124-2146	1086
	Y1-5R	5'-TGTATTAGAATGTAATTGAACTTT-3'	3209-3187	
D	Y1-5F	5'-AAAGTTCATTACATTCTAATACA-3'	3187-3209	647
	Y1-3R	5'-GGACTCAAATTGAAAGGGCTCGA-3'	269- 247	
E	Y1-1F	5'-TCCACTTTATTCCAGGCCTGTCC-3'	3511-3533	323
	Y1-3R	5'-GGACTCAAATTGAAAGGGCTCGA-3'	269- 247	

^aNakagome *et al.* (9), Nakahori *et al.* (10)

hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-NaOH buffer (pH 7.2), and suspended in 0.5 ml of the buffer.

Separation of X- and Y-bearing human sperm was carried out in a free-flow electrophoretic apparatus (ACE 710, Hirschmann, Munich) according to the method previously described (5, 6).

Human Y-bearing sperm were detected by fluorescence staining with quinacrine mustard dihydrochloride (Sigma, St. Louis, MO) according to the method of Barlow and Vosa (1) with a slight modification (5).

DNA was extracted from the remaining sperm of each fraction and from the unseparated specimen. The sperm, approximately 5×10^4 cells, were incubated in 300 μ l of a lysis buffer for 90 min at 37°C. The buffer was contained 100 mM NaCl, 10 mM Tris (tris-hydroxymethylaminomethane)-HCl buffer (pH 8.0), 9 mM EDTA (ethylenediaminetetraacetic acid), 1.5% SDS (sodium dodecyl sulfate), 40 mM DTT (dithiothreitol), and 187 μ g proteinase K. Organic extraction was performed as follows: 300 μ l of water-saturated phenol/chloroform (1:1) was added to each polypropylene tube. Then, the mixture was vortexed 4 times for 20 s each time and centrifuged at 12,000 rpm for 10 min in a TOMY MC-15-A. The phenol extraction was repeated once and followed by extraction with Sevag solution (24:1, chloroform: isoamyl alcohol). After the organic extractions, the DNA in the aqueous layer was precipitated overnight at -20°C by the addition of 2 vol of 99.9% ethanol. The DNA

was recovered by centrifugation, resuspended in 70% ethanol for desalting, and then suspended in 60 μ l of water.

About 100 ng of the sperm DNA from each fraction obtained by free-flow electrophoresis was amplified enzymatically in a 100- μ l reaction mixture. The mixture was put in a capped 0.5 ml polypropylene tube containing a 1 μ M concentration of a given set of primers. The reaction mixture contained primers for DYZ 1 (see Table 1), 200 μ M deoxynucleotides (Pharmacia), 2 units of *Thermus aquaticus* DNA polymerase (*Taq* polymerase) (Perkin-Elmer Cetus), 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl buffer (pH 8.4), and 20 μ g/ml gelatin, and was overlaid with mineral oil. PCR was performed in a programmable thermal cycler PJ-1000 (Perkin-Elmer Cetus) for 25 cycles. Each cycle consisted of 1 min of denaturation at 94°C, 2 min of annealing at 60°C, and 2 min of polymerization at 72°C. At the end of the 25th cycle, the samples were held at 72°C for 7 min and then cooled down. Samples were then subjected to analysis by agarose gel electrophoresis either immediately or after standing overnight at -20°C.

Eighteen microliters of the PCR product was mixed with 2 μ l of a loading buffer containing 40% sucrose, 0.25% bromophenol blue and 0.25% xylene cyanol, and electrophoresed in a 1% agarose gel (SeaKem-LE) containing ethidium bromide using TBE buffer (45 mM Tris-borate and 1 mM EDTA) at 90 V for 2 h.

Five different kinds of primer-sets were used to

Fig. 1 Ethidium bromide-stained gels of control PCR experiments for five different segments in DYZ 1 locus (A-E, Cf. Table 1). See text for details. M represents molecular size markers consisting of 4,870, 2,016, 1,360, 1,107, 926, 658, 489, 267, 80 bp (pHY marker: Takara Shuzo, Kyoto, Japan).

Table 2 Characteristics of Sperm Fractionated by Free-Flow Electrophoresis

	Fr. 16 (near cathode)	Fr. 21 (near anode)
Number of sperm	4.2×10^6	3.5×10^6
F-body test (%)	81.4	1.6
PCR (cont.=1.0)	1.15	<0.008

amplify the corresponding segments in DYZ 1 (Table 1). Eight different female blood samples, 8 different male blood samples, and 8 different semen samples were used. Fig. 1 shows one of the typical results in which some segments (B, C, D) were highly male specific, whereas the others (A, E) were shared with female DNA. Multiple bands were observed in both the D and E reactions that were performed with male blood and sperm DNA, or female blood and male blood DNA, respectively. The results obtained for blood cell DNA were in agreement with previous results (9). Sperm DNA exhibited same profiles as male blood cell DNA. We used the DYZ 1B (1,024 bp) fragment primer-set for subsequent experiments because it was highly sensitive and highly specific for male blood cell DNA and sperm DNA, but did not react with female DNA.

Fractionated sperm were counted and smeared for F-body test, and the remainder was subjected to the extraction of DNA for PCR experiments.

Purified human sperm were separated into two peaks under the electric field as described previously (3, 5-7). Table 2 shows a typical result of the cell count, percentage of F-body positive sperm, and densitometric analysis of agarose gel after PCR for 5×10^4 cells (densitometric analysis) when sperm were electrophoretically separated into two peaks. Fraction 16, which was a peak near the cathode, was highly F-body positive, suggesting that this fraction was rich in Y-bearing sperm. On the other hand, fraction 21, which was the peak near the anode, was almost negative for F-body, suggesting the presence of few Y-bearing sperm in this fraction.

Fig. 2 shows the results obtained by PCR using DYZ 1B. Single band (1,024 bp) of DYZ 1 was condensed in fraction 16. Fraction 21, on the other hand, was almost negative for DYZ 1. Although the results obtained by PCR were not necessarily proportional to the amounts of template DNA because the control of amplification conditions is

Fig. 2 PCR experiments with unseparated (C) and electrophoretically separated human sperm (16, fraction number 16 near cathode; 21, fraction number 21 near anode) DNA. Each fraction contained DNA from 5×10^4 sperm. M represents the same molecular size markers as in Fig. 1.

difficult, our preliminary experiment for sperm DNA showed that the amount of PCR product was proportional to the amount of template DNA to a certain extent under some limited experimental conditions: Our template DNA was derived from the same number of sperm (5×10^4 cells), and the DNA loaded was less than 100 ng for 25 amplification cycles. The reproducibility of the PCR was confirmed in a calibration experiment from 50 ng to 1 μ g.

The present results are consistent with our previous results (5–7) but conflict with the result of Engelmann *et al.* (3); i.e., in our hands X-bearing sperm were more negatively charged than Y-bearing

sperm. The PCR technique proved useful to evaluate the existence of the target sequence in a small amount of DNA sample. This is the first paper showing the application of the PCR to confirm the separation of X- and Y-bearing sperm.

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