Mark S. Nelson,[1] *M.S.; Lisa L. Turner;*[1]
and Emily G. Reisner,[2] *Ph.D.*

A Feasibility Study of Human Leukocyte Antigen (HLA) Typing for Dried Bloodstains

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ABSTRACT: This paper constitutes a feasibility report on the use of the human leukocyte antigen (HLA) system for the typing of dried bloodstains. Antigens tested include the HLA-A2, A3, A10, B7, B8, and B14 alleles. An aging study conducted on A3 positive bloodstains showed that HLA-A3 could be reliably detected on bloodstains stored up to 30 days at 22°C. Unlike most earlier reports on HLA typing of bloodstains, no cross-reactivity problems were detected with the antisera used in this study. In addition to the successful typing of bloodstains, we were also able to type fresh, neat seminal and saliva stains in the A2 and A10 antigenic systems.

KEYWORDS: forensic science, human leukocyte antigen, antigen systems, genetic typing

The extreme polymorphism exhibited by the human leukocyte antigen (HLA) system makes it a very attractive system for forensic science use. It is presently being used for parentage determination, but the difficulty of working with blood material other than fresh lymphocytes or platelets has hindered its adaptation to the examination of bloodstained materials.

To date, the feasibility of HLA typing bloodstains has been examined by four groups. Rittner and Waiyawuth [*1*] studied the A1, B7, and A10 antigens using a two-stage extraction and absorption method coupled with cytotoxicity testing. In a later paper [*2*], the same authors recommend the substitution of the microcomplement fixation test for the lymphocytotoxicity method. Newall [*3*] presented a shorter one-stage extraction and absorption method to study the A2 and B5 antigens. A second paper by Newall [*4*] discusses the addition of the A9 antigenic system and describes typing seminal stains. Hodge et al [*5*] concentrated on testing only the A1 antigenic system. Lotterle [*6*] tested the A1, A3, A9, B7, and B8 antigens. All authors reported some degree of problems with cross-reactivity in the HLA system. Many performed aging studies and reported an upper limit of acceptable results ranging from 30 [*1*] to 230 days [*5*].

This report deals with a feasibility study undertaken by the North Carolina State Bureau of Investigation (NCSBI) and the HLA Laboratory of Duke University Medical Center to determine how HLA testing could best be used and organized to provide reliable evidence in the identification of bloodstains and stains of other body fluids.

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[1]Forensic scrologist and intern, respectively, North Carolina State Bureau of Investigation, Raleigh, NC.

[2]Director, HLA Laboratory, Duke University Medical Center, Durham, NC.

Materials and Methods

Collection of Blood Samples

Fresh blood samples were collected from consenting individuals, whose HLA types were known, by venipuncture into heparinized vacutainer tubes. Bloodstains were prepared by pipetting whole blood onto clean white cotton sheeting which was then allowed to air dry. Case samples were of material left after routine examination had been completed by the NCSBI laboratory. The stain panel is shown in Table 1.

Preparation of Lymphocytes

Whole blood was diluted volume/volume with barbitol buffer (complement fixation test dilution tablets, Oxoid Ltd., England) and passed through a nylon column designed to remove granulocytes and platelets. Approximately 5 mL of Ficoll-Hypaque (specific gravity 1.077) was carefully pipetted underneath the diluted blood. The blood sample was then centrifuged at 2700 rpm for 20 min and the lymphocyte interface between the plasma and Ficoll-Hypaque layer (which contains the red blood cells) was carefully removed. The lymphocytes were washed with barbitol buffer and then centrifuged at 1100 rpm for 10 min to form a cell button. The supernatant was decanted and the button was resuspended in 0.5 mL of barbitol buffer.

The lymphocyte preparation was then examined in a hemacytometer and the concentration was adjusted to between 2×10^6 and 2.5×10^6 cells/mL with at least 80% of the lymphocytes being viable. A minute amount of 1% trypan blue was added on a wooden applicator stick to make the lymphocyte preparation visible on the microtest plates [7].

Antisera

Seven antisera were used in these experiments:

LS	anti A10 (25 and 26)
VB	anti B8
JM	anti B8
GR	anti A2
LK	anti B14
DAL	anti B7
WMC	anti A3

The first five antisera were from multiparous women; DAL and WMC were from volunteers immunized before 1970. All seven antisera have been extensively tested and screened to insure specificity. All sera were recalcified plasma and were stored at −20°C.

HLA Typing of Bloodstains

Bloodstains were typed via the one-stage extraction and absorption technique described by Newall [3] with several modifications. A 2-mm square of bloodstained material was placed in each of three tubes. Ten microlitres of neat antisera were added to the first tube, the next tube received 10 μL of antisera diluted 1:2 by volume with barbitol buffer, and the last tube received 10 μL of antisera diluted 1:4 by volume with barbitol buffer. The stains were incubated for 3 to 4 h at 4°C. After incubation, 1 μL of the supernatant was removed from each tube and placed into separate wells on a microtest plate.

In addition, 1 μL of the neat, 1:2, and 1:4 antisera was added to separate wells on the microtest plate to serve as a titer control. ALG, a positive control designed to lyse all lym-

TABLE 1—*Stain panel.*

Stains	HLA Antigens	
	A	B
SS	3	7
BF	2,3	8,14
MM	3	8
MA	1,2	8,12
ER	1,w31	12
TK	2,w24	40,63
LH	28,29	w44,40
MN	2,26	7
DS	1,2	8,27
GG	3,w24	w49,27
EB	3,w24	7
AS	1,2	5,7
HR	1	7.17

phocytes, was added to a separate well on each plate and 1 μL of barbitol buffer was also added to a well on the microtest plate to serve as a negative control.

After adding the unabsorbed antisera, the controls, and the bloodstain supernatants to the microtest plate, 1 μL of the lymphocyte preparation was added to each well. The plate was then agitated to mix the lymphocytes with the antisera and controls and the plate was incubated for 30 min at 22°C. Each well was filled with barbitol buffer in a wash stage, and left for 10 min to allow the white cells to settle. The plates were then "flicked" to remove the antisera and buffer from the wells.

Next 5 μL of cold rabbit complement was added to each well and the test plate was incubated at 22°C for 1 h. After complement fixation, the plates were "flicked" to remove complement and approximately 10 μL of 2% ethylenediaminetetraacetic acid (EDTA)/1% trypan blue were added to each well for 10 min. The EDTA stops the action of the rabbit complement and the trypan blue stains lysed cells. The plates were then "flicked" to remove the EDTA/trypan blue and 5 μL of barbitol buffer was added to each well. Plates were viewed under a microscope at ×150 and cell lysis was scored using the system of Amos et al [7] shown at the bottom of Table 3.

Results and Discussions

HLA antigens were selected for this study by two criteria: (1) that an adequate supply of high titered, monospecific antisera was available in our laboratory and (2) that the antigens have good population discrimination power. These reasons resulted in the selection of the HLA-A2, A3, A10, B7, B8, and B14 antigens. Table 2 gives the phenotype frequencies of each antigen [8].

Although one would like to perform as complete an HLA typing as possible on bloodstains, using only the six antigens tested in this study would give a positive result (that is, at least one of the six positive) in 95% of samples from whites. The panel is not as adequate as it stands for samples from blacks since about 25% of the black population can be predicted to be negative for all six factors. This does show, however, that it is possible to construct a small panel of antisera to "guarantee" positive results.

The HLA-A3 antigen was the primary antigen studied and was examined in terms of method development, cross-reactivity, and aging effects. The results shown in Table 3 are representative samples of the results obtained for this antigen. Interpretation of the results in Table 3 is simplified if one looks at the results for the control titration of the WMC antisera. At neat strength of the antisera 95 to 100% lymphocyte lysis occurs, at a 1:2 dilution

TABLE 2—*HLA phenotype frequencies.*

Antigen	White Populations, %	Black Populations, %
A2	48.0	34.0
A3	23.0	20.0
A10	15.0	6.0
B7	25.0	19.0
B8	22.0	5.0
B14	9.0	5.0

TABLE 3—*HLA A3 antigen.*

Donor	HLA Phenotype		Antisera Dilution[a]		
			Neat	1:2	1:4
MA	A1,2	B8,12	6	4	2
ER	A1,w31	B12	5	4	1
TK	A2,w24	B40,w63	6	5	2
DS	A1,2	B8,27	4	5	3
MN	A2,26	B7	6	5	2
GG	A3,24	B27,w49	2	2	1
MM	A3	B8	2	1	1
SS	A3	B7	2	1	1
BF	A2,3	B8,14	2	2	1
WMC	antisera	...	6	5	1
ALG	positive control	...	6	...	...
BB	negative control	...	1	...	...

[a]Scoring system used:
1 = 0–15% cell lysis,
2 = 15–20% cell lysis,
3 = 20–30% cell lysis,
4 = 30–60% cell lysis,
5 = 60–95% cell lysis, and
6 = 95–100% cell lysis.

60 to 95% lysis occurs, and at a 1:4 dilution only 0 to 15% lysis occurs. One would expect A3 negative bloodstains to react similarly to the WMC antisera control and A3 positive stains to show an inhibition of lymphocyte lysis. Since the HLA-A3 antigen may be cross-reactive with the A1 antigen positive stains, negative stains carrying the cross-reactive antigen A1, and negative stains carrying neither A1 or A3 were tested. In all cases, A3 positive stains showed complete inhibition of the undiluted antisera; A3 negative stains, whether A1 positive or negative, showed little or no inhibition of lymphocyte lysis.

Although cross-reactivity between HLA antigens has been observed in typing fresh lymphocytes and was also reported by investigators testing dried blood stains [*1, 3, 5, 6*] we observed no reactions attributable to cross-reactivity in our studies. We believe we avoided the problem for dried bloodstain testing in exactly the same manner it is prevented for fresh lymphocytes, that is, through the use of selected, carefully characterized reagents. For example, serum WMC, used extensively by our laboratory for clinical testing, has never exhibited cross-reactivity.

Rittner and Waiyawuth [*1*] and subsequent workers have required at least 50% inhibition of the antisera to report a stain as being positive for a given antigen. Using their formula of

$$\% \text{ Inhibition} = 100 - \frac{\% \text{ cells lysed in the presence of antigen}}{\% \text{ cells lysed in the absence of antigen}} \times 100$$

approximately 80 to 85% inhibition occurs in the A3 positive stains. However, we believe that the use of three dilutions produces much clearer and more dramatic results than a calculation of percent inhibition.

Aging studies showed that HLA-A3 antigens can be consistently typed correctly up to 30 days stored at 4 or 22°C (Table 4). After this time, the results could be variable. Positive stains incubated at 37°C for four days before testing gave completely negative results.

Reactions obtained for the HLA-A2, A10, B7, B8, and B14 antigenic systems gave results similar to those shown for the A3 antigens. Representative results obtained for the HLA-B8 antigen are shown in Table 5 to illustrate how interpretation of results can be influenced by the antisera titer. The antisera VB is a rather weak reagent lysing only 20 to 30% of cells when undiluted, JM is a stronger antisera and lyses 60 to 95% of positive cells at neat strength. Although positive and negative stains can be delineated even with the weaker antisera, interpretation is much easier when the antisera is of a higher titer. It should be noted, however, that extremely high titered reagents can give false negatives (that is, show no inhibition by positive stains) if they are not diluted. This point emphasizes the need to use carefully characterized reagents for this procedure.

Representative results obtained for the anti A2 reagent are shown in Table 6 and are included to illustrate the use of a moderately strong antibody that lyses between 60 to 95% of cells when undiluted for blood, semen, and saliva stains. This reagent correctly discriminated between A2 positive and A2 negative stains for these body fluids. Similar results were obtained for blood and seminal stains in the HLA-A10 system.

Only a limited number of actual case samples were tested during the feasibility phase of this research. In general, the results of these tests agree with data previously obtained for other polymorphic systems. However, at this early stage of test development we suggest that only positive results be interpreted.

TABLE 4—*Results of stain aging study.*[a]

		Antisera Dilution (WMC)[b]					
		Neat		1/2		1/4	
Age (days)	Stain	RT	R	RT	R	RT	R
6	BF (A3+)	3,1	1,1	3,3	1,2	3,3	2,2
7		2,1	1,2	1,1	2,2	2,2	2,1
12		2,3	2,2	1,1	2,1	2,1	1,1
18		1,2	2,1	1,1	2,2	1,1	1,1
26		1,1	1,1	1,2	1,1	1,1	1,2
35		1,6	6,6	1,1	1,1	1,1	1,1
47		6,1	6,6	6,2	1,1	1,1	1,1
12	MM (A3+)	1,3	3,3	1,1	2,2	1,1	1,1
19		1,1	1,2	1,2	1,1	1,1	1,2
40		4,5	6,5	1,5	1,1	1,1	1,1
48		1,1	1,1	1,1	1,1	1,1	1,1
6	ER (A3-A1+)	4,4	nd,4	4,4	4,4	3,3	5,4
7		4,4	4,5	4,3	4,3	1,2	3,3
12		5,6	4,5	4,4	4,4	2,3	1,1
18		6,5	6,5	5,4	3,3	nd,1	1,1
26		4,6	6,5	5,5	5,5	2,1	2,3
47		5,5	4,6	3,3	4,4	2,1	1,1

[a]Refer to Table 3 for scoring system.
[b]R = stored at 4°C, RT = stored at 22°C, and nd = not determined.

TABLE 5—*HLA B8 antigen.*

Donor	HLA Phenotype		Antisera Dilution[a] Neat	1:2	1:4
MN	A2,26	B7	4	3	3
ER	A1,w31	B12	4	3	2
SS	A3	B7	3	2	3
TK	A2,w24	B40,w63	3	3	2
LH	A28,29	B40,w44	3	2	2
MM	A3	B8	2	1	1
MA	A1,2	B8,12	1	2	1
BF	A2,3	B8,14	1	1	1
VB	antisera	...	3	3	2
ALG	positive control	...	6	...	...
BB	negative control	...	1	...	...
ER	A1,w31	B12	6	2	2
SS	A3	B7	6	5	2
BF	A2,3	B8,14	2	1	1
MA	A1,2	B8,12	2	1	1
JM	antisera	...	5	5	2
ALG	positive control	...	6	...	...
BB	negative control	...	1	...	...

[a]Refer to Table 3 for scoring system.

TABLE 6—*HLA A2 antigen.*

Donor	HLA Phenotype		Antisera Dilution[a] Neat	1:2	1:4
SS	A3	B7	4	4	3
EB	A3,w24	B7	4	1	1
MN	A2,26	B7	1	1	1
AS	A1,2	B5,7	2	1	1
MN	A2,26	B7	1	1	1 semen
MN	A2,26	B7	2	1	1 saliva
HR	A1	B7,17	4	1	1 semen
GR	antisera	...	5	3	1
ALG	positive control	...	6	...	...
BB	negative control	...	1	...	...

[a]Refer to Table 3 for scoring system.

Conclusions

One can conclude from this study that present technology for HLA typing bloodstains is feasible and workable. The use of the technique demands access to high-titered, well-characterized HLA reagents and a test cell panel of well-characterized lymphocytes. Both these conditions preclude the use of this technique in the average forensic science laboratory; however, a specialized laboratory or an HLA laboratory with a forensic science branch could easily provide such testing.

Questions remaining to be answered are to check potential cross-reactivity problems more thoroughly and with a larger stain donor panel, perform an aging study on each antigen used, run a greater number of case samples to check the effect of forensic science problems (for ex-

ample, putrefaction), and to conduct more extensive research into the usefulness of HLA typing on mixtures of body fluids. Experiments to answer these questions are being designed.

References

[1] Rittner, C. and Waiyawuth, V., "HL-A Typing in Dried Blood Stains. 1. Specificity and Reliability of the Inhibition Test," *Journal of Immunogenetics*, Vol. 1, No. 2, April 1974, pp. 99-111.

[2] Rittner, C. and Waiyawuth, V., "HL-A Typing in Dried Blood Stains. II. Comparative Studies on Microcytotoxicity and Microcomplement Fixation Tests," *Journal of Immunogenetics*, Vol. 2, No. 4, Aug. 1975, pp. 211-222.

[3] Newall, P. J., "The Identification of HLA-A2 and HLA-B5 Antigens in Dried Bloodstains," *Canadian Society of Forensic Sciences Journal*, Vol. 12, No. 1, March 1979, pp. 1-16.

[4] Newall, P. J., "The Identification of HLA-A9 in Dried Stains of Blood and Body Secretions," *Canadian Society of Forensic Sciences Journal*, Vol. 14, No. 3, Sept. 1981, pp. 104-112.

[5] Hodge, D. G., Wolf, E., Lincoln, P. J., Festenstein, H., and Dodd, B. E., "The Detection of the HLA-A1 Antigen in Bloodstains," *Medicine, Science and the Law*, Vol. 20, No. 3, July 1980, pp. 213-220.

[6] Lotterle, J., "HLA-Typisierung von Blutspuren. Untersuchungen mit einem Absorptionstest," *Zeitschrift fur Rechtsmedizin*, Vol. 87, No. 3, Oct. 1981, pp. 217-224.

[7] Amos, D. B., Pool, P., and Grier, J., "HLA-A, HLA-B, HLA-C, and HLA-DR," in *Manual of Clinical Immunology*, 2 ed., N. R. Rose and H. Friedman, Eds., American Society for Microbiology, Washington, DC, 1980, pp. 978-986.

[8] Miller, W. V., "HLA Serotyping in Cases of Disputed Paternity," in *Paternity Testing*, H. Silver, Ed., American Association of Blood Banks, Washington, DC, 1978, pp. 55-67.

Address requests for reprints or additional information to
Dr. E. G. Reisner
P.O. Box 3934
Duke University Medical Center
Durham, NC 27710