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Critical Revision of Presumptive Tests for Bloodstains

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Introduction

Advances in the polymerase chain reaction-based (PCR-based) forensic nuclear DNA procedures allow analysis of bloodstains containing as little as 200 picograms (pg) of DNA. It is possible that a 25-µL bloodstain diluted to approximately 1:3500 would contain sufficient DNA for PCR-based analysis. The analysis of mitochondrial DNA (mtDNA) requires less template DNA.

To achieve this, preliminary steps in a bloodstain investigation should provide precise and reliable results. An inadequate interpretation of stains may invalidate a criminal investigation. Normally the stain investigation should follow these steps:

1. Visually examine the stain or substance. Ideally, the visual aspect of the bloodstain is sufficiently expressive to point toward certain conclusions. In the event of doubts, these may be resolved in subsequent stages.

2. Perform presumptive tests using chemical reagents. DNA typing can be performed on stains that have presumptively tested positive for the presence of blood and have a visual appearance of blood. Further serological examinations can be performed if sufficient material remains.

3. Perform confirmatory tests to determine if blood is present. Confirmatory tests are less sensitive than presumptive and species-origin tests. Therefore, it is possible to obtain a negative result for a confirmatory test in the presence of blood. Hence, a negative confirmatory test does not negate the presence of blood.

4. Employ a species-origin test, if sufficient material remains, to determine if a stain (which may or may not have tested positive for the confirmatory test) contains human protein.

5. Employ DNA analysis to determine the genotype and the gender of the bloodstain's donor.

As an example, a case presents a stain that is thought to be a bloodstain after visual inspection. Chemical tests are performed, and a negative result is obtained. An investigator would normally conclude that it is not a bloodstain or that only an imperceptible amount of blood is present. Therefore, Steps 4 and 5 would not be performed.

Our study questions the dependability usually attributed to presumptive tests. Throughout the process, we found that a stain that was clearly a bloodstain gave a negative result in the presumptive test. This result was obviously a false negative, a nonlegitimate negative that might have detained a perfectly viable and necessary investigation.

False positives in presumptive tests have been studied by a number of authors (Cox 1991; Culliford and Nickols 1964), but we found no studies that show experimental proof of obtaining false negatives. The first experiments we performed to study false negatives resulted in the affirmation that when lemon juice is added to a blood sample, its acid content may prevent the detection of a positive test result (Verdú Pascual and Gisbert Grifo 1995). Because we knew that the chemical process on which presumptive tests are based is an oxidation-reduction reaction in which a reagent (o-tolidine,

tetramethylbenzidine, phenolphthalein, and leucomalachite green) is oxidized by hydrogen peroxide in the presence of peroxidases, we concluded that the substance capable of interfering in the test must be a reduction compound.

Given that the aim of our study was to demonstrate that reduction substances contaminating a bloodstain may prevent a positive test result from being observed, ascorbic acid was chosen as the substance to be used after the components of lemon juice were analyzed (Windholz et al. 1996). For this reaction, the reduction power of the contaminator had to be adequate, and the variations produced depended on the type of reagent used for the test (tolidine and phenolphthalein). Nevertheless, should this working hypothesis be confirmed, it can be concluded that all tests grouped as catalytic tests entail the possibility of producing false negatives. Our study focused on false negatives by applying the tolidine test on a bloodstain (Castelló 1997; Gisbert 1998).

The next step is to extend our study to other reagents used in presumptive tests. If the cause of the interference produced in the test proves correct, similar results using other reagents can be expected. We plan to continue seeking an explanation of interferences in the test reaction. In our initial study, however, the following aims were pursued:

- test that the addition of ascorbic acid to a bloodstain produces a false negative;
- determine the effect of the contaminant on the different sample types used (liquid, stain, and print);
- determine if false negatives are obtained regardless of the reagent employed, allowing for the possibility that false-negative results are caused by contamination; and
- evaluate possible differences in the results obtained using different reagents.

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Materials and Methods

Reagents	*Material For*
O-tolidine (Probus)	Volume measurement
Tetramethylbenzidine (Probus)	Preparation and study of samples
Leucomalachite green (Merck)	Blood extraction
Phenolphthalein (Panreac)	Also:
Ethanol (Panreac)	Analytical balance
Potassium hydroxide (Panreac)	Centrifuge
Powdered zinc (d'Hemio)	Oven
Hydrogen peroxide (Prolabo)	Refrigerator
Glacial acetic acid (Probus)	Observation chamber
Ascorbic acid (Merck)	Pressure roller
Distilled water	

Tests Using Tolidine:

1. Collection: We obtained blood specimens by means of

venipuncture;

2. Reagent preparation: We added 1.6 g of o-tolidine base to 40 mL of absolute ethanol. To this we added 30 mL of glacial acid and 30 mL of distilled water.

3. Specimen preparation: We prepared blood solutions. Concentration is expressed in milliliters of blood per total volume in milliliters. We added 1 mL of the blood sample to a test tube, followed by 9 mL of distilled water, for a final solution concentration of 1:10. We diluted 1 mL of 1:10 solution to 10 mL to yield a corresponding 1:100-concentration solution. We used the same procedure, in turn, to obtain sample concentrations of 1:1000, 1:10000, and 1:100000.

4. Acid solution preparation: Using the normal concentration of lemon juice in ascorbic acid, 0.38 mg/mL (Jimenez et al. 1994), we prepared a base aqueous solution to test the bloodstain and to obtain diluted solutions with a smaller concentration of the contaminant, from 2.e-3 M to 2.e-7 M.

5. Blood-specimen preparation with the addition of the contaminant: Each test tube sample contained 1 mL of the blood solution and 1 mL of the different ascorbic acid solutions.

6. Bloodstain preparation on semiporous filter paper: We applied five drops of the sample on filter paper.

7. Study of the presumptive test: We added two drops of reagent to the samples (fresh solution [0.1 mL], dried stain, and print), and then if no color change was observed, we added two drops of a 3 percent solution of hydrogen peroxide.

> 7.1. *Control test:* We ensured that no positive reactions were caused by the contaminant.
>
> 7.2. *Analysis of liquid, stain, and print samples:* We obtained prints by dampening filter paper with distilled water and applying pressure over the stain. The test was performed on the print leaving a stain on the paper.

Tests Using Tetramethylbenzidine, Leucomalachite Green, and Phenolphthalein:
We repeated the procedure previously described using the reagents tetramethylbenzidine, leucomalachite green, and phenolphthalein, but the tests were only performed on stains on filter paper. We prepared the reagents as described by Eckert and James (1989).

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Results

Tests Using Tolidine:
The results we obtained are shown in Table 1. This table was used to construct Figures 1A, 1B, and 1C.

Tests Using Tetramethylbenzidine, Leucomalachite Green, and Phenolphthalein:
The results we obtained are shown in Table 2. Figure 2 provides a comparison of the effectiveness of the reagents in samples

contaminated by ascorbic acid.

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Evaluation of the Results

When added to a blood sample, ascorbic acid gives rise to a false negative upon application of the presumptive test using tolidine as a reagent. The effect of the contaminator may vary as follows:

- With the fresh solution samples, the first negative readings corresponded to samples of 1:2000 blood solution.
- In the case of 1:200000 blood solution samples, we observed negative readings throughout the concentration interval when using the contaminant.
- In the case of dried stain samples, our results indicate that the contaminant does not interfere with the outcome of the test when using blood concentrations in the range of 1:2 to 1:20000.
- In the print samples, we noted no interference with the reaction for blood concentrations in the 1:2 to 1:2000 interval.
- We recorded negative results after performing tests with samples at a concentration of 1:20000 in blood.
- We performed no tests on 1:200000 specimens because, on applying the assay to a print without any contaminant, the outcome was found to be negative already.

The second experiment we performed demonstrates the possibility of obtaining false negatives with reagents frequently used in presumptive tests. Just as false positives are sometimes encountered, our tests affirm that false-negative results may be obtained as a result of sample contamination.

We proved that there are differences in sensitivity with respect to the action of the contaminant among different reagents. Following analysis of the tests performed on stains on paper, we found that false negatives were detected using tetramethylbenzidine with low-concentration blood samples (1:400000), whereas o-tolidine and leucomalachite green produced the same with samples that were slightly more concentrated (1:200000) and phenolphthalein gave false negatives in samples concentrated at 1:2000.

One possible explanation is that the reducer competes for oxygen in the reagent and prevents oxidation. Alternately, the ascorbic acid may also reduce the reagent after it has been oxidized by the hydrogen peroxide, thus preventing the characteristic color from appearing because of the breakdown of the reagent and its rapid disappearance.

The presence of ascorbic acid may not be the only cause of false results. It is possible for a bloodstain to go unnoticed if it has had sufficient contact with a product with a high reduction strength (detergents and foods). Evidence examiners should also consider that a variety of circumstances, such as washing, rain, heat, and time, may reduce the concentration of blood in the sample, with small amounts of

contaminant being sufficient to give rise to a false negative in the presumptive test. Examiners may, therefore, encounter stains that look like blood, but these stains would not be recognized as such in a presumptive test. This would give rise to a false negative in an otherwise reliable presumptive test, with the result that important evidence may be lost.

The frequency with which contamination-related problems are encountered is unknown, but such problems do exist. Therefore, it is impossible to enumerate the specific circumstances in which special care should be taken to avoid such problems. As we noted in the introduction, the important point is that presumptive tests performed on a bloodstain may give rise to a negative result, thereby truncating subsequent tests.

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Summary

We studied false-negative results in bloodstain presumptive tests using blood samples to which contaminating substances were added. As a result, we found that a reduction compound (ascorbic acid) added to the blood sample may give rise to a false-negative result for the test. This occurred regardless of the type of sample used (liquid, stain, or print), although different degrees of sensitivity were observed.

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