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Application of ELISA-ABC method to the identification of minute human bloodstains

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Summary. Bloodstained threads (1 cm in length) were tested to identify human origin by a direct ELISA-ABC method using biotinylated antibody against human HbA_0. By this method human bloodstains were clearly distinguishable from bloodstains of other species including Japanese monkey. The minimum detection limit of bloodstains prepared from undiluted human whole blood was 1:5, 120 (28 ng Hb) and that of bloodstains from diluted human whole blood was 1:640 ~ 1:1, 280. Human Hb was more easily detectable in bloodstains prepared from diluted human blood after extraction with 5% ammonia than after extraction with phosphate-buffered saline.

Key words: Bloodstains – Human origin identification – Anti-HbA_0 – ELISA – Avidin-biotin complex (ABC) system

Zusammenfassung. Es wurde eine direkte ELISA-Methode unter Verwendung des Avidin-Biotin-Komplexes zur Identifizierung von menschlichem Blut an einem verdächtigen Blutfleck (ein Faden mit einer Länge von 1 cm) angewandt. In dieser ELISA-ABC-Methode wurde ein Biotin-gebundener IgG-Antikörper (Ziege) gegen Human-HbA_0 verwandt. Mit dieser Methode war ein menschlicher Blutfleck von dem Blutfleck japanischer Affen und anderer Tiere sehr leicht unterscheidbar. Die minimalen Grenzen der postiven Reaktionen erwiesen sich beim Blutfleck mit unverdünntem Blut als 1:5, 120 (28 ng Hb) verdünnte Extraktionsflüssigkeit und beim Blutfleck mit verdünntem Blut als 1:640 ~ 1:1, 280 verdünntes Blut. Der Nachweis von Menschen-Hb im Blutfleck mit verdünntem Blut wurde mit 5% Ammoniak-Extraktionsflüssigkeit leichter erbracht als mit Flüssigkeit des Phosphatpuffersalzes.

Schlüsselwörter: Blutfleck – Menschenblutidentifizierung – Anti-HbA_0 – ELISA – Avidin-Biotin (AB)-Komplex-System

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Introduction

The ELISA technique, first introduced by Engvall and Perlmann [1], is very sensitive, and several systems have been applied to studies in forensic medicine. Almost all the ELISA methods for species identification of bloodstains are indirect ELISA procedures using a primary antibody against human serum proteins [2–7]. As human secretions (e.g., saliva, semen, sweat, etc.) contain serum proteins, false-positive reactions can cause problems when these ELISA methods are used. Moreover, positive reactions are observed with bloodstain extracts from mice and rats, because an enzyme-labelled sheep anti-mouse IgG is used as the secondary antibody [3]. The indirect ELISA methods are also time-consuming. In a previous study [8], a new ELISA-ABC method was reported using biotinylated antibody against human HbA_0, in which the avidin-biotin complex (ABC) system [9] was adopted in place of the secondary antibody. Consequently, it was possible to make a clear distinction between human blood and blood of other species, and only 3 h was necessary to obtain the results. In the present study, the ELISA-ABC method was applied to the identification of minute human bloodstains and the detection limits of human Hb extracted with various buffers were compared. The present ELISA-ABC method can be useful for the direct determination of human origin from minute bloodstains which become progressively insoluble due to Hb denaturation.

Materials and methods

Biotinylated antibody against human HbA_0. A biotin-labelled goat antibody against human HbA_0 was prepared according to the method described previously [8].

Bloodstains and bloodstain extracts. Human blood samples (haemoglobin levels 14.1 ~ 16.9 g/100 ml) were obtained from six normal adults and diluted with aqua dest. (1:10 ~ 1:5, 120). Hb concentrations were measured spectrophotometrically and calculated using a standard curve of pure Hb (Acuglobin, Ortho Diagnostic

Table 1. Species specificity of the ELISA-ABC method using a biotinylated antibody against human HbA_0

Bloodstain source	Highest and lowest OD values by ELISA-ABC-PO method				Highest and lowest OD values by ELISA-ABC-AP method			
Human	1.683	(1:10)[a]	0.300	(1:10,240)	1.144	(1:80)	0.085	(1:10,240)
Japanese monkey	0.176	(1:1)	0.024	(1:5,120)	0.076	(1:10)	0.010	(1:1,280)
Dog	0.102	(1:1)	0.007	(1:10,240)	0.045	(1:1)	0	(1:640)
Cat	0.089	(1:1)	0.005	(1:10,240)	0.060	(1:1)	0	(1:640)
Rat	0.183	(1:1)	0.005	(1:5,120)	0.055	(1:10)	0	(1:640)
Rabbit	0.083	(1:10)	0.007	(1:5,120)	0.042	(1:80)	0	(1.:1,280)
Cow	0.032	(1:1)	0.005	(1:5,120)	0.022	(1:1)	0	(1:640)
Horse	0.052	(1:1)	0.005	(1:5,120)	0.027	(1:1)	0	(1:640)
Pig	0.050	(1:1)	0.016	(1:5,120)	0.022	(1:1)	0	(1:640)
Goat	0.023	(1:640)	0.001	(1:10)	0.005	(1:1)	0	(1:1)
Chicken	0.158	(1:1)	0.014	(1:5,120)	0.066	(1:1)	0	(1:320)
Goose	0.175	(1:1)	0.015	(1:5,120)	0.065	(1:1)	0	(1:320)
Dove	0.170	(1:1)	0.015	(1:5,120)	0.055	(1:1)	0	(1:320)
Blank (PBS)	0.053				0.078			

[a] Dilutions of bloodstain extracts in parentheses

System, USA). Diluted or undiluted blood (1 µl) was applied to a cotton thread (No. 3/0) 1 cm in length, allowed to dry at room temperature and stored for 1 week at room temperature. Bloodstains of 12 species of animals were also prepared in a similar manner.

Bloodstains were extracted in the following buffers; 0.012 *M* phosphate-buffered saline (PBS, pH 7.2), 0.06 *M* veronal buffer (pH 8.6), 0.1 *M* carbonate buffer (pH 9.5) and 5% ammonia solution (pH 11.6). Bloodstained threads were extracted in 100 µl buffer and stirred using a thin glass rod until the thread was separated into fibrils. The extract was serially diluted with the extraction buffer to dilutions of 1:10 ~ 1:10, 240.

Reagents and buffers. Reagents and buffers used in this study were as reported in a previous study [8].

Standard ELISA-ABC procedure. The wells of flat-bottomed microplates (Nunc-Immuno Plate; Nunc, Denmark) were coated with 100 µl bloodstain extract for 15 min at room temperature. After discarding the supernatant 200 µl blocking solution was added to each well and left at room temperature for 15 min. The plates were washed three times with washing buffer using an Immuno Washer NK-100 (Inter Med, Japan), 100 µl biotinylated anti-human HbA_0 solution (diluted 1:100) was added to each well and incubated for 15 min at 37°C followed by 15 min at room temperature.

After washing ten times, 1 drop of ABC reagent (ABC-PO system) or ABC-AP reagent (ABC-AP system) was added to each well and incubated for 15 min at room temperature. The plates were again washed ten times and development was carried out in the dark with 100 µl PO substrate solution for 15 min or AP substrate solution for 30 min. The ABC-PO reaction was terminated by the addition of 100 µl 1 *M* H_2SO_4 and the optical density (OD) was measured at 492 nm using an EIA reader (Titertek Multiskan MCC; Flow Laboratories, Finland); for the AP substrate, 100 µl 1 *M* NaOH was added to stop the reaction and the absorbance was measured at 405 nm.

A reagent 'blank' value was automatically subtracted from all sample readings. A positive result was recorded at OD values greater than 0.5 for the ABC-PO and greater than 0.2 for the ABC-AP system.

Results

Human bloodstain extracts yielded positive reactions in dilutions up to 1:5, 120 (28 ng Hb) by the ELISA-ABC-PO method (OD values 0.531 and 0.562). All animal bloodstain extracts, including Japanese monkey, gave negative reactions with OD values less than 0.183. The highest and lowest OD values obtained from PBS extracts of human and animal bloodstains are listed in Table 1. Figure 1 shows the results obtained from the PBS extracts of human and animal bloodstains by the ELISA-ABC-PO method. Similar results were also obtained from the 5% ammonia extracts.

Whole bloods of human, Japanese monkey and dog were serially diluted with aqua dest. to concentrations ranging from 1:10 to 1:5, 120, and 1 µl of each dilution was applied to a cotton thread 1 cm in length. Bloodstains made from diluted human blood were extracted with 100 µl extraction solution and tested by the ELISA-ABC-PO method. As shown in Fig. 2, slightly higher OD values were obtained from bloodstains at dilutions 1:20 ~ 1:320 after extraction with 5% ammonia. The minimum detection limits with PBS or veronal buffer

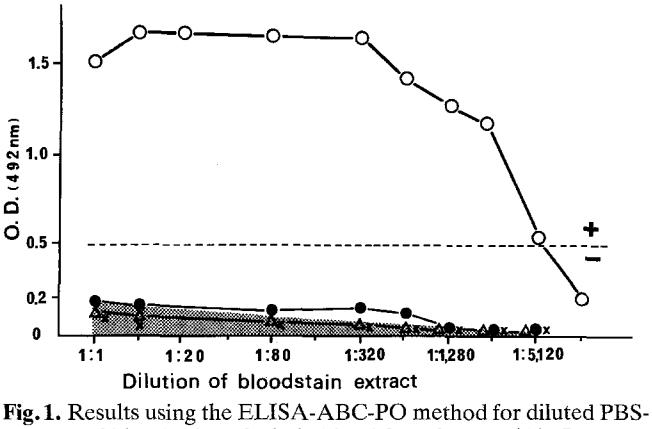

Fig. 1. Results using the ELISA-ABC-PO method for diluted PBS-extracts of bloodstains of whole blood from human (○), Japanese monkey (●), dog (△), cat (X), and other animals (▨), i.e., rat, rabbit, cow, horse, pig, goat, chicken, goose, and dove

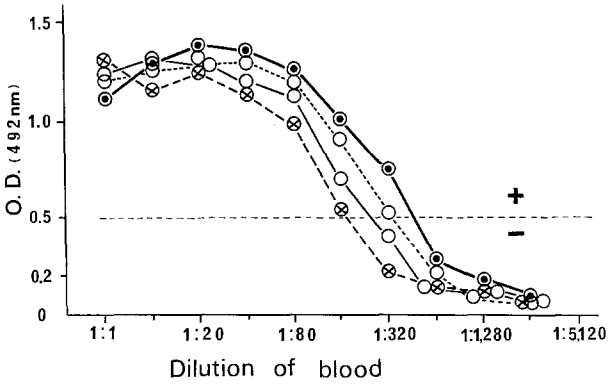

Fig. 2. Differences in OD values in PBS (⊗---⊗), veronal buffer (○——○), carbonate buffer (○-----○) and 5% ammonia (◉——◉) extracts of bloodstains of diluted human blood

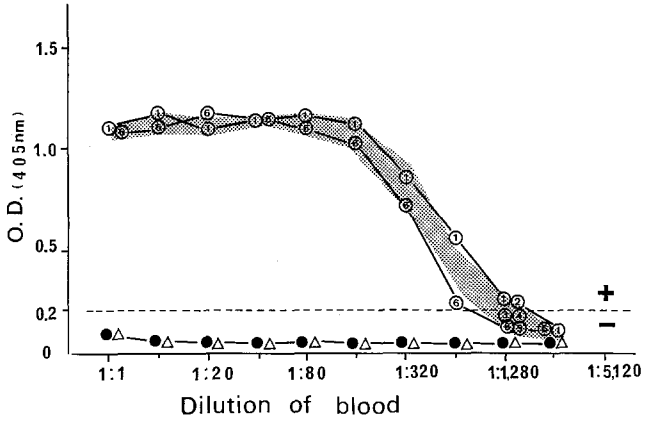

Fig. 3. Results using the ELISA-ABC-AP method for 5% ammonia extracts of bloodstains of diluted bloods from six normal adults (donor 1, ① 16.9 gHb/100 ml; donor 2–5 ▨ 16.5 ~ 15.6 gHb/100 ml; donor 6, ⑥ 14.1 gHb/100 ml), Japanese monkey (●), and dog (△)

were 1:160 (0.88 μg Hb) and with carbonate buffer or 5% ammonia solution 1:320 (0.44 μg Hb).

Bloodstains prepared from diluted blood from six normal adults (16.9 ~ 14.1 gHb/100 ml), Japanese monkey and dog were extracted with 5% ammonia solution and tested by the ELISA-ABC-AP method (Fig. 3). All human bloodstain extracts showed positive reactions at dilutions up to 1:640 (0.22 μg), and two samples (nos. 1 and 2) out of six yielded positive reactions at 1:1, 280 (0.11 μg Hb). All animal bloodstain extracts gave negative reactions with OD values lower than 0.064.

Discussion

Almost all ELISA methods for species identification of bloodstains are indirect ELISA procedures using antisera or antibodies against human serum proteins as the primary antibody [2–7], and almost all are time-consuming. The ELISA-ABC method described here uses biotinylated antibody against human HbA_0, and ABC or ABC-AP reagent instead of the enzyme-labelled secondary antibody. A positive reaction is therefore a direct indication of human blood, because the biotinylated IgG antibody against human HbA_0 does not react with human sera and bloods of other animals including Japanese monkey [8]. In fact, the difference in OD values was so significant that human bloodstains were clearly distinguishable by the present ELISA-ABC method (Table 1). Minimum OD values for 0.5 (ABC-PO) and 0.2 (ABC-AP) were established on the following bases. The colour of the postive reactions at these OD values was easily recognized with the naked eye and the values were at least twofold greater than the highest OD values from animal bloodstains (ABC-PO rat: 0.183; ABC-AP Japanese monkey: 0.076). As human blood specificity is of primary importance in medico-legal testing of bloodstains, faint or weak colour development (OD value 0.3 ~ 0.4 for ABC-PO and 0.15 for ABC-AP) from human bloodstains was regarded as inconclusive.

In addition to the high specificity for human blood, the sensitivity of this method is superior to that of the inhibition ELISA method, in which anti-human Hb serum was used as the primary antibody (range 1 ~ 10 μg Hb) [10]. By the ELISA-ABC method, positive reactions were obtained from bloodstain extracts at dilutions up to 1:5, 120 and the minimum detection limit of human Hb was 28 ng. The detection limits of Hb from human bloodstains were similar for some extraction solutions by both ELISA-ABC methods using the ABC-PO or ABC-AP system.

In bloodstains made from diluted human blood, human Hb was more sensitively detected with 5% ammonia extracts than with PBS and the ELISA-ABC-AP method proved to be more sensitive than the ELISA-ABC-PO method. By the ELISA-ABC-PO method the minimum detection limits from bloodstains of diluted blood were 1:160 for the PBS or veronal buffer and 1:320 for the carbonate buffer or 5% ammonia (Fig. 2). By the ELISA-ABC-AP method the minimum detection limits of 5% ammonia extracts of bloodstains from diluted bloods of six normal adults ranged from 1:640 to 1:1, 280 (Fig. 3). Nevertheless, the detection limit in human bloodstains of the diluted blood was lower than that of bloodstains of undiluted human whole blood. This could be due to easier adhesion of diluted blood to cotton thread and/or to denaturation or progressive insolubilization of a dilute Hb on drying. Similar results have also been found by a microprecipitation method [11] in which human Hb was detectable in picogram quantities [12].

According to Kind and Cleevely [13] and Chism [14], 5% aqueous ammonia solution was very useful for extraction of ABO blood group antigens from old bloodstains. In the present study it was also found that 5% ammonia was suitable for the extraction of Hb from faint bloodstains. These results encouraged the development of a new ELISA-ABC method for the simulataneous identification of human origin and ABO blood grouping from minute bloodstains. The ABC technique allows a short incubation time owing to the extraordinarily high affinity between biotin and avidin [15]. It takes only 3 h to identify human blood by the ELISA-ABC method.

Thus, the ELISA-ABC method described here seems to have a practical application for criminal examinations.

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