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Differentiation Between Human and Chimpanzee in Bloodstains by Enzyme-linked Immunosorbent Assay (ELISA) Using Antihuman Serum*

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Summary. An enzyme-linked immunosorbent assay (ELISA) for species identification of human bloodstains using two commercially available antisera against human serum is described. Human bloodstains were to be distinguished from those of chimpanzees and other animals using raw antisera, and the differentiation between human and chimpanzee became more evident when those antisera were absorbed with a small amount of chimpanzee plasma. Human bloodstains could clearly be identified by the present method even after 4 weeks of aging at room temperature.

Key words: Bloodstains, species identification – Enzyme-linked immunosorbent assay (ELISA), spezies identification – Species identification, ELISA

Zusammenfassung. Ein enzymgekoppelter Immunabsorptions-Test (ELISA) für die Speziesidentifizierung menschlicher Blutspuren unter Verwendung zweier kommerzieller Anti-Seren gegen menschliches Serum wird beschrieben. Menschliche Blutspuren waren unterscheidbar von solchen des Schimpansen und solchen anderer Tiere unter Verwendung von Rohseren, und die Differenzierung zwischen Mensch und Schimpanse wurde eindeutiger, wenn diese Anti-Seren mit einer kleinen Menge von Schimpansenplasma absorbiert wurden. Menschliche Blutspuren konnten durch die gezeigte Methode klar identifiziert werden, dies sogar nach vierwöchiger Lagerung bei Raumtemperatur.

Schlüsselwörter: Immunabsorptionstest (ELISA), Speziesdifferenzierung in Blutspuren – Blutspuren, Speziesdifferenzierung

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Introduction

Species identification of human bloodstains is important in forensic practice. For that purpose, various immunologic methods, such as ring test [1], indirect hemagglutination inhibition test [2], or latex agglutination inhibition test [3] using antisera against human blood proteins have been used. Recently, Tamaki and Kishida [4] applied enzyme-linked immunosorbent assay (ELISA) using antisera against human albumin or other blood proteins to species identification of human bloodstains. Other sensitive ELISA methods using both monoclonal and polyclonal antibodies have been also reported [5–7]. However, none of the above methods, including sensitive ELISA methods, could differentiate between human and chimpanzee in bloodstains.

In the present study, a new ELISA method using commercially available anti-human serum distinguishing human from chimpanzee in bloodstains is presented.

Materials and Methods

Antisera

Two lots of rabbit anti-human serum were purchased from MBL Laboratory (Nagoya, Japan) and Handai-biken (Osaka, Japan). Alkaline phosphatase-labeled goat anti-rabbit IgG was purchased from Kirkegaard and Perry Laboratory (Gaithersberg, MD, USA).

Specimens

Heparinized blood samples were obtained from humans, apes (chimpanzee), Old World monkeys (Japanese monkey, rhesus monkey, and crab-eating monkey), New World monkeys (night monkey and tufted capuchin monkey), prosimians (grand galago and ring-tailed lemur) and other animals (cow, goat, dog, cat, and rat). Pieces of filter paper (Toyokagaku, No. 2) were stained with these blood samples, dried at room temperature, and subjected to analysis. Human bloodstains left for 1–4 weeks at room temperature were also analyzed. From blood samples of humans and chimpanzees, plasma was further prepared by centrifugation.

Procedures

Unless otherwise mentioned, all the steps below were performed at room temperature. The wells of a polystyrene microtiter plate (129B, Dynatech Ltd., Sussex, England) were coated with 100 µl each of 100-fold diluted human plasma in 50 m*M* sodium carbonate buffer (pH 9.6). After incubation for 10 min, the wells were washed three times with 0.01 *M* phosphate-buffered saline (pH 7.6) containing 0.05% Tween 20 (PBS-Tween). To each well was added 100 µl of a 1:1 mixture of 50–1000-fold diluted antiserum and 50–5000-fold diluted test plasma in PBS containing 1% bovine serum albumin (PBS-BSA) which had been allowed to react for 30 min. When bloodstains were analyzed, a piece of the stains, 5 × 5 mm in area, was directly reacted with 240 µl of 100–1000-fold diluted antiserum for 60 min, and then 100 µl of the solution was added to each well. After incubation for 10 min, the wells were washed three times with PBS-Tween, and 100 µl of 1000-fold diluted enzyme-labeled goat anti-rabbit IgG in PBS-BSA was added to each well. After incubation for 60 min, the wells were washed three times with PBS-Tween. Finally, a reaction medium (100 µl; 1*M* diethanolamine containing 1 m*M* magnesium chloride, pH 9.3) and a substrate solution (10 µl; 100 m*M* of p-nitrophenyl phosphate in 0.001 *N* HCl) were added to each well and incubated for 60 min at 37°C. At the end of incubation, 50 µl of the mixture was added to 600 µl of a stop solution (0.35 *N* sodium

hydroxide), and the absorbance at 405 nm was measured with a Hitachi 557 spectrophotometer. Determinations were made in duplicate.

Results

Pooled human plasma in various protein concentrations were analyzed in the present inhibition ELISA using four different dilutions of the antisera. Typical standard curves with MBL anti-human serum are shown in Fig. 1. Using a 50- or 100-fold dilution of the antiserum, the ELISA reactions decreased linearly with increase in the concentration of human plasma from 0.1 mg/ml. With a 500- or 1000-fold diluted antiserum, human plasma in much lower concentrations caused the inhibition although the original ELISA reactions became weaker. Another lot of the antiserum obtained from Handai-biken gave similar results.

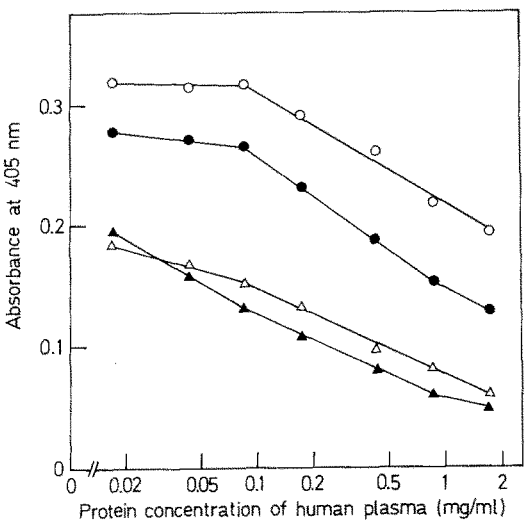

Fig. 1. Standard curves of human plasma samples in the present ELISA method using MBL anti-human serum. Antiserum dilutions used were as follows: ○ 1:50; ● 1:100; △ 1:500; ▲ 1:1000

Table 1. Inhibition of the ELISA reactions by human and animal bloodstains using two lots of raw antihuman sera

Species	(*n*)	Inhibition (%)					
		Dilution of antiserum (MBL)			Dilution of antiserum (Handai-biken)		
		1:100	1:500	1:1000	1:100	1:500	1:1000
Human	(5)	61.1 ± 5.1[a]	89.8 ± 1.2	93.2 ± 0.6	60.2 ± 0.9	81.3 ± 1.3	88.7 ± 1.4
Chimpanzee	(4)	26.2 ± 4.5	57.3 ± 3.5	70.3 ± 3.7	20.7 ± 2.0	49.3 ± 4.5	61.9 ± 4.5
Old World monkeys	(3)	<10	<20	<30	<10	<20	<30
New World monkeys	(2)	<10	<15	<20	<10	<10	<20
Prosimians	(2)	<10	<15	<20	<5	<5	<15
Other animals	(5)	<5	<10	<10	<5	<5	<10

[a] Mean ± standard deviation (SD)

Table 2. Inhibition of the ELISA reactions by human and animal bloodstains using two lots of antihuman sera absorbed with ⅕ volume of chimpanzee plasma

Species	(*n*)	Inhibition (%)					
		Dilution of antiserum (MBL)			Dilution of antiserum (Handai-biken)		
		1:100	1:500	1:1000	1:100	1:500	1:1000
Human	(5)	81.0 ± 2.2[a]	87.1 ± 5.1	93.1 ± 1.4	79.8 ± 2.4	87.9 ± 3.1	98.1 ± 1.1
Chimpanzee	(4)	13.2 ± 2.9	37.8 ± 5.7	47.5 ± 3.3	18.4 ± 0.4	33.2 ± 1.2	66.3 ± 4.4
Old World monkeys	(3)	<10	<20	<30	<10	<20	<25
New World monkeys	(2)	<10	<10	<20	<10	<10	<20
Prosimians	(2)	<10	<10	<20	<5	<5	<10
Other animals	(5)	<5	<5	<10	<5	<5	<10

[a] Mean ± standard deviation (SD)

Table 3. Inhibition of the ELISA reactions by aged human bloodstains using two lots of antihuman sera absorbed with ⅕ volume of chimpanzee plasma

Age of stain	Inhibition (%)	
	Dilution of antiserum (MBL) 1:100	Dilution of antiserum (Handai-biken) 1:100
1 week	80.2 ± 0.9[a]	77.7 ± 1.8
2 weeks	80.1 ± 0.8	77.7 ± 2.5
3 weeks	79.6 ± 1.1	76.9 ± 2.1
4 weeks	79.9 ± 1.0	76.1 ± 2.3

[a] Mean ± standard deviation (SD)

Inhibition of the ELISA reactions by bloodstains of various species was investigated. As shown in Table 1, human bloodstains inhibited the reactions most, and chimpanzee ones follow. Bloodstains of other species inhibited the reaction only slightly although there appeared some relationships between the inhibition rate and the phylogenic order: inhibition generally decreased by the order of Old World monkeys, New World monkeys, prosimians, and other animals. When antisera absorbed with ⅕ volume of chimpanzee plasma were used, human bloodstains were clearly distinguished from chimpanzee ones by the present ELISA (Table 2). Difference in inhibition rate between human bloodstains and chimpanzee ones was greatest when 100-fold diluted antisera were used.

Human bloodstains aged for at least 4 weeks at room temperature inhibited the ELISA reactions to the same extent as fresh ones (Table 3).

Discussion

Authors have reported a simple method for identification of human bloodstains by the indirect hemagglutination inhibition test using chromic chloride-treated red blood cells and commercially available anti-human serum [2]. The method

could easily differentiate bloodstains of humans from those of non-human primates except chimpanzees.

Recently, ELISA methods using monoclonal [5] or polyclonal [6, 7] antibodies against human blood proteins have been applied for identification of human bloodstains. Although these methods are sensitive, neither of them could differentiate human bloodstains from chimpanzee ones. In the present study, we devised a new ELISA method for identification of human bloodstains using commercially available anti-human serum. The method could differentiate between human and chimpanzee bloodstains using raw antisera, and the differentiation became more evident when the antisera were absorbed with ⅕ volume of chimpanzee plasma.

The reason why our method can differentiate human bloodstains from chimpanzee ones and other ELISA methods cannot is not clear now. Some differences in experimental conditions might be responsible to the apparently discrepant results. Especially, application of a relatively low dilution of the antisera (100-fold) appears to be important (see Tables 1 and 2). Since either of the two antisera tested gave similar results, the antisera used appeared not to be critical, and most of the commercial antisera may be used in the present ELISA system.

Aging experiments showed that the antigenicity of human bloodstains was stable for 4 weeks at least. Therefore, the present method seems to be useful for forensic science practice.

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