TECHNICAL NOTE

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The Identification of Human Bloodstains by Means of a Micro-Thin-Layer Immunoassay Procedure

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ABSTRACT: A method for the identification of human bloodstains using a micro-thin-layer immunoassay (TIA) procedure is presented. The equivalent of approximately 0.01 μL of human blood may be detected using this method with anti-human hemoglobin. Blind studies and stability studies indicate that the method is sufficiently specific and sensitive to be of forensic science value.

KEYWORDS: pathology and biology, blood, immunoassay, human bloodstain identification, anti-human hemoglobin

The use of thin-layer immunoassay (TIA) as a method of immunological assay has been described in a series of reports [*1-4*]. The method allows antigen-antibody reactions to occur on plastic surfaces and to be visualized in situ with water vapor. The identification of human bloodstains by means of TIA performed on polystyrene surfaces has been described previously [*5*]. In this report we describe a micro-TIA procedure on polyvinyl chloride (PVC) for the identification of human bloodstains with anti-human hemoglobin serum (AHHb).

Materials and Methods

Lyophilized AHHb containing 0.1% sodium azide was obtained from Miles Laboratories and Cappel Laboratories. The AHHb was reconstituted according to the supplier's directions. All subsequent dilutions were made with saline.

Flat bottom, flexible polyvinyl chloride Cooke Microtiter® plates containing 96 wells were used.

Samples of human, dog, and rabbit whole blood were diluted (1:10) with water. The resultant mixtures were centrifuged and all subsequent dilutions of the supernatant were made with water. Two series of human, dog, and rabbit bloodstains were prepared: 5 μL of blood was applied to filter paper, and 5 μL of blood was applied uniformly to a cotton thread 50 mm in length. All samples were dried thoroughly with cold air and then stored either at room temperature (22 to 25°C) or at −20°C for periods from 1 h to five months prior to use.

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Cat, cow, dog, horse, and sheep bloodstains used in one of the blind studies had been stored at −20°C for approximately six months prior to their use.

Semen and saliva stains were prepared by applying these fluids to filter paper and drying them thoroughly with cold air. Portions (5 by 5 mm) of these stains were analyzed within 24 h of their preparation.

Stain Extraction

Three different extraction procedures were used depending on the study being conducted:

(1) Stains prepared with a known volume of either human, dog, or rabbit blood (5 μL) or 5- by 5-mm semen and saliva stains were extracted with 500 μL of saline for 1 h. Aliquots of the resulting bloodstain extracts as well as 1:10 and 1:100 dilutions of these extracts were analyzed. Extracts of the semen and saliva stains were not diluted for analysis.

(2) Stains containing an unknown amount of either human or heterologous animal blood were extracted with ten drops of saline until a pale yellow extract was produced. This extract was then analyzed.

(3) Stained threads, 0.5, 1, 2, and 4 mm in length, were extracted in 500 μL of saline for 1 h. Aliquots of these extracts were analyzed.

Blind Studies

Two blind studies were conducted. In the first, 1- by 1-mm bloodstains were extracted by means of stain extraction Procedure 2 and in the second, 1-mm bloodstained threads were extracted by means of stain extraction Procedure 3. In both studies, these extracts as well as extracts of unstained substrate material were assigned a code number and submitted to an analyst who was told nothing concerning the nature of the samples to be analyzed.

Procedure

Approximately 0.1 mL of the sample to be analyzed (bloodstain extract, bloodstain extract dilution, or whole blood hemolysate dilution) was incubated in a square-bottom polyvinyl chloride well for 2 min at room temperature to produce the antigen monolayer. Following the incubation approximately 0.1 mL of water was added to the well and removed immediately with gentle suction. This washing procedure was repeated three additional times in rapid succession, after which the monolayer surface was dried thoroughly with gentle suction and a stream of cold air.[2] The monolayer surface was not dried thoroughly until after the final wash. Following the formation of the antigen monolayer, approximately 0.1 mL of bovine serum albumin (BSA), 44 mg/L, was incubated for 2 min at room temperature. Following this incubation the well was washed four times and dried as described above. A 3-μL sample of AHHb was then applied to the monolayer surface and incubated for 15 min at room temperature. Following this incubation the plate, with the AHHb application still in place, was inverted and the monolayer surface was exposed to water vapor at 60°C (±2°C) for 1 min, after which the well was washed four times and dried as before. Visualization of the antigen/antibody reaction was achieved by exposing the exterior bottom surfaces of the wells to water vapor at 60°C (±2°C) for 15 s, after which the wells were inverted immediately and the monolayer surfaces exposed to water vapor at 60°C (±2°C) for 1 min. The monolayer surface was then viewed microscopically. Generally, 15 to 30 samples were analyzed simultaneously.

[2]The following alternate washing procedure may be used. Fill the wells with water and then immediately remove the water by inverting the plate and flicking it. Repeat three times, and then dry the monolayer surface thoroughly with cold air.

Results and Discussion

The results of the TIA analyses of known dilutions of human, dog, and rabbit whole blood hemolysates are presented in Table 1. These data demonstrate that both Miles and Cappel undiluted AHHb cross-reacted to a significant degree with dog and rabbit blood. However, the cross-reactivity was minimized or eliminated when dilutions of AHHb were used. These data were used to determine the greatest AHHb dilutions that met the following criteria: the highest titer against human blood and the greatest difference in titer between the human and heterologous animal bloods. Based on these criteria the following dilutions were selected for further evaluation with bloodstains: Miles AHHb, 1:2, 1:4, and 1:8; Cappel AHHb, undiluted, 1:2, and 1:4.

Table 2 contains the data obtained from the analysis of stain extracts and dilutions prepared from 5-μL bloodstains by stain extraction Procedure 1. These data demonstrate a titer and specificity of the AHHb dilutions similar to those observed in the analysis of the whole blood hemolysates. The titer of each AHHb dilution tested was one to two orders of magnitude greater for human blood than for either of the heterologous species, indicating that the specificity of the reaction was improved by using appropriate dilutions of AHHb and

TABLE 1—*Titer expressed as the reciprocal of the highest dilution of whole blood hemolysates giving positive results with the corresponding AHHb dilution.*

Blood	Undiluted	1:2	1:4	1:8	1:16
		MILES SERUM			
Human	10 000	10 000	10 000	10 000	NR[a]
Dog	1 000	100	NR	NR	NR
Rabbit	10	10	NR	NR	NR
		CAPPEL SERUM			
Human	10 000	10 000	100	10	10
Dog	100	10	10	NR	NR
Rabbit	10	NR	NR	NR	NR

[a]NR = no reaction at the lowest blood dilution tested, that is, 1:10.

TABLE 2—*Titer expressed as the reciprocal of the highest dilution of bloodstain extracts giving positive results with the corresponding AHHb dilution.*

Blood	Undiluted	1:2	1:4	1:8
		MILES SERUM		
Human	NT[a]	100	100	100
Dog	NT	10	UN[b]	NR[c]
Rabbit	NT	UN	NR	NR
		CAPPEL SERUM		
Human	100	10	10	NT
Dog	UN	UN	NR	NT
Rabbit	NR	NR	NR	NT

[a]Not tested.
[b]Undiluted stain extract, that is, 5-μL bloodstain, extracted with 500-μL of saline.
[c]No reaction with the lowest dilution used, that is, undiluted stain extract.

bloodstain extracts. Since only an aliquot of the bloodstain extract dilutions (approximately 0.1 mL) was used for analysis, the positive results obtained with the 1:100 dilution of these extracts represent the detection of approximately 0.01 μL of human blood by this procedure. Undiluted extracts of 5- by 5-mm semen and saliva stains yielded negative results.

Extraction Procedure 2 was employed for the analysis of 1- by 1-mm human and heterologous bloodstains whose volume was unknown. The pale yellow extract initially prepared by this procedure was assumed, based on visual color comparison, to be approximately equivalent to a 1:1000 dilution of the whole blood hemolysates. Therefore, a 1:10 dilution of these extracts, which produced colorless solutions, was assumed to provide a satisfactory dilution that would eliminate interference from the heterologous species. These

TABLE 3—*Smallest stain[a] giving positive results with the corresponding AHHb dilution.*

	Miles Serum		Cappel Serum	
Blood	1:4	1:8	Undiluted	1:2
Human	0.5	0.5	0.5	0.5
Dog	4	NR[b]	2	4
Rabbit	NR	NR	NR	NR

[a]All values given are thread lengths in mm; stained threads contained approximately 0.1 μL of blood per millimetre.
[b]No reaction with the largest stained thread tested, that is, 4 mm.

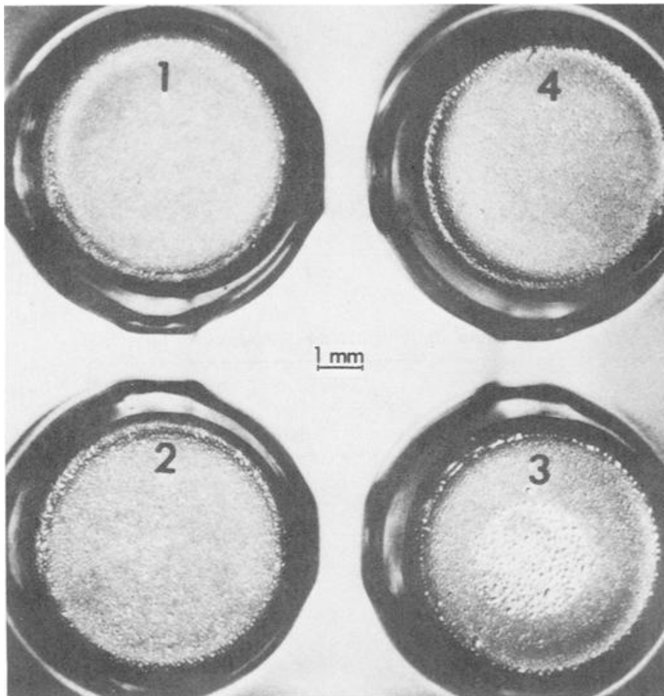

FIG. 1—*Results obtained from the analysis of 0.5-mm threads:* (1) *unstained thread;* (2) *dog;* (3) *human; and* (4) *rabbit. A 1:4 dilution of Miles AHHb serum was used.*

colorless dilutions were analyzed with both Miles AHHb (1:4 and 1:8) and Cappel AHHb (undiluted and 1:2). By means of this procedure, human bloodstains were differentiated from cat, cow, dog, horse, and sheep bloodstains. There were no false-positive or false-negative results.

The results obtained from the analysis of bloodstained threads extracted by Procedure 3 are presented in Table 3. The data demonstrate that human blood was detectable in a 0.5-mm stained thread, which contained approximately 0.05 μL of blood, with all the AHHb dilutions used. Differentiation of human blood from dog and rabbit blood was achieved by the use of small (0.5 to 1 mm) stained threads. Although smaller stained thread samples were not tested, the detection limit for human blood may be estimated as approximately 0.01

TABLE 4—*Blind study with 1- by 1-mm stains.*

	Number of Samples	
Serum	Tested	Identified As Human Blood
Human blood		
Miles	13	13
Cappel	4	4
Cat blood		
Miles	4	0
Cappel	2	0
Cow blood		
Miles	4	0
Cappel	2	0
Dog blood		
Miles	5	0
Cappel	4	0
Horse blood		
Miles	4	0
Cappel	2	0
Sheep blood		
Miles	4	0
Cappel	2	0
Unstained samples		
Miles	11	0
Cappel	5	0

TABLE 5—*Blind study with 1-mm bloodstained threads.*

	Number of Samples	
Serum	Tested	Identified as Human Blood
Human		
Miles	5	5
Cappel	9	9
Dog		
Miles	5	0
Cappel	6	0
Rabbit		
Miles	2	0
Cappel	4	0
Unstained threads		
Miles	4	0
Cappel	7	0

μL since only one fifth of the 500 μL extract of the stain was used for each analysis. Typical results obtained from the analysis of 0.5-mm threads are presented in Fig. 1.

The results of the two blind studies in which more than 120 samples were analyzed are presented in Tables 4 and 5. There were no false-positive or false-negative results obtained in these studies.

Human bloodstains stored at room temperature for 18 months have been identified successfully by the use of TIA.

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References

[1] Elwing, H., Nilsson, L., and Ouchterlony, O., "Visualization Principles in Thin-Layer Immunoassays (TIA) on Plastic Surfaces," *International Archives of Allergy and Applied Immunology*, Vol. 51, No. 4, 1976, pp. 757-762.

[2] Elwing, H., Nilsson, L., and Ouchterlony, O., "A Simple Spot Technique for Thin-Layer Immunoassays (TIA) on Plastic Surfaces," *Journal of Immunological Methods*, Vol. 17, No. 2, 1977, pp. 131-145.

[3] Elwing, H., Nilsson, L., and Ouchterlong, O., "A Precipitate Adsorption on Surface Technique: A Combination of Immunodiffusion and Thin-Layer Immunoassay," *International Archives of Allergy and Applied Immunology*, Vol. 55, No. 1, 1977, pp. 82-85.

[4] Jeansson, S., Elwing, H., and Nilsson, L., "Thin-Layer Immunoassay for Determination of Antibodies to *Herpes simplex* Virus," *Journal of Clinical Microbiology*, Vol. 9, No. 3, 1979, pp. 317-322.

[5] Lappas, N. T., "The Identification of Human Bloodstains by Means of Thin-Layer Immunoassay: A Preliminary Report," *Journal of the Forensic Science Society*, in press.

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