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ORIGINIAL ARTICLE

Comparison of the effects of two presumptive test reagents on the ability to obtain STR profiles from minute bloodstains

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STR profiling

Abstract *Background:* Bloodstains often constitute the major physical evidence in criminal investigations. In many cases, the bloodstains found by the crime scene examiner are minute, possibly because of dissimulation efforts by the perpetrator to eliminate evidence that reveals his identity. In such cases, short tandem repeat (STR) detection procedures must be performed using the same minute bloodstain evidence on which presumptive tests had been performed earlier. In the present study, two of the most often used presumptive test reagents, phenolphthalein and leucomalachite green, were tested to determine their effects on the ability to obtain STR profiles from minute bloodstains.

Methods: Dried minute bloodstains obtained from 10 donors were treated with phenolphthalein and leucomalachite green. After various times, genomic DNA was extracted from the treated samples using a QIAamp DNA Micro Kit. DNA was quantified with real-time PCR using a Quantifiler Kit. STR loci were amplified using an AmpFLSTR Identifiler Plus Kit, and the amplified products were separated via capillary electrophoresis in a 3130 Genetic Analyzer.

Results: Full DNA profiles were obtained from all minute bloodstain samples treated with phenolphthalein when extracted after intervals ranging from 1 h to 1 week. In contrast, the DNA in minute bloodstain samples treated with leucomalachite green was severely degraded, especially after relatively long intervals, leading to poor partial DNA profiles.

Conclusion: Phenolphthalein is recommended as a safe presumptive test reagent for the detection of blood evidence recovered from crime scenes that might subsequently undergo DNA profiling analysis.

1. Introduction

Blood detected at crime scenes constitutes a major part of the physical evidence in a criminal investigation. It is often decisive in crime elucidation. Adequate collection and preservation of blood samples are critical for establishing associations between the perpetrator, victim, and crime scene.[1]

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Perpetrators hide or wash blood evidence in an attempt to remove traces which can indicate their identity. In such cases, forensic experts direct considerable effort towards finding biological evidence at the crime scene. If found, the evidence could be a minute bloodstain located behind objects or in the grooves of furniture.

Stains suspected of containing blood or another body fluid are initially analysed with presumptive biochemical tests for constituents of body fluids. Stains that yield positive presumptive test results are subjected to further analyses.[2] Some presumptive test reagents can destroy the genetic material assessed in conventional genetic marker analyses.[3]

With the advent of short tandem repeat (STR) analysis of biological evidence recovered from crime scenes, it is necessary to determine whether presumptive test reagents have an adverse effect on the STR loci tested. The most common presumptive test reagents used to identify blood are phenolphthalein and leucomalachite green. This study evaluated the effects of the two presumptive test reagents on the ability to obtain STR profiles from minute bloodstain evidence.

2. Materials and methods

2.1. Samples

Minute bloodstain samples were prepared after withdrawing 1 cm^3 of venous blood from each donor (10 donors were included in the study). Informed consent was obtained from all donors. Blood was immediately deposited on a piece of white 100% cotton without the addition of anticoagulants and allowed to dry for 90 min at room temperature. Bloodstains were cut using sterilized stainless steel scissors into 3-mm^2 pieces to simulate the minute bloodstain evidence recovered from crime scenes. Research samples were divided into two categories. The first category was treated with phenolphthalein, and the second category was treated with leucomalachite green for four time intervals: 1 h, 1 day, 3 days and 1 week. Positive control samples consisting of minute bloodstains were analysed without the application of any reagent. One negative control sample, consisting of a piece of white 100% cotton identical to the cloth used as a substrate in the other samples was included. In total, 91 samples were processed.

2.2. Preparation of reagents

2.2.1. Phenolphthalein

The reagent was prepared by dissolving 1 g of phenolphthalein and 10 g of potassium hydroxide in 50 ml of deionized water. The solution was refluxed over 10 g of zinc granules until it was colourless.[4] The test reagent was prepared by adding 5 ml of the stock solution to 20 ml of ethanol. To the stains, 50 μl of the diluted working solution was applied, followed by 50 μl of freshly prepared 3% hydrogen peroxide.

2.2.2. Leucomalachite green

The reagent was prepared from a dry mixture of 0.1 g of leucomalachite green and 0.32 g of sodium perborate. The mixture was added to 6.6 ml of glacial acetic acid diluted with 3.3 ml of deionized water.[5] To the stains, 50 μl of the solution was applied, followed by 50 μl of freshly prepared 20% hydrogen peroxide.

2.3. DNA extraction

Total genomic DNA was extracted from bloodstain samples (3 mm^2) using a QIAamp DNA Micro Kit (Qiagen) according to the "*Isolation of Genomic DNA from Dried Blood Spots*" protocol.[6] The extracted DNA was stored at 4 °C for less than 2 weeks until further processing.

2.4. DNA quantitation

Two microlitres of each extracted sample was quantified with a Quantifiler Kit (Life Technologies) according to manufacturer's protocol.[7] using a Real-time PCR 7500 system (Applied Biosystems).

2.5. DNA amplification

The AmpFLSTR® Identifiler Plus® PCR Amplification Kit was used to amplify the following autosomal STR loci: D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, amelogenin, D5S818, and FGA. DNA was amplified according to manufacturer's recommendations using a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems). AmpFLSTR® Control DNA 9947A (Applied Biosystems) was also amplified. PCR amplification was performed in a final volume of 25 μl containing PCR reaction mix (10 μl), a primer set (5 μl), and extracted DNA template (10 μl). Initial incubation at 95 °C for 11 min was followed by 28 cycles of denaturation (94 °C for 20 s), and annealing and extension (59 °C for 3 min). A final extension at 60 °C for 10 min followed by a final hold at 4 °C was included at the end.[8] Amplified samples were stored at 4 °C for less than 2 weeks until further processing.

2.6. STR genotyping

One microlitre of PCR product from each sample was mixed with 8.6 μl of Hi-Di formamide (Sigma–Aldrich, St. Louis, MO, USA) and 0.4 μl of Genescan-500 LIZ size standard (Applied Biosystems, Warrington, Great Britain). The mixture was denatured at at 95 °C for 3 min and cooled to 4 °C for 3 min. Electrophoresis was performed on an ABI 3130 Genetic Analyzer using POP4 polymer (Applied Biosystems, Fosters City, CA, USA). Samples were injected for 5 s at 15 kV, and then run at 15 kV for 25 min at a constant temperature of 60 °C. The raw data were collected using 3130 Data Collection software, version 3.0 and analysed using GeneMapper ID-X software, version 1.0. Genotypes were determined by compar-ing the size of the unknown fragments to the allelic ladders provided by the manufacturer.[8]

3. Results

3.1. DNA analysis

DNA extracted from the samples after different time intervals was analysed with real-time PCR to compare the DNA con-

Table 1 Concentration of total genomic DNA extracted from positive control samples.

Positive control samples	DNA concentration (ng/μl)
1	1.59
2	1.07
3	3.76
4	1.99
5	1.55
6	2.36
7	0.526
8	1.22
9	0.687
10	1.57
Mean	1.6323

centrations in the positive control samples with the DNA concentrations in samples treated with the two presumptive test reagents. Tables 1–3 show the concentrations of total genomic DNA extracted from positive control samples, samples treated with phenolphthalein, and samples treated with leucomalachite green respectively.

As shown in Tables 1–3, when comparing the mean DNA concentration of the positive control samples (1.6323 ng/μl) with that of samples extracted 1 h after treatment with the presumptive test reagents, we observed a significant decrease in the DNA concentration in samples treated with phenolphthalein (0.6036 ng/μl) and an even greater decrease in samples treated with leucomalachite green (0.1390 ng/μl). The decrease in the DNA concentration was due to the degradation of genomic DNA in the minute bloodstain samples treated with the presumptive test reagents. The DNA concentration in samples treated with the presumptive test reagents decreased as the time of exposure increased. Figs. 1–4 compare the DNA concentrations of samples treated with phenolphthalein or leucomalachite green to the DNA concentrations of positive control samples after 1 h, 1 day, 3 days, and 1 week, respectively.

Multiplex fluorescent PCR was performed using the Identifiler Plus® PCR Amplification Kit containing 15 polymorphic autosomal STR loci and amelogenin as a gender-determining locus, followed by capillary electrophoresis of amplified fragments and detection of STR genotypes in all samples. Table 4 shows the results of the STR typing of the 10 positive control samples included in the study. In bloodstain samples not treated with any presumptive test reagent, all STR loci analysed were successfully detected after 10 min.

The number of STR loci detected after different time intervals in samples treated with phenolphthalein or leucomalachite green is shown in Table 5.

Table 2 Concentration of total genomic DNA extracted from samples treated with phenolphthalein.

Sample treated with phenolphthalein	DNA concentration (ng/μl)			
	After 1 h	After 1 day	After 3 days	After 1 week
1	0.3740	0.0271	0.0228	0.0234
2	0.4820	0.0383	0.0823	0.1170
3	0.6100	0.1590	0.0805	0.1700
4	0.2180	0.1030	0.0769	0.0300
5	1.2700	0.1040	0.1790	0.1040
6	1.5200	0.4700	0.3840	0.0396
7	0.4210	0.1240	0.1070	0.0366
8	0.4050	0.4680	0.1370	0.1110
9	0.5360	0.1750	0.1900	0.1140
10	0.2000	0.0582	0.0635	0.0089
Mean	0.6036	0.1727	0.1323	0.0755

Table 3 Concentration of total genomic DNA extracted from samples treated with leucomalachite green.

Sample treated with leucomalachite green	DNA concentration (ng/μl)			
	After 1 h	After 1 day	After 3 days	After 1 week
1	0.0748	0.0549	0.0526	0.0292
2	0.0642	0.0929	0.0202	0.0172
3	0.2450	0.1740	0.1750	0.0895
4	0.0546	0.0558	0.0197	0.0632
5	0.3740	0.0961	0.1410	0.0791
6	0.0161	0.1390	0.0696	0.0085
7	0.0155	0.0072	0.0207	0.0883
8	0.2160	0.0572	0.1290	0.0114
9	0.0635	0.0810	0.0125	0.0191
10	0.2660	0.0466	0.0216	0.0196
Mean	0.1390	0.0805	0.0662	0.0425

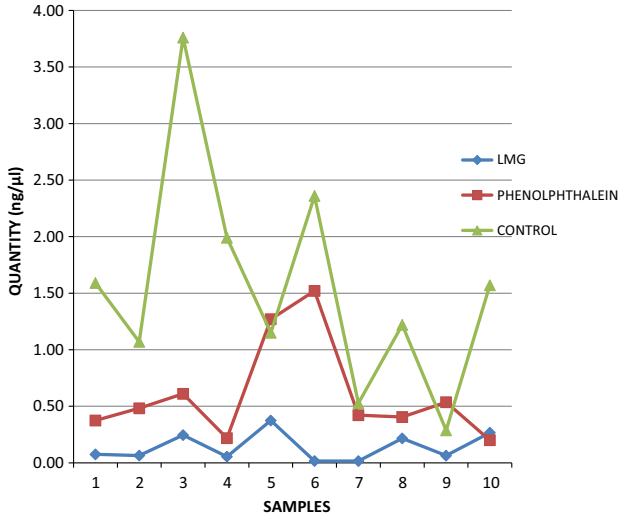

Figure 1 DNA concentration after 1 h in samples treated with phenolphthalein, samples treated with leucomalachite green (LMG), and positive control samples.

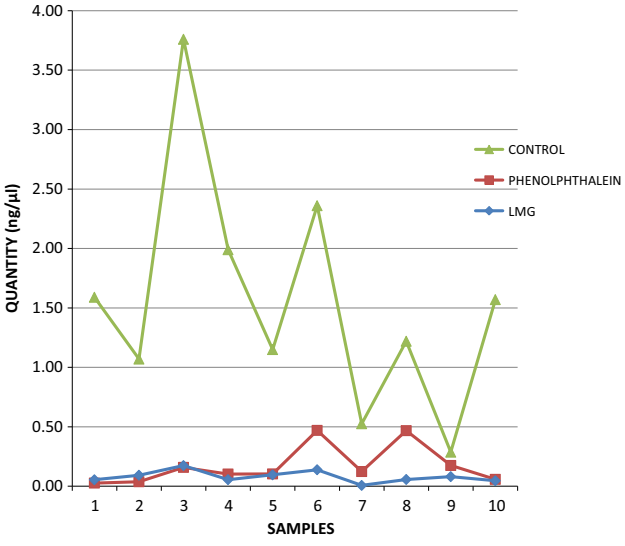

Figure 2 DNA concentration after 1 day in samples treated with phenolphthalein, samples treated with leucomalachite green (LMG), and positive control samples.

3.2. Statistical analysis

To compare the percent of STR loci detected with the presumptive test reagents, a Z-test was performed to assess the level of significance (0.01) using the following two hypothesis[9]:

Null hypothesis H_0: The percent of STR loci detected in the samples treated with phenolphthalein = the percent of STR loci detected in the samples treated with leucomalachite green.

Alternative hypothesis H_1: The percent of STR loci detected in the samples treated with phenolphthalein > the percent of STR loci detected in the samples treated with leucomalachite green.

The results are shown in Table 6.

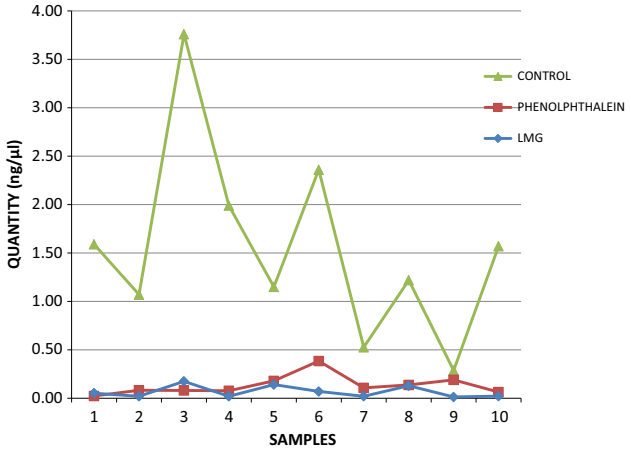

Figure 3 DNA concentration after 3 days in samples treated with phenolphthalein, samples treated with leucomalachite green (LMG), and positive control samples.

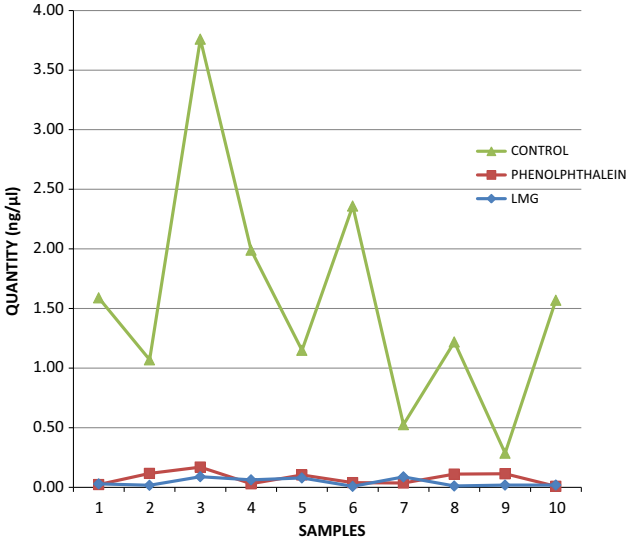

Figure 4 DNA concentration after 7 days in samples treated with phenolphthalein, samples treated with leucomalachite green (LMG), and positive control samples.

From the statistical analyses (Table 6), we found that the *P*-value was less than the statistical level of significance (α = 0.01) for all time intervals when samples treated with phenolphthalein and samples treated with leucomalachite green were compared. This led to the acceptance of the alternative hypothesis (H_1), which states that the percent of STR loci detected in samples treated with phenolphthalein is significantly higher than the percent of STR loci detected in samples treated with leucomalachite green.

4. Discussion

Presumptive tests are a necessary part of the analysis of evidentiary materials recovered from crime scenes. Given that some

Table 4 STR typing results of positive control samples.

STR/Sample	1	2	3	4	5	6	7	8	9	10
D8S1179	13,15	13,14	10,15	10,14	10,13	10,15	14,16	15,15	12,13	14,15
D21S11	29,29	31,32.2	28,30	29,30	28,30	28,30	28,30	28,29	29,30	28,23.2
D7S820	9,11	10,11	10,11	8,8	10,10	10,10	8,9	11,11	8,11	10,11
DSF1PO	11,12	11,11	11,11	11,12	12,12	11,12	10,12	10,12	10,10	10,11
D3S1358	15,15	16,16	15,16	13,16	14,16	16,17	15,16	15,16	16,17	15,17
THO1	7,7	7,9	7,9.3	7,6	6,9	7,9	8,9	7,9.3	8,9	6,7
D13S317	11, 12	9,11	11,13	12,13	8,11	11,13	11,12	12,14	11,12	12,12
D16S539	9,10	11,11	9,11	9,9	9,11	9,12	11,13	9,12	11,11	11,12
D2S1338	17,24	18,24	18,24	16,25	17,23	17,18	19,20	17,24	20,24	17,24
D19S433	13,16	11,12.2	13,15.2	12,12	15.2,16	13,16	14,15	13,16	12,14.2	13,13
vWA	15,17	17,20	15,16	17,17	14,16	15,16	15,17	15,16	14,17	15,18
TPOX	8,10	8,11	9,10	8,10	8,8	9,11	8,9	9,10	8,9	8,9
D18S51	15,15	15,16	15,16	14,16	15,17	15,16	15,16	15,16	13,22	15,16
Amelogenin	X,X	X,X	X.X	X,X	X,X	X,Y	X,Y	X,Y	X,Y	X,Y
D5S818	8,13	10,11	13,13	10,11	10,11	12,13	11,12	13,13	9,13	12,13
FGA	24,25	24,24	23,26	19,21	22,25	23,23	21,25	23,23	21,23	19,19

Table 5 Number of STR loci detected after different time intervals in samples treated with presumptive test reagents.

Partial profile (3–8) STR loci samples	Partial profile (9–15) STR loci samples	Full profile samples	Time interval	Presumptive test reagent
0 samples	0 samples	10 samples	1 h	Phenolphthalein
0 samples	0 samples	10 samples	1 day	
0 samples	0 samples	10 samples	3 days	
0 samples	0 samples	10 samples	1 week	
0 samples	3 samples	7 samples	1 h	Leucomalachite green
0 samples	4 samples	5 samples	1 day	
0 samples	4 samples	5 samples	3 days	
0 samples	5 samples	4 samples	1 week	

Table 6 *Z*-test for comparing the percent of STR loci detected in samples treated with phenolphthalein and samples treated with leucomalachite green.

P-value	*Z*	Difference in percent between full profile samples (%)	Percent of samples with full profiles (%)	Number of samples with full profiles	Total number of samples	Reagent	Duration of exposure
0.005	2.592	30	100	10	10	Phenolphthalein	1 h
			70	7		Leucomalachite green	
0.000	3.512	50	100	10		Phenolphthalein	1 day
			50	5		Leucomalachite green	
0.000	3.512	50	100	10		Phenolphthalein	3 days
			50	5		Leucomalachite green	
0.000	3.963	60	100	10		Phenolphthalein	1 week
			40	4		Leucomalachite green	

presumptive tests can interfere with conventional genetic marker analyses, such that they yield no or inconclusive results, deleterious effects might also occur in the STR analysis of body fluid stains. As DNA typing gains widespread implementation in the forensic science community, it is essential to understand the effects of the most common presumptive test reagents.[2]

This study evaluated the effect of two widely used presumptive test reagents on the ability to obtain STR profiles from minute bloodstain evidence. The results showed that both presumptive test reagents, phenolphthalein and leucomalachite green, degraded the DNA contained in minute bloodstains (Fig. 5). The extent of degradation increased as the time of exposure before DNA extraction increased (Table 7).

Despite the degradation of DNA by phenolphthalein, the amount of extracted DNA was sufficient to produce full profiles after amplification with the AmpFLSTR® Identifiler Plus® PCR Amplification Kit. However, after the treatment with leucomalachite green, degradation was extensive, resulting in partial profiles for the majority of samples mixed with

Table 7 Extent of DNA degradation induced by phenolphthalein and leucomalachite green in minute bloodstain evidence.

Duration of DNA exposure	Percent degradation by phenolphthalein	Percent degradation by leucomalachite green
1 h	61.12	91.05
1 day	88.88	94.82
3 days	91.48	95.74
7 days	95.14	97.26

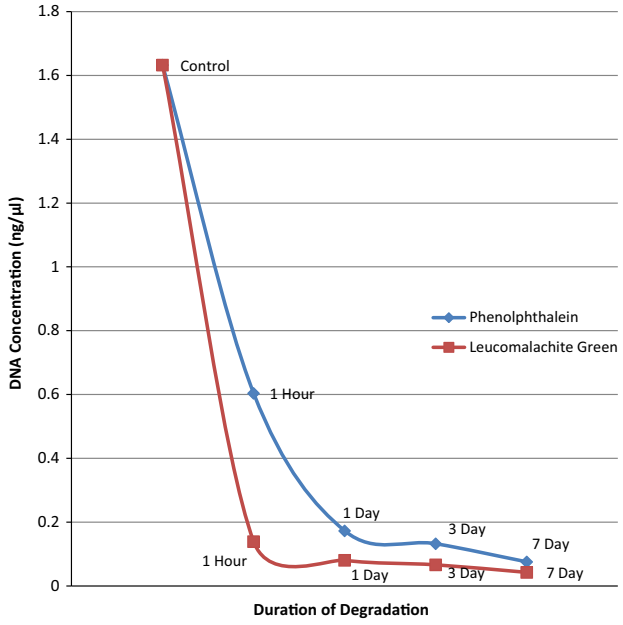

Figure 5 Effects of phenolphthalein and leucomalachite green on DNA degradation after different time intervals.

the pre- sumptive test reagent and thus adversely affecting the eviden-tiary power of STR typing in the legal arena.

Conclusion

Our results recommend the use of phenolphthalein as a pre-sumptive test reagent when analysing evidentiary blood samples that will undergo DNA analysis with STR profiling. In such cases, the use of leucomalachite green is not recommended.

Funding

None.

Informed consent

Informed consent was obtained from all donors.

Conflict of interest

The authors declare no conflict of interest.

Ethical approval

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