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Human identification and sex determination of dental pulp, bone marrow and blood stains with a recombinant DNA probe

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Summary. Recombinant DNA hybridizing specifically to a 300 nucleotide repeat DNA sequence (BLUR8) of human specificity and to human repeat DNA sequence (pHY10) on the Y chromosome was used for human identification and sex determination of degraded DNA samples of blood stains, dental pulp, and bone marrow. This radioactive technique enabled reliable and sensitive human and sex determination from blood stains that were more than 80 years old. Less than 1 piece of 0.5 cm length thread of blood stain was enough for both tests. DNA from relatively fresh dental pulp and bone marrow was clearly identified. The human identification test, which could recognize up to 0.3 ng DNA correctly, was 3 to 5 times more sensitive than the sex determination test.

Key words: Human identification – Sex determination – Dot hybridization

Zusammenfassung. Rekombinante DNA, welche spezifisch mit einer repititiven DNA-Sequenz menschlicher Spezifität und mit einer repititiven DNA-Sequenz (pHY10) des Y-Chromosoms hybridisiert, wurde für die Identifizierung der menschlichen Spezies und für die Geschlechtsbestimmung degradierter DNA aus Blutspuren, Zahnpulpen und aus Knochenmark benutzt. Diese radioaktive Technik ermöglicht zuverlässige und empfindliche Bestimmung der menschlichen Spezies und des Geschlechts aus Blutspuren mit einem Alter von mehr als 80 Jahren. Weniger als ein blutspurenhaltiger Baumwollfaden von 0,5 cm Länge war ausreichend, um beide Tests durchzuführen. DNA aus relativ frischer Zahnpulpa und aus Knochenmark wurde eindeutig identifiziert. Der Identifikationstest auf die menschliche Spezies, welcher bis zu 0,3 Nanogramm DNA korrekt erkannte, war drei- bis fünfmal empfindlicher als der Geschlechtsbestimmungstest.

Schlüsselwörter: Identifizierung der menschlichen Spezies – Geschlechtsbestimmung – Dot-Hybridisierung

Introduction

Serological identification of denatured samples in forensic practice is frequently troublesome in sensitivity and specificity. Human identification has been at-

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tempted using specific antisera with the techniques of Ouchterlony [1], ELISA [2, 3] and latex particle agglutination [4], but these techniques still have some difficulties in differentiating humans from anthropoids. Sex determination has been performed with the techniques of quinacrine hydochloride analysis of portions of Y chromosome [5, 6] and sex hormone analysis [7]. In recent years advances in recombinant DNA techniques have been remarkable, and investigation of complete identification of an individual has now been achieved [8, 9]. Recently Tyler et al. [10] demonstrated that the pHY2.1 probe could be used for sex determination of fresh blood stains using dot hybridization, and Gill [11] demonstrated sex determination of blood stains up to 4 years old using pYZ2 probe with the radioactive labeling method. In forensic practice, human and sex determination of test materials such as blood stain, dental pulp and bone marrow should be done before blood grouping.

In the preceding paper, we described the use of a human specific sequence probe (BLUR8) and a Y chromosome sequence specific probe (pHY10) to identify human blood and relatively fresh blood stains [12]. This paper describes the application of these probes to dental pulp, bone marrow and to extremely old blood stains with dot-blot hybridization.

Materials and methods

Preparation of samples

General procedures used for bacterial transformation, plasmid DNA isolation and DNA isolation from whole blood were described by Yamada et al. [13]. All blood stains were prepared on cotton fabrics and allowed to dry at room temperature until use. Except the test of aged blood stain, one-month old stains were used. Preparing blood stains to cotton fabrics, 1 ml whole blood gave a stain of approximately 150 cm^2 in size. 10 µl of blood stain (150 mm^2 in size) consisted of 80 pieces of 1 cm length cotton threads. We estimated that the amount of DNA in a blood stain from 10 µl whole blood to be approximately 250 ng DNA. We usually cut out 150 mm^2 (10 × 15 mm) stain and cut the fabric into pieces DNA extraction.

Teeth were split into two halves with an electric chisel to expose the dental pulp, and the mass of pulp cells was cut into small pieces for extraction. The materials used were permanent teeth extracted after surgical treatment which were normal or almost normal teeth. These were stored at room temperature up to about one month (see Table 1). Aged dental pulp was also used in this experiment. Powdered samples less than 50 µg scratched from the inner surface of the teeth were used for the extraction. Bone marrow of aged ulna and femur was scratched out, and 10 mg powdered sample used for the extraction.

Extraction of DNA

Samples to be extracted were incubated 2 h at 37°C in 0.01 *M* Tris-HCl pH 7.4, 0.05 M EDTA containing 0.5% N-lauryl sarcosine sodium salt, 20µg/ml proteinase K (Boehringer Mannheim, West Germany) and 0.001 *M* dithiothreitol. DNA was extracted with three times phenol/chloroform (1:1), twice with chloroform and was precipitated with ethanol. The DNA pellet was dried under vacuum and dissolved in 10 m*M* Tris-HCl pH 7.4 containing 1 m*M* EDTA and stored at 4°C until use. The amount of DNA was measured by spectrophotometer scanning between 340 nm to 240 nm, and calculated at 260 nm absorbance. An optical density (OD_{260}) of 1.0 was calculated as corresponding to 50 µg/ml DNA. At the same time, the purity of the samples was checked with the ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}). The OD_{260}/OD_{280} of our extractions were between 1.8 to 2.0.

Table 1. The weight of dental pulp and DNA of relatively fresh teeth about one month after extraction

Lapse of time after extraction	Age	Sex	Position of the teeth	Dental pulp weight (mg)	DNA weight (μg)
0 day	19	♀	4	7.9	18.6
0 day	29	♀	4	8.8	30.3
3 days	21	♀	8	10.5	33.2
3 days	18	♀	8	52.5	161.7
3 days	21	♂	8	26.6	114.2
3 days	33	♀	8	17.2	87.6
3 days	33	♀	8	6.4	57.6
4 days	40	♂	5	6.2	38.2
4 days	22	♀	8	17.4	46.6
4 days	22	♀	8	15.2	38.6
5 days	39	♂	5	4.8	20.4
5 days	40	♂	8	20.0	97.4
10 days	23	♂	8	9.1	49.1
10 days	22	♂	5	16.4	55.3
10 days	22	♂	5	20.3	29.7
14 days	22	♀	8	30.5	89.1
27 days	24	♂	8	26.3	48.2
27 days	13	♀	4	3.6	9.1
33 days	47	♂	7	1.6	8.1
34 days	54	♀	3	2.8	2.9
34 days	28	♀	8	3.4	27.0

Labeling of probes

Human specific repeat sequence probe (BLUR8) developed by Deininger et al. [14] and Y specific probe (pHY10) by Nakahori et al. [10] were kindly supplied by the Japanese Cancer Research Resources Bank. The probes were radioactively labeled with [^{32}P]dCTP(2′-deoxycytidine-5′-triphosphate, 3000 μCi/mmol) using the Multiprime DNA labeling system according the protocol supplied by Amersham. This system gave high specific activity (2–3 $\times$ 10^9/μg of probe DNA) by using random sequence hexanucleotides to prime DNA synthesis.

Dot blot hybridization

After boiling for 2 min, DNA samples were chilled on iced water for 2 min and applied a nitrocellulose membrane (8 $\times$12 cm, Schleicher und Schuell) using a Hybri-slot manifold (BRL, slot dimension 0.5 $\times$ 4.0 mm) for each analysis. The DNA analysis procedure with ^{32}P-dCTP was carried out mainly using the protocol supplied by Schleicher and Schuell. The filters were lightly soaked for few minutes in 6 $\times$ SSC (1 $\times$SSC = 0.15 mol/l sodium chloride, 0.015 mol/l sodium citrate) and baked in a vacuum oven at 80°C for 1.5 h. Prehybridization was done at 50°C (pHY10 probe) and at 42°C (BLUR8 probe) for at least 1 h in a solution containing 50% deionized formamide, 5 $\times$ SSPE (1 $\times$ SSPE = 0.15 *M* sodium chloride, 0.01 *M* sodium dihydrogen phosphate, 1 m*M* EDTA), 0.2 mg/ml salmon sperm DNA (Sigma type III) 0.1% sodium dodecylsulfate (SDS, Nakarai Chemical Co., extra pure), 5 $\times$ Denhardt's solution [1 $\times$ Denhardt's solution = 0.2 g/l Ficoll (Sigma, type 400), 0.2 g/l polyvinylpyrrolidone (PVP, Kodak), 0.2 g/l bovine serum albumin (Sigma, type V). The filters were hybridized

with at least 1×10^9 cpm/μg of probes (5 ng DNA probe for one filter, 8×12 cm) labeled by Multiprime at 50°C (pHY10 probe) and at 42°C (BLUR8 probe) for 18 h in the same solution as for prehybridization except a 0,1 mg/ml concentration of salmon sperm DNA. The filters were washed twice for 10 min at room temperature with a solution containing $2 \times$ SSPE, 0.1% SDS, then washed twice at 55°C for 15 min with $0.1 \times$ SSPE, 0.1% SDS, in $0.1 \times$ SSPE for few minutes and then air dried. Autoradiography was carried out at −80°C using Fuji X-ray film for periods between a half and 1 day.

Results

Human identification and sex determination of relatively fresh and aged blood stains

Figure 1 shows the autoradiogram of DNA extracted from blood stains of human male origin (lines A, B, and C) and human female origin (line D) of one month old, respectively. With BLUR8 probe (lines A and B), human DNA could be identified up to slot 7 which corresponded to 0.0125 μl human whole blood and approximately to 0.31 ng human DNA. The pHY10 probe experiment (lines C and D) showed lower sensitivity than BLUR8 probe, i.e. the minimum DNA quantity from whole blood stain was approximately 1.5 ng. The DNA of female origin showed no hybridized dark bands on line D.

Figure 2 shows the hybridized results of human aged blood stains with BLUR8 (lines A and B) and pHY10 (lines C and D). Every sample showed clear and strong reactions except the samples of line A, 1 and 3. Ten pieces of stain thread 1 cm in length were used for the test (Fig. 2). The sample of male origin prepared in 1883 (lines A and C, slot 1) showed faint dark masses at lines A and C. The sample of male origin prepared in 1919 (lines A and C, slot 3) showed no reaction. The others showed clear dark bands correctly.

Human identification and sex determination of dental pulp

Table 1 shows the weights (mg) of dental pulp tissue of relatively fresh teeth left for about one month at room temperature after extraction of the teeth and the

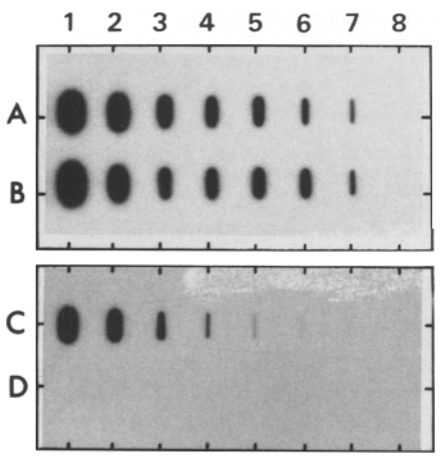

Fig. 1. Autoradiograms of DNA extracted without (*A*) or with (*B*) carrier DNA from human blood stains hybridized with human BLUR8 probe (*A, B*) and of DNA extracted with carrier DNA from human male (*C*) and female (*D*) blood stains hybridized with human pHY10 probe. The first slot contained DNA from 8 pieces of 1 cm length thread of blood stain; slot 2, 5 pieces of 1 cm; slot 3, 1 piece of 1 cm; slot 4, 1 piece of 5 mm length; slot 5, 1 piece of 4 mm; slot 6, 1 piece of 2 mm; slot 7, 1 piece of 1 mm; slot 8, 1 piece of 1 cm of blank thread, respectively

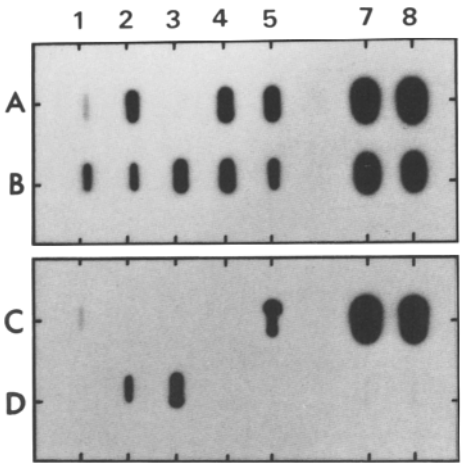

Fig. 2. Autoradiograms of DNA from aged human blood stains hybridized with human BLUR8 probe (*A* and *B*) and with human pHY10 probe (*C* and *D*). The DNA was extracted from 10 pieces of 1 cm length thread of blood stain. The first slot (1) of lines *A* and *C* contained DNA of male blood stain on prepared in 1883. Slot 1, lines *B* and *C*, female in 1905; slot 2, lines *A* and *C* female in 1908, slot 2, lines *B* and *D*, male in 1918; slot 3, lines *A* and *C*, male in 1919; slot 3, lines *B* and *D*, male in 1922; slot 4, lines *A* and *C*, female in 1922; slot 4, lines *B* and *D*, female in 1922; slot 5, lines *A* and *C*, male in 1927; slot 5, lines *B* and *D*, female in 1930, respectively. The 7th and 8th slots of lines *A* and *C* contained 200 ng DNA of male origin as a control. The 7th and 8th slots of lines *B* and *D* also contained 200 ng DNA of female origin as a control

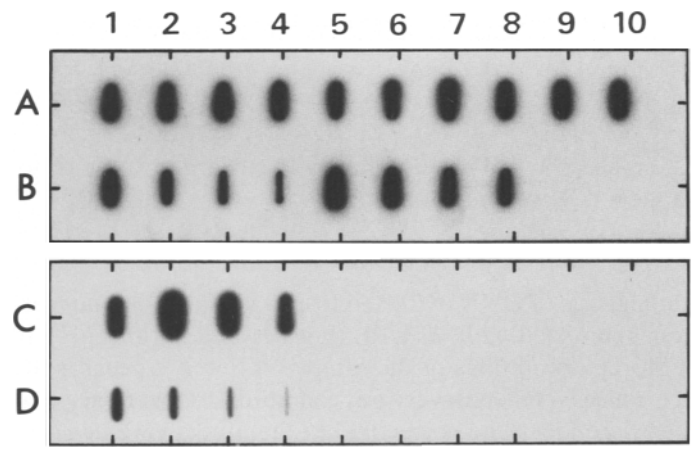

Fig. 3. Autoradiograms of DNA extracted from relatively fresh dental pulp hybridized with human BLUR8 probe (*A* and *B*), and with human pHY10 probe (*C* and *D*). Lines *A* and *C*, slots 1 to 5 show the hybridized results of the dental pulp DNA of male origin of 1, 21-year old (3 days after extraction); 2, 40-year old (4 days); 3, 23-year old (10 days); 4, 24-year old (27 days); 5, 47-year old (33 days), respectively. Lines *A* and *C*, slots 6 to 10 show the hybridized results of the dental pulp DNA of female origin; 6, 18-year old (3 days after extraction); 7, 22-year old (4 days), 8; 22-year old (14 days); 9, 13-year old (27 days); 10, 54-year old (34 days), respectively. Lines *B* and *D*, slots 1 to 4 were applied with DNA of male origin as controls; 1, 200 ng; 2, 100 ng; 3, 50 ng; 4, 25 ng, respectively. Lines *B* and *D*, slots 5 to 8 were applied with DNA fo female origin as controls; 5, 200 ng; 6, 100 ng; 7, 50 ng; 8, 25 ng, respectively

weights (μg) of DNA recovered from the same teeth. Two hundred ng of extracted DNA from these teeth (Table 1) were applied onto nitrocellulose membrane and hybridized with BLUR8 (Fig. 3, lines A and B) and pHY10 (Fig. 3, lines C and D). Every sample, including one 35-years old was identified correctly. As dental pulp cells disapeared with aging, the inner surface of teeth was scratched, and a small amount of powder (about 10–100 μg in weight) was col-

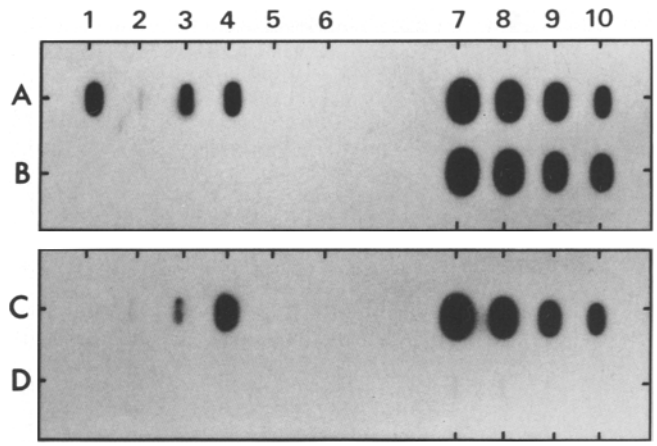

Fig. 4. Autoradiograms of DNA extracted from aged bone marrow, hybridized with human BLUR8 probe (*A* and *B*) and with human pHY10 probe (*C* and *D*). The DNA extracted from scratched powder from inner surface of bones was applied onto the nitrocellulose paper. Lines *A* and *C* were applied with bone marrow DNA of slot 1, female origin prepared in 1962; slot 2, male in 1962; slot 3, male in 1979; slot 4, male in 1982; slot 5, male in 1982; slot 6, female in 1987, respectively. Lines *A* and *C*, slots 7 to 10 were applied with DNA of male origin as controls; 1, 200 ng; 2, 100 ng; 3, 50 ng; 4, 25 ng, respectively. Lines *B* and *D*, slots 7 to 10 were applied with DNA of female origin as controls; 1, 200 ng; 2, 100 ng; 3, 50 ng; 4, 25 ng, respectively

lected. Four out of 11 samples showed positive human identification and sex determination, but the others did not (data not shown).

Human identification and sex determination of aged bone marrow

Figure 4 shows autoradiograms of DNA extracted from aged human bone marrow of ulna and femur hybridized with BLUR8 (lines A and B) and pHY10 (lines C and D). The storage conditions of the samples differed especially the samples in slots 5 and 6 which were left in very wet conditions for over one year. Three out of 6 samples were identified correctly, but the others gave negative results (Fig. 4). The samples from a male prepared in 1962 (slot 2) showed very faint dark mass at both lines A and C. The samples on slots 5 and 6 showed no reaction at all with either probe.

Discussion

BLUR 8 probe [14] is now well known to hybridize specifically to a consensus of a 300 nucleotide repeat DNA sequence distributed throughout the human genome. The extremely high specificity of this probe was confirmed previously [16] in which no-cross reaction with the DNA of apes was observed. Full sequences and characteristics of pHY10 were investigated by Nakahori et al. [15]. This pHY10 was a 3,564 bp EcoR1 fragment, and 229 out of 713 pentanucleotides were TTCCA, and 297 were single base substitutes. Gill et al. [8] used a 2.47 kb Y probe named DYZ2 which was originally reported by Schmidtke and Schmid [17]. The Probe pHY10 used in this experiment was present on the more proximal region of the long arm. Tyler et al [10] used a Y chromosome

probe resembling our probe, but they did not mention the quantitative change of DNA with aging in detail. Gill [18] demonstrated positive sex determination of blood stains up to 4 years old using pYZ2 probe with a radioactive labeling method. Pääbo reported the cloning and the sequencing of the human specific sequence (Alu family) from the human remains of the 2400-year-old mummy of a child [19].

The advantage of our DNA extraction method from blood stains was mentioned in the previous paper [12]. The labeling with [^{32}p] dCTP Multiprime usually gave specific activity of 2–3 $\times 10^9$ cpm/μg of DNA probe. This relative activity was about 5–10 times higher than that of nick translation. It resulted in short exposure time in autoradiography, usually a half to one day exposure. With the Multiprime labeling method, only 5 ng of labeled DNA probe was needed for one hybridization filter (8 × 12 cm).

Carrier DNA added before ethanol precipitation procedure (Fig. 1, line B) seemed slightly more effective by which the precipitated DNA could be observed with the naked eye. With BLUR8 probe, human DNA could be identified up to slot 7 which corresponded to 0.0125 μl human whole blood and also approximately to 0.31 ng of human DNA. The test for human identification usually gave 3–5 times more sensitive results than that for sex determination (Fig. 1, lines A, B and C). The samples from female origin (Fig. 1, line D) showed no cross reaction at all with pHY10 probe. When hybridization was done at 42°C, very high cross-reaction occured which was not removed by washing at 65°C (data not shown). This may be one of the reasons why Gill showed high cross-reaction with Y specific probe using dot-hybridization [18]. An extremely old blood stain prepared in 1883 was not so clearly identified (Fig. 2, slot 1). However, we were able to determine the sex of 10 pieces of 1 cm length threads of blood stains up to 80 years old.

Sufficient DNA could be recovered from dental pulp tissue of relatively fresh teeth about one month after extraction (Table 1). The amount of DNA varied considerably with the storage, age and lapse of time after extraction of teeth. DNA was easily and clearly identified in these samples. (Fig. 3). For dot-hybridization, 200 ng of DNA was enough for the test, and 3 μg of DNA for DNA fingerprinting. It is possible for fingerprinting tests to be perfomed from 1–50 times and 10 to 800 times with dot-hybridization on DNA from only one tooth (Table 1). Less than 50 ng DNA was recovered from aged dental pulp cells over 35-years old. It was very difficult to recover the samples from the inside of aged teeth. Even using 10 mg of powdered sample from bone marrow cells, only three out of six samples showed successful DNA extraction (Fig. 4). The ambient conditions of samples will be one of the most important factors. It was corroborated that DNA may be seriously degraded in wet or hot conditions, and no hybridized spot can be observed as seen in slots 5 and 6 (Fig. 4).

All the samples of female origin showed negative spots with pHY10 probe. The human identification test indicated 3–5 times more sensitivity than that of sex identification. For reliable tests of every unknown material, we recommend that both tests always be performed for each sample. If the result of human identification test was distinct, the sample would offer definite information about sex determination.

The time required for Multiplime probe labeling method was usually two days, one day for DNA extraction and hybridization and the other day for washing and autoradiography. For introduction of this technique in the forensic practice, it would require the basic facilities of isotope probe labeling. On the other hand, the non-radioactive photobiotin method has some problems but will be expected to be improved as discussed in a recent paper by Yokoi et al. [12].

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