TECHNICAL NOTE

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The Effect of Luminol on Presumptive Tests and DNA Analysis Using the Polymerase Chain Reaction

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ABSTRACT: This study was designed to test the following factors involved with processing luminol treated bloodstained evidence: 1) The reactivity of other presumptive chemical color tests, phenolphthalin (PT) and tetramethylbenzidine (TMB), following the application of the light emitting luminol presumptive test. 2) The effect of different cleanings of various bloody substrates on the luminol test. 3) The effect of different cleanings of various bloody substrates on the ability to obtain DNA suitable for PCR testing. 4) The ability to extract DNA from luminol treated bloodstained substrates using three extraction techniques. 5) The effect of spraying washed and unwashed bloodstains on various substrates with luminol on the ability to correctly type the DNA using PCR. Our findings indicated that luminol did not adversely effect the PCR testing and did not interfere with the PT and TMB presumptive tests for blood. It was determined that the substrate and the method of cleaning were the major factors affecting DNA yield and the ability to type the bloodstains using PCR based technologies.

KEYWORDS: forensic science, blood test, DNA typing, polymerase chain reaction, DQA1, LDLR, GYPA, HBGG, D7S8, GC, Polymarker, short tandem repeats, vWA, FGA, D3S1358, luminol, phenolphthalin, tetramethylbenzidine

Luminol, 3-aminophthalhydrazide, has become a popular presumptive test for blood (1–3), especially in crime scene investigation. In previous research it has been shown that the treatment of bloodstains with luminol can have an effect on the typing of bloodstains using conventional serological typing (4–6), however it does not have an adverse effect on the subsequent analysis of these bloodstains using DNA analysis (4,7,8). In those studies, results were obtained from bloodstains after luminol application using the restriction fragment length polymorphism technique (RFLP) (4,7) and from the DQA1 typing test which utilizes the polymerase chain reaction technique (8). The current research addresses several other issues involved with using luminol at crime scenes as well as subjecting the DNA obtained from these stains to the next generation of DNA typing test—flourescent-based STR analysis.

Like the presumptive chemical color tests phenolphthalin (PT) (9) and tetramethylbenzidine (TMB) (10), the luminol reaction is driven by the peroxidase-like activity of heme. The heme in blood catalyzes the oxidation of luminol in alkaline solution. A positive luminol test is indicated by the emission of light. Two formulations for luminol testing have been used extensively, Luminol I described by Grodsky (11) and Luminol II described by Weber (12). In addition to using luminol to locate traces of blood, it can be used to find bloodstained areas which may have been washed, and to examine large areas for traces of blood in a short period of time. Luminol can also be used to detect blood that has flowed between floor cracks, in back of baseboards or door casings, or has soaked into carpet backings. Until recently, finding trace amounts of blood lead only to presumptive results because restriction fragment length polymorphism (RFLP) DNA analysis could not be performed on the samples due to either the small sample size or the degraded conditions of the samples. With the development of polymerase chain reaction (PCR) DNA techniques, locating traces of blood at a crime scene using luminol testing can lead to conclusive PCR DNA results. Since using luminol at crime scenes has become so widespread, this study was designed to test the following factors involved with processing bloodstained evidence: Comparison of other presumptive chemical color tests to luminol testing; the effect of various substrates and cleaning methods on the luminol reaction; and the effect of different extraction methods on the ability to obtain typeable DNA from samples which were tested with luminol.

Materials and Methods

Sample Application and Cleaning

Seven surfaces were utilized in this study: shag carpet, linoleum floor tile, bare wood, varnished wood, concrete, and painted sheetrock placed in a horizontal position (H) and painted sheetrock placed in a vertical position (V). Each surface was divided into quadrants and a bloody handprint was placed in each quadrant resulting in a total of 28 tests. After drying overnight, the bloody handprints were treated in the following manner: Quadrant I received no washing; Quadrant II was washed with a sponge and warm tap water;

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Quadrant III was washed with a sponge and Neutrad® labware soap (Fisher Scientific, Pittsburg, PA) in warm tap water; and Quadrant IV was washed with a sponge and 10% bleach. The washed areas were allowed to air dry prior to presumptive testing.

Phenolphthalin Testing

A dry cotton-tipped swab was vigorously rubbed over each quadrant. One or two drops of ethanol was added to the swab followed by one or two drops of the phenolphthalin stock solution (9). After waiting a few seconds to allow time for observing a false positive color change, one or two drops of hydrogen peroxide were added. The development of a pink color within 10 sec was a positive result for the indication of blood. A known bloodstain was used as a control to ensure the reagents were working properly.

Tetramethylbenzidine Testing

A dry cotton-tipped swab was vigorously rubbed over each quadrant. One or two drops of tetramethylbenzidine solution (10) (Sigma Chemical Co.) was added to the swab followed by one or two drops of hydrogen peroxide. The development of a green-blue color within 10 s was a positive result for the indication of blood. A known bloodstain was used as a control to ensure the reagents were working properly.

Luminol Application

In a dark room, the surfaces were sprayed with Luminol I (3.5 g Sodium Perborate, 0.5 g Luminol, 25 g Sodium Carbonate in 500 mL water) and luminescent areas were marked. While the surfaces were still wet, the presumptive tests were performed again as previously described on the areas which showed luminescence. After the surfaces had air dried the presumptive tests were performed a third time.

Sample Collection

Three samples were collected from each quadrant of each of the seven surfaces (a total of 84 samples). Samples from the tile and concrete were collected by swabbing the surface with a sterile cotton-tipped swab wetted with stain extraction buffer [1 M Tris, 0.5 M Ethylenediaminetetraacetic acid (EDTA), 5 M NaCl, 20% Sodium Dodecyl Sulfate (SDS)]. Samples from the carpet were collected by cutting fibers from the mesh backing. Samples from the sheetrock and wood were cut from the test surface using a scalpel. All the samples were not collected with the same method; since the best technique for obtaining a sample was not necessarily the same for the different substrates. The methods of sample collection described above are methods which have performed well on case samples in our laboratory.

DNA Extraction

The DNA from a sample from each quadrant was extracted using the following three methods (28 samples extracted using each method): 1) Organic extraction as described by Laber (13), 2) Chelex extraction as described by Walsh (14), and 3) Organic extraction with Centricon clean up as described by Gross (15).

Sample Quantitation

All extracted DNA samples were quantitated by slot blot analysis (16) using the Quantiblot kit (Applied Biosystems, a division of Perkin-Elmer, Branchburg, NJ).

HLA-DQA1 and Polymarker Amplification and Typing

Approximately 2–5 ng of DNA was amplified using the Amplitype® HLA-DQα kit (17) and the Amplitype® PM kit (18) (Applied Biosystems). Amplification, utilizing a Thermal Cycler 480, was performed according to manufacturer's guidelines. The amplified products were typed using reverse dot blot hybridization. Black and white photos were taken of the developed strips. Typing results were compared to the known types previously obtained from the blood donor.

STR Amplification and Typing

Approximately 2 ng DNA was amplified using the AMPF/STR Blue PCR Amplification kit (Applied Biosystems, Foster City, CA). This kit co-amplifies three short tandem repeats, D3S1358 (19), vWA (20), and FGA (21). Amplification, using a Geneamp PCR System 9600, was performed as outlined in the AMPF/STR Users Manual (22). The fluorescently tagged amplified products were analyzed on polyacrylamide gels using a 377 DNA Sequencer (Applied Biosystems). Final concentrations in the gel solution were as follows: 5% Long Ranger DNA Sequencing acrylamide (FMC, Rockland, ME), 1 X Tris-Borate-EDTA, 8 M Urea, 0.001% N,N,N′,N′ tetramethylethylenediamine (TEMED) and 0.05% ammonium persulfate. Gels were cast between the glass plates using a sliding-plate gel caster (23) (The Otter™ Sequencing Gel Casters, Owl Scientific, Inc., Woburn, MA) and allowed to polymerize for 2 h to overnight. Gels were prerun for approximately 1 h (1000 V, 35 mA, 50 W, 51°C) prior to sample loading. One and one half (1.5) μL of amplified product was mixed with 0.5 μL internal standard (GENESCAN-350, DNA labeled with 6-carboxy-X-rhodamine (ROX) purchased from Applied Biosystems) and 4.5 μL loading buffer (2 X TBE, 20 mM EDTA, 20 mg/mL Blue Dextran, 8 M urea). This mixture was denatured at 95°C for 2 min and snap cooled in an ice water bath for 3 min. One and one half (1.5) μL of this mixture was loaded onto the gel. Gels were allowed to run for approximately 2 h (3000 V, 60 mA, 200 W, 51°C). Allele sizes were analyzed in real time using the Local Southern Method (ABI 377 Gene Sequencer Analysis v 1.1 software, Applied Biosystems). Data were analyzed using the Genotyper 2.0 software (Applied Biosystems) and compared to the known types previously obtained from the blood donor.

Results and Discussion

Luminol Testing

Quadrant I (not washed): All surfaces had bright luminescent handprints. Quadrant II (washed with warm tap water): A luminescent pattern of parts of the handprint was detected on the bare wood, varnished wood and cement. All other surfaces had large bright luminescent areas, but no visible patterns. Quadrant III (washed with soap and warm tap water): All surfaces had large bright luminescent areas, but no visible patterns. To determine if this luminescence was due to a substance in the soap, an area of tile without any blood present was washed in the same manner and treated with luminol. No luminescent areas were present, indicating the soap was not interferring with the test. Quadrant IV: (washed with bleach): A very faint handprint was visible on the bare wood, varnished wood and horizontal sheetrock. Areas of faint luminescence were visible on the vertical sheetrock, tile and cement. Only the edges of the washed area on the carpet showed any luminescence.

TABLE 1—*Presumptive test results after luminol treatment.*

Surface	Treatment	PT			TMB		
		B	W	D	B	W	D
Carpet	Not cleaned	+	+	+	+	+	+
	Cleaned w/ H_2O	+	+	+	+	+	+
	Cleaned w/ soap and H_2O	+	+	+	+	+	+
	Cleaned w/ 10% bleach	+	+	w	–	–	–
Tile	Not cleaned	+	+	+	+	+	+
	Cleaned w/ H_2O	+	+	+	+	+	+
	Cleaned w/ soap and H_2O	+	+	+	+	+	+
	Cleaned w/ 10% bleach	+	–	–	w	–	–
Bare Wood	Not cleaned	+	+	+	+	+	+
	Cleaned w/ H_2O	+	+	+	+	+	+
	Cleaned w/ soap and H_2O	+	+	+	+	+	+
	Cleaned w/ 10% bleach	+	+	+	+	+	+
Varnished Wood	Not cleaned	+	+	+	+	+	+
	Cleaned w/ H_2O	+	+	+	+	+	+
	Cleaned w/ soap and H_2O	+	+	+	+	+	+
	Cleaned w/ 10% bleach	+	+	+	+	+	+
Sheetrock-Horizontal	Not cleaned	+	+	+	+	+	+
	Cleaned w/ H_2O	+	+	+	+	–	–
	Cleaned w/ soap and H_2O	+	+	+	+	–	–
	Cleaned w/ 10% bleach	–	–	–	–	–	–
Sheetrock-Vertical	Not cleaned	+	+	+	+	+	+
	Cleaned w/ H_2O	+	+	+	w	–	–
	Cleaned w/ soap and H_2O	+	+	+	+	+	–
	Cleaned w/ 10% bleach	w	w	+	–	–	–
Concrete	Not cleaned	+	+	+	+	+	+
	Cleaned w/ H_2O	+	+	+	+	+	+
	Cleaned w/ soap and H_2O	+	+	+	+	+	+
	Cleaned w/ 10% bleach	+	+	+	+	+	+

PT = Phenolphthalin
TMB = Tetramethylbenzidene
+ = positive result
– = negative result
w = weak result
B = Before luminol application
W = Immediately after luminol application
D = Allowed to dry after luminol application

Phenolphthalin Testing versus Tetramethylbenzidine Testing

Results for the presumptive tests using PT and TMB are given in Table 1. Prior to luminol testing, both PT and TMB performed well in detecting the blood on all surfaces which had not been washed. Both tests gave positive results 7/7 times. Similar results were obtained on the surfaces which had been washed with either water or soap and water. There were 14/14 positive results for both tests, although a weak result was obtained for the sheetrock-V tested with TMB. The testing of the surfaces which had been washed with bleach gave strong positive results with PT on 5/7 surfaces; the sheetrock-V was a weak positive and the sheetrock-H was negative. For those same surfaces tested with TMB, 3/7 gave strong positive results; one weak positive (tile) and three negative results (carpet, sheetrock-V, and sheetrock-H). The results for the PT test after the luminol treatment, both while the surface was still wet from the luminol treatment and after being allowed to dry, were similar to the results obtained before the luminol treatment. The only surface which gave different results after luminol treatment was the tile which was washed with bleach. Prior to luminol testing, the PT test on tile gave a weak positive result, while after luminol treatment the PT results were negative. Four of the surfaces which gave positive results with the TMB test before luminol treatment gave negative results after treatment (both wet and dry), and one of the surfaces which gave positive results before luminol treatment and after luminol treatment (wet) gave negative results once the surface had dried.

DNA Extraction

DNA was obtained from Quadrant I (no cleaning) of all surfaces using each of the three extraction methods, except for the varnished wood. For the varnished wood, DNA was obtained only by the organic method. Since DNA was not obtained using the organic/centricon method, it may indicate that the difference was in the sampling and not in the extraction technique. There was a significant effect from the cleaning of the surfaces on the ability to obtain DNA. DNA was obtained from the water washed quadrants of the bare wood, the carpet and the tile. DNA was obtained from the soap and water washed quadrants of the carpet and the bare wood. The bare wood was the only surface from which DNA was obtained from the bleach washed quadrants. It should be noted that for all samples for which DNA was obtained, there was either visible blood staining or discoloration present. These results demonstrate that cleaning of the surface and the type of surface on which the blood is deposited are both important factors in determining whether DNA is obtained. Since DNA was obtained from all surfaces which were treated with luminol but were not washed in any way, this shows that the application of the luminol directly on the blood does not have an adverse affect on the DNA. This observation is similar to that observed by Hochmeister et al. (7). For the 12 samples where DNA was obtained for the Organic/Centricon method, the average amount obtained was 43 ng (range 10.5 to 90 ng). For the 11 samples where DNA was obtained for the Organic method, the average amount obtained was 35 ng (range 0.8 to 100 ng). For the 6 samples which DNA was obtained for the Chelex method, the average amount obtained was 18 ng (range 6 to 50 ng). These results indicate the Organic and Organic/Centricon extraction methods both give significantly higher yields of DNA than the Chelex extraction method.

HLA-DQA1/PM Amplification and Typing

The samples extracted from Quadrant I were used for this part of the study. Sufficient DNA was obtained for amplification from 6/7 surfaces extracted by all three extraction methods. Although DNA was obtained by using the organic extraction for the varnished wood, the total amount of DNA obtained was below the minimum amount of input DNA for amplification. For the 18 samples (6 surfaces, 3 samples each) for which sufficient DNA was obtained, the only sample which did not amplify was the DNA extracted using

the Chelex method from the blood on the concrete. All samples which amplified gave the same HLA-DQA1/PM DNA types as those obtained from the known blood of the donor for these experiments. The 17 samples that amplified using HLA-DQA1/PM were then subjected to STR typing.

STR Amplification and Typing

Of the 17 samples which were correctly typed using the HLA-DQA1/PM systems, 15 of these also amplified using the AMPF*l*STR BLUE kit. The sample extracted from the tile using the Chelex method gave a partial profile, giving results for the D3S1358 and vWA loci, but not the FGA locus. The one sample which did not amplify at all was the sample organically extracted from the bare wood. All samples which amplified gave the same genotype, as determined using the Genotyper software, as obtained from the known blood of the donor for these experiments.

Conclusion

This study demonstrates and is in agreement to previously reported data (8) that luminol does not have an adverse affect on subsequent DNA typing using PCR. Furthermore, it was shown that the surface on which the blood was deposited does have an affect on whether or not DNA could be obtained. The most deteriorating substrate tested was the varnished wood. It appears that a component within the varnish is degrading the DNA, since typeable DNA was obtained from the bare wood. Based on our results, we conclude that bloodstains treated with luminol will yield true and accurate results using PCR testing. Therefore, luminol can be used to locate traces of blood, areas which have been washed, as well as to examine large areas in a short period of time without compromising the potential for subsequent PCR DNA typing.

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