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A Sequence of Tests of Minute Human Blood Stains for Human Origin Identification and ABO Blood Grouping

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Summary. A series of examinations is presented for human origin identification and ABO blood grouping of doubtful minute human blood stains. A blood-stained thread (0.5 cm in length) was first tested to identify human origin by microprecipitation method and then the ABO blood type was determined by both a modified absorption-elution test and a modified mixed agglutination. In the continuous tests, the maximum limits of positive reactions of the microprecipitation method, the modified absorption-elution test, and the modified mixed agglutination were 1:640, 1:160, and 1:2,560 diluted blood, respectively. A and B agglutinogens were more sensitively determined than H agglutinogen. Hemagglutinogens of blood stains on cotton threads were more easily detected than those of polyester ones.

Key words: Forensic serial examination – Species identification, blood stains – ABO blood grouping, blood stains – Microprecipitation method, absorption elution, mixed agglutination – Blood stains

Zusammenfassung. Es werden drei kontinuierliche Verfahren zur Identifizierung von menschlichem Blut und zur Bestimmung der ABO-Blutgruppen an einem verdächtigen winzigen Blutfleck (ein Faden mit einer Länge von 0,5 cm) beschrieben. Nach der Menschenblutbestimmung des verdächtigen Blutflecks mittels Mikropräzipitationsmethode wurden die ABO-Blutgruppen durch eine modifizierte Absorptions-Elutions-Methode bestimmt, und schließlich wurde eine verbesserte Mischzellagglutination zur Blutgruppenbestimmung am verdächtigen Blutfleck ausgeführt. Die maximalen Grenzen der positiven Reaktionen lagen bei folgenden Blutverdünnungen: (1) Mikropräzipitationsmethode 1:640; (2) modifizierte Absorptions-Elutions-Methode 1:160 und (3) verbesserte Mischzellagglutination 1:2560. Die Empfindlichkeit der Feststellung von A- und B-Agglutinogen auf dem Blutfleck war höher als die von H-Agglutinogen. Der Nachweis von Hämagglutinogenen des Blutflecks wurde auf dem Baumwollfaden leichter erbracht als bei dem Blutfleck auf dem Polyesterfaden.

Schlüsselwörter: Rechtsmedizinische Serienuntersuchung – Menschenblutidentifizierung, Blutfleck – ABO-Gruppenbestimmung, Blutfleck – Mikropräzipitationsmethode, Absorptions-Elutions-Methode, Mischzellagglutination – Blutfleck

Identification of human origin and determination of ABO blood groups of a blood stain are an important routine work in forensic medical practice. In Japan, doubtful blood stains are generally first examined by a preliminary test and then by a test for identification of human origin, and finally ABO blood groups of the stain are determined. In the case of a minute doubtful blood stain, if the material to be tested for ABO blood groups is different from the material used for human origin identification, determination of the blood groups may turn out false since ABO blood grouping is carried out on material without any human blood. It is necessary that the same material tested by a method for human origin identification is continuously examined by tests for ABO blood grouping. Katsura et al. [1] reported on a method of continuous determination of human origin and ABO blood groups on a single fibril stained with human blood. Determination of ABO blood groups from a minute stain is so difficult that a blood stain should be tested by multiple methods to obtain a distinct result. This paper presents three continuous methods for human origin identification and the blood groupings: (1) microprecipitation method [2], (2) modified absorption-elution test [3, 4], and (3) modified mixed agglutination [5, 6].

Materials and Methods

Anti-human Hemoglobin Serum

Specific anti-human HbA_0 serum was prepared as follows: Goats were immunized s.c. with a mixture of 10 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 [7]. Crude goat antiserum obtained after 10 days from the last injection (total injected HbA_0: 160 mg) was specified by absorption with a large amount of dog hemolysate conjugated to Sepharose 4B (Pharmacia) gel and a small amount of Japanese monkey hemolysate.

Anti-A, Anti-B, and Anti-H Hemagglutinins

Anti-A and anti-B sera with a titer of 1:256 (Dade Co.) were used. Two anti-H agglutinins with the same titer as anti-A or anti-B were prepared as follows: One was Ulex anti-H lectin (anti-HU) fractionated by DEAE cellulose chromatography of an ethanol precipitate from *Ulex europaeus* seeds [8]. The other was chicken anti-H (anti-HC) obtained by i.v. immunization with 10% human erythrocyte suspension of group O once a week for 4 weeks [9]. Heterohemagglutinin of crude chicken antisera was absorbed with human erythrocytes of group AB, and mucilaginous substance of the serum was completely eliminated with nylon and cotton fibers.

Blood Stains

Human blood (groups A, B, AB, and O) was diluted twofold. Half a milliliter was spread on a clean cotton and polyester cloth piece (5 × 6 cm), allowed to dry at room temperature, and stored for 3 months. Blood stains of various animals were also prepared.

Identification of Human Blood by the Microprecipitation Method

Antiserum-agarose plate for microprecipitation was prepared as follows: 2 g agarose A-37 (Nakarai Chemicals) was dissolved in 100 ml of 0.05 *M* veronal buffer (pH 8.6) containing 0.1% sodium azide at 100°C. One volume of specific anti-human HbA_0 serum was mixed with an equal volume of the agarose solution at 55°C. The mixture was immediately poured into a frame (1 mm thick, 15 × 15 mm in size) adhered to a slide glass and covered with a cover glass. The anti-human-HbA_0-agarose plate was stored in a moist chamber until the test.

After removing the cover glass, four threads (0.5 cm in length) were taken out of the blood stain and applied directly onto the anti-human-HbA_0-agarose plate. The plate was allowed to stand in a moist chamber and observed with a dark field condenser of Nikon "Dry" or of a stereoscopic microscope. If the thread contains human blood, white precipitate appears around the thread (Fig. 1).

ABO Blood Grouping by Means of Absorption-Elution

Washing of the Threads. After observation on microprecipitation reaction in the anti-human-HbA_0-agarose plate, the threads were picked up with a sharp pincer and distributed to each of the test tubes (1.5 × 10 cm). The tubes were put into a water bath at 56°C and filled with saline at 56°C. The supernatant was aspirated and emptied carefully. The washing of the threads was repeated three times, and for this washing a total of 30 min was required. After complete removal of saline in the tubes, they were heated to fix hemagglutinogens on the threads in an air-oven at 100°C for 15 min.

Sensitization with Hemagglutinins. The heated tubes were cooled to room temperature, and each two drops of anti-A, anti-B, anti-HU, and anti-HC sera were added to the respective tubes. All tubes were kept at room temperature for 1 h and then at 4°C for 2 h.

Elution. After removing the unabsorbed agglutinins, the threads in the tubes were washed five times by a jet of cold saline from a washing bottle. After removal of saline of the last washing, two drops of normal saline were added to the tubes and warmed in a water bath at 56°C for

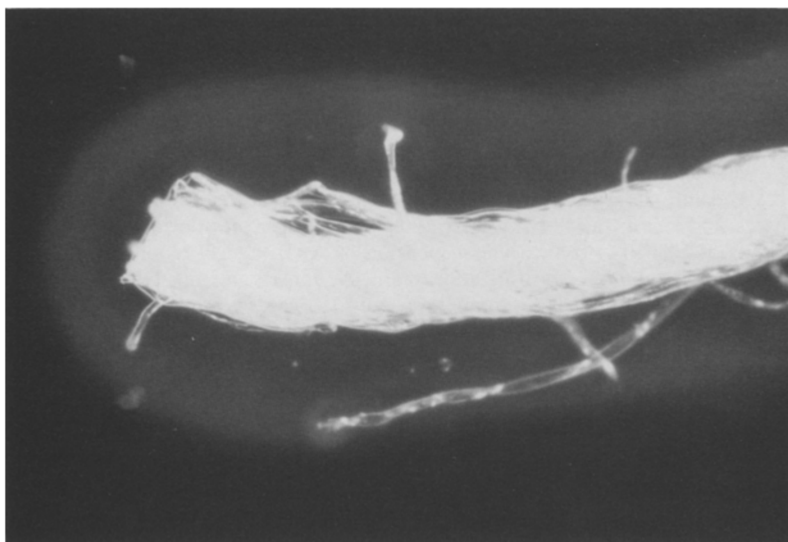

Fig. 1. Positive microprecipitation reaction around a thread stained with 1:80 diluted human blood. × 100

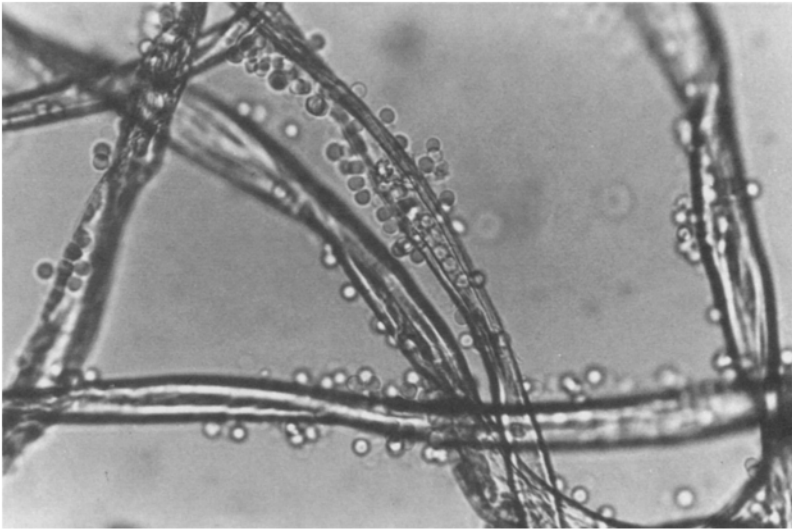

Fig. 2. Positive hemagglutination reaction of fibrils stained with 1:80 diluted human blood of group B in the modified mixed agglutination using anti-B serum and erythrocytes of group B. ×200

10 min. While the threads were warm, they were transferred separately to each of the flat-bottom wells (16 mm in diameter, 10 mm in depth) of a plastic hemagglutination plate with 80 wells (Tomy Seiko Co.) for the last test.

Hemagglutination. One drop of 0.5%–1% erythrocyte suspension of the A, B, and O blood groups each was added to the respective tubes holding the eluate only. The tubes were shaken and centrifuged for 1 min at 1,000 rpm (about 150 *g*), and agglutination was observed with a magnifying mirror.

ABO Blood Grouping by Modified Mixed Agglutination

The threads in the wells of the hemagglutination plate were carefully unfastened into fibrils with two dissecting needles so as to rub the surface of the fibrils as little as possible. The wells holding fibrils were filled with normal saline at a time, and only the saline was carefully removed from the wells using a thin pipette. The fibrils were sensitized with two drops of anti-A, anti-B, anti-HU, and anti-HC, respectively, for 2 h at room temperature. After removal of uncombined antisera and lectin, the fibrils were washed three times with normal saline in the wells. Three drops of 5%–10% erythrocyte suspension of A, B, and O blood groups were added to the wells holding the fibrils sensitized with anti-A, anti-B, and anti-HU or anti-HC, respectively. The plate was kept at room temperature for 2 h. After removal of free erythrocytes by gentle washing with normal saline, hemagglutination on the fibrils was observed with a microscope (Fig. 2).

Results

Identification of Human Blood by the Microprecipitation Method

Threads with human blood diluted up to 1:10 showed positive microprecipitation within 15 min after applying the threads on the anti-human-HbA_0-agarose

Table 1. The detection limits of human blood from a thread stained with diluted human blood by microprecipitation

Blood stains of	Blood stains of groups of			
	O	A	B	AB
Cotton	1:640	1:640	1:640	1:640
Polyester	1:640	1:640	1:640	1:640

Table 2. The detection limit of ABH hemagglutinogens of a thread stained with diluted human blood by modified absorption-elution test

Blood stains of	Sensitized with	Blood stains of groups of			
		O	A	B	AB
Cotton	anti-A	–	1:80	–	1:80
	anti-B	–	–	1:160	1:80
	anti-HU	1:5	1:1	1:1	–
	anti-HC	1:5	1:1	1:1	–
Polyester	anti-A	–	1:20	–	1:10
	anti-B	–	–	1:20	1:10
	anti-HU	–	–	–	–
	anti-HC	–	–	–	–

plate. If the reaction appears strongly, it can be observed with the naked eye. On threads with 1:160 ~ 1:320 diluted blood, positive reactions were shown within 1 h and the reaction of threads with 1:640 diluted blood appeared in a period from 3 to 24 h (Table 1). Blood stains with 1:1,280 or more diluted blood did not show the reaction. In control tests, threads without human blood or with each of the animal bloods of Japanese monkey, canine, bovine, horse, pig, sheep, cat, rat, chicken, and goose did not show a positive reaction even after 2 days.

ABO Blood Grouping by the Modified Absorption-Elution Test

Determination limits of ABO blood grouping of the threads are presented in Table 2. A and B hemagglutinogens of cotton threads (0.5 cm in length) with 1:80 ~ 1:160 diluted blood and polyester ones with 1:10 ~ 1:20 could be correctly determined. H hemagglutinogen of cotton threads with 1:1 ~ 1:5 diluted blood was detected, but not polyester threads.

ABO Blood Grouping by the Modified Mixed Agglutination

The test was performed on the same threads as those that had been tested by both the microprecipitation and the modified absorption-elution test. Determi-

Table 3. The detection limits of ABH hemagglutinogens of a thread stained with diluted human blood by modified mixed agglutination

Blood stains of	Sensitized with	Blood stains of groups of			
		O	A	B	AB
Cotton	anti-A	–	1:1,280	–	1:1,280
	anti-B	–	–	1:2,560	1:1,280
	anti-HU	1:160	–	1:1	1:1
	anti-HC	1:1,280	1:20	1:40	1:20
Polyester	anti-A	–	1:320	–	1:320
	anti-B	–	–	1:640	1:320
	anti-HU	1:5	–	–	–
	anti-HC	1:80	1:10	1:10	1:5

nation limits of the blood group of cotton and polyester threads were 1:1,280 ~ 1:2,560 diluted blood and 1:320 ~ 1:640, respectively, as shown in Table 3. Determination limits of H hemagglutinogen of cotton threads with group O blood was 1:1,280 by the test with anti-HC and eight times sensitive in blood dilution than the limit tested with anti-HU. A, B, and H hemagglutinogens of cotton threads could be more sensitively detected than those of the polyester threads.

In the serial examination of human origin and the blood grouping by the modified absorption-elution test or mixed agglutination followed the micro-precipitation, the determination limits of the blood groups decreased one step in two-fold dilution of the blood in comparison with the limits obtained by a single test of the blood grouping.

Discussion

Immunoelectrosyneresis [10, 11] and anti-human globulin inhibition test [12, 13] are frequently used for identification of human origin of doubtful blood stains. In this paper, human blood was identified by the microprecipitation method which is able to detect several pg of hemoglobin in a single erythrocyte [14]. In the serial examination, a preceding test should not disturb the following ones with the appearance of non-specific reactions or reduction of sensitivity. Heterohemagglutinin was scarcely or not detected in goat anti-human-HbA_0 serum for the microprecipitation, and no influences on the blood grouping were observed. Moreover, goat anti-human-HbA_0 serum attached to the threads in the microprecipitation method was completely removed after washing of the threads with normal saline at 56°C.

Determination limits of the blood grouping in the continuous examination reduced in one step in two-fold blood dilution as compared with a limit obtained by a single blood grouping. This was caused by the fact that heat-unfixed

threads were used for human origin identification and washed with warm saline to remove the goat anti-human-HbA_0 serum. The reduction of the determination limits of the blood grouping is not so important in comparison with the blood grouping on a blood stain without human origin identification. The reduction of the grouping limits is a small matter because the modified mixed agglutination is a very sensitive test and its determination limits are above the limits of human origin identification by the microprecipitation method. In the blood grouping, however, it is better that a more sensitive test, i.e., the modified mixed agglutination, follows the modified absorption-elution test.

It is known that preliminary screening tests, e.g., tetramethylbenzidine test or leukomalachite green test, inhibit serologic reactions for human blood identification and ABO blood grouping of a blood stain. Katsura et al. [15] reported that doubtful minute blood stains recognized with the naked eye should be tested directly by the microprecipitation method without preliminary screening tests because the sensitivity of the microprecipitation method exceeds the recognition limits of doubtful blood stains with the naked eye.

The time required in the continuous examination in this paper was 10 h in general which is not much for scientific crime research examination. The most important advantage of the serial examination is that determination of ABO blood group is carried out by two blood grouping tests on the doubtful blood stain identified as human origin. The blood group of the stain is more correctly determined by a series of examinations in comparison with independent tests.

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