Z Rechtsmed (1985) 95:227–233

Zeitschrift für
Rechtsmedizin

Polymorphism of EsD by Isoelectric Focusing: Description of the New Allele EsD*Kofu and Phenotyping in Bloodstains

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Summary. The polymorphism of EsD was investigated in 1115 unrelated Japanese individuals by isoelectric focusing. Besides the three common phenotypes two heterozygotes EsD 7-1 and EsD 7-2 were observed. The gene frequencies were: EsD*1 = 0.6234, EsD*2 = 0.3663, and EsD*7 = 0.0103. In addition, a rare variant was detected in a probandus living in the city of Kofu. The family analysis suggested the hereditary occurrence of a new allele EsD*Kofu.

The isoelectric focusing method was successfully applied to phenotyping EsD in bloodstains; each phenotype was demonstrated at 37°C for up to 2 weeks, at room temperature for up to 9 weeks, and at 4°C for over 20 weeks after stain formation.

Key words: Enzyme polymorphism, EsD – Isoelectric focusing, EsD – EsD*Kofu – Bloodstains, EsD phenotyping

Zusammenfassung. Bei 1115 nicht verwandten japanischen Individuen wurden die EsD-Typen mittels isoelektrischer Fokussierung untersucht. Neben den drei häufigen Phänotypen wurden zwei Heterozygoten EsD 7-1 und EsD 7-2 beobachtet. Die Genfrequenzen betrugen: EsD*1 = 0,6234, EsD*2 = 0,3663 und EsD*7 = 0,0103. Außerdem wurde eine seltene Variante bei einem in der Stadt Kofu lebenden Probandus gefunden. Die Familienanalyse zeigte das Vorkommen eines neuen Allels EsD*Kofu und dessen Erblichkeit.

Die Isoelektrofokussierungsmethode wurde mit Erfolg zur EsD-Typisierung an Blutspuren angewandt; jeder Phänotyp konnte bei 37°C bis zu 2 Wochen, bei Zimmertemperatur bis zu 9 Wochen und bei 4°C über 20 Wochen nach Beginn der Lagerung nachgewiesen werden.

Schlüsselwörter: Enzympolymorphismus, EsD – Isoelektrofokussierung, EsD – EsD*Kofu, neues Allel – Blutspuren, EsD-Typisierung

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The genetic polymorphism of human esterase D (EsD) was first discovered by Hopkinson et al. (1973). Using starch gel electrophoresis and the substrate 4-methylumbelliferyl acetate, they demonstrated three common phenotypes: EsD 1, EsD 2-1, and EsD 2 determined by two codominant autosomal alleles EsD*1 and EsD*2.

Subsequent studies revealed the existence of a number of rare alleles in various populations: EsD*3 (Bender and Frank 1974), EsD*4 (Berg et al. 1976), EsD*Mamelodi (Hitzeroth et al. 1976), EsD*0 (Marks et al. 1977), EsD*3Negrito (Omoto et al. 1978), EsD*3·1 (Suzuki et al. 1978), EsD*5 (Martin 1979), EsD*6 (Radam et al. 1980), EsD*7 (Siege and Schwehn 1983), EsD*Copenhagen (Dissing and Eriksen 1984), EsD*Düsseldorf (Henke and Basler 1984), and EsD*Yamaguchi (Yuasa et al. 1985b). Using isoelectric focusing, the EsD*5 allele was later shown to be relatively common in white people, while the EsD*7 allele was reported to occur with appreciable frequency in Japanese by Nishigaki and Itoh (1984). Although these isoelectric focusing methods provided a better separation of the products of EsD*5 or EsD*7 from those of EsD*1 and EsD*2, it was rather difficult to distinguish between the products of EsD*1 and EsD*2, and several technical improvements have been undertaken to overcome this problem (Bär et al. 1984; Budowle 1984; Divall 1984; Finney et al. 1985; Henke et al. 1985).

We have recently recognized the isoelectric method developed by Yuasa et al. (1985a) to be quite suitable for phenotyping EsD, especially in identifying the products of EsD*7. In the present study, the polymorphism of EsD in a Japanese population was investigated with this technique, and a new rare variant was found. Furthermore, the method was successfully applied to phenotyping EsD in bloodstains.

Materials and Methods

Blood Samples

Venous blood samples were collected from 1115 unrelated residents of Yamanashi Prefecture. Fresh hemolysates were prepared from washed red cells by freezing and thawing. They were pretreated with an equal volume of 0.05 *M* dithiothreitol (DTT: Sigma Chemical Co., USA) for 15 min at room temperature and absorbed on 5 × 6 mm pieces of filter paper (Toyoroshi No. 2, Tokyo, Japan).

Bloodstains

Blood samples from 20 individuals with known phenotypes were dropped on filter paper (Toyoroshi No. 2) and allowed to dry for a few hours at room temperature. The bloodstains thus made were stored at 37°C in a thermostatic chamber, at room temperature, and at 4°C in a refrigerator, and examined at 1-week intervals over a period of 20 weeks. The stains were cut in 5 × 6 mm pieces and moistened with 10 µl 0.05 *M* DTT for 30 min at room temperature just before analysis.

Isoelectric Focusing

Isoelectric focusing was performed with an LKB 2117 Multiphor apparatus (Bromma, Sweden) on polyacrylamide gel as developed by Yuasa et al. (1985a). The gel plate (230 × 110 × 0.5 mm) was composed of 20 ml stock solution (5.25% acrylamide/0.25% N, N'-methyl-

enebisacrylamide), 1 ml Ampholine pH range 4.0–6.5 (LKB), 0.3 ml 0.01% riboflavin, and 2.5 g sucrose. The electrode paper strips were soaked with 1 *M* phosphoric acid for anode and with 0.2 *M* sodium hydroxide for cathode. The specimens were applied onto the gel surface 2 cm from the cathode. Electrofocusing was conducted at a constant voltage of 1000 V for 80 min. During focusing the gel plate was cooled by circulating water at 4° C.

Enzyme Staining

The staining solution for EsD was prepared by the method of Hopkinson et al. (1973). A sheet of filter paper (Toyoroshi No. 2), measuring 230 × 60 mm, was soaked with a mixture of 2 ml acetone and 20 ml 0.05 *M* acetate buffer (pH 5.2) containing 3 mg 4-methylumbelliferyl acetate (Koch-Light Laboratories Ltd., Berks, England). The gel plate was covered with the above filter paper and incubated for 5 min at 37° C. The isoenzyme bands were visualized under long-wave UV light.

Results and Discussion

Population Study

Figure 1 shows the isoelectric focusing patterns of six phenotypes of red cell EsD. The three common phenotypes EsD 1, EsD 2-1, and EsD 2 as well as two heterozygotes EsD 7-1 and EsD 7-2 were clearly identified. Both EsD 7-1 and EsD 7-2 types exhibited four distinct isoenzyme bands: anodal two corresponded electrophoretically with the EsD 1 or EsD 2 isoenzyme, and cathodal two were considered to be the products of the EsD*7 allele. Our sample included no homozygous EsD 7 type. Meanwhile we have found an unusual isoenzyme pattern in the blood sample from a 56-year-old man, which, to our knowledge, has not been reported anywhere.

The blood samples from members of the family with the above unusual pattern were analyzed using isoelectric focusing together with the use of conventional starch gel electrophoresis, and the results are given in Figs. 2 and 3. The variant phenotype was characterized by three pairs of isoenzyme bands, one pair of which corresponded in electrophoretic mobility with the EsD 1 isoenzyme and the other two pairs migrated toward the anode. The banding profile of this variant resembles that of the rare EsD 6-1 type, but these two types

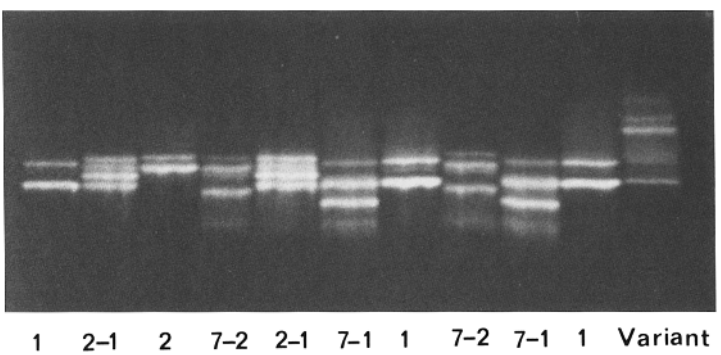

Fig. 1. Isoelectric focusing patterns of six phenotypes of red cell EsD. The anode is at the *top*

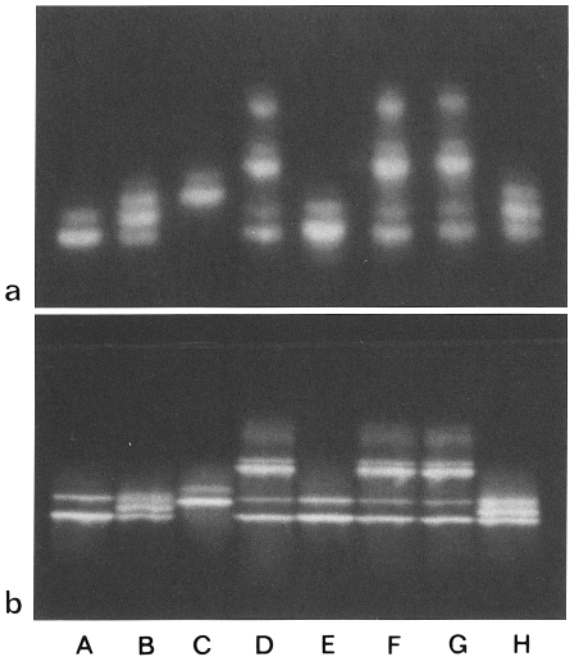

Fig. 2a, b. EsD isoenzyme patterns of members of the family with EsD*Kofu allele as revealed by starch gel electrophoresis according to the method of Coates et al. (1975) (**a**) and by the present isoelectric focusing method (**b**). The anode is at the *top*. *A* Reference, EsD 1; *B* and *H* reference, EsD 2-1; *C* reference, EsD 2; *D* probandus, EsD Kofu-1; *E* wife, EsD 1; *F* daughter, EsD Kofu-1; *G* daughter, EsD Kofu-1

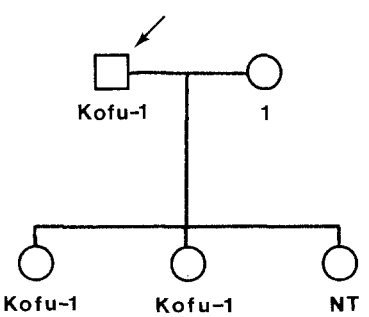

Fig. 3. Pedigree of the family with EsD*Kofu allele. *Arrow* indicates the probandus; *NT* not tested

are distinctly different in that the intermediate double bands of our variant move far more anodally than the EsD 2 isoenzyme, particularly in the separation by isoelectric focusing, while the middle band of the EsD 6-1 type is located more cathodally as shown previously by Radam et al. (1980). The results of the family analysis strongly suggest the hereditary occurrence of a new variant allele at the EsD locus. We may propose to designate tentatively this allele as EsD*Kofu after the name of the city where the family resides. The present variant pheno-

Table 1. Distribution of EsD types in a Japanese population

Phenotype	No. observed (%)	No. expected
1	430 (38.57)	432.9
2-1	511 (45.83)	508.8
2	150 (13.45)	149.5
7-1	18 (1.61)	14.3
7-2	5 (0.45)	8.4
7	0 (0.00)	0.1
Kofu-1[a]	1 (0.09)	
Total	1115 (100.00)	1114.0

Gene frequencies: EsD*1 = 0.6234, EsD*2 = 0.3663, EsD*7 = 0.0103; $\chi^2 = 2.47$; $df = 3$; $0.50 > P > 0.30$

[a] Omitted for the calculation of gene frequencies

type appears to be the heterozygous products of EsD*1 and EsD*Kofu and may be expressed as EsD Kofu-1.

Table 1 presents the distribution of EsD types in 1115 blood samples from Japanese individuals. The observed and expected numbers are in equilibrium according to the Hardy-Weinberg law. The EsD*7 frequency in the present sample (0.0103) is almost similar to that reported in a western part of Japan (0.0109) by Yuasa et al. (1985b), but higher than that reported in a central part of Japan (0.008) by Nishigaki and Itoh (1984). The occurrence of the EsD*7 allele with relatively high frequency and the absence of the EsD*5 allele in Japanese suggest that this allele may be characteristic for Mongoloids. Further population surveys in other Asiatic regions will solve this problem.

Phenotyping in Bloodstains

In medicolegal investigations the polymorphism of EsD is one of the most useful genetic markers for the grouping of bloodstains. Since isoelectric focusing has recently been introduced for use in the analysis of EsD types, several attempts have been made to demonstrated this enzyme polymorphism from bloodstains (Horscroft and Sutton 1983; Budowle 1984; White 1984; Finney et al. 1985; Yuasa et al. 1985a).

Table 2 summarizes the results for the determination limits of EsD types in bloodstains stored at 37° C, room temperature, and 4° C obtained by the present isoelectric method. Our sample included five EsD 1, six EsD 2-1, five EsD 2, two EsD 7-1, and two EsD 7-2. Figure 4 shows the EsD patterns in bloodstains stored at room temperature for 4 weeks.

All the bloodstains examined were successfully typed for EsD at 37° C for periods of up to 2 weeks, at room temperature for periods of up to 9 weeks and at 4° C even for periods in excess of 20 weeks. Our results are superior to the determination limits reported by other workers (Horscroft and Sutton 1983; Budowle 1984; White 1984; Finney et al. 1985). With increasing time of storage, the bands of the heterozygous types EsD 2-1, EsD 7-1, and EsD 7-2 became fainter and more indistinct than those of the homozygous types EsD 1 and EsD 2.

Table 2. Positive results for the determination limits of EsD types in 20 bloodstains stored at 37°C, room temperature, and 4°C obtained by isoelectric focusing

Phenotype	No. tested	Temperature	Age of bloodstains (weeks)																			
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	5	37°C	5	5	5	5	0															
2-1	6		6	6	6	5	0															
2	5		5	5	5	5	0															
7-1	2		2	2	2	0	0															
7-2	2		2	2	0	0	0															
1	5	Room temperature	5	5	5	5	5	5	5	5	5	5	5	2	0							
2-1	6		6	6	6	6	6	6	6	6	6	4	1	1	0							
2	5		5	5	5	5	5	5	5	5	5	5	4	3	0							
7-1	2		2	2	2	2	2	2	2	2	2	1	1	1	0							
7-2	2		2	2	2	2	2	2	2	2	2	1	1	0	0							
1	5	4°C	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
2-1	6		6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
2	5		5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
7-1	2		2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
7-2	2		2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

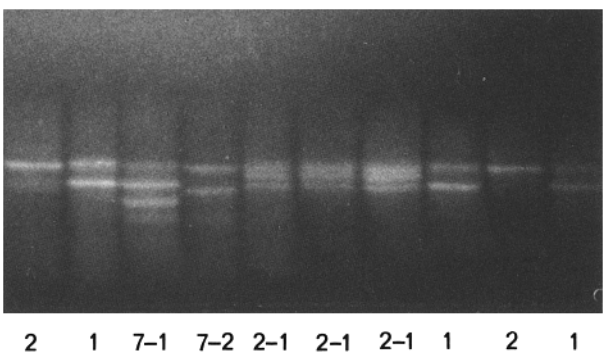

Fig. 4. Isoelectric focusing patterns of EsD in bloodstains stored at room temperature for 4 weeks. The anode is at the *top*

The present stability study shows that the isoelectric method developed by Yuasa et al. (1985a) permits EsD phenotyping in bloodstains for fairly long storage periods. The simplicity, easiness, and reliability of the technique make it the recommended method for the determination of EsD types from bloodstains in medicolegal practice.

Acknowledgements. We are very grateful to Dr. I. Yuasa, Dept. of Legal Medicine, Tottori University School of Medicine, for his valuable advice and helpful suggestion. The blood samples were kindly provided by Dr. T. Kagami, Yamanashi Prefectural Central Hospital.

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Received August 13, 1985