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Effects of Presumptive Test Reagents on the Ability to Obtain Restriction Fragment Length Polymorphism (RFLP) Patterns from Human Blood and Semen Stains

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ABSTRACT: Some of the commonly used presumptive test reagents for identification of blood and semen could potentially affect the recovery of intact high-molecular-weight deoxyribonucleic acid (DNA) from evidentiary samples. Thus, the capability of performing restriction fragment length polymorphism (RFLP) analysis on evidentiary samples could be compromised.

In order to investigate the potential effects of presumptive test reagents on the DNA present in these samples, bloodstains on cotton and glass were exposed directly to luminol, benzidine, phenolphthalein, *o*-tolidine, and leucomalachite green, while semen stains and vaginal swabs containing semen were exposed directly to bromochloroindolyl phosphate (BCIP) and sodium thymolphthalein monophosphate (STMP) reagents.

The yield gels for DNA quality and quantity and RFLP results indicated that bloodstains exposed to luminol, benzidine dissolved in ethanol, and phenolphthalein, as well as semen stains and vaginal swabs exposed to BCIP and STMP yield RFLP patterns consistent with that of the uncontaminated control. Except for the phenolphthalein treatment, the quantity of extractable, high-molecular-weight DNA obtained was comparable with that of untreated stains. Therefore, evidentiary material purposely or inadvertently contaminated with these reagents can be successfully typed. However, stains exposed to benzidine dissolved in glacial acetic acid, leucomalachite green, and *o*-tolidine failed to yield high-molecular-weight DNA or to produce any RFLP patterns.

KEYWORDS: criminalistics, deoxyribonucleic acid (DNA), body fluids, presumptive tests, identification of blood, identification of semen, RFLP

The analysis of stains suspected of containing blood or semen is initiated by carrying out presumptive biochemical tests for constituents of these body fluids. Stains that yield positive presumptive test results are subjected to further analyses. The most common presumptive tests employed in the potential identification of blood are luminol, leuco-

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malachite green, phenolphthalein, *o*-tolidine, and benzidine, while bromochloroindolyl phosphate (BCIP) and sodium thymolphthalein monophosphate (STMP) are used for the presumptive identification of semen stains. It is known that some of these presumptive test reagents can have a destructive effect on conventional genetic marker analyses [*1*]. Therefore, presumptive testing is usually performed by swabbing or excision of only a small portion of a stain.

With the advent of restriction fragment length polymorphism (RFLP) analysis of body fluid stains, it became pertinent to determine whether or not these presumptive test reagents would have an adverse effect on the high-molecular-weight deoxyribonucleic acid (DNA) in evidentiary samples and thereby impact RFLP results.

Materials and Methods

Blood and Semen Specimens

Fifty and 100-μL aliquots of blood, drawn by venipuncture into red stopper tubes (without preservative) were placed on glass and 100% cotton substrates and air-dried at ambient temperature for 24 h after deposition. Thirty and 50-μL aliquots of semen were placed on cotton and allowed to air-dry for 24 h at ambient temperature. Vaginal swabs containing semen were collected from volunteer donors, dried with a swab dryer, and stored at ambient temperature for 24 h.

A total of 235 samples were processed.

Reagents

Luminol—Luminol was prepared by two methods [*1,2*]. Both used 0.1 g of luminol and 5 g of sodium carbonate dissolved in 100 and 90 mL, respectively, of distilled water (final luminol concentration, 0.1%). In the first protocol, 0.7 g of sodium perborate was added immediately prior to use, while in the second, 10 mL of 3% hydrogen peroxide was added immediately prior to use.

Benzidine—Benzidine was also prepared by two methods [*2,3*]. The first used 0.25 g of benzidine dissolved in 175 mL of absolute ethanol and 0.5 mL of glacial acetic acid (final benzidine concentration, 0.14%). This solution was added to the stain, followed by the addition of freshly prepared 3% hydrogen peroxide. The second method dissolved 1 g of benzidine in 10 mL of glacial acetic acid. The reagent was applied to the stain, followed by the addition of fresh 20% hydrogen peroxide.

Phenolphthalein—This reagent was prepared by dissolving 2 g of phenolphthalein and 20 g of potassium hydroxide in 100 mL of distilled water. The solution was refluxed over 20 g of zinc granules until it was colorless [*2*]. The test reagent was prepared by adding 5 mL of this stock solution to 20 mL of ethanol. The diluted working solution was applied to the stains, followed by the addition of freshly prepared 3% hydrogen peroxide.

Orthotolidine—*o*-Tolidine stock solution was prepared by dissolving 4 g of the reagent in 100 mL of methanol. A working solution was prepared by mixing equal parts of the stock solution, glacial acetic acid, and distilled water [*4*]. The working solution was applied to the stains, followed by the addition of freshly prepared 20% hydrogen peroxide.

Leucomalachite green—This 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 distilled water [*5*]. This solution was applied to the stains, followed by the addition of freshly prepared 20% hydrogen peroxide.

BCIP—This reagent was prepared by dissolving 0.25 g of BCIP in a few drops of dimethylsulfoxide (DMSO). Then this solution was added to 500 mL of 0.01*M* sodium acetate buffer, pH 5.5, containing 0.2 g of thimerosal (final BCIP concentration 0.05%) [*2,6*].

STMP—STMP was prepared as a three-step test [*2,7*]. The first reagent was prepared by the addition of 1.85 g of STMP to a sodium citrate buffer (2.0 g of citric acid monohydrate (26.17 g of trisodium citrate dihydrate in 1 L of distilled water, titrated to pH 5.95).

N-2-hydroxyethylpiperazineethanesulfonic acid (HEPES)-buffered saline (HBS) was prepared by adding 0.842 g of sodium chloride (NaCl) and 0.238 g of HEPES to 100 mL distilled water, titrated to pH 7.2.

The color developer was prepared by dissolving 0.2 g of sodium hydroxide (NaOH) and 0.53 g of sodium carbonate in 100 mL of distilled water. HBS and STMP were added to the stains and vaginal swabs, followed by addition of the color developer.

Methods

Using an aerosol sprayer or a dropper bottle, each test reagent was added to the stains and swabs to produce a strong positive result. Special care was taken to ensure that the entire surface of the stains and swabs was covered with the presumptive test reagent. At timed intervals (one day through four weeks), samples were subjected to RFLP analysis by a previously described method [*8,9*]. Loci D10S28, D2S44, D17S79, D1S7, and D4S139 were typed in this analysis.

Results

After extraction of DNA from the stains, one day through four weeks after contamination, high-molecular-weight DNA was detectable under ultraviolet (UV) light in a 1% agarose gel containing ethidium bromide from the stains exposed to luminol, benzidine dissolved in ethanol, and phenolphthalein, as well as from semen stains and vaginal swabs exposed to BCIP and STMP (Fig. 1).

Semiquantitative information from these gels demonstrated that these test reagents did not appear to have an effect on the qualitative and quantitative recovery of high-molecular-weight DNA—the exception was phenolphthalein-treated stains, which had an apparent lower recovery yield of high-molecular-weight DNA. In all cases where there was detectable high-molecular-weight DNA, the generated RFLP patterns were identical to those of the untreated control samples (Fig. 2).

Bloodstains treated with benzidine dissolved in glacial acetic acid, *o*-tolidine, and leucomalachite green produced no or little detectable DNA on the test gel. The small amount of DNA on the test gels was present only in the low-molecular-weight region (indicating degraded DNA), and clearly the DNA was insufficient to produce a RFLP pattern.

Conclusions

Presumptive tests are a necessary part of the analysis of evidentiary material. Since it is known that some of these tests can interfere with conventional genetic marker analyses, yielding no or inconclusive results, such deleterious effects also may occur for the RFLP analysis of body fluid stains. As DNA typing gains widespread implementation in the forensic science community, it is essential to be aware of the effects that can be produced by the most common presumptive test reagents.

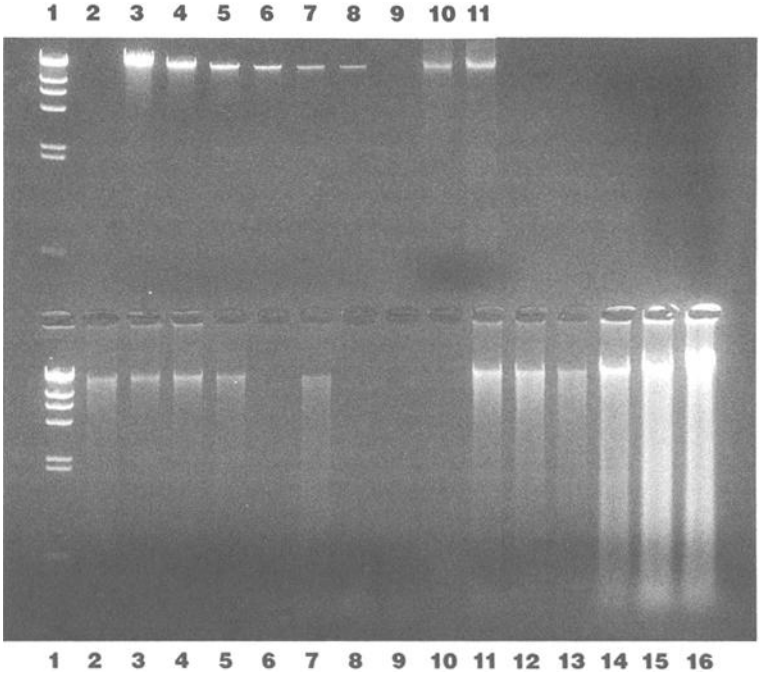

FIG. 1—*Test gel.* Upper part of the gel: *Lane 1 = 200 ng of lambda*/Hind *III digest; Lane 2 = blank; Lanes 3 through 8 = 600, 300, 150, 75, 37, and 19 ng of lambda DNA, respectively; Lane 9 = blank; Lanes 10 and 11 = 100 and 200 ng of human DNA from K562 cell line, respectively.* Lower part of the gel: *Lane 1 = 200 ng of Lambda*/Hind *III digest; Lane 2 = untreated control bloodstain; Lane 3 = bloodstain treated with luminol-perborate; Lane 4 = bloodstain treated with luminol/hydrogen peroxide; Lane 5 = bloodstain treated with benzidine dissolved in ethanol; Lane 6 = bloodstain treated with benzidine dissolved in glacial acetic acid; Lane 7 = bloodstain treated with phenolphthalein; Lane 8 = bloodstain treated with orthotolidine; Lane 9 = bloodstain treated with leucomalachite-green; Lane 10 = blank; Lane 11 = untreated control semen stain; Lane 12 = semen stain treated with BCIP; Lane 13 = semen stain treated with STMP; Lane 14 = untreated vaginal swab—male fraction; Lane 15 = vaginal swab treated with BCIP—male fraction; Lane 16 = vaginal swab treated with STMP—male fraction. Extracted DNA was resolubilized in 36 μL TE-buffer; 4 out of 36 μL were loaded on the test gel.*

The results demonstrate that direct testing of blood stains with benzidine dissolved in glacial acetic acid, *o*-tolidine, and leucomalachite green can have a negative effect on the ability to produce RFLP patterns. Treatment with phenolphthalein reduces the amount of extractable high-molecular-weight DNA from bloodstains. Conversely, benzidine dissolved in ethanol and luminol did not interfere with the ability to recover high-molecular-weight DNA and to produce RFLP patterns. Direct testing of vaginal swabs or semen stains with BCIP and STMP yielded RFLP patterns identical to those of the untreated control samples.

This study demonstrates that evidentiary body fluid stains purposely or inadvertently contaminated with luminol, benzidine dissolved in ethanol, or phenolphthalein still may be successfully typed by RFLP procedures. Direct testing of vaginal swabs or semen stains with BCIP or STMP has no effect on a subsequent RFLP analysis. In spite of these findings, we recommend that analysts continue the prudent practice of testing small portions of an evidentiary stain prior to submission for RFLP analysis, as is currently done for conventional genetic marker analysis.

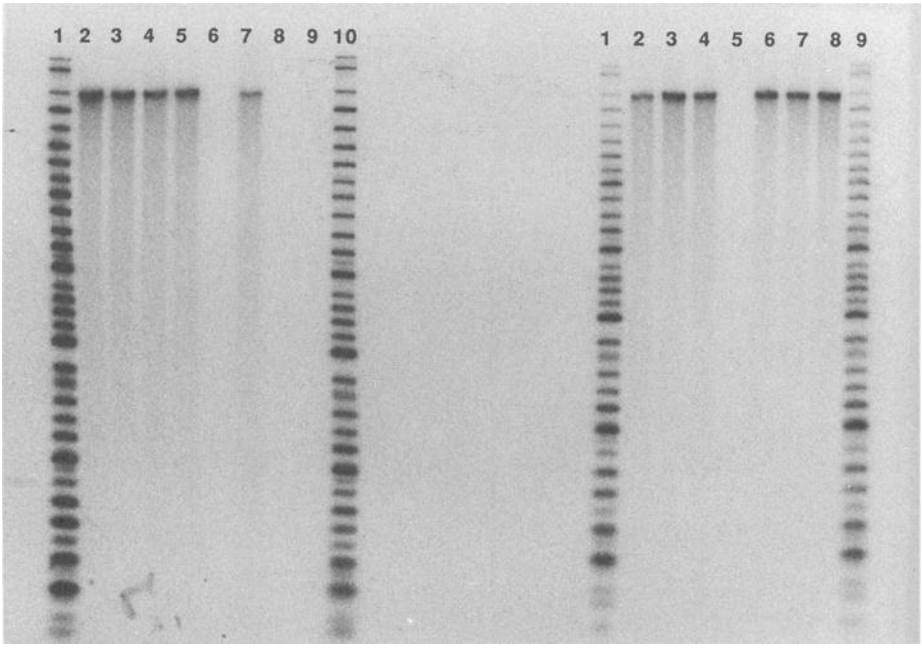

FIG. 2—*RFLP profiles demonstrating the effects of presumptive test reagents.* Left autoradiogram: *Lane 1 = 23.0 kb size standard (life codes); Lane 2 = untreated control bloodstain; Lane 3 = bloodstain treated with luminol-perborate; Lane 4 = bloodstain treated with luminol/hydrogen peroxide; Lane 5 = bloodstain treated with benzidine dissolved in ethanol; Lane 6 = bloodstain treated with benzidine dissolved in glacial acetic acid; Lane 7 = bloodstain treated with phenolphthalein; Lane 8 = bloodstain treated with orthotolidine; Lane 9 = bloodstain treated with leucomalachite-green; Lane 10 = 23.0 kb size standard (life codes).* Right autoradiogram: *Lane 1 = 23.0 kb size standard (life codes); Lane 2 = untreated control semen stain; Lane 3 = semen stain treated with BCIP; Lane 4 = semen stain treated with STMP; Lane 5 = blank; Lane 6 = untreated vaginal swab—male fraction; Lane 7 = vaginal swab treated with BCIP—male fraction; Lane 8 = vaginal swab treated with STMP—male fraction; Lane 9 = 23.0 kb size standard (life codes). All lanes contain about 500 ng of human genomic DNA. The autoradiography exposure time was 48 h. The individual typed shows homozygosity for the locus D4S139.*

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