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QUANTITATIVE ANALYSIS OF SPECIES IDENTIFICATION TESTS OF BLOODSTAINS USING ANTI-HUMAN SERUM

HIROSHI OKAJIMA and YOSHINAO KATSUMATA

Department of Legal Medicine, Nagoya University School of Medicine, Nagoya, Japan

ABSTRACT

Immunological reactions of bloodstains of human and animals to anti-human serum were analysed quantitatively to determine which technique is the most effective to differentiate human bloodstains from those of animals. The lower threshold of counter-electrophoresis was 10—25 μg protein/ml and increased only slightly with increasing age of the bloodstains, while that of the ring test and immunodiffusion decreased greatly. In counter-electrophoresis, absorption of anti-human serum by Japanese monkey serum resulted in a marked decrease in the cross reaction with animals, and still showed no change in the lower threshold for human samples. The present results show that counter-electrophoresis is especially useful for the identification of human bloodstains.

Key Words: Quantitative analysis, Species identification, Anti-human serum, Counter-electrophoresis.

INTRODUCTION

Species identification of bloodstains is very important for forensic science practice. Immunological techniques such as precipitin ring test, double immunodiffusion test, and counter-electrophoresis using anti-human hemoglobin or anti-human serum are generally used.[1)~4)] Since the exact amount of blood contained in the stains is usually unknown, the amount of the specimen to be used should be arbitrarily decided by each examiner. For the well-trained examiner, deciding an appropriate amount of specimen is not difficult in most cases. However, some cross reactions of antibodies to animal materials, especially those of the monkey are inevitable, and the human antigens in stains may deteriorate. Thus, precautions should be taken for false positive or false negative reactions, and it is useful to have some quantitative indicator of the amount of blood in specimens.

In the present study, protein concentrations of the bloodstain extracts of human and animals were determined. Also, the sensitivities of the three techniques frequently used for species identification were analysed quantitatively on the basis of protein concentration using commercially available anti-human serum.

MATERIALS AND METHOD

Anti-human serum

An IgG fraction of anti-human serum derived from rabbits or goats was purchased from MBL Laboratory (Nagoya, Japan). In some experiments, these antisera were absorbed with

Correspondence: Department of Legal Medicine, Nagoya University, 65 Tsuruma-cho, Showa-ku, Nagoya 466, Japan

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Japanese monkey serum at the volume ratio of 1/5 to 1/20.

Specimens

Heparinized blood samples were obtained from humans, apes (chimpanzee and white handed gibbon), Old World monkeys (rhesus monkey, Japanese monkey, and patas monkey), New World monkeys (tufted capuchin monkey), prosimian (grand galago) and other animals (cattle, cat, goat, dog, sheep, guinea pig, rat, and chicken). Pieces of filter paper (Whatmann No.2) were stained with these blood samples (20μl blood from human and 10 μl blood from animals), dried at room temperature, and subjected to analysis. Some of these bloodstains were left under indirect illumination for various durations at room temperature for aging experiments.

Preparation of blood extracts

Each bloodstain was cut to a size of about 1 × 1 cm, and put into a test tube containing 2ml (for human) or 1ml (for animals) of physiological saline. The test tubes were incubated at 25°C in a temperature-controlled water bath for 60 min. After incubation, the tubes were shaken briefly, and the extracts were removed. Protein concentrations of the extracts were determined by the method of Lowry *et al.*,[5] and the extracts were diluted to the concentrations of 1 μg-4mg/ml. Recovery of the proteins in the extracts was calculated from the protein amount contained in them and that contained in the original human blood (20μl) used for preparation of the bloodstain.

Ring test

Approximately 40 μl of anti-human serum was put into a small tube (inner diameter 0.3 cm, height 4.5 cm) and the specimen was added. The tubes were incubated at 25°C, and the precipitin line was observed at 30 min and 60 min.

Double immunodiffusion test

The test was performed according to the method of Yakulis and Heller.[6]

On agarose gel plate (7 × 7 cm, 1 mm thick), a hexagonal pattern was punched out, maintaining 3 mm between one well and another (each 3 mm bore). A central well was filled with 4 μl of the anti-human serum, and the specimens of the same volume were placed in the six peripheral wells. The plate showing precipitin lines after 24 hr of incubation at room temperature in a wet box was washed with saline overnight, and stained with brom thymol blue.

Counter-electrophoresis

Counter-electrophoresis was performed using a modification of Culliford's method.[1] Two parallel lines of wells (3 mm bore) were punched out on an agarose gel plate in the direction of electrophoresis, approximately 3 mm apart. Four microliters of antigen was placed in the cathodal wells and 4 μl of anti-serum, in the anodal wells. Electrophoresis was performed at 10 mA for 20 min, and after the plate was washed with physiological saline for 24 hr, the precipitin lines were carefully examined.

The recommended procedure for identification of human bloodstains is as follows: 1) Prepare bloodstain extracts containing appropriate amounts of proteins with physiological saline. The amount of bloodstain used depends on the recovery of the proteins, and larger amounts should be used for aged bloodstains. Usually, 1 cm × 1 cm of the stain is extracted with 1 ml of physiological saline for one hour at room temperature. 2) Determine the protein concentration of the extract according to the method of Lowry *et al.* 3) Dilute the extract to concentrations of 50 μg and 100 μg protein/ml with physiological saline. 4) Detect human serum proteins by

counter-electrophoresis using anti-human serum absorbed with Japanese monkey serum.

RESULTS

Ten human blood samples tested 130 ± 20 mg protein/ml. Animal blood samples contained similar amounts of proteins. When bloodstains were extracted with physiological saline, the recovery of the proteins gradually decreased with the increase in their ages, especially after 3 months (Fig. 1). The time required for maximum recovery increased with age as well. The sensitivity of the three techniques, precipitin ring test, counter-electrophoresis, and double immunodiffusion test, was analysed quantitatively on the basis of the protein concentration of the bloodstain extracts. For the identification of human bloodstains using a rabbit anti-human serum, the lower threshold of the ring test was 10 μg protein/ml, that of counter-electrophoresis was 10–25μg protein/ml and that of double immunodiffusion test was 50 μg protein/ml. Similar results were obtained using goat anti-human serum. The lower threshold of the ring test or the immunodiffusion test, expressed as the minimum protein concentration showing positive reactions, decreased markedly with age, while that of the counter-electrophoresis decreased only slightly (Table 1).

Cross reactions of the bloodstain extracts of various animals with rabbit anti-human serum in the ring test, counter-electrophoresis and immunodiffusion test were summarized in Table 2. In the ring test, monkeys generally showed strong cross reactions, and cattle, cat, goat, dog and sheep showed moderate cross reactions. Guinea pig and rat displayed weak cross reactions, and chicken did not show any reaction. Similar cross reactions were observed in the other two methods, although the sensitivities of the two were somewhat lower than that of the ring test.

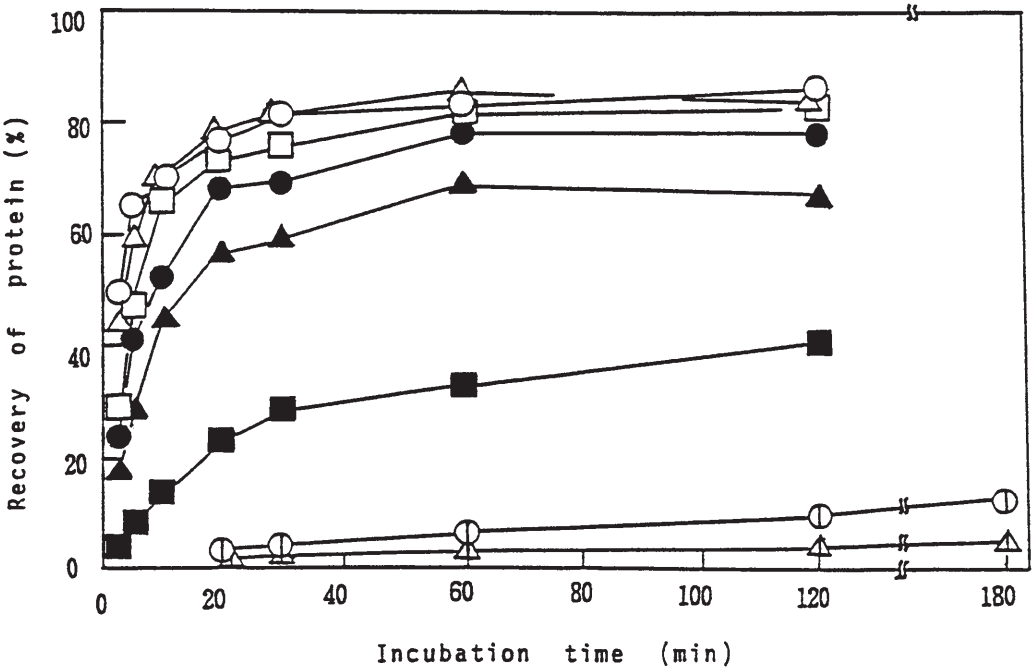

Fig. 1. Recovery of proteins from aged bloostains. Ages of the bloodstains were ○, 1 day; △, 1week; □, 1 month; ●, 2 months; ▲, 3 months; ■, 6 months; ⦶, 9 months; ⧋, 1 year.

Table 1. Sensitivity of the three immunochemical methods for detection of human serum in the extracts of aged human bloodstains using rabbit anti-human serum

Aging periods	Minimum protein concentration showing positive reaction (μg/ml)			
	Ring test		Counter-electrophoresis	Immunodiffusion
	30 min	60 min		
1day	10	10	10—25	50
1 week	10	10	10—25	50
1 month	25	10	10—25	50
2 months	25	25	10—25	100
3 months	50	25	10—25	150
6 months	50	50	25	150
9 months	75	50	25	200
1 year	75	75	25	200

Table 2. Cross reaction of animal bloodstain extracts with rabbit anti-human serum in the three immunochemical methods (ring test, counter-electrophoresis, immunodiffusion)

Species	Minimum protein concentration showing positive reaction (μg/ml)			
	Ring test		Counter-electrophoresis	Immunodiffusion
	30 min	60 min		
Human	10	10	10—25	50
Chimpanzee	25	10	25—75	100—250
White-handed gibbon	25	25	25—75	100—250
Rhesus monkey	25	25	50—100	100—250
Japanese monkey	25	25	50—100	100—250
Patas monkey	25	25	50—100	100—250
Tufted capuchin monkey	25	25	50—100	100—250
Grand galago	50	25	50—100	100—250
Cattle	25—50	25	100—250	250—500
Cat	50	25	100—250	250—500
Goat	50	25	100—250	250—500
Dog	50	50	100—250	250—500
Sheep	100	50	100—250	250—500
Guinea pig	250—500	250	1000	2000—3000
Rat	500	250	1000	2000—3000
Chicken	—	—	—	—

When the antiserum was absorbed with one twentieth volume of Japanese monkey plasma, the cross reactions of the specimens of monkeys and animals except apes disappeared as shown in Table 3. The sensitivity of the ring test for human samples decreased from 10 μg/ml to 100—250 μg/ml, and that of immunodiffusion, from 50 μg/ml to 100—250 μg/ml. On the other hand, the sensitivity of the counter-electrophoresis for human samples did not change by the absorption, and the difference between human samples and ape samples became evident. The experiments using the antiserum absorbed with larger volumes (one tenth or one fifth) of Japanese monkey plasma gave similar results.

Table 3. Cross reaction of animal bloodstain extracts with rabbit anti-human serum in the three immunochemical methods (ring test, counter-electrophoresis, immunodiffusion)

Species	Minimum protein concentration showing positive reaction (μg/ml)			
	Ring test		Counter-electrophoresis	Immunodiffusion
	30 min	60 min		
Human	250	100—250	10—25	100—250
Chimpanzee	250	250	75—100	250—500
White-handed gibbon	250	250	75—100	250—500
Rhesus monkey	—	—	—	—
Japanese monkey	—	—	—	—
Patas monkey	—	—	—	—
Tufted capuchin monkey	—	—	—	—
Grand galago	—	—	—	—
Cattle	—	—	—	—
Cat	—	—	—	—
Goat	—	—	—	—
Dog	—	—	—	—
Sheep	—	—	—	—
Guinea pig	—	—	—	—
Rat	—	—	—	—
Chicken	—	—	—	—

Japanese monkey serum was added to anti-human serum at the volume ratio of 1 : 20

Immunoelectrophoresis of the extracts of human bloodstains showed that the immunological reaction of the fractions moving in the cathodal direction specifically decreased in the extract derived from the stain aged for 6 months (Fig. 2).

DISCUSSION

Since the protein concentrations of human blood samples in the present investigation were fairly consistent, we could assume the amount of blood in the bloodstain extract from the

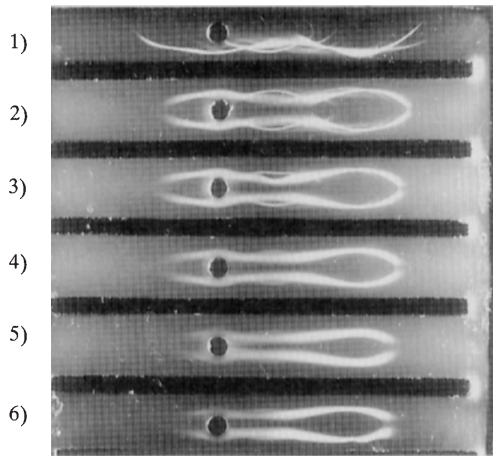

Fig. 2. Immunoelectrophoresis of fresh human plasma (1), and human bloodstain extracts of various ages (2, 1 day; 3, 1 month; 4, 3 months; 5,6 months; 6, 1 year.) developed with anti-human serum.

amount of the proteins recovered in it: 200 μg protein represents approximately 1 μl of blood. Of course, we must be careful because the blood contains many kinds of proteins having different antigenicity and solubility, and various diseases affect the total amount of blood proteins. Furthermore, the possibility of contamination by other proteins should be carefully evaluated in each case. Therefore, the protein amount in the sample gives only a rough approximation of the amount of original blood. Such problems are inevitable in the forensic sciences. However, more objective information about the forensic samples in question should always be the goal, and the protein amounts in the bloodstain extract could well become a useful indicator for species identification tests. Thus, we recommend the procedure described in the Materials and Methods.

When human bloodstains were aged, both the recovery of blood proteins by the extraction medium and their antigenicity decreased gradually. Therefore, the immunological reactions of a definite area of human bloodstains largely decreased with age. It can be assumed that the denaturation of the blood proteins was responsible for the decrease in both solubility and antigenicity. Immunoelectrophoresis of the aged human bloodstains showed that the solubility or the immunogenicity of the fractions moving in the cathodal direction (mainly IgG) decreased pronouncedly after 6 months of age, while that of other proteins did not. It should be noted that the decrease in the antigenicity of blood proteins was very small when analysed by counter-electrophoresis (Table 1).

This phenomenon can be reasonably explained by the fact that the counter-electrophoresis detects proteins moving in the anodal direction that were soluble even after 6 months of age.

Significant cross reactions of animal bloodstains against rabbit anti-human serum were observed with the ring test, counter-electrophoresis and immunodiffusion test using raw anti-human serum. Absorption of the antiserum by 1/20 or more volumes of Japanese monkey serum caused complete disappearance of the cross reactions of animals except apes (Table 3). Although absorption caused a large decrease in the sensitivity of the ring test and the immunodiffusion test, it did not cause any decrease in the sensitivity of the counter-electrophoresis. Consequently, the present counter-electrophoresis could detect human serum proteins efficiently even in aged bloodstains.

REFERENCES

1) Culliford, B. J.: Precipitin reactions in forensic problems. *Nature,* 201, 1092—1094 (1964).
2) Ohya, I., Tsugawa, N., Sawada, H. and Yada, S.: Medico-legal application of precipitation-electrophoresis. *Rep. Nation. Res. Inst. Police Sci.*, 23, 14—220 (1970).
3) Hara, M., Inoue, T., Koyanagi, Y. and Yamasaki, H.: Comparative study of sensitivity between ring probe and precipitation reaction in agar gel. *Jap. J. Legal Med.*, 22, 1—4 (1968).
4) Oda, N.: Investigation on precipitin reaction of the purpose of practical application. *Rep. Nation. Res. Inst. Police Sci.*, 22, 79—88 (1969).
5) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J.: Protein measurement with the folin phenol reagent. *J. Biol. Chem.*,193, 265—275 (1951).
6) Yakulis, V. J. and Heller, P.: Rapid slide technic for double diffusion agar precipitin test. *Amer. J. Clin.* Path., 31, 323—325 (1959).