F. Samuel Baechtel,[1] Ph.D.; Kathryn W. Presley,[1] M.S.; and Jill B. Smerick,[1] M.S.

D1S80 Typing of DNA from Simulated Forensic Specimens

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ABSTRACT: The reliability of a D1S80 typing procedure has been evaluated using simulated forensic specimens. D1S80 alleles were detectable in DNA recovered from bloodstains exposed to sunlight for up to 20 weeks. However, D1S80 alleles were undetectable in semen stains after six weeks sunlight exposure. Analysis of blood and semen that had been deposited on a variety of substrates and examined over a twenty-week period, revealed no systematic influence of substrate on the ability to type D1S80. A study in which body fluids were exposed to household chemical substances, such as bleach, acids, oil, and gasoline, indicated that only HCl and bleach had a deleterious effect on the ability to type D1S80. In addition, personal care chemical products were without effect on D1S80 allele patterns derived from semen. Exposure of blood and semen to four different species of microorganisms resulted in no alteration of D1S80 genotype patterns in these body fluids. D1S80 genotypes could be reliably determined even when body fluids from different individuals were mixed. DNA from no animals other than humans and higher primates could be amplified at locus D1S80 when the DNA had been isolated through an organic procedure.

These studies, in concert with the reports of others, indicate that the procedures for the amplification and detection of genetic variation at locus D1S80 are suitable for use on forensic evidentiary materials.

KEYWORDS: pathology and biology, polymerase chain reaction, D1S80, body fluid stains, DNA typing

The polymerase chain reaction (PCR) [*1,2*] has become an extraordinarily useful process in the forensic laboratory for the genetic analysis of biological materials. Polymorphic loci that are amplifiable by the PCR often lack the discriminating power of variable number of tandem repeat (VNTR) loci detected by the restriction fragment length polymorphism (RFLP) analysis. However, the advantages of utilizing such loci lie in the rapidness and simplicity of PCR test procedures and the ability to enable the acquisition of genetic information from DNA specimens that are limited in both quantity and quality [*3*]. The PCR has been applied to the detection of both sequence [*1–6*] and length polymorphisms [*7–9*] in DNA.

One of the polymorphic regions that can be amplified readily by the PCR is the VNTR found at locus D1S80 [*10*]. Since the initial report on the amplifiability of this locus [*9*], the forensic utility of DNA typing at the D1S80 has been assessed in a number of laboratories [*9,11–16*]. Before a caseworking laboratory can adopt the procedures for the DNA typing of forensic evidence at locus D1S80, it is necessary to satisfy certain acknowledged validation requirements [*17–18*]. In the present study we have evaluated the ability of our D1S80 typing procedure [*19*] to yield results that are reliable when used for the analysis of biological specimens that have sustained environmental challenges. In addition, we have examined the reliability of the typing technique when applied to the analysis of specimens which contain mixed body fluids from different individuals. The results of the present work, in combination with other studies [*9,11–16,25*], have shown that our approach to the PCR-based typing of DNA at locus D1S80 is valid and acceptable for use on forensic evidence.

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[1]Research Chemist and Chemists, respectively, Forensic Science Research and Training Center, FBI Laboratory, FBI Academy, Quantico, VA.

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Materials and Methods

Sample Preparation and Amplification

Blood and semen specimens were obtained from volunteer donors. Blood was collected by venipuncture into tubes that did not contain anticoagulant. Immediately after collection, the blood was poured into a sterile plastic container and, without delay, was used to prepare bloodstains. After donation into sterile plastic tubes, semen was stored at −20°C until thawed for experimental use. Saliva was collected from some of the donors of blood and semen. In these cases, saliva was collected in a sterile plastic tube and used immediately for stain preparation.

DNA was recovered from blood, semen, and saliva stains by methods previously reported [*20*]. Where appropriate to the experiment, a differential DNA recovery procedure was carried out. Extracted DNA was quantified by the slot-blot procedure of Waye et al. [*21*] and was amplified by the PCR. The PCR was carried out in 50 μL reaction volumes containing 5 ng template DNA at a maximum, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin (Tris, KCl, $MgCl_2$, and gelatin were obtained from Perkin Elmer, Branchburg, NJ, as a 10X concentrate), 1 nmole of each of the four deoxyribonucleotide triphosphates, 12.5 pmoles of each primer [*9*], 8 μg bovine serum albumin (BSA), and 2.5 units Taq DNA polymerase. BSA (catalog number A3550) was obtained from Sigma Chemical Company, St. Louis, MO. The PCR reaction mixtures were placed into a Perkin Elmer 9600 thermal cycler and were subjected to 27 cycles of denaturation at 95°C for 10 s, primer annealing at 67°C for 10 s, and primer extension at 70°C for 30 s. The PCR products were separated by polyacrylamide gel electrophoresis previously described [*11,19–20*] except that 4.5 μL amplified product was applied to each applicator tab. The amplified alleles and the ladder on the gel were revealed by silver staining [*11*].

D1S80 allele designations were determined by reference to a

D1S80 allelic reference ladder [19] that was subjected to electrophoresis in parallel with the experimental specimens. The D1S80 reference ladder contained alleles 16 through 37 and allele 41. The D1S80 genotypes of the blood, semen, and saliva donors were as follows: Blood: 17/18, 28/31, 29/31, 18/31, and 24/28; semen: 25/28, 18/24, 29/32, and 18/22; saliva: 17/18, 18/31, and 29/31.

Application of Environmental Abuse to Body Fluid Stains

Exposure to Sunlight—Replicate 5 μL bloodstains, using blood of four donors, and 5 μL semen stains, using semen from four donors, were prepared on washed cotton sheeting. One panel of stains, one-third of the total, was placed on the roof of our laboratory building, facing southward without shade, during a time period from late March until mid-August (20 weeks). The temperature ranged from −5°C to 37°C during this time period and the percent relative humidity ranged from 52 to 85. Another panel of stains (one-third of the total) also was placed on the roof during this time period, but was deliberately shaded from direct sunlight. Both of these panels were protected from rainfall, but were covered in a manner that exposed them to atmospheric variations in humidity and temperature. The remaining panel of stains was maintained in the laboratory at room temperature and ambient lighting conditions. Duplicate stains representative of blood donor one and semen donor one were removed from each of the test panels at days 1, 3, 5, 9, and 11. Likewise, duplicate stains representative of blood donor two and semen donor two were removed at days 1, 2, 4, 8, 10, and 12. In a similar manner, blood and semen stain donors three and four had their stains removed from the panels at day 1 and weeks 2, 6, 10, 14, and 20; and day 1 and weeks 4, 8, 12, and 16 respectively. All cut stains were stored at −70°C until processing of all the stains in this study could be begun.

Deposition Substrates—Replicate 5 μL bloodstains and 5 μL semen stains, using body fluids from the same donors described in the sunlight exposure experiment, were prepared on six substrates: old nylon panties, recently painted wallboard, used leather athletic shoes, used pile carpet, old denim bluejeans, and a piece of dressed pinewood. The substrate panels were maintained at room temperature for 20 weeks and duplicate stains were removed from each substrate following the same schedule described for the sunlight exposure study. All cut stains were stored at −70°C until processing of all the stains in this study could be initiated.

Chemicals—Some common substances were evaluated for their influence on D1S80 typing patterns. Washed cotton sheeting panels were stained with used automobile motor oil, unleaded gasoline, or a coating of Virginia red clay. The following day, replicate 5 μL aliquots of blood from two donors, semen from two donors, and saliva from two donors were applied to each of the stained panels. Twenty-four hours later, each body fluid stain was cut out and processed as described above.

In a separate phase of this study, 5 μL aliquots of blood, semen, and saliva were applied to washed cotton sheeting panels and permitted to dry. The following day, individual stain panels were dipped 10 times into 100 mL of the following solutions: full-strength laundry bleach, a 0.1% (V/V) liquid laundry detergent, 0.4 N NaOH, 1% acetic acid, or 1 M HCl. Then each panel was rinsed by dipping 10 times into 100 mL deionized water. After an overnight drying period, the stain areas were cut and processed as described.

Microorganisms—Cultures of four microorganisms, *E. coli* (American Type Culture Collection [ATCC], Rockville, MD, number 35270); *S. epidermidis* (ATCC number 155); *B. subtilis* (ATCC number 6051); and *C. albicans* (ATCC number 18804) were utilized in this study. *E. coli*, *S. epidermidis*, and *B. subtilis* were cultured in LB medium [22] at temperatures recommended by the ATCC for each strain. *C. albicans* was cultured in YM broth [22] at 25°C.

In part one of this study, replicate 5 μL bloodstains and 5 μL semen stains were prepared on washed cotton sheeting and permitted to dry overnight at room temperature. The next day, 5 μL volumes of overnight cultures of each microorganism were applied separately to the dried blood and semen stains. Control stains containing only microorganisms in culture fluid were prepared on the cotton sheeting. The contaminated stains and control stains were maintained at room temperature for seven days and then processed as described above.

In part two of this study, DNA was isolated from an overnight culture of each microorganism [23] and quantified by ultraviolet spectrophotometry. Various quantities of the bacterial or yeast DNA were combined with 5 ng human DNA and the mixtures used as templates for D1S80 amplification. Controls consisted of bacterial or yeast DNA mixed and no human DNA.

Miscellaneous Contaminating Substances—Additional substances were tested for their potential effects on the ability to type correctly D1S80 from liquid semen specimens. Five mL semen was pipetted into a Trojan Plus 2 latex condom. This brand of condom contains nonoxynol-9 as a spermicide. The semen was mixed in the condom and duplicate portions of semen were removed for storage at −20°C until analysis was begun. This testing procedure was done for two semen donors, using separate condoms.

In another experiment, semen specimens from two donors were diluted in serial 10-fold steps with either Massengill Fresh Mountain Breeze douche; Massengill Vinegar and Water douche; or with sterile deionized water. Five μL aliquots of each dilution were taken for analysis.

An over-the-counter vaginal antifungal agent, Gyne-Lotrimin, and a vaginal contraceptive insert, Semicid, were evaluated for their effects on D1S80 typing. A single Gyne-Lotrimin insert and a single Semicid insert were each dissolved in 10 mL of sterile deionized water. The Gyne-Lotrimin insert did not dissolve completely, but remained as a fine suspension. Ten μL aliquots of the Gyne-Lotrimin suspension were mixed with 10 μL aliquots of semen from two donors and 10 μL aliquots of the Semicid solution also were mixed with 10 μL aliquots from each of two semen donors. Ten μL portions of each of the four mixtures were taken for analysis of D1S80.

Detection of D1S80 Alleles in Mixed Body Fluid Stains

Three categories of mixed body fluid stains were prepared and the detectability of D1S80 alleles within those stains was determined. Mixtures of blood and blood; blood and semen; and semen and saliva were prepared in microcentrifuge tubes in a series of volume to volume ratios. Immediately after mixing, duplicate 5 μL portions of each fluid mixture were pipetted onto washed cotton sheeting and permitted to dry. After seven days at room temperature, the stains were removed and processed.

Detection of D1S80 Alleles in Solid Tissue Extracts

Samples of liver, bone, muscle, spleen, kidney, and a bloodstain were obtained during postmortem analyses of four individuals. These samples were stored at −20°C until thawed for processing. Duplicate pieces of tissue were cut from each sample. Wet weights of these pieces ranged from approximately 8.0 mg to 126 mg. After weighing, each tissue piece was minced with a sterile scalpel blade to increase its surface area (bone samples were not minced). The bloodstain cutting and the minced solid tissues were placed in microfuge tubes and the DNA recovered as described [*20*] for bloodstains. D1S80 profiles were determined for each of the tissues.

Differential Recovery of DNA from Vaginal Swabs

Vaginal swabs were obtained from a single donor. Semen from a single donor was diluted in serial two-fold steps from 1:1 to 1:256 using phosphate-buffered saline. Twenty μL aliquots of neat semen and of each semen dilution were applied to duplicate vaginal swabs. Swabs were permitted to dry overnight at room temperature. Each swab was processed by dissecting the swab material from the applicator stick and placing the material into a microfuge tube. The material was extracted for 2 h at 37°C after addition of 500 μL of a solution containing 400 μL TNE (10 mM Tris, 100 mM NaCl, and 1 mM EDTA; pH 8.0), 25 μL 20% N-lauroylsarcosine, 75 μL deionized water, and 100 μg proteinase K. After incubation, the swab material was removed from the tube and placed into a plastic basket insert (part number 9201, Costar, Cambridge, MA). The basket was returned to the original extraction tube and the apparatus centrifuged in a Model HSC 10K (Savant Instruments, Farmingdale, NY) at maximum speed for 5 min. The supernatant fluid, considered to be the lysed cell fraction (that is, female fraction), was carefully pipetted away from any pellet material and placed in a new microcentrifuge tube. The pellet was washed three times by repetitive resuspension in pellet wash buffer (10 mM Tris, 10 mM EDTA, 50 mM NaCl, and 2% Sodium dodecyl sulfate, pH 7.5), followed by centrifugation at maximum speed for 5 min and disposal of the supernatant wash fluid. After washing, the putative sperm pellet was resuspended in a solution containing 150 μL TNE, 50 μL 20% N-lauroylsarcosine, 40 μL 0.39 M dithiothreitol, 150 μL deionized water, and 100 μg proteinase K. The swab head was suffused with the same volume of the same solution as was used for the pellet. The putative sperm pellet and the swab head were incubated at 37°C for 2 h. The swab head was placed in a clean basket insert and centrifuged at maximum speed for 5 min. The supernatant fluid was retained for analysis. The DNA in each of the three swab fractions was recovered by methods described by Comey et al. [*20*]. DNA recovered from each fraction was typed as described.

Amplification of DNA from Nonhuman Animal Sources

Dried bloodstains were available for the following animals: Celebes ape, gorilla, Debrazza monkey, Geoffrey spider monkey, Japanese macaque, orangutan, horse, pig, dog, domestic cat, swine, and lion. Bloodstain specimens were available for multiple dogs and cats. For each stain a cutting of 1 cm^2 in area was taken for processing. DNA was recovered from the stain and quantified by yield gel [*24*] since the slot-blot procedure could not be used. A large quantity of DNA was taken for amplification from each animal's blood. Except for three animals, 124 ng of DNA were taken for each amplification mixture. Because of low yield, only 28 ng DNA were used in each amplification for the horse, orangutan, and one dog blood sample. Amplification of each animal's DNA was set up in duplicate. One member of each duplicate pair received only the DNA from the animal being tested. The other member received 5 ng K562 DNA in addition to the animal DNA. All tubes were amplified under conditions established for human DNA.

Results

Exposure to Sunlight

The ability to correctly type D1S80 from blood and semen stains that had been subjected to prolonged sunlight exposure was assessed over a 20-week period. As shown in Table 1, D1S80 could be typed from the bloodstains that had been exposed to direct sunlight, as well as from those stains kept in the shade or in the laboratory throughout the 20-week trial. One specimen, out of a total of 132, exhibited extra bands. The replicate of this stain did not exhibit extra bands. One specimen, of genotype 18/31, that had been exposed to sunlight for 16 weeks, demonstrated preferential amplification in that the 18 allele was present (although very weak) and the 31 allele was absent. The replicate of this stain demonstrated both the 18 and 31 alleles.

D1S80 could not be typed from semen stains throughout the entire 20-week exposure period (Table 2). Semen stains subjected to direct sunlight became untypeable after six weeks of exposure. Semen stains maintained in the shade, but outside, were typeable for up to 10 weeks. Control semen stains that had been maintained in the laboratory at ambient temperatures remained typeable for the entire 20-week experimental period. One specimen, out of a total of 132 specimens exhibited an extra band. The replicate of this stain did not show an extra band.

Deposition Substrates

The results of D1S80 typings for blood and semen stains that had been prepared on different substrates and harvested for analysis

TABLE 1—*Amplification success for bloodstains maintained in the sunlight, shade, or at room temperature.*

Time[a]	Number of successful amplifications/Number of amplifications attempted		
	Sunlight	Shade	Laboratory
1 d	8/8	8/8	8/8
2 d	2/2	2/2	2/2
3 d	2/2	2/2	2/2
4 d	2/2	2/2	2/2
5 d	2/2	2/2	2/2
8 d	2/2	2/2	2/2
9 d	2/2	2/2	2/2
10 d	2/2	2/2	2/2
11 d	2/2	2/2	2/2
12 d	2/2	2/2	2/2
2 w	2[b]/2	2/2	2/2
4 w	2/2	2/2	2/2
6 w	2[b]/2	2/2	2/2
8 w	2/2	2/2	2[b]/2
10 w	2/2	2/2	2/2
12 w	2/2	2/2	2/2
14 w	2/2	2/2	2/2
16 w	2[c]/2	2/2	2/2
20 w	2/2	2/2	2/2

[a]d = days; w = weeks.
[b]Extra bands were observed in one of the two specimens.
[c]Preferential amplification noted for one specimen.

TABLE 2—*Amplification success for semen stains maintained in the sunlight, shade, or at room temperature.*

Time[a]	Number of successful amplifications/Number of amplifications attempted		
	Sunlight	Shade	Laboratory
1 d	8/8	7/8	8/8
2 d	2/2	2/2	2/2
3 d	2/2	2/2	2/2
4 d	2/2	2/2	2/2
5 d	2/2	2/2	2/2
8 d	2/2	2/2	2/2
9 d	2/2	2/2	2/2
10 d	2/2	2/2	2/2
11 d	2/2	2/2	2/2
12 d	2/2	2/2	2/2
2 w	2/2	2/2	2/2
4 w	1[b]/2	2/2	2/2
6 w	0/2	2/2	2/2
8 w	0/2	2/2	2/2
10 w	0/2	2/2	2/2
12 w	0/2	0/2	2/2
14 w	0/2	0/2	2/2
16 w	0/2	0/2	2/2
20 w	0/2	0/2	2/2

[a]d = days; w = weeks.
[b]Weak, extra bands were noted in the specimen that amplified.

TABLE 4—*Amplification success for semen stains prepared on various substrates.*

Time[a]	Number of successful amplifications/Number of amplifications attempted					
	Nylon	Wallboard	Leather	Denim	Carpet	Wood
1 d	7/8	8/8	8/8	8/8	8/8	7/8
2 d	2/2	2/2	2/2	2/2	1/2	2/2
3 d	2/2	2/2	2/2	2/2	2/2	2/2
4 d	2/2	2/2	2/2	2/2	2/2	2/2
5 d	2/2	2/2	2/2	2/2	2/2	2/2
8 d	2/2	2/2	2/2	2/2	2/2	2/2
9 d	2/2	2/2	2/2	2/2	2/2	2/2
10 d	2/2	2/2	2/2	2/2	2/2	2/2
11 d	1/2	2/2	1/2	2/2	2/2	2/2
12 d	2/2	2/2	2/2	2/2	2/2	2/2
2 w	2/2	2/2	2/2	2/2	2[b]/2	2/2
4 w	2/2	2/2	2/2	2/2	2/2	2/2
6 w	2/2	2/2	2/2	2/2	2/2	2/2
8 w	2/2	2/2	0/2	2/2	2/2	2/2
10 w	2/2	2/2	2/2	2/2	2/2	2/2
12 w	2/2	2/2	2/2	2/2	1/2	2/2
14 w	2/2	2/2	2/2	2/2	2/2	2/2
16 w	2/2	2/2	2/2	2/2	2/2	2/2
20 w	2/2	2/2	2/2	2/2	2/2	2/2

[a]d = days; w = weeks.
[b]Extra bands were observed in one specimen.

over a 20-week period are shown in Tables 3 and 4. The data in these tables reveal that there was no systematic loss in the ability to determine the D1S80 genotypes of the blood or semen stains as either a function of the deposition substrate or as a function of stain age.

Of the 528 blood and semen stains that were prepared and processed in this series of experiments, 70 stains failed to amplify during the initial attempt. Upon a second amplification attempt 46 specimens yielded D1S80 products. Thus, 24 stains failed to amplify despite two attempts. Twenty of the 24 double amplification failures were extracts of blood stains. Five specimen extracts exhibited extra bands. The extra bands in one specimen were due to the inadvertent placement of two stains in the same area of the cloth substrate during stain preparation. Thus, only four stain extracts in this study displayed extra bands.

TABLE 3—*Amplification success for bloodstains prepared on various substrates.*

Time[a]	Number of successful amplifications/Number of amplifications attempted					
	Nylon	Wallboard	Leather	Denim	Carpet	Wood
1 d	8/8	8/8	8[b]/8	8/8	8/8	7/8
2 d	2/2	2/2	1/2	1/2	0/2	2/2
3 d	2/2	2/2	2/2	2/2	1/2	2/2
4 d	1/2	2/2	2/2	2/2	2/2	2/2
5 d	2/2	2/2	2/2	2/2	2/2	2/2
8 d	1/2	2/2	2/2	2/2	1/2	2/2
9 d	2/2	2/2	2/2	2/2	2/2	0/2
10 d	2/2	2/2	2/2	2/2	1[c]/2	2/2
11 d	1/2	2/2	2/2	2/2	2/2	2/2
12 d	2/2	2/2	2/2	2/2	1/2	2/2
2 w	2/2	2/2	2/2	2[c]/2	2/2	2/2
4 w	2/2	2/2	2/2	2/2	2/2	2/2
6 w	2/2	2/2	2/2	2/2	2/2	2/2
8 w	2/2	2/2	2/2	2/2	2/2	2/2
10 w	2/2	2/2	2/2	1/2	2/2	1/2
12 w	2/2	2/2	2/2	2/2	1/2	2/2
14 w	2/2	2/2	2/2	2/2	2/2	2/2
16 w	0/2[d]	2/2	2/2	2/2	1/2	2/2
20 w	2/2	2/2	2/2	2/2	2/2	2/2

[a]d = days; w = weeks.
[b]Extra bands were observed in two specimens.
[c]Extra bands were observed in one specimen.
[d]Allele patterns in both specimens were inconclusive because of contamination with reference ladder.

Nineteen stain extracts in the substrate study did not demonstrate the presence of DNA at the lower detection limit of the slot-blot quantification procedure of 156 pg DNA. Despite the apparent low level of DNA, amplifications were attempted with 20 μL of each of these stain extracts. Twelve of these extracts yielded typeable results that were consistent with the expected genotype, although the allele patterns were visibly weak. The remaining extracts failed to amplify during both attempts at the PCR.

Chemical Contamination

Blood, semen, and saliva were exposed to several chemical or natural substances to determine if these substances might adversely affect D1S80 typing patterns. Only two of the chemical substances tested had a negative effect on D1S80 stain profiles. As seen in Table 5, the application of full-strength household bleach or 1M HCl to blood, semen, and saliva stains totally abrogated the ability to obtain D1S80 profiles. None of the other treatments affected D1S80 allele profiles. Body fluid stains prepared on dirt-stained cotton remained typeable at locus D1S80. These results differ from those obtained for locus DQα [3]. However, in the case of the DQα study, powered soil was mixed directly with blood, leading to an inability to obtain amplified product. In the present study, the body fluids were applied to soil-stained cloth, which apparently is not a form of contamination as deleterious to amplification.

Microorganisms

Aliquots of viable cultures of three bacterial species, *E. coli, S. epidermidis,* and *B. subtilis,* and one fungal species, *C. albicans,*

TABLE 5—*Detection of D1S80 alleles in stains exposed to chemical contaminants.*

Contaminant	Alleles detected in stains[a]				
	Blood		Semen		Saliva
	Donor 1	Donor 2	Donor 1	Donor 2	Donor 1
None	17/18	18/31	25/28	18/24	29/31
Oil	17/18	18/31	25/28	18/24	29/31
	17/18	18/31	25/28	18/24	29/31
Gasoline	17/18	18/31	25/28	18/24	29/31
	17/18	18/31	25/28	18/24	29/31
Dirt	17/18	18/31	25/28	18/24	29/31
	17/18	18/31	25/28	18/24	29/31
Bleach	NEG	NEG	NEG	NEG	NEG
	NEG	NEG	NEG	NEG	NEG
Laundry Detergent	17/18	18/31	25/28	18/24	29/31
	17/18	18/31	25/28	18/24	29/31
NaOH	17/18	18/31	25/28	18/24	29/31
	17/18	18/31	25/28	18/24	29/31
Acetic Acid	17/18	18/31	25/28	18/24	29/31
	17/18	18/31	25/28	18/24	29/31
HCl	NEG	NEG	NEG	NEG	NEG
	NEG	NEG	NEG	NEG	NEG

[a]Results are shown for tests of duplicate stains, except when no contaminant was present.

were applied to fresh duplicate stains of blood from two donors and semen from two donors. Duplicate stains of each microorganism also were prepared at the same time using the same volume of culture fluid as was applied to the body fluid stains. D1S80 analysis of DNA recovered from these stains revealed the presence of only the D1S80 alleles characteristic of the blood or semen donor (Table 6). Control stains consisting only of the microorganisms demonstrated no amplification products.

In a second part of this study, DNA was isolated from active cultures of each microorganism and mixed in various ratios with 5 ng human genomic DNA. These mixtures were then amplified under conditions established for D1S80. Table 7 indicates that the amplification of the human genomic DNA was unaffected by the presence of high levels of bacterial or fungal DNA. All human genomic samples exhibited the expected alleles upon amplification and typing. Samples in which the bacterial or fungal DNA was the only potential amplification template showed no products when amplified under D1S80 conditions.

TABLE 6—*Exposure of blood and semen stains to microorganisms.*

Contaminating microorganism	Alleles detected in stain[a]				
	Blood		Semen		Micro-organism only
	Donor 1	Donor 2	Donor 1	Donor 2	
None	28/31	18/31	25/28	18/24	Not
	28/31	18/31	25/28	18/24	applicable
C. albicans	28/31	18/31	25/28	18/24	None
	28/31	18/31	25/28	18/24	None
S. epidermidis	28/31	18/31	25/28	18/24	None
	28/31	18/31	25/28	18/24	None
E. coli	28/31	18/31	25/28	18/24	None
	28/31	18/31	25/28	18/24	None
B. subtilis	28/31	18/31	25/28	18/24	None
	28/31	18/31	25/28	18/24	None

[a]Results are shown for tests of duplicate stains.

TABLE 7—*Lack of effect of microbial DNA presence on amplification of a human DNA template.*

Organism	μg microbial DNA per amplification	Alleles detected	
		Human DNA template	No human DNA template
C. albicans	150	18/31	None
	100	18/31	None
	50	18/31	None
	0	18/31	Not Done
S. epidermidis	150	18/31	None
	100	18/31	None
	50	18/31	None
	0	18/31	Not Done
E. coli	150	18/31	None
	100	18/31	None
	50	18/31	None
	0	18/31	Not Done
B. subtilis	150	18/31	None
	100	18/31	None
	50	18/31	None
	0	18/31	Not Done

Miscellaneous Substances

Two liquid semen specimens were placed in a rubber condom containing the spermicide nonoxynol-9. Testing of duplicate samples removed from each condom revealed D1S80 allele patterns that were identical to untreated specimens from these two donors. When semen was mixed with a solution prepared from Semicid, an over-the-counter spermicide that also contains nonoxynol-9, the expected D1S80 allele patterns were seen. These observations corroborate those of Hochmeister et al. [25] that nonoxynol-9, a common constituent of over-the-counter contraceptive products, has no effect on DNA typing patterns at this locus.

In another experiment, liquid semen from the same two donors was diluted in serial ten-fold steps with sterile water, or with two types of douche fluid. The data of Table 8 show the results of this study. The data are expressed as the reciprocal of the greatest dilution of each semen specimen that yielded a typeable D1S80 allele pattern. It can be seen that neither douche solution had a significant effect on typing at the D1S80 locus.

Finally, Gyne-Lotrimin, an anti-fungal vaginal insert, when dissolved in water and mixed with liquid semen, had no effect upon the ability to obtain typeable D1S80 patterns from the semen.

Mixed Body Fluids

It was of interest to evaluate the ability of the D1S80 typing procedure to detect alleles in mixtures of body fluids from two donors whose D1S80 genotypes differed. The mixtures tested were:

TABLE 8—*Typing of D1S80 from semen after dilution with douche solutions.*

Semen Diluent	Reciprocal of the greatest dilution of semen that exhibited detectable alleles	
	Donor 1	Donor 2
Sterile Water	10^{-3}	10^{-3}
Douche 1[a]	10^{-3}	10^{-2}
Douche 2[b]	10^{-4}	10^{-2}

[a]Massengill *Fresh Mountain Breeze* douche.
[b]Massengill *Vinegar and Water* douche.

TABLE 9—*Detection of D1S80 alleles in stains containing blood from different donors.*

Relative fluid volume		Alleles detected[a]			
Donor 1	Donor 2	Test Pair 1	Test Pair 2	Test Pair 3	Test Pair 4
1	0	18/31	18/31	25/28	24/28
		18/31	18/31	25/28	24/28
1	1	18/25/28/31	18/29/31/32	24/25/28	24/28/29/32
		18/25/28/31	18/29/31/32	24/25/28	24/28/29/32
1	5	18/25/28/31	18/29/31/32	24/25/28	24/28/29/32
		18/25/28/31	18/29/31/32	24/25/28	24/28/29/32
1	20	25/28	29/32	24/28	24/28/29/32
		18/25/28/31	18/29/31/32	24/28	24/28/29/32
20	1	18/31	18/29/31/32	24/25/28	24/28/29/32
		18/25/28/31	18/29/31/32	24/25/28	24/28/29/32
5	1	18/25/28/31	18/29/31/32	24/25/28	24/28/29/32
		18/25/28/31	19/29/31/32	24/25/28	24/28/29/32
0	1	25/28	29/32	24/28	29/32
		25/28	29/32	24/28	29/32

[a]Results are shown for tests of duplicate stains.

blood mixed with blood (four donor combinations tested); semen and saliva (four donor combinations tested); and semen and blood (three donor combinations tested). For the combinations in which semen was one of the body fluids, no attempt was made to differentially separate sperm cell DNA from somatic cell DNA.

Table 9 contains the results of D1S80 tests in which bloods from two donors were mixed in several fixed ratios. Alleles of the minor contributor in each of the test pairs could always be detected when diluted 1:6 by the major component. In five of 16 specimens in which the minor blood contributor had been diluted 1:21, the alleles of the minor contributor could not be detected. The alleles of the minor contributor were seen in the remaining 11 specimens of this combination ratio.

Table 10 shows the D1S80 typing results when semen and saliva from donors were mixed in several volume ratios. Except for one fluid mixture, the alleles of the saliva donors in all three test pairs were undetectable when the saliva had been diluted as little as 1:1 by semen. In one of six specimens in which saliva had been diluted 1:1 by semen, the alleles of the saliva donor were observed. In contrast, D1S80 alleles characteristic of the semen donor could still be detected in all three test pair combinations even though the semen had sustained a dilution of 1:21 by saliva.

Typing results for mixtures of blood and semen from different donors are shown in Table 11. A comparison of the dilutions at which alleles characteristic of one donor were no longer detectable revealed that the blood-derived alleles were detectable when the blood had been diluted 1:1 with semen. For none of the body fluid mixtures composed of 1 part blood and five or more parts of semen could the alleles of the blood donor be detected. The alleles of three of the four semen donors could be detected in mixtures in which the semen had been diluted 1:21 with blood. Alleles of the fourth semen donor were detectable only up to a 1:5 dilution with blood.

Solid Tissue Extracts

DNA was recovered from five different tissues from each of four individuals, and used as a template source for amplification at locus D1S80. A dried blood exemplar from each donor also was tested. The D1S80 genotypes were identical in each of the tissues from a single individual and were consistent with the genotype obtained for the exemplar bloodstain for each individual (results not shown). That DNA profiles are consistent within the tissues of an individual has been demonstrated for this locus [*15*] and for other loci [*13,26*] as well.

Differential Recovery of DNA from Vaginal Swabs

It was of interest to ascertain the approximate minimum quantity of semen that could be present on a vaginal swab and exhibit detectable D1S80 alleles after a differential separation procedure had been carried out on the swab. DNA was recovered from semen-inoculated vaginal swabs in a differential procedure that was designed to yield three fractions: a non-sperm fraction enriched in DNA from the swab donor; a fraction enriched in sperm cell DNA; and a fraction representative of the DNA remaining in the swab after the differential recovery procedure had been completed. Semen was applied to the swabs in dilutions that ranged from neat semen to a dilution of 1:256.

TABLE 10—*Detection of D1S80 alleles in stains containing semen and saliva from different donors.*

Relative fluid volume		Alleles detected[a]		
Saliva	Semen	Test Pair 1	Test Pair 2	Test Pair 3
1	0	17/18	29/31	18/31
		17/18	NEG	18/31
1	1	25/28	29/32	18/22
		25/28	29/32	18/22/31
1	5	25/28	29/32	18/22
		25/28	29/32	18/22
1	20	25/28	29/32	18/22
		25/28	29/32	18/22
20	1	17/18/25/28	29/31/32	18/22/31
		17/18/25/28	29/31/32	18/22/31
5	1	17/18/25/28	29/31/32	18/22/31
		17/18/25/28	29/31/32	18/22/31
0	1	25/28	29/32	18/22
		25/28	29/32	18/22

[a]Results are shown for tests of duplicate stains.

TABLE 11—*Detection of D1S80 alleles in stains containing blood and semen from different donors.*

Relative fluid volume		Alleles detected[a]			
Blood	Semen	Test Pair 1	Test Pair 2	Test Pair 3	Test Pair 4
1	0	NEG	28/31	29/31	18/31
		17/18	28/31	29/31	18/31
1	1	17/18/25/28	18/24/28/31	29/31/32	18/22/31
		17/18/25/28	18/24/28/31	29/31/32	18/22/31
1	5	25/28	18/24	29/32	18/22
		25/28	18/24	29/32	18/22
1	20	25/28	18/24	29/32	18/22
		25/28	18/24	29/32	18/22
20	1	17/18	18/24/28/31	29/31/32	18/31
		17/18	18/24/28/31	29/32	18/31
5	1	17/18/25/28	INC	29/31/32	18/22/31
		17/18/25/28	18/24/28/31	29/31/32	18/22/31
0	1	25/28	18/24	29/32	18/22
		25/28	18/24	29/32	18/22

[a]Results are shown for tests of duplicate stains.

The data of Table 12 show that all of the non-sperm DNA fractions exhibited D1S80 alleles characteristic of the swab donor. Although none of the non-sperm DNA fractions exhibited the alleles of the semen donor in this study, in actual casework one would not expect such perfect separations in all cases because prelysed sperm cells could be present initially in the stain or vaginal swab. One of the sperm cell DNA fractions from a single swab contained D1S80 alleles characteristic of both the swab donor and the semen donor. Semen donor alleles could be detected in the sperm cell fractions of swabs out to a semen dilution of 1:32. Semen donor alleles were detected in one swab at a semen dilution of 1:256. Given that each swab was inoculated with 20 μL of semen suspension, a 1:32 fold dilution would be equivalent to approximately 0.6 μL of undiluted semen.

Reextraction of the swab material after acquisition of the sperm fraction revealed that the alleles of both the semen donor and the swab donor could be readily detected. Alleles characteristic of both individuals could be seen up to a semen dilution of 1:16. Thus, the extracted swab material can remain a probative source of DNA that could be used to confirm results found in the male and female swab fractions.

Amplification of DNA from Nonhuman Animal Sources

DNA was recovered from the blood of 24 animals (11 species) and used as potential templates for the amplification of D1S80. Of the 24 animals tested, only three demonstrated amplification fragments. The gorilla demonstrated a D1S80 genotype of 14/17 (the position of the 14 allele was estimated since this allele was not present in our reference ladder). These major bands were accompanied by two weak bands, 18 and 24. The orangutan exhibited a single allele fragment which was smaller than the human 14 allele. Finally, both swine specimens demonstrated a single allele that was much larger in size than the human 41 allele. Amplifications of all animal DNA preparations were attempted with animal DNA alone and with animal DNA supplemented with 5 ng human K562 cell line DNA. The following animal DNA specimens failed to show amplification products at locus D1S80 when unsupplemented with K562 DNA: celebes ape, deBraza monkey, Geoffrey spider monkey, Japanese macaque, horse, dog, cat, and lion. In all cases, when animal DNA was mixed with K562 DNA, the 18/29 genotype of K562 was demonstrable. It is not surprising that conditions for the amplification of locus D1S80 in humans will also amplify DNA regions in higher primates. Other studies have shown that DNA probes to human VNTR loci commonly cross-react with DNA of animals that are phylogenetically close to humans [*27*].

We have observed (data not shown) that when cat or dog DNA is extracted from blood stains by the Chelex procedure [*28,29*] and amplification at locus D1S80 attempted, fragment bands can be detected. Unrelated cats always exhibited fragments corresponding to alleles 22 and 24; and unrelated dogs exhibited fragments corresponding to alleles 20 and 27. The basis for the appearance of these fragments which appear when DNA has been extracted by the Chelex procedure is unknown. Their occurrence can be avoided by extracting DNA by the organic procedure.

TABLE 12—*Differential recovery of sperm and non-sperm DNA from vaginal swabs.*

Fold semen dilution	Equivalent volume of semen (μL) per swab	Fraction	Alleles detected[a] Swab 1	Swab 2
1	20	Non-Sperm	18,31	18,31
		Sperm	29,32	29,32
		Swab	18,29,31,32	18,29,31,32
2	10	Non-Sperm	18,31	18,31
		Sperm	29,32	29,32
		Swab	18,29,31,32	18,29,31,32
4	5	Non-Sperm	18,31	18,31
		Sperm	29,32	29,32
		Swab	18,28,31,32	18,29,31,32
8	2.5	Non-Sperm	18,31	18,31
		Sperm	29,32	29,32
		Swab	18,29,31,32	18,29,31,32
16	1.25	Non-Sperm	18,31	18,31
		Sperm	29,32	None
		Swab	18,29,31,32	18,29,31,32
32	0.63	Non-Sperm	18,31	18,31
		Sperm	29,32	None
		Swab	18,31	18,31
64	0.313	Non-Sperm	18,31	18,31
		Sperm	None	None
		Swab	18,31	18,31
128	0.156	Non-Sperm	18,31	18,31
		Sperm	None	None
		Swab	18,31	18,31
256	0.078	Non-Sperm	18,31	18,31
		Sperm	18,29,31,32	None
		Swab	18,31	18,31

[a]Swab donor was D1S80 genotype 18/31 and the semen donor was genotype 29/32.

Discussion

In the present study, five areas of validation have been experimentally addressed that apply to the amplification and detection of polymorphic variation at locus D1S80. The areas evaluated were: (1) the ability of the typing system to yield reliable results with body fluid stains that have sustained environmental, microbiological, and chemical abuse; (2) the ability of the typing system to yield reliable results from body fluids that have been deposited on different substrates; (3) the sensitivity of detection of alleles in mixtures of body fluids from different individuals; (4) the consistency of D1S80 typing patterns among various tissues within an individual; and (5) use of nonhuman animal DNA as a potential template source for the D1S80 amplification procedure. The results of this study, when combined with other reports [*9,11–20*], convincingly demonstrate the reliability of the methods we have used for typing forensic biological evidence at locus D1S80.

The D1S80 locus is robust, for only a few of the agents chosen to mimic environmental, microbiological, or chemical abuse of body fluid stains had a deleterious effect on D1S80 typing results. D1S80 amplification products could not be produced from semen stains that had been exposed to sunlight for up to six weeks or up to 10 weeks in the shade. Blood and semen stains that been exposed to HCl or bleach could not be typed at this locus.

There was a solitary occurrence of preferential amplification in these studies. Preferential amplification has been observed with both sequence and fragment length polymorphic regions [*30,31*] and is characterized by different amplification efficiencies for the two alleles in a heterozygous DNA specimen. There are several mechanisms that account for preferential amplification, including degradation of template and presence of polymerase inhibitors in the template source. In the present instance of preferential amplification, the stain donor was genotype 18/31 and the stain had been exposed to sunlight for 16 weeks. Despite the fact that this donor

was used to prepare a total of 179 bloodstains in the course of these studies, only once was preferential amplification seen with her blood. Preferential amplification was never seen with any other of the eight body fluid donors used in these studies. Given the potential for occurrence of preferential amplification, albeit remote, care must be exercised when interpreting weak, single band patterns in case work. One probably should declare a questioned specimen extract pattern inconclusive when it displays a weakly amplified single band that matches the smaller of two bands from an exemplar specimen.

It was significant that none of the substrates upon which blood and semen were deposited systematically affected the ability to successfully type D1S80 from those body fluid specimens. There were random instances of amplification failure upon an initial attempt (<5% of the stain extract failed to type); however, many of these samples were successfully amplified during a second try at the same template level. This suggests that specimens that fail to amplify initially should not be abandoned before a second amplification failure has been observed. Also during this series of studies it was recognized that D1S80 amplification products can be obtained from specimens that appear to lack DNA at a level detectable by the slot-blot procedure. Although it may be possible to obtain D1S80 information from such weak extracts, it would seem unwise to use such information for case interpretation because of potential stochastic sampling effects. In fact, when this typing procedure is applied to casework specimens, at least 400 pg DNA per 20 μL of specimen extract must be available before amplification will be attempted.

The observation that blood, semen, and saliva stains could still be genetically typed at locus D1S80 after the stains had been washed in laundry detergent suggests that biological materials deposited onto clothing under normal circumstances might well survive laundering to become visibly occult stains that possess genetic information.

On a volume basis, semen possesses considerably more DNA than blood, and blood more DNA than saliva. Thus, it should not be surprising that the ability to detect D1S80 alleles from two contributors in a mixed body fluid stain would be a function of the types of body fluids mixed and the volume ratio of one fluid to the other. When the stain mixtures were composed of blood from two individuals who did not share a common allele, the genotype of the minor contributor to the stain was apparent by the diminished intensity of their alleles. When the two individuals shared an allele in a stain of mixed bloods, or when the body fluid stain mixtures were composed of semen and saliva or blood and semen, the intensity differences among alleles were less conducive to an accurate assignment of alleles to major and minor contributors.

The lack of effect of microbial presence on DNA typing patterns is consistent with similar studies carried out at the DQα locus [*3*] and loci detectable by RFLP procedures [*27*], but inconsistent with the observations of Lienert and Fowler [*32*]. The latter group observed both inhibition of amplification and allele "dropout" when DNA from several microorganisms was mixed with human DNA and amplified at locus D17S30 or at the ApoB variable number tandem repeat. The lack of such an effect in the present study might be further evidence of the robust nature of the D1S80 locus and the methods used to detect its polymorphic variation.

Out of more than 1200 specimens that were extracted, quantified, amplified, and subjected to electrophoresis in this series of studies, only seven specimens exhibited extra bands (the extra bands were attributable in the eighth specimen extract). In all cases except one, these extra bands were very much weaker than the primary allelic bands. In the one exception, the extra bands were of almost equal intensity to the expected allelic bands. The persistence of these bands was confirmed by reamplification of the specimen extracts. Interestingly, the presence of contaminating DNA in these seven specimens could not be demonstrated when the extracts were amplified and typed in the AmpliType PM system (data not shown). For these experiments, amplified products of the DQα locus were not typed. This observation suggests that the level of contamination in these stain extracts was very low and suggests that the D1S80 typing system might be more sensitive than that of the AmpliType PM system (without DQα).

Extra bands appeared in four extracts from the substrate deposition study. Because old clothing, discarded leather athletic shoes, and used household carpet items were used as some of the deposition substrates, the possibility cannot be ruled out that cellular material, characteristic of prior users of these items, was not present and underlay some of the regions where body fluids were deliberately applied. In fact, one of the adventitious bands detected in a stain deposited on the leather athletic shoes was allele 18. Retrospectively, the donor of the shoes was typed at locus D1S80 and found to possess an 18/18 genotype. Clothing and other items that humans contact in the course of their daily activities undoubtedly can harbor cellular material. When body fluids have been deposited onto such items as a result of violent crime, it is not unreasonable to expect that genetic factors characteristic of normal cellular deposition might be seen in addition to the genetic factors contributed by the evidentiary fluids or tissue

Three of the extracts that displayed extra bands were seen in the study of sunlight exposure effects. New, washed, cotton bedsheet material was used as substrate for the blood and semen stains in this study, so incumbent genetic material can be ruled out as a source of the extra alleles. A plausible explanation for the random appearance of these alleles is the inadvertent contamination of either the stain panels or the extracted DNA prior to amplification.

Specimens displaying extra bands were seen only in the first two validation studies conducted (sunlight exposure and substrate effects). For the majority of specimens, the visual intensity of the extra bands were very much weaker than the primary allelic bands. These two studies alone accounted for 660 of the slightly more than 1200 specimens processed in the entire validation study. The cutting, extraction, quantification, and amplification of such a large number of specimens in parallel offered opportunity for inadvertent contamination. It is notable that in the remainder of the validation studies, in which there were fewer specimens per study, there were no incidents of extra bands seen among the more than 540 specimens processed. The lesson from these observations would appear to be that only a few specimens should be processed at one time if one wishes to eliminate a potential for inadvertent contamination. It should be noted that these validation studies did not follow the procedural policy by which case evidence specimen processing is governed. In our case working laboratory, only a single PCR case is handled at one time, and the known and questioned specimens are processed at separate times to eliminate the potential for cross-contamination.

With the completion of the D1S80 studies described in the current paper, this laboratory has now validated typing procedures for seven genetic loci amplifiable by the PCR [*5,6*], and for a number of polymorphic loci detectable through restriction fragment length polymorphism analysis [*27*]. Other laboratories have contributed their own results [*3–6,12–16,19,20,25–27*] to the fund of knowledge that supports DNA typing of biological evidence in

the forensic laboratory. A common theme within these studies is the lack of false positive or false negative typing results when the procedures are carried out in a controlled fashion and the results are interpreted within the proper guidelines. In fact, it could be argued that for DNA derived from body fluid and tissue specimens subjected to environmental duress, the precepts which we learned from RFLP validation studies [*27*] and from early validation studies at locus DQα [*5*], could have been successfully used to predict the typing behavior at loci validated since, including D1S80. It seems clear that future validations of DNA typing procedures could be grossly reduced in scope without compromising the forensic utility of the procedures.

In conclusion, the reliability of the D1S80 typing procedure we have developed has been evaluated in an eclectic blend of experimental studies that were designed to simulate some of the categories of biological materials that are received by a forensic laboratory. The studies have confirmed that the test procedure yields the correct typing results when applied to specimens of this nature. This typing procedure also has been evaluated with old, non-probative case evidence (manuscript in preparation), where it has continued to demonstrate its effectiveness and utility. The D1S80 test system will be a valuable addition to the collection of tools available to the forensic laboratory for the examination of biological evidence.

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Address requests for reprints or additional information to
F. Samuel Baechtel, Ph.D.
DNA Analysis Unit
JEH FBI Building
Room 3328
10th and Pennsylvania Ave.
Washington, DC 20535