TECHNICAL NOTE

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A Rapid Dot-Blot Method for Species Identification of Bloodstains

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ABSTRACT: A very simple and rapid test for species identification is reported. Extracts of bloodstains were applied to a synthetic porous membrane and dried. The membrane was then quenched with glycine buffered saline containing BSA and Tween 20. A suspension of colloidal gold particles (GP) coated with rabbit antiserum to human IgG was poured onto, gently whirled and aspirated through the membrane. Spots from the human and monkey bloodstains became red, whereas those from other species of animals remained unstained. This test was completed within 3 to 4 min, and the antibody-coated GP reagent was prepared within 20 min using a very small quantity of antiserum. Cellulose acetate membranes of 0.45 μm or more in pore size were appropriate to this test.

KEYWORDS: pathology and biology, human identification, bloodstain, colloidal gold, immunologic staining, immunoblotting

Various test methods have been applied for species identification of biological stains. The classical ring precipitation test of Uhlenhuth [*1,2*] is still being used in many laboratories in Japan. Various immunodiffusion techniques, such as the Ouchterlony test [*3*], electroimmunodiffusion [*4*] and microprecipitin tests on a thin agar film [*5,6*] are routinely used in some laboratories. Precipitation on a synthetic porous membrane [*7*] and the latex agglutination tests [*8*] have also been reported to be sensitive and specific. However, our attempts to obtain results using these techniques required at least 20 to 30 min, even in the fastest cases.

This report describes a very simple and rapid test method for the species identification of bloodstains. This method can be used for many tests in forensic science and clinical laboratories.

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Materials and Methods

Antisera and Bloodstains

Eight antihuman IgG antisera were procured commercially or prepared in our laboratory. The results of this study were obtained with a commercial antiserum (Behringer, imported through Hoechst Japan, Tokyo). Twelve rabbit antisera to human hemoglobin (Hb) prepared in our laboratory were also used. All bloodstains were prepared as follows. Blood containing anticoagulant and blood diluted with water (10 μl) were spotted on filter papers (No. 1, Toyo-Roshi, Tokyo), which were dried and kept completely dry in our laboratory until use. The stains on the filter papers were cut out with scissors and extracted with an appropriate quantity of a glycine-buffered saline containing 0.1% sodium azide (0.1 M, pH 8.3, GBS). The blood from the crab-eating macaque was used as monkey blood.

Purification of Antibody Activity by Absorption of Antiserum

For the purification of antiserum to human IgG, a 1% suspension of heavy latex particles (SDL-59, pore size 0.9 μm, specific gravity 1.12; Takeda Chemical Inc., Osaka) was added to a mixture of 1/50 bovine, 1/50 canine and 1/100 chicken serum. After standing for 2 h at room temperature, the particles were washed three times with GBS by centrifugation at 10 000 rpm and resuspension. The final sediments of the latex particles were mixed with 40 times in volume of the antisera to be absorbed, allowed to stand at room temperature for 30 min, and spun down to collect the transparent supernatants. Antisera to human Hb was absorbed with a mixture of animal sera and animal Hb solutions.

Gold Particles (GP) Coated with Antiserum

A suspension of 0.01% GP, 15 nm in diameter, was prepared by a modification of the method of Frens [9]. Briefly, chloroauric acid (Nakarai, Kyoto) was dissolved in deionized and distilled water, which had been passed through a filter (Toyo-Roshi, pore size 0.2 μm). All vessels were rinsed with the filtered water before use. The 0.01% solution (500 mL) thus prepared was heated directly over a flame until boiling point, 50 mL of 1% trisodium citrate (for Immunogold Silver Staining; Janssen, Belgium) was added, and the mixture was kept boiling for an additional 15 min. The yellow color of the chloroauric acid solution turned light blue-black and then red. The resulting GP colloid solution could be stored for at least 6 months in aseptic conditions at 5°C. We coated the GP by two methods, one of which we called the "standard method" for this study. The 0.01% GP and an appropriate dilution of the antiserum to human IgG (dilutions around 1:640) were mixed in a tube, and allowed to stand overnight at room temperature and for another 2 days at 5°C. This was followed by centrifugation for 30 min at 15 000 rpm. to precipitate the GP. After aspiration of the supernatant, the GP were resuspended in the original volume of fresh GBS and stored at 5°C until use. Precipitate that formed in the GP suspension after storage was removed by filtration through a cellulose acetate membrane of pore size 0.2 μm (Toyo-Roshi). GP were coated with anti-Hb in a similar fashion. The other coating method, "simplified method" was carried out by mixing 0.01% GP suspension with an appropriate quantity of antihuman IgG, and allowing it to stand for over 20 min at room temperature. The quantity of the antihuman IgG immune serum was determined by a series of preliminary experiments. In this study, the 1:350 to 1:500 dilution in GBS was mixed with an equal volume of the 0.01% GP. This reagent mixture was also stored at 5°C.

Synthetic Porous Membrane and Filter Paper

The membranes were supplied by Toyo-Roshi Co. (Tokyo). The synthetic membranes were of nitrocellulose (NC; with pore sizes of 0.1, 0.2, 0.3, 0.45, 0.65, 0.8, 1.0, 3.0, and 5.0 μm), cellulose acetate (CA; with 0.2, 0.45, 0.8, and 3.0 μm), polyether sulfone (0.2 and 0.45 μm) and nylon (0.2 and 0.45 μm). The circles on the membranes were drawn with a ball-point pen. The synthetic porous membrane and thick filter paper were punched using a die of 36 mm in diameter.

Species Identification Test

Blood or other biological stains were extracted in a tube containing the GBS. The extracts (1 to 3 μL) were applied to the membrane using thin capillaries, and were dried spontaneously or by blowing cool to slightly warm air from a hair dryer over them. Hot air was not used because it might have narrowed the pore size of the membrane. To filter paper fitted in an aspiration device as shown in Fig. 1, was added 1.5 mL of a quenching solution (GBS plus 1% bovine serum albumin and 0.1% Tween 20). The membrane was then placed on the wet filter paper. When the membrane had become wet through contact with the filter paper, the components A and B in Fig. 1 were tightened in order to secure the synthetic porous membrane and the filter membrane. Quenching

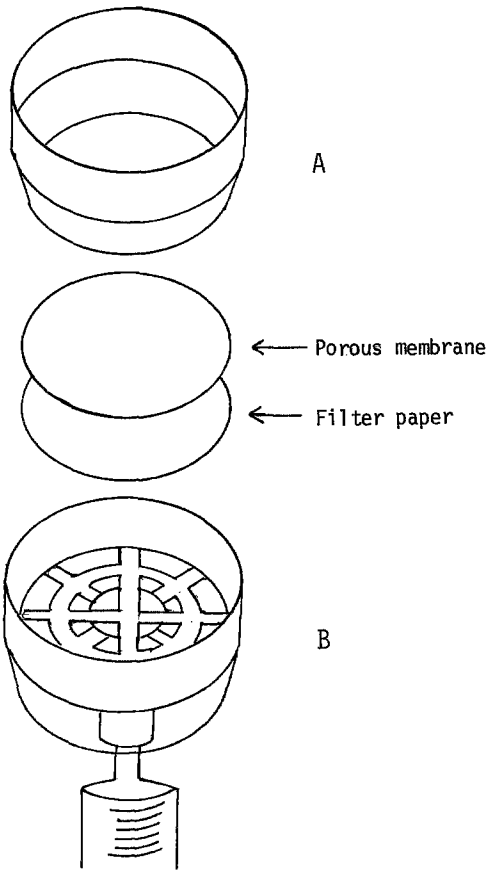

FIG. 1—*Aspiration device with filter paper and synthetic porous membrane.*

buffer (0.5 mL) was then added and aspirated by suction with a disposable syringe connected to the bottom of the aspiration apparatus. The GP suspension coated with antihuman IgG antiserum (1 mL) was poured onto the membrane, and gently whirled for about 30 s followed by slow aspiration. The human bloodstain extracts turned red after this procedure. If needed, the GP suspension in the syringe could be added again to the membrane and again aspirated, and this process repeated two or three times, to improve the coloration of the spots. Finally, fresh quenching solution (about 2 mL) was aspirated to increase the whiteness of the background. The procedure for this test was completed within 3 to 4 minutes; and the results were determined with the naked eye.

Results

Table 1 shows how the detection limit, the whiteness of background and the kinds and pore sizes of the synthetic porous membranes were related. Use of NC membranes with pore sizes of 0.8 μm or more gave a clear background. Sensitivity toward human bloodstains was good when NC membranes were used. The NC membranes nonspecifically adsorbed Hb, although tests with the membranes of large pore sizes prevented the occurrence of this to some degrees. CA membrane fixed proteins weakly, and the extracts diffused to broader areas. The CA membrane did not adsorb Hb, and gave a clear background. Polyethylene sulfone membranes adsorbed proteins very weakly and showed low activity in this test. The backgrounds of nylon membranes underwent strong staining, which was not removed by washing with the quenching solution. When rabbit antisera to human Hb were used, they failed to show either good specificity or sufficient sensitivity with any of these membranes.

Figure 2 presents actual examples of NC and CA membranes stained with antiserum to human IgG.

TABLE 1—*Summarized results of the species identification test with various synthetic membranes.*

Synthetic membrane	Pore size (μm)	Detection[a] limit	Nonspecific adsorption of GP by animal hemoglobin	Nonspecific staining of background
NC	0.1	100	very strong	strong
	0.2	300	very strong	strong
	0.3	1000	strong	moderate
	0.45	1000	strong	moderate
	0.65	1000	moderate	moderate
	0.8	1000	weak	weak
	1.0	1000	weak	weak
	3.0	1000	weak	weak
	5.0	1000	weak	weak
CA	0.2	30	trace	weak
	0.45	100	none	none
	0.8	100	none	none
	3.0	100	none	none
Polyether sulfone	0.2	30	weak	no
	0.45	30	weak	no
Nylon	0.2	100	very strong	very strong
	0.45	100	very strong	very strong

[a]The detection limits are shown by the maximum dilutions of human blood in the prepared bloodstains. Blood specimens were placed on a filter paper and stored for one week at room temperature before extraction with GBS.

TABLE 2—*Specificity of the method using a CA membrane of pore size 5.0 μm.*

Antihuman IgG for coating GP	Results with bloodstain extract[a]										
	Human	Monkey	Dog	Cat	Sheep	Goat	Cow	Horse	Mouse	Chicken	Rabbit
Unabsorbed 1:500 rabbit antiserum	+ + +	+ + +	+ +	+ +	–	–	+ +	+	–	+	–
1:500 rabbit[b] antiserum absorbed with animal serum coated latex	+ + +	+ + +	–	–	–	–	–	–	–	–	–

[a]All blood specimens were diluted to 1:50 to 1:100.
[b]Absorbed with latex particles coated with a mixture of bovine, canine, and chicken sera.

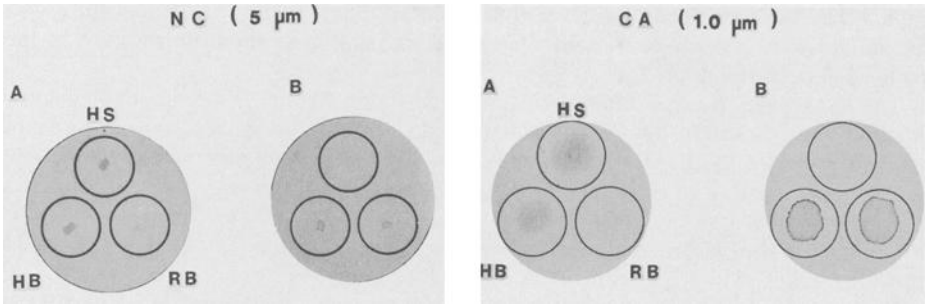

FIG. 2—*Results with CA (pore size 1.0 μm) and NC (pore size 5.0 μm) membranes. Each membrane received 2 μl of 1:10 diluted human serum (HS), 1:10 diluted human bloodstain extract (HB) and 1:10 diluted rabbit bloodstain extract (RB). After staining with antihuman IgG coated GP, followed by washing, stains of HS and HB became red, whereas RB spots wholly or partially lost their hemoglobin color (A). On controls (B) the membranes received the serum and the bloodstain extracts. Neither staining nor washing processes were performed.*

Table 2 shows the specificity of testing by the aspiration method with CA membranes. The human and monkey bloodstains were not differentiated by using GP reagents coated with a purified antihuman IgG antiserum. The bloodstains from other mammalian and chicken bloodstains gave negative reactions.

As Table 3 shows, test results were similar with the reagents prepared by both standard and simplified coating methods. The test reagent made by the simplified coating method was somewhat more sensitive immediately after preparation. The life spans of the GP reagents prepared by the simple and standard coating methods were 6 and over 14 months, respectively.

Discussion

The test method described first in this report resembles the dot immunoblotting technique [*10–13*] because of the use of porous membranes. However, our method is far faster than the immunoblotting method. The sensitivity of the test is sufficient for practical application. Although good results were not obtained with GP coated with antisera to

TABLE 3—*Sensitivity[a] after storage of two GP reagents prepared by the standard and simplified methods, and tested with a CA membrane of pore size 3 μm.*

Storage period	Preparation method	
	Standard	Simplified
1 day	300	1000
1 week	300	300
1 month	300	300
3 months	300	300
6 months	300	300
9 months	300	100
12 months	300	30
14 months	100	10

[a]Shown by the dilution of human serum. These test reagents caused similar degrees of cross-reaction with monkey serum. No cross-reaction occurred with the other animal sera shown in Table 2.

human Hb, we concluded that the use of antisera to human IgG is sufficient for use as an initial, rapid test for bloodstains. No particular skill is required on the part of the technician performing the test.

The test reagent for this study can be prepared within 20 min by the simplified coating method. The detection limit of its reactivity do not differed markedly from those of reagent prepared by the standard sensitization method. CA membranes of 0.45 μm or larger in pore size were concluded to be the best choice for this test. These membranes do not have the drawback of nonspecific adhesion of Hb, although their sensitivity is lower than that of NC membranes. NC membranes are thought to be inappropriate to this test because the nonspecific adhesion of Hb, though very weak, is unavoidable.

The specificity of this test is inferior to that of the agglutination-inhibition test using antigen coated latex particles [*8*], which can differentiate human bloodstains from the bloodstains of the monkey, but is similar to the specificity of methods using antibody coated latex particles, of ELISA and of the ring interfacial test. The principle of the test can be applied to other tests in forensic and clinical laboratories.

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