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Comparative Studies Between Counterimmunoelectrophoresis and Microprecipitation Method of Identification of Human Minute Bloodstains

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Summary. A minute bloodstain on a thread 2 mm in length was tested to identify human origin by counterimmunoelectrophoresis (CIE), modified CIE, and microprecipitation method (MPM), using anti-human HbA serum. The detection limits expressed as the highest dilution of human blood on the thread in positive reaction were 1:160, 1:320, and 1:320 in CIE, modified CIE, and MPM, respectively. About 1 h was required to obtain the results. In CIE and modified CIE, the detection limits diminished according to the reduction of the samples from one half to one-eighth of the thread, but not in MPM.

Key words: Human blood identification – Counterimmunoelectrophoresis – Bloodstains, microprecipitation method

Zusammenfassung. Es wurden die Empfindlichkeiten mittels der Gegenstromimmunoelektrophorese (GIE), der modifizierten GIE und der Mikropräzipitationsmethode (MPM) zur Identifizierung von menschlichem Blut an einem verdächtigen winzigen Blutfleck (ein Faden mit einer Länge von 2 mm) mit Anti-HbA Serum gemessen und verglichen. Die minimalen Grenzen der positiven Reaktionen erwiesen sich durch GIE als 1:160 verdünntes Blut, durch die modifizierte GIE 1:320 und durch MPM 1:320. Diese positiven Reaktionen wurden nach etwa 1 h vom Untersuchungseinsatz gewonnen. Die festgestellten Empfindlichkeiten mittels GIE und der modifizierten GIE verminderten sich entsprechend der Abname der Menge von Blutfleck, von einer Hälfte bis ein Achtel vom Faden mit einer Länge von 2 mm, während sie bei MPM nicht sanken.

Schlüsselwörter: Blutidentifizierung, Gegenstromimmunoelektrophorese, Mikropräzipitationsmethode – Spurenanalyse, Blutfleck

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Introduction

It is one of the most important routine works to identify bloodstains in the criminal investigation. For this purpose, various methods, such as Ascoli's ring test [1, 2], precipitation in gel [3–5], immunoelectrophoresis [6], anti-human globulin inhibition test [7], sensitized latex method [8], micro-thin-layer immunoassay [9], radioimmunoassay [10] , and isoelectric focusing [11] are applied. Ascoli's ring test is commonly used for identification of bloodstains in Japan.

Ascoli's ring test and the microprecipitation method developed by Katsura [12, 13] were comparatively studied on identification of human origin from bloodstains [14]. As the results, the amount of hemoglobin required to identify human origin by microprecipitation method was smaller than that by Ascoli's ring test.

A combination method of electrophoresis and precipitation was developed and named electrosyneresis by Bussard and Huet in 1959 [15]. Culliford [16] adapted electrosyneresis to forensic problems. Grunbaum [17], Dorrill and Whitehead [18], and Divall [19] used the method (cross-over electrophoresis) for identifying bloodstains. Electrosyneresis has recently also been called counter-immunoelectrophoresis (CIE) [20].

In the present study, the detection limits of the minute bloodstains and the required time were comparatively studied in the microprecipitation method, CIE, and the modified CIE.

Preliminary Tests

Agaroses and agars with various electric osmosis values were tested to select for counterimmunoelectrophoresis (CIE). A gel solution was made by mixing equal volumes of 2% agarose or agar and 0.06 *M* veronal buffer (pH 8.6), and poured into a glass tray $9.5 \times 7 \times 0.15$ cm in size. Pairs of wells 2.5 mm in diameter about 5 mm apart were made, and anti-human HbA serum and diluted human hemolysate were put in the cathodal and anodal wells, respectively. Electrophoresis was carried out at 1–5 mA/cm for 30 or 45 min.

No hemolysates with low or high Hb concentrations were demonstrated by CIE using agarose with an electric osmosis of less than 0.1-mr or more than 0.3-mr. Agarose with 0.1–0.2-mr electric osmosis was very suitable for the CIE. Low Hb concentration of less than 22.5 μg/ml did not form a precipitation line by the electrophoresis at 1 mA/cm, and a high Hb concentration of more than 360 μg/ml did not show a line more than at 5 mA/cm. Positive precipitation was observed in a wide range from 11.25 μg/ml to 720 μg/ml of Hb concentration at 1.4 mA/cm, and a minimum Hb amount for the reaction was 56 ng.

Materials and Methods

Anti-human HbA Serum. Anti-human HbA serum was obtained from goats immunized s.c. with a mixture of 10 mg or 20 mg human HbA_0 fractionated by DEAE-Sephadex column chromatography and Freund's complete adjuvant once or twice a week for 2 months. The crude anti-humam HbA goat serum was specified by adsorption with Sepharose 4B conjugated

with a large amount of dog hemolysate and a small amount of cat and goose hemolysates. Anti-human HbA serum reacted strongly with human adult hemolysate and weakly with fetal one. Moreover, the antiserum did not precipitate with human serum, semen, saliva, and hemolysates from animals, such as canine, cat, rat, swine, bovine, horse, rabbit, chicken, goose, and dove, except Japanese monkey.

Blood Stain. Blood from healthy adults diluted to 1:10 (1.4 g/100 ml Hb) was twice serially diluted with aqua dest., and 1 µl of the diluted blood was applied on cotton threads (no. 30) 1 cm in length. A thread 2 mm in length was cut off from the bloodstain thread and unfastened with needles into fibrils. Other than the 2 mm bloodstain thread, each of nearly a half, a quarter, and one-eighth of the thread was tested.

Microprecipitation Method (MPM). Antiserum agarose mixture was prepared by mixing a equal volume of 2% agarose A 37 (Nakarai Chemicals) veronal buffer solution (0.05 *M*, pH 8.6) and specific anti-human HbA serum at 55°C. The mixture was poured into a glass frame, and an antiserum-agarose plate of 1.5 × 1.5 × 0.1 cm in size was made. Bloodstain threads to be tested were directly put on the antiserum-agarose plate and allowed to stand in a moist chamber. Microprecipitation in the antiserum-agarose plate was observed with a dark-field condenser of Nikon "Dry".

Counterimmunoelectrophoresis (CIE). Agarose GP-42 or agarose LE (Nakarai Chemicals) with electric osmosis of 0.1–0.2-mr was used for CIE. The bloodstain thread 2 mm in length was put into a small test tube and 10 µl of Hirschfeld veronal buffer (0.06 M, pH 8.6) [16] was added, and stood with stirring from time to time. Five microliters of the extract of the bloodstain thread and the anti-human HbA serum were applied into the cathodal and anodal wells of the agarose plate, respectively, and electrophoresed at 1.4 mA/cm for 45 min.

Modified CIE. A bloodstain thread 2 mm in length or the smaller sample was directly put into the gel 5 mm apart from the antibody well, and extraction of Hb from the thread or the smaller sample was performed in the gel without adding any buffer solution. Electrophoresis was carried out at 1.4 mA/cm for 45 min.

Results

A detection limits of human blood is expressed as the highest dilution of human blood stained to the threads in positive precipitation reaction.

The detection limits of 1-week-old bloodstain threads by three methods were comparatively summarized in Table 1. In MPM, positive reaction reached the minimum (1:320) 1 h after the application of the thread to the anti-HbA serum agarose plate, and the detection limits did not reduce even in the smallest sample (one-eighth of the thread). In the modified CIE, a minimum detection limit (1:640) of the two bloodstain threads was obtained after 30 min extraction of the threads in the gel plate; however, that of one thread was 1:320 and reduced from 1:320 to 1:160 in extraction periods of more than 3 or 6 h. It took 3 h or more to obtain the minimum detection limit (1:320) of the one thread by using CIE. Detection limits of the smaller bloodstain threads diminished twofold serially according to the reduction of the samples in methods of CIE and modified CIE (Fig. 1).

In general, it took much more time to obtain the minimum detection limits of 3-week-old bloodstain threads as compared with those of 1-week-old ones. In 3-week-old bloodstain threads stained with 1:160 diluted blood or their smaller samples, positive reaction was observed 1 h after the application by MPM (Fig. 2), and precipitation of bloodstain threads or the smaller samples with 1:320 di-

Table 1. Detection limits of human hemoglobin from 1-week-old bloodstains tested by three methods

Method	Volume of bloodstain	Diffusion or extraction time (h)					
		0	0.5	1	3	6	Overnight
MPM	2 threads	n.t.	1:160	1:320	1:320	1:320	1:320
	1 thread	n.t.	1:160	1:320	1:320	1:320	1:320
	½ thread	n.t.	1:160	1:320	1:320	1:320	1:320
	¼ thread	n.t.	1:160	1:320	1:320	1:320	1:320
	⅛ thread	n.t.	1:160	1:320	1:320	1:320	1:320
CIE	2 threads	n.t.	1:160	1:320	1:640	1:640	1:640
	1 thread	n.t.	1:80–160	1:160	1:320	1:320	1:320
	½ thread	n.t.	1:80	1:80	1:160	1:160	1:160
	¼ thread	n.t.	1:40	1:40–80	1:80	1:80	1:80
	⅛ thread	n.t.	1:20–40	1:20–40	1:40	1:40	1:40
Modified CIE	2 threads	1:320	1:640	1:640	1:320	1:320	1:160
	1 thread	1:160	1:320	1:320	1:160–320	1:160	1:80
	½ thread	1:80	1:160	1:160	1:160	1:80	1:40
	¼ thread	1:40	1:80	1:80	1:80	1:40	1:20
	⅛ thread	1:20	1:40	1:40	1:20–40	1:20–40	1:10

Detection limits are shown by blood dilution. A thread: 2 mm in length, MPM: Microprecipitation method; CIE: Counterimmunoelectrophoresis; n.t.: not tested

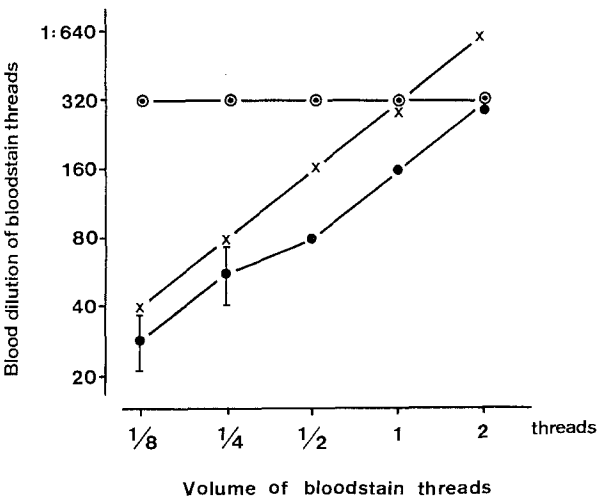

Fig. 1. Comparison of detection limits of 1-week-old bloodstain threads at 1 h after the application by means of microprecipitation method (⊙), counterimmunoelectrophoresis (●) and modified counterimmunoelectrophoresis (×)

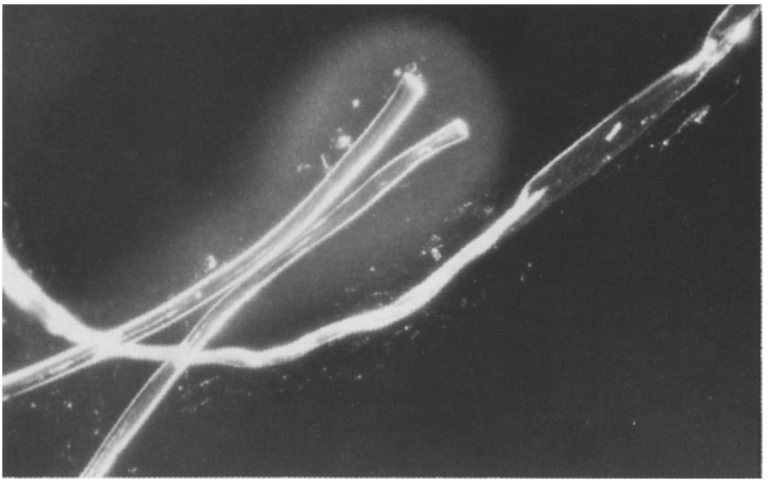

Fig. 2. Positive microprecipitation around the fibrils of one-eighth of a 3-week-old bloodstain thread with human blood diluted 1:160. Observation: 1 h after the application, ×200

luted blood appeared 3 or 6 h after the application. The minimum detection limit (1:160 or 1:320) of the one bloodstain thread was obtained 1 or 3 h after extraction of the thread in the gel of the agarose plate by the modified CIE, and reduced after extraction of more than 6 h. It required an overnight extraction of the bloodstain thread to obtain the minimum detection limits by CIE. In the modified CIE and CIE, detection limits of the smaller samples of the 3-week-old bloodstain thread diminished at the same rate as those of the 1-week-old bloodstains.

Discussion

Positive precipitation of a tested sample by use of anti-human HbA serum directly indicates that human blood is in the sample because anti-human HbA serum does not react with human serum, saliva, semen, and many animal bloods except for the blood of a monkey. Culliford [16] and Divall [19] reported that 10^{-8} g of serum protein and serum diluted up to 1:500,000 were detected by CIE using antiserum against human serum, respectively. In the preliminary test of the present study, a minimum Hb amount for the positive reactions was 56 ng by CIE. Taki [21] quantitatively determined HbA_1 amounts of individual erythrocytes of adults by MPM, and reported that HbA_1 weight in the erythrocytes ranged from 4.7 to 62.6 pg and averaged 23.7 pg. Antigen diffuses into antibody-gel only in MPM, so that any kind of agarose is available. On the other hand, CIE is based on the rules of gel-electrophoresis in which antigen and antibody are caused to migrate toward the opposite sides of each other, so that care must be taken to select the best agarose and electric condition suitable for the test system. The gels with an electric osmosis of less than 0.1-mr or more than 0.3-mr, especially agar gels, gave unsatisfactory results in this study.

The minimum detection limit (1:320) of one thread (2 mm in length) with the bloodstains by MPM was the same as that by CIE and modified CIE, and the detection limit increased to 1:640 blood dilution when two bloodstain threads were tested by CIE and modified CIE (Table 1). However, remarkable differences in the three methods were found in the tests on the smaller samples of the bloodstain thread. In CIE and modified CIE, the detection limits reduced to low levels in proportion to the volumes of the samples diminished, but not in MPM (Fig. 1). When the smallest sample, one-eighth of the bloodstain thread 2 mm in length, was used for the test, the minimum detection limits reduced to 1:10–40 blood dilution in CIE and modified CIE, while they remained at 1:160–320 in MPM.

For the practice of criminal investigation, it is important to know the result of a test after a short time. In MPM, the time required to identify human blood from bloodstain threads was about 1 h when the antiserum-agarose plate had been prepared previously. This plate is usable for at least 6 months, if it is covered with a cover glass or a piece of vinyl sheet and stored in a moist chamber at 4°C until the test. Modified CIE required so much time to MPM, and CIE took even more time. Positive reaction is usually obtained within several minutes in MPM, when fresh and undiluted or slightly diluted bloodstains are examined. Moreover, bloodstain threads after the test by MPM can be used consecutively for determining ABO blood group [22]. Therefore, MPM could be recommended as a routine method to identify human blood from a minute bloodstain.

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