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Sex Identification in Fresh Blood and Dried Bloodstains by a Nonisotopic Deoxyribonucleic Acid (DNA) Analyzing Technique

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ABSTRACT: Deoxyribonucleic acid (DNA) specimens were prepared from blood or bloodstain extracts, and the content of a Y-chromosome specific DNA fragment was investigated by the Southern hybridization method using a nonisotopic staining technique. Thus obtained patterns of male DNA showed a clear band, whereas broad stains with some faint bands appeared on the patterns of DNA from both sexes. This method is expected to be a new powerful mean of forensic medical examination.

KEYWORDS: forensic science, deoxyribonucleic acid (DNA), blood, human identification

Sex identification is important in forensic medical examination. It has been mostly attempted by the investigation of leucocyte nuclear drumsticks [*1*], sex chromatins [*2-4*], or fluorescence of the Y-chromosome [*5*]. These morphological methods can determine the sexes only from relatively fresh specimens.

The Y-chromosome specific structure of deoxyribonucleic acid (DNA) has been studied by several researchers [*6-8*], and sex determination by the recombinant DNA technique has been performed with amniotic fluids, bloodstains, and other biological materials [*9-15*].

We report here a method of sex determination from blood or bloodstain specimens by analyzing the Southern hybridization pattern of DNA, which expectedly has a broad applicability because of the adoption of a nonisotopic technique.

Materials and Methods

Probes

Principal experiments were carried out using a plasmid pHY10R/pBR322 and a 3.564-kilobase (kb) pair EcoRI fragment (pHY10), both containing the complete nucleotide se-

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quence, which represents a major component of the human Y-chromosome specific repeated DNA family (DYZ1) [16]. For autoradiographic determination, the probes were radioactively labeled with ^{32}P-dATP (Amersham Japan Co., Tokyo) by the nick translation to a specific activity of approximately 5×10^8 cpm/μg before use. For the nonisotopic detection, the biotin labeled probes were prepared using biotin-11-dUTP (BRL, Maryland) by the nick translation and stored at $-20°C$ until use.

Isolation of DNA from Blood and Bloodstain Extract

Male and female blood specimens were obtained from Japanese adults by venipuncture and an anticoagulant was added. One volume of the blood was mixed with three volumes of a cold lysing solution (10m*M* potassium bicarbonate [$KHCO_3$], 0.155*M* ammonium chloride [NH_4Cl], 0.1m*M* ethylenediaminetetraacetate [EDTA], pH 7.3) and allowed to stand for 20 min at 4°C. After centrifugation (1600 $\times$ *g* for 10 min) at 4°C, the leucocytes were resuspended in 1/5 in volume of TEN buffer (150m*M* sodium chloride [NaCl], 10m*M* Tris-HCl pH 7.8, 1m*M* EDTA). The suspension was mixed with an equal volume of proteinase K solution (0.1 mg/mL of 0.4% sodium dodecyl sulfate (SDS) added TEN) and incubated for 15 min at 65°C and then overnight at 37°C. This mixture was consecutively extracted with phenol, phenol-chloroform (1 : 1), and chloroform. DNA was precipitated by the addition of ethanol and the precipitates grown were resuspended in TE buffer (10m*M* Tris-HCl pH 8.0, 1m*M* EDTA). This buffer was also used as the diluent of DNA. DNA concentration was determined by the absorbancy of 260 nm.

As for the preparation of bloodstains, cotton sheets were immersed with blood specimens and completely dried at room temperature. About 1 cm^2 of the sheets absorbed 50 μL of the blood. After one to two months from preparation, the bloodstains (about 1 cm^2) were cut into small pieces and extracted by adding 200 μL of the proteinase K solution and incubating for 15 min at 65°C and then overnight at 37°C. Thus provided extracts were treated as was the leucocyte fractions from blood specimens.

Southern Hybridization

Procedures were similar to those described by Maniatis et al. [17]. EcoRI (Takara Shuzo Co., Kyoto) was selected for the digestion of DNA specimens because of the prominent appearance of the 3.4-kb band. The digested DNA fragments (about 1.5 μg) were electrophoresed in a 0.75% agarose gel (108 by 60 by 5 mm) containing 0.5 μg/mL of ethidium bromide at the constant voltage of 100 V using Mupid-2 equipment (Advance Co., Tokyo). The electrophoresis gel plate included a sample of HindIII digested lambda phage DNA for molecular weight markers. After the completion of electrophoresis, the distribution of DNA on the gel plate was observed under ultraviolet light illumination. The DNA fragments were then transferred to a nylon membrane (Gene-Screen Plus, NEN/DuPont, MA) according to the manufacturer's instruction. The membrane was prehybridized for 5 h at 65°C in a hybridizing solution containing 6$\times$ SSPE (SSPE: 0.18*M* NaCl, 10m*M* NaH_2PO_4 pH 7.4, 1m*M* EDTA), 2$\times$ Denhardt's solution, 0.5% SDS, and 100 μg/mL of salmon sperm DNA (Sigma, MO). For the autoradiographic detection, hybridization was carried out by adding the radiolabeled probe (approximately 8×10^6 cpm/mL) and overnight incubation at 65°C. The membrane was twice washed with 2$\times$ SSPE containing 0.1% SDS for 15 min at 65°C, and twice in 0.2$\times$ SSPE containing 0.1% SDS for 15 min at the same temperature. The patterns of hybridization were visualized by the sensitization of Kodak XAR-5 film in a casette with intensifying screens at $-80°C$. In the case of nonisotopic detection, the biotin labeled probe (8 ng/mL) was added to nucleic acid target sequences, immobilized on the nylon membrane, and hybridized by overnight incubation at 65°C. The probe-target hybrids were developed and detected with a nonradioactive nucleic acid detection system

(BluGENE®, BRL). Briefly, the membrane was washed, blocked with bovine serum albumin, treated with streptavidin-alkaline phosphatase conjugate, and finally stained with the dye solution.

Results

The appropriate type of probe for the forensic science investigation of DNA specimens was determined by a series of preliminary studies. We concluded that use of the plasmid was adequate because of good sensitivity. A clear male-specific band was produced by using the EcoRI fragment purified from the plasmid; however, sensitivity was somewhat low. Although cross-reactive bands were produced by using the plasmid among female specimens, the occurrence of these bands evinced the presence of sufficient DNA on the electrophoresed lane. This means the facility of feminity estimation by using the plasmid as probe.

Figures 1, 2, and 3 show three kinds of patterns of identical DNA specimens from male and female individuals. The patterns in Fig. 1 are the fluorescence of ethidium bromide added gel, which shows a similar degree of sensitivity between the male and female specimens. Figures 2 and 3 present the autoradiographic and nonisotopically stained hybridization patterns on a nylon membrane, of which DNA components had been transferred from the gel, shown in Fig. 1. Both these patterns were closely similar to each other. A 3.4-kb band was found to appear on the patterns of male DNA. The detection limit of this 3.4-kb band by the autoradiographically visualized pattern after the exposure period of 5 h was 0.04-μg DNA/lane (1:64 dilution or 1-μL blood/lane). The bands in the nonisotopically stained patterns were clear and narrow, although inferior to the autoradiographic pattern in sensitivity. The detection limit of the 3.4-kb band was 0.16-μg DNA/lane or 4-μL blood/lane. In the nonisotopically stained patterns, a faint repetitious band of lower kilobase value appeared close to the main 3.4-kb band. Some broad stains with rather faint band(s) were found at the range of 0.5 to 23 kb on the patterns of both sexes.

Figure 4 shows the nonisotopically stained hybridization patterns of male and female DNA. The 3.4-kb band appeared on the patterns of all male specimens. A faint 3.4-kb band

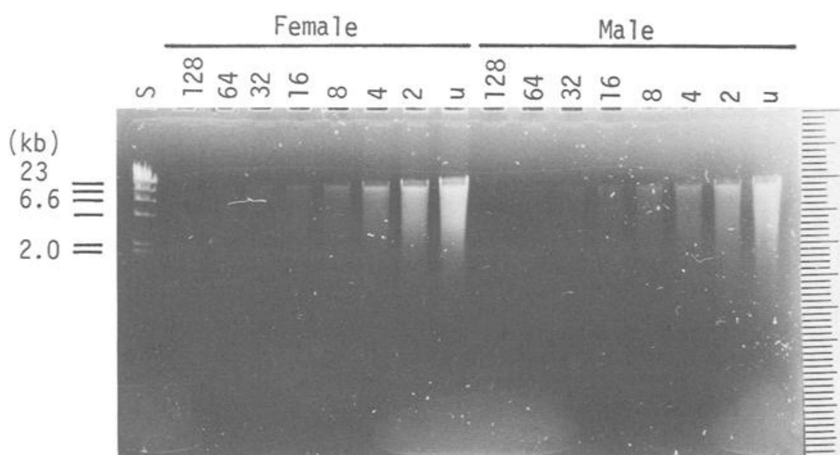

FIG. 1—*Fluorescence of ethidium bromide added gel. Numbers indicate dilutions of EcoRI treated DNA fractions. Undilutions* (u) *contained about 1.5-μg DNA/lane. Lane* S *is for molecular weight standards.*

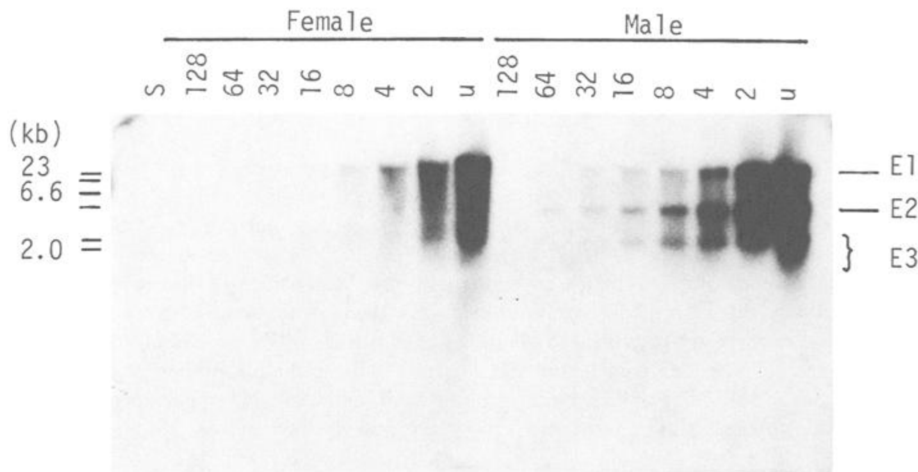

FIG. 2—*Autoradiographically obtained hybridization patterns of EcoRI treated and serially diluted DNA from a male and a female blood.* E2, *3.4-kb band;* E1 *and* E3, *bands common to male and female DNA.*

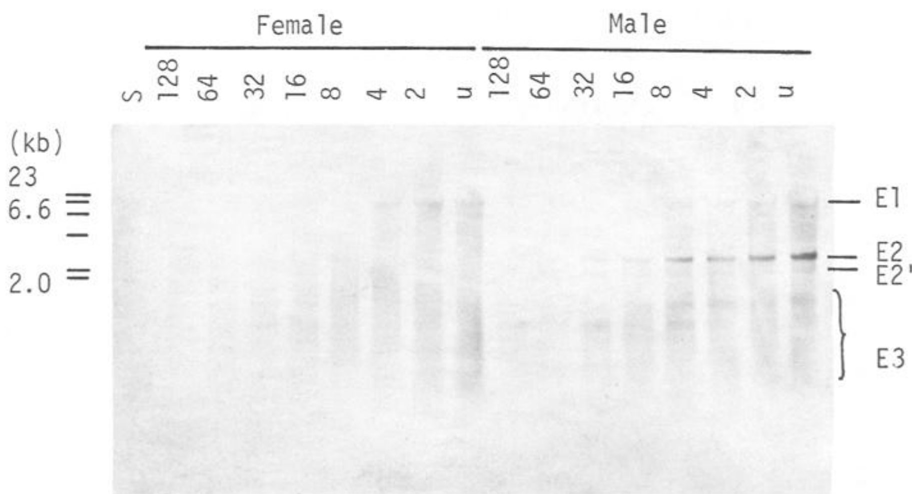

FIG. 3—*Hybridization patterns of serially diluted EcoRI treated DNA from a male and a female blood visualized by nonisotopic staining.* E2′, *repetitious band of band E2.*

infrequently appeared on the patterns of female specimens. However, the density of this band was apparently light compared to those for male specimens.

Figure 5 presents the hybridization patterns obtained by the nonisotopic staining of DNA from bloodstains. The patterns were identical to those with the DNA from fresh blood specimens in a parallel test. The requisite volume of bloodstains on a cotton sheet were about 1 cm^2, and the DNA from this size of bloodstain was sufficient for test of about ten times.

We performed blind tests for sex determination from bloodstains by the nonisotopic detection method. The DNA specimens were concluded to be male, when the 3.4-kb band was

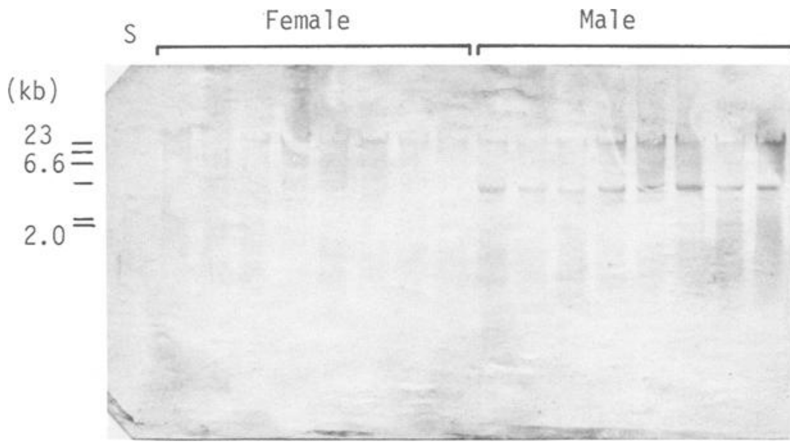

FIG. 4—*Hybridization patterns of DNA fractions from eight male and eight female individuals' blood visualized by nonisotopic staining. DNA specimens from about 15- to 50-μL blood/lane were applied.*

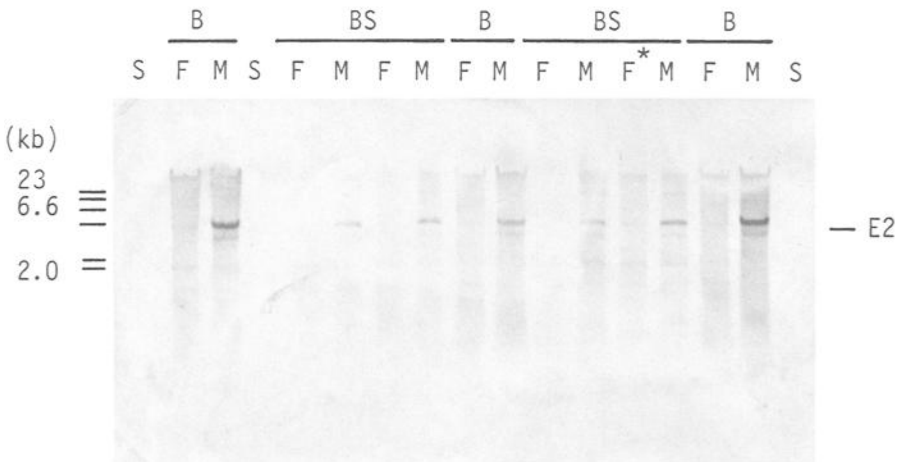

FIG. 5—*Hybridization patterns of DNA preparations from stains of blood* (BS) *on about 0.4-cm² cotton sheets. The bloodstains were prepared with the specimens from four male* (M) *and three female* (F) *individuals. Control DNA preparations of blood from persons of known sexes* (B) *(about 1.0 μg/ lane) were pararelly tested. The lane S as for molecular weight standards. *, a faint 3.4-kb band produced by female specimen.*

apparently dense compared to other bands. Femaleness of the specimens was determined, if both of the following criteria were satisfied: (1) demonstration of sufficient DNA on electrophoresed lane by the fluorescence of ethidium bromide added gel or nonisotopic detection method or both, and (2) no appearance of 3.4-kb band or apparently weak occurrence of this band compared to other bands. As shown in Table 1, maleness was determined by using all of undiluted and ten times diluted DNA specimens, whereas femaleness could be proved only with undiluted DNA specimens. Although sexes were not determined with highly diluted DNA specimens, no mistake in sex determination results occurred.

TABLE 1—*Results of blind tests with DNA preparations from bloodstains.*[a]

	Dilution of DNA from Bloodstain	Number Tested	Fluorescence of Electrophoresed DNA		Nonisotopic Southern Hybridization			Determined Sex		
					Dominance of					
			(+)	(−)	3.4-kb Band	Other Band	No Band	Male	Female	Undetermined
Male	Undil	10	10	0	10	0	0	10	0	0
	×10	10	5	5	10	0	0	10	0	0
	×100	6	0	6	2	0	4	2	0	4
	×1000	4	0	4	0	0	4	0	0	4
Female	Undil	11	11	0	0	10	1	0	11	0
	×10	7	2	5	0	0	7	0	2	5
	×100	3	0	3	0	0	3	0	0	3

[a]Undiluted DNA preparation from 1-cm^2 bloodstain was dissolved in 20 μL of TE buffer and diluted with this buffer. Three microlitres of undiluted or diluted DNA preparations were applied to each lane of electrophoresis.

Discussion

Tyler et al. [14] have reported a sex determination method for bloodstains by a simple spot test based on the principle of recombinant DNA technique. Although their method is useful as a screening test, confirmative results were not obtained in our laboratory, because relatively large quantities of female DNA caused nonspecific staining. The method of this study could offer affirmative sex determination results owing to the separation of components to cause specific and nonspecific reactions by electrophoresis. The DNA was estimated to be female, if the hybridized pattern showed presence of the nonspecifically reacted components and lack of the male-specific band. Investigation of ethidium bromide added and electrophoresed gel under ultraviolet light could assure the presence of sufficient DNA on the gel plate. Appearance of the faint 3.4-kb band among the patterns of a few female specimens did not lead to misinterpretation of results. Male and female specimens could be clearly discriminated by comparing the intensities of the 3.4-kb band and other portions of the hybridization patterns.

The nonisotopic method for this study was considerably sensitive. Tests could be accomplished with small quantities of DNA contained in about 50-μL blood or a 1-cm^2 bloodstain on a cotton sheet, although less sensitive compared to the autoradiographic method. The processing time of this nonisotopic method (3 to 4 h) is short compared to the autoradiographic method. The biotin labeled probe can be stored at least one year without remarkable loss of reactivity. Use of specifically designed laboratory and expensive equipment is unnecessary. Thus, this nonisotopic method for sex identification could be availed as a routine forensic science test of biological materials.

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