J Forensic Sci, January 2007, Vol. 52, No. 1
doi:10.1111/j.1556-4029.2006.00324.x
Available online at: www.blackwell-synergy.com

TECHNICAL NOTE

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Evaluation of Six Presumptive Tests for Blood, Their Specificity, Sensitivity, and Effect on High Molecular-Weight DNA

ABSTRACT: Luminol, leuchomalachite green, phenolphthalein, Hemastix®, Hemident™, and Bluestar© are all used as presumptive tests for blood. In this study, the tests were subjected to dilute blood (from 1:10,000 to 1:10,000,000), many common household substance, and chemicals. Samples were tested for DNA to determine whether the presumptive tests damaged or destroyed DNA. The DNA loci tested were D2S1338 and D19S433. Leuchomalachite green had a sensitivity of 1:10,000, while the remaining tests were able to detect blood to a dilution of 1:100,000. Substances tested include saliva, semen, potato, tomato, tomato sauce, tomato sauce with meat, red onion, red kidney bean, horseradish, 0.1 M ascorbic acid, 5% bleach, 10% cupric sulfate, 10% ferric sulfate, and 10% nickel chloride. Of all the substances tested, not one of the household items reacted with every test; however, the chemicals did. DNA was recovered and amplified from luminol, phenolphthalein, Hemastix®, and Bluestar©, but not from leuchomalachite green or Hemident™.

KEYWORDS: forensic science, luminol, leuchomalachite green, phenolphthalein, Hemastix®, Hemident™, Bluestar©, presumptive tests, sensitivity, specificity, DNA recovery

Blood is the most common and perhaps the most important form of evidence in the world of criminal justice today (1). Blood evidence associated with a crime can provide essential information that may help solve a case, collaborate witness testimony, define a scene of crime, link a suspect and scene, or simply point the investigation in a new direction (1,2). Therefore, it is important to identify any stain that could potentially be blood at a crime scene. Obvious bloodstains should never be contaminated with any reagent (3). When encountered with a potential bloodstain that cannot be identified immediately, several questions enter the mind of an investigator or forensic scientist. These include "What is that stain?"; "Could it be blood?"; or if a stain is expected or suspected, and is absent, "Was there blood here at one time?" Cox (4) describes the attributes that a good presumptive test for blood should have: it should be sensitive, specific, quick, simple, and safe.

More recently, these questions have expanded to include "Whose blood is this?"; "Can it be excluded from a control or known sample?"; and "Is there enough genetic material here to obtain a complete DNA profile?" It is therefore obvious that within a forensic context, the most important components of blood are those that can be used for blood identification and to individualize it (5).

In order for these presumptive tests for blood to function properly, they must detect a component of blood, which ideally should not be commonly found in the everyday environment. Therefore, most presumptive tests for blood rely on the peroxidase-like activity of hemoglobin. This is a component of blood that is not commonly found in the everyday environment, although there are other substances found in fruits and vegetables that perform a similar function.

In the past half-century, several studies have been conducted on the sensitivity and specificity of presumptive tests for blood, and their effect on subsequent DNA analysis.

In the past 50 years, there have been many tests conducted on the sensitivity of presumptive blood tests (4,6–15). The findings of these studies are in great contradiction with each other. Sensitivities for luminol range from 1:200 (11) to 1:100,000,000 (6); from 1:200 (11) to 1:100,000 for leuchomalachite green (LMG) (8); and from 1:2,000 (12,13) to 1:10,000,000 for phenolphthalein (9). The various differences in the sensitivities reported by different researchers of presumptive blood tests are probably caused by differences in reagent concentrations, methods of preparation of samples, reagents and results, and in the type of material containing the blood (4). Grodsky et al. (8) also add that dried bloodstains are not comparable with the same amount of blood dissolved in a solution. They further add that many of the discrepancies observed are probably due to the presumptive test reagents being added directly to a dilute blood solution, thereby also diluting the reagent, while in other cases the dilute blood solutions are dried first and then tested with full-strength reagents (8).

In the past half century, many tests have been conducted on the specificity of presumptive blood tests. These tests for specificity include changing substrates, adding material and chemicals to the bloodstains, and testing to see whether the reagents will react with substances other than blood (4,7,11,13,14,16–21). Grodsky et al. (8) believe that studies involving the various presumptive blood tests indicate that there is a degree of interference with some of them that effectively prevents their effective use as a test for the

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Received 5 Feb. 2006; and in revised form 12 July 2006 and 2 Aug. 2006; accepted 1 Sept. 2006; published 8 Dec. 2006.

presumptive identification of blood. Therefore, this must be addressed and examined with experimentation.

The ideal presumptive blood test is one that is specific to blood (more specifically to human blood), has a high sensitivity, will meet the Frye standard, and will not damage underlying DNA so that a full DNA profile can be obtained after the reagent's use (5,22). New reagents will be tested with the ones most commonly used by police and forensic scientists throughout the world: Kastle–Meyer (KM), leuchomalachite green, and luminol (23,24). The ease of transport, ease of use, working life, and storage will be determined and discussed for the three new reagents.

Current literature reports differing sensitivities for the various blood detection tests, often conflicting in their results. Therefore, the sensitivity limits of the reagents will be tested and the limits will be determined.

The specificity of the new reagents will be tested with substances commonly known to interfere with traditional reagents, or those that could be mistaken for blood spatter in some situations.

DNA will be collected and PCR performed to determine whether the reagents have limits less than, equal to, or exceeding that of current DNA detection techniques.

Materials and Methods

Samples

Blood samples were taken from an anonymous donor. All equipment used to extract, store, apply, and manipulate the blood for the experiments was sterile. The equipment was either open from sterile packaging or autoclaved at 120°C for 20 min.

Blood from the donor was used for all experiments and for positive controls. The blood was extracted by creating a small lancet wound in the finger of the donor and was not subject to any form of anticoagulants or other contaminants.

Reagents

Luminol (3-aminophthalhyrazidem), LMG, and phenolphthalein KM were prepared according to Strathclyde University, Centre For Forensic Science guidelines. Hemastix® (instructions included with reagent), Hemident™ (MacPhails™ Reagent; instructions included with reagent), and Bluestar© (instructions included with reagent) were from commercially available kits provided by WA Products (Essex, U.K.) (product codes: B23014, B23013, B23014). All reagents were used according to the manufacturer's guidelines.

Positive controls were taken by applying the reagent to a blood-stained piece of filter paper. Negative controls were performed by applying the reagents to a fresh piece of filter paper with no trace of blood. The positive control was retained for further DNA testing.

Sensitivity Testing

Autoclaved bottles (125°C for 20 min) and distilled H_2O were used. Water was measured using a graduated cylinder and blood was added using a Gilson pipette. Differing low concentrations of blood were achieved by making a stock solution of blood and distilled water. Solutions of 1:10,000; 1:100,000; 1:1,000,000; 1:5,000,000; and 1:10,000,000 were prepared.

A set of 25 1 cm × 1 cm pieces of filter paper were placed in each of the diluted blood solutions for each of the presumptive reagents tested. The pieces of filter paper were then removed and allowed to dry for 72 h. Each of the pieces of filter paper was then tested with its corresponding reagent to see whether the blood present was detectable. The reagents were added directly to the 1 cm^2 pieces of filter paper. The time taken for the reagent to register a positive result was determined and recorded. Tests were considered negative if reagents failed to react within 4 min of exposure to the blood-stained filter paper. The treated pieces of filter paper that had not reacted with any reagents were retained for subsequent DNA analysis.

Specificity Testing

Substances found to give false positives previously as reported by other authors, or substances which could be mistaken for blood, were tested. The tests were also carried out on saliva and semen.

The six different reagents were tested against saliva, semen, potato, tomato, tomato sauce, tomato sauce with meat, red onion, red kidney bean, horseradish, 0.1 M ascorbic acid, 5% bleach, 10% cupric sulfate, 10% ferric sulfate, and 10% nickel chloride.

For each of the presumptive reagents tested, a large piece of filter paper (approximately 100 cm^2) was exposed to each of the substances being tested in 25 separate sample stains. These were allowed to dry for a minimum of 18 h. Each of the pieces of filter paper, and subsequent stains, were then tested with their corresponding reagent to see whether the substance caused a reaction. The time taken for the reagent to register a positive result was determined and recorded. Tests were considered positive if there was any color change, and were considered negative if there was no observable color change within 4 min of exposure to the stained filter paper.

DNA Testing

The Chelex method of DNA purification and recovery was used. The protocol consisted of sterile distilled water, 20% Chelex suspension, and extraction buffer. For each sample, 0.5 mL Sterile H_2O was pipetted to a 0.5 mL Eppindorf tube. A small 3 mm^2 section of the positive control (which had been exposed to the reagents) was added to the tube. For the sensitivity testing, the entire 1 cm × 1 cm section of the filter paper was added to the tube. For the controls, a 3 mm^2 section of bloodstain was placed in the tube.

All the samples were incubated at room temperature for 25 min with occasional inverting. They were then centrifuged at maximum for 2 min. Each tube had 0.35 mL of the supernatant removed and then the pellet was resuspended. Fifty microliters of 20% Chelex was added to each tube and they were then incubated at 56°C for 30 min. Samples were then vortexed for 10 sec, boiled for 10 min, and then vortexed for a further 10 sec. Samples were then centrifuged at maximum for 2 min. The supernatant was removed and retained in a separate Eppendorf tube and the pellet was discarded. The retained supernatant was stored frozen.

A full commercial DNA profiling kit will not be used as the amount of information that a full 10 or 14 loci (using SGMPlus™ or IdentifilerPlus™, Applied Biosystems, Foster City, CA) profile would provide is not needed in this study. Instead, two STR loci from a well-used commercial kit, SGMPlus™, will be amplified. The STR loci to be used are D19S433 and D2S1338, the smallest and largest loci, respectively (25). This will allow for both ends of the spectrum to be amplified, as larger products are more likely to drop out in degraded DNA than smaller loci are. Therefore, if only D19S433 amplifies and D2S1338 drops out, it would mean that partial amplification could likely be obtained from a commercial kit. If both D19S433 and D2S1338 amplify, then this should

indicate that a commercial STR typing kit would be able to obtain a full profile off the samples.

As the exact primer sequence used by Applied Biosystems is not known, different primers were used. The primer sequences were obtained from UniSTS (26), which is a comprehensive database of sequence-tagged sites (STSs) defined by PCR primer pairs and are associated with additional information such as genomic position, genes, and sequences (26). The primer information for the two loci was given as:

D19S433

Forward: 5′-HEX-CCTGGGCAACAGAATAAGAT-3′
Reverse: 5′-TAGGTTTTTAAGGAACAGGTGG-3′

D2S1338

Forward: 5′-HEX-CCAGTGGATTTGGAAACAGA-3′
Reverse: 5′-ACCTAGCATGGTACCTGCAG-3′.

Primer sets for D19S433 and D2S1338 were each run in a separate PCR of 25 μL total; 2 μL of each primer was used and 5 μL of template DNA. PCR was performed on a Perkin Elmer GeneAmp PCR System 2400 (Boston, MA). Thirty two cycles of 94°C for 30 sec; 55°C for 30 sec; 72°C for 1 min and 30 sec; and a final extension of 45 min at 72°C were performed.

Eleven experimental samples were run. Six correspond to all of the positive controls, 3 were from the dilution sets 1:10,000, 1:100,000, and 1:1,000,000, a positive control from the blood donor, and a negative control.

An ABI PRISM® 310 Genetic Analyzer (Applied Biosystems) was used to analyze all samples.

Statistical Tests

The test used to compare the different reagents was the χ^2 test for consistency in a $2 \times K$ table.

Results and Discussion

The positive control of luminol reacted instantly with blood, giving a blue luminescence appearing at the site of the deposition; this persisted for over 1 min. The negative control did not react on addition of the reagent. Grodsky et al. (8) believe that luminol's only serious disadvantage, other than interference, is its requirement of near or complete darkness in order to perceive the chemiluminescence.

The LMG-positive control reacted within a few seconds of application of the H_2O_2, with a blue/green color appearing at the site of blood deposition. The negative control did not react on addition of the H_2O_2 but if left out will develop a green ring around where reagents were deposited.

The phenolphthalein KM positive control reacted within a few seconds of application of the H_2O_2, with a pink color appearing at the site of blood deposition. The negative control did not react on addition of the H_2O_2; however, there was a reaction after several minutes (greater than the 4 min timed) with a pink color developing around the edges of the area of reagent deposition.

The Hemastix® reagent strips-positive control reacted instantly on application to the blood by turning dark gray/green; the site where the reagent strip touched the filter paper also turned dark green/blue where there was blood. The negative control did not react on addition of the H_2O and there was no reaction on the filter paper; however, there was a reaction after several minutes (greater than the 4 min timed), with the reagent pad turning light green.

The ease of transport and use of the Hemastix® reagent strips is excellent. The strips are easily stored and transported and there is no risk of chemical spills or solution breakdown or contamination, all that is required is some distilled water (tap water would most likely also be fine) and a desiccant (provided with the strips) with the reagent strips. They are easy to use, and easy to transport. There is a range of color reactions to compare with on the container, for accurate reading of the strips. According to the manufacturer, storage is provided in the container and has a life of 6 months from initial opening, and about a year if unopened.

The Hemident™ (MacPhail's reagent)-positive control reacted within a few seconds of application of the H_2O_2, with a dark green/blue color appearing at the site of blood deposition. The negative control did not react on addition of the H_2O_2 but if left out will develop a green ring around where reagents were deposited.

If used according to the manufacturer's guidelines, the Hemident™ test is easy to transport and use. The ampoules are not easily broken, and the case provides a convenient disposal vessel. There are no instructions for any special storage conditions, or any expiry date indicated.

The Bluestar©-positive control reacted instantly, with a blue luminescence appearing at the site of blood deposition; this dissipated within 30 sec. The negative control did not react on addition of the reagent.

The reagent was easy to prepare from the two tablets, which were mixed directly together into a spray bottle with water (tap water can be used). The tablets can be brought to a scene separately, so there is no risk of leaking bottles of reagents. Bluestar© was extremely easy to use and was effective by just spraying over an entire area for full coverage. The working life of the solution is a problem as it is quite short once the solution is mixed and may only be reactive for a few hours. The tablets come in two separate foil-wrapped packages, but a warning is given that the product is stored under pressure and it should not be stored in the home or car without proper precautions according to the manufacturer. There is no expiry date given with the tablets, indicating that they are stable if stored with the proper precautions. The only problem, much like luminol, is that the product requires complete or near-complete darkness to be effective.

Sensitivity

The approximate numbers of erythrocytes, leukocytes, and hemoglobin molecules, as given by Marieb (27), were calculated for each of the five dilution factors and are shown in Table 1. Table 2 illustrates the results obtained for the sensitivity portion of the experiment.

The luminol reagent reacted instantly, with both the 1:10,000 and 1:100,000 dilution factors producing a blue luminescence. The luminescence lasted for close to a minute. However, both dilution factors were much less intense than the positive control of whole blood. The reaction with the 1:100,000 dilution factor was extremely faint. There was no reaction with dilutions of 1:1,000,000, 1:5,000,000, or 1:10,000,000 within the 4 min of timed experimentation.

LMG reacted at a dilution factor of 1:10,000. All samples except one showed a positive reaction within 1 min. The samples turned a green color within 1 sec of the application of the LMG reagent and the H_2O_2. A single sample did not show a positive reaction within the 4 min of timed experimentation. The LMG reagent did not show a positive reaction at dilution factors of 1:100,000, 1:1,000,000, 1:5,000,000, or 1:10,000,000 within the 4 min of timed experimentation.

TABLE 1—*Distribution of blood cells for the different dilution factors, calculated from the values given in Marieb (27).*

Blood 1 mL = 1 mm^3	Erythrocytes		Hemoglobin		Leukocytes	
	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum
1:l	4,300,000	5,200,000	1.075E+15	1.3E+15	4000	11,000
10:l	43,000,000	52,000,000	1.075E+16	1.3E+16	40,000	110,000
1:10,000	430	520	1.075E+11	1.3E+11	0.4	1.1
1:100,000	43	52	10,750,000,000	13,000,000,000	0.04	0.11
1:1,000,000	4.3	5.2	1,075,000,000	1,300,000,000	0.004	0.011
1:5,000,000	0.86	1.04	215,000,000	260,000,000	0.0008	0.0022
1:10,000,000	0.43	0.52	107,500,000	130,000,000	0.0004	0.0011

The phenolphthalein reagent registered a positive reaction for all samples at a dilution factor of 1:10,000. The samples turned pink after 45 sec of the introduction of the reagent and H_2O_2. At a dilution factor of 1:100,000, three of 25 samples showed a positive reaction: two of them at 1 min and 30 sec, and the third at 2 min and 30 sec. The phenolphthalein reagent did not show any reaction with dilution factors of 1:1,000,000, 1:5,000,000, or 1:10,000,000 within the 4 min of timed experimentation.

The Hemastix® reagent strips reacted with the 1:10,000 dilution by first causing a color reaction with the filter paper. The filter paper changed to a green color where the Hemastix® was pressed within a few seconds. The actual Hemastix® took between 30 and 60 sec to register a reaction. Eighteen Hemastix® were positive for +25 erythrocytes; the remaining seven registered positive for +80 erythrocytes.

The Hemastix® reagent strips reacted with the 1:100,000 dilution by first causing a color reaction with the filter paper. At 1 min, one of the samples showed a color change on the filter paper of a green color. The rest of the samples showed this same reaction at between 1 min and 45 sec and 2 min. At 3 min and 45 sec, 17 of the reagent strips were a very light shade of green, corresponding with a trace 10 hemolyzed sample according to the instructions. Four of the strips registered +25 erythrocytes at the same time. The remaining four strips registered a negative result at 4 min.

The Hemastix® reagent strips did not react with the 1:1,000,000, 1:5,000,000, and 1:10,000,000 dilutions. There was no color change on the filter paper or on the reagent strips.

There were no previous literature values for the sensitivity of Hemastix® although the package claims to be able to detect blood in urine down to 10 erythrocytes, which equates to between a 1:100,000 and 1:1,000,000 dilution factor (Table 1). This is consistent with the results obtained in this experiment, although the strips should be read at 60 sec and a reaction was not observed on the strips until between 3 and 4 min after initial application to the stain.

The Hemident™ reagent reacted with most of the samples at the 1:10,000 dilution. One sample reacted before the addition of the H_2O_2 with a color change to green; two other samples did not register a reaction. The samples that did react showed a green/blue color change at the edges of the filter paper, predominantly in the corners, which occurred within 1 min of addition of the H_2O_2.

The Hemident™ reagent did not react with most of the samples at the 1:100,000 dilution. Two of the samples showed a positive result at 4 min. The remainder did not show any reaction. There was no reaction with the samples diluted to 1:1,000,000, 1:5,000,000, or 1:10,000,000 within the 4 min of timed experimentation.

There were no previous literature values for the sensitivity of Hemident™, but the package claims a capability of identifying one part per million of blood (28). The findings of this study do not confirm this. Hemident™ is slightly more sensitive than leuchomalachite green, but does not even approach the sensitivity it claims to have. Two samples showed a positive reaction at 1:100,000 dilution, which is 10 times more sensitive than leuchomalachite green, but this was not consistent over all 25 samples, and it is still one-tenth of the sensitivity claimed.

The Bluestar© reagent reacted instantly with the 1:10,000 with a blue luminescent glow but faded within a few seconds. The 1:100,000 dilution showed slight reactivity, with five of the 25 samples showing a very faint positive, which faded in a few seconds. However, both dilution factors were much less intense than the positive control of whole blood. There was no reaction with dilutions of 1:1,000,000, 1:5,000,000, or 1:10,000,000 within the 4 min of timed experimentation.

The Bluestar© reagent has no previous tested sensitivities, although the company claims sensitivity to 1:1,000 dilution (29). This was not found to be consistent with this study as luminescence was detected at 1:100,000 dilution of blood in water, 100 times more sensitive than what is claimed by the company. This luminescence was faint and short-lived, but was still detectable.

Specificity

Table 3 gives the specificity results for all reagents.

TABLE 2—*Sensitivity results for the six different reagents.*

Dilution	Reagent					
	Luminol	LMG	KM	Hemastix®	Hemident™	Bluestar©
1:10,000	1	1	1	1	1	1
1:100,000	1	NR	2	2	4	1
1:1,000,000	NR	NR	NR	NR	NR	NR
1:5,000,000	NR	NR	NR	NR	NR	NR
1:10,000,000	NR	NR	NR	NR	NR	NR

The shortest reaction time is shown here.

A positive reaction was any sort of color change to the stain (or reagent strip in the case of Hemastix®); 0, color change before all reagents were added; 1, color change within 1 min of all reagents being added; 2, color change within 1–2 min of all reagents being added; 3, color change within 2–3 min of all reagents being added; 4, color change within 3–4 min of all reagents being added;

NR, indicates that there was no reaction within the 4 min of timed experimentation; KM, Kastle–Meyer; LMG, leuchomalachite green.

TABLE 3—*Specificity results for the six different reagents.*

Substance	Reagent					
	Luminol	LMG	KM	Hemastix®	Hemident™	Bluestar©
Saliva	NR	NR	NR	1 (3)	NR	NR
Semen	NR	NR	1 (25)	NR	0 (25, white)	NR
Potato	NR	0 (6, green)	3 (7)	1 (25)	NR	1 (25)
Tomato	NR	NR	NR	1 (23)	NR	1 (25)
Tomato sauce	NR	NR	4 (6)	NR	NR	NR
Tomato sauce w/meat	NR	NR	NR	4 (6)	NR	1 (25)
Red onion	NR	0 (5, pink)	0 (25, yellow)	1 (21)	0 (6, pink)	1 (25)
Red kidney bean	NR	NR	2 (5)	NR	NR	1 (25)
Horseradish	NR	NR	4 (25)	NR	NR	1 (25)
1 M Ascorbic acid	NR	NR	0 (25, yellow)	NR	1 (11)	1 (25)
Bleach solution 5%	NR	NR	3 (2)	NR	1 (5)	1 (25)
10% Cupric sulfate	1 (25)	0 (25, blue)	0 (25, blue)	1 (25)	0 (25, blue)	1 (25)
10% Ferric sulfate	1 (25)	0 (25, orange)	0 (25, yellow)	1 (25)	0 (25, red/brown)	1 (25)
10% Nickel chloride	1 (25)	0 (25, blue)	0 (25, green)	1 (25)	0 (25, green)	NR

The shortest reaction time is shown here. Numbers in parentheses indicate the number of samples that reacted out of 25 and the color change observed if different from that of a reaction with blood.

A positive reaction was any sort of color change to the stain (or reagent strip in the case of Hemastix®); 0, a color change before all reagents were added; 1, indicates a color change within 1 min of all reagents being added; 2, color change within 1–2 min of all reagents being added; 3, color change within 2–3 min of all reagents being added; 4, color change within 3–4 min of all reagents being added; NR, indicates that there was no reaction within the 4 min of timed experimentation.

There was a reaction between the luminol reagent and 10% cupric sulfate, 10% ferric sulfate, and 10% nickel chloride indicated by a blue chemiluminescence. Both the 10% cupric sulfate and 10% ferric sulfate showed an immediate reaction on addition of the luminol. The 10% nickel chloride also showed a reaction on addition of the luminol; however, the intensity of this reaction was far less than for the ferric and cupric sulfates and it did not occur instantly. The reaction took several seconds to become visible.

Luminol's reaction with the metal salts was expected as Grodsky et al. (8) noted that the common substances that interfere with luminol are copper-containing surfaces.

Contrary to the literature findings, this study found that luminol only reacted with blood and the metal salts. Bleach gave no reaction, but this could be because the bleach solution was only 5% concentration, and that it was not tested right away but first allowed to dry for at least 18 h. Kent et al. (20) noted that when bleach-treated blood is left for several days, the interference by bleach is diminished. The negative reaction observed may be due to the storage time of the sample. Luminol was expected to react with the potato and horseradish as it has been used to study vegetable peroxidase reactions, such as the horseradish peroxidase reaction (30), and Albrecht noted that fresh potato juice caused luminescence (16). This could once again be due to the substances' drying time before testing.

LMG showed a reaction with several of the substances tested. However, the results of these reactions would not be mistaken for a reaction with blood. All of the substances that reacted did so after the addition of the LMG reagent but before the addition of the H_2O_2. This agrees with the findings of Alvarez de Toledo and Valero, who noted that many chemical oxidants may yield the reaction in the absence of H_2O_2 (31). Blood only reacts after the addition of the hydrogen peroxide and then only at the site of blood deposition. Therefore, none of the substances tested react in the same manner as blood and could not be mistaken for a reaction with blood.

Several substances reacted with the phenolphthalein reagent. Semen stains showed a very light pink color change at 45 sec, which grew stronger as the timing approached 2 min. Seven potato samples showed a slight pink color change within 2 min of introduction of the reagents. Six tomato sauce samples showed a pink color at 3 min and 45 sec. Red onion samples turned yellow after the addition of the KM reagent, but before the addition of H_2O_2. The horseradish samples all showed a very slight pink color change at 3 min. The 0.1 M ascorbic acid samples turned yellow after the addition of the phenolphthalein reagent, but did not show any further color change on addition of the H_2O_2. Two of the 5% bleach samples showed a slight pink color at 2 min, but the rest did not show a color change within the 4 min of timed experimentation. On addition of the phenolphthalein reagent, the 10% cupric sulfate samples turned blue. On addition of the H_2O_2, the samples instantly turned brown and foamed. All 25 of the 10% cupric sulfate samples then developed an intense pink color around the stain, 11 within 1 min and the remaining 14 within 2 min and 30 sec. The 10% ferric sulfate samples turned yellow/brown on addition of the phenolphthalein reagent and the stains had dark edges. The samples foamed on addition of H_2O_2. At 1 min and 30 sec, 13 of the samples showed a pink color developing around the stain, and two additional samples showed this same color change at 3 min. The remaining samples did not develop any color around the stains in the 4 min of timed experimentation. The 10% nickel chloride turned light green on addition of the phenolphthalein reagent. This color deepened on addition of the H_2O_2. Eight of the samples developed a pink color around the stain at 3 min. The rest of the samples failed to react within the 4 min of timed experimentation.

The reaction of the phenolphthalein reagent with other substances differs from Pinker (32), who did not find even one substance that would give a true positive reaction with phenolphthalein. This does not correspond with the current findings as semen caused a reaction at 45 sec, that grew stronger with time. The potato stains reacted the same way, as did tomato sauce, red kidney beans, horseradish, and 5% bleach, which all reacted at some point within the 4 min of timed experimentation. This time delay in reaction is much like the time delay observed for dilute blood samples (1:100,000) and therefore, any of these stains could be conceivably mistaken for very dilute blood.

The remaining substances reacted before all the reagents were added, or did not form the pink color as an expected bloodstain would. Both the red onion and the 0.1 M ascorbic acid samples turned yellow on addition of the phenolphthalein reagents but before the H_2O_2 was added. The metal salts also all reacted before

the H_2O_2 was added by turning blue (10% cupric sulfate), yellow/brown (10% ferric sulfate), and light green (10% nickel chloride).

There was a reaction between the Hemastix® reagent strips and saliva, potato, tomato, tomato sauce with meat, red onion, 10% cupric sulfte, 10% ferric sulfate, and 10% nickel chloride. The Hemastix® reagent strips reacted with three of the saliva samples turning the paper green. One reacted within 1 min, and the remaining two samples reacted within 2 min. The actual reagent strips did not show a reaction within the 4 min of timed experimentation, nor did any of the remaining 22 samples. All of the potato stains produces a color reaction by turning green within 15 sec, which darkened to blue as time progressed. There was no reaction with the actual reagent strips within the 4 min of timed experimentation. The tomato samples reacted within 1 min by turning a very light green, which darkened as time passed. Two of the stains did not react, and most of the reagent pads did not react within the 4 min of timed experimentation. Two of the pads showed a very slight green color change at 4 min. Six of the tomato sauce with meat samples reacted at 3 min. The six stains turned green around the edges at 3 min. The rest of the sample stains as well as all of the reagent pads did not react within the 4 min of timed experimentation. Twenty-one of the red onion samples turned a very light green at 1 min with the color darkening to dark green/blue as time progressed. Three of the reagent pads show a green line running horizontally, the remaining pads and 4 stains did not react within the 4 min of timed experimentation. The 10% cupric sulfate samples showed an instant blue/green color on the stain. The reagent pads appear as trace (spots of green) non-hemolyzed and progress to a dark green/blue uniform color. The 10% ferric sulfate samples all turned instantly green/blue at the center of the stain, which progressed to brown and then yellow along the outer margins of the stain. The reagent pads showed a small trace (spots of green color) at 4 min. The 10% nickel chloride sample stains all turned instantly green/blue on application of the reagent. The reagent pads were all negative after the 4 min of timed experimentation except for one, which showed a green/blue color at the end of the strip.

The Hemastix® reagent strips were quite reactive with eight of the substances tested. All of the substances showed a green color, which may or may not have progressed to blue. This is the same reaction observed on blood samples, except that when exposed to blood the reagent strips also showed a reaction. This was not the case for saliva, potato, and the tomato sauce with meat, which did not react with the reagent pads.

The samples that did show a reaction on the actual reagent pads were tomato, red onion, 10% cupric sulfate, 10% ferric sulfate, and 10% nickel chloride. These samples showed the same reaction as dilute blood samples would, and in the case of the 10% nickel chloride samples, showed a color change indicative of whole blood.

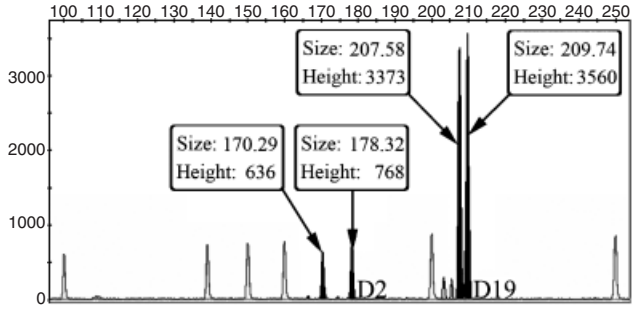

FIG. 1—*Positive control DNA. Loci D2S1338 and D19S433 are shown with peak size and height.*

The Hemident™ reagents did show a color reaction with semen, red onion, 0.1 M ascorbic acid, 5% bleach, 10% cupric sulfate, 10% ferric sulfate, and 10% nickel chloride. All of the semen stains turned white on addition of the first reagent, but there was no further reaction on addition of the H_2O_2. Six of the red onion samples turned pink on addition of the first reagent but there was no further reaction on addition of the H_2O_2 during the 4 min of timed experimentation. Two of the 0.1 M ascorbic acid samples turned instantly positive with a blue/green color. Within 30 sec, nine other samples a slightly positive, with a light blue/green color developing and increasing with intensity as time progressed. The remaining 14 0.1 M ascorbic acid samples did not react with the reagents during the timed experiment. Five of the 5% bleach samples turned light blue/green along their margins 30 sec after addition of the H_2O_2; the remaining 20 samples did not react with the reagents within the 4 min of timed experimentation. The 10% cupric sulfate samples all turned slightly blue on addition of the first reagent, and 12 developed an instant blue/green color around their edges on addition of the H_2O_2. A further five samples showed the same blue/green edges after 1 min and this color intensified with time, but no other samples reacted. The 10% ferric sulfate sample all turned brown/red on addition of the first reagent and instantly turnedgrass green on addition of the H_2O_2. This color darkened to blue/green over time. The 10% nickel chloride samples all turned very light green after the addition of the first reagent, but there was no further color change on addition of the H_2O_2 during the 4 min of timed experimentation.

TABLE 4—*DNA results for the various presumptive tests, positive and negative controls, and dilution series.*

	D2S1338		D19S433	
	Peak 1 (Height)	Peak 2 (Height)	Peak 1 (Height)	Peak 2 (Height)
Positive control	170.29 (636)	178.32 (768)	207.58 (3373)	209.74 (3560)
Luminol	170.40 (1528)	178.34 (1596)	207.63 (4984)	209.81 (4510)
LMG	N/R	N/R	N/R	N/R
KM	170.39 (489)	178.34 (436)	207.57 (580)	209.84 (400)
Hemastix®	170.38 (1934)	178.28 (1814)	207.52 (1873)	209.59 (1938)
Hemident™	N/R	N/R	N/R	N/R
Bluestar®	170.31 (3685)	178.27 (2652)	207.36 (4840)	209.46 (4300)
1:10,000	N/R	N/R	N/R	N/R
1:100,000	N/R	N/R	N/R	N/R
1:1,000,000	N/R	N/R	N/R	N/R
Negative control	N/R	N/R	N/R	N/R

KM, Kastle–Meyer; LMG, leuchomalachite green; N/R, no result.

TABLE 5—χ^2 *test for consistency results for sensitivity samples with 95% confidence.*

Sensitivity	$v = 4$ $\alpha = 0.05$ $\chi^2_4(0.05) = 9.49$ Luminol	LMG	KM	Hemastix®	Hemident™	Bluestar®
Luminol		*18.12*	*12.04*	0.00	*12.76*	8.89
LMG	*18.12*		2.73	*18.12*	2.00	4.41
KM	*12.04*	2.73		*12.04*	0.11	0.43
Hemastix®	0.00	*18.12*	*12.04*		*12.76*	8.89
Hemident™	*12.76*	2.00	0.11	*12.76*		0.92
Bluestar®	8.89	4.41	0.43	8.89	0.92	

The null hypothesis was that the two samples originate from two populations with the same distributions. Numbers in italics reject the null hypothesis at 95% confidence; KM, Kastle–Meyer; LMG, leuchomalachite green.

The Hemident™ reagent reacted with several of the substances tested. Semen, red onion, 0.1 M ascorbic acid, 5% bleach, 10% cupric sulfate, 10% ferric sulfate, and 10% nickel chloride all showed a color reaction with one or both of the reagents. Semen, red onion, 10% cupric sulfate, 10% ferric sulfate, and 10% nickel chloride all reacted after the addition of the first reagent and would therefore not be mistaken for a possible bloodstain.

The 0.1 M ascorbic acid and 5% bleach samples reacted with a blue/green color on addition of the H_2O_2 as would blood. The 5% bleach samples showed a color change around the margins of the stain, which is not indicative of blood, which reacts on top of the actual stain. The 0.1 M ascorbic acid reacted as blood would for 11 of the 25 samples.

The Bluestar© reagent reacted with potato, tomato, tomato sauce with meat, red onion, red kidney bean, horseradish, 0.1 M ascorbic acid, 5% bleach, 10% cupric sulfate, and 10% ferric sulfate indicated by a blue chemiluminescence upon application.

DNA Analysis

Figure 1 shows the results for the positive control DNA, with the D2 and D19 loci clearly visible. This demonstrates the functionality of the primers and indicates that they would react with any viable DNA obtained from samples exposed to the presumptive tests. These results can be seen in Table 4.

Luminol, phenolphthalein, Hemastix®, and Bluestar© all achieved amplification at both loci tested, which corresponded to the alleles found on the positive control. All four tests gave amplification, although Bluestar© claimed that it destroyed DNA (29). Phenolphthalein had a much reduced peak height compared with the other three tests. This is consistent with Hochmeister et al. (24), who found that phenolphthalein reduces the amount of extractible high-molecular-weight DNA. LMG and Hemident™ did not achieve amplification.

No DNA results were obtained from any of the dilution series. This could be because there is such a small amount of template DNA that in order to achieve detectable amplification product, it would need several more PCR cycles.

Statistical Interpretation

The results of the χ^2 test for consistency can be seen for sensitivity and specificity in Tables 5 and 6, respectively. The null hypothesis was that the two samples originate from two populations with the same distributions.

The samples that come from populations with the same distributions as each other do not necessarily react with the same substances or at the same rates. Therefore, what one test might react with, another test from a similar population would not react, or if it did it may do so at a different rate. The same distribution comes from the number of substances other than blood that the given reagent will react with.

Conclusion

It is almost never necessary to apply presumptive test reagents directly to dried bloodstain evidence (33,34). However, with extremely small samples, or when testing large areas, it may be necessary to expose the potential bloodstains directly to presumptive tests. Based on this, the best overall presumptive blood test in this study was luminol. It had the greatest sensitivity and specificity. It did not destroy the DNA, and it could be reapplied. Its only drawback is that it must be used in near or complete darkness. Leuchomalachite green was found to be as specific to blood as luminol, but its sensitivity was 10 times less, and it destroyed the DNA. Phenolphthalein had equal sensitivity to most of the other tests, but was extremely unspecific, and the amount of recoverable DNA is reduced when this test is used. Hemastix™ were easy to transport and use, were sensitive, but not very specific although specificity could be increased if the strips were looked at rather than the reaction on the stain. DNA was recovered from stains exposed to Hemastix™. Hemident® was specific and sensitive, but destroyed DNA and so cannot be used where subsequent DNA analysis is needed. Bluestar© had good sensitivity, but very poor specificity. The need for complete darkness for use further complicates this because even if a stain did not look like blood, it would react in the same way and could be mistaken for blood.

The dilutions of blood did not show any amplification of DNA, but this could be because of the small quantity of template DNA and the low number of cycles of PCR.

TABLE 6—χ^2 *test for consistency results for specificity samples with 95% confidence.*

Specificity	$v = 13$ $\alpha = 0.05$ $\chi^2_{13}(0.05) = 22.36$ Luminol	LMG	KM	Hemastix®	Hemident™	Bluestar©
Luminol		13.76	*83.08*	*58.12*	14.59	*184.17*
LMG	13.76		*65.01*	*35.07*	18.44	*155.28*
KM	*83.08*	*65.01*		*127.26*	*65.05*	*144.46*
Hemastix®	*58.12*	*35.07*	*127.26*		*63.69*	*123.91*
Hemident™	14.59	18.44	*65.05*	*63.69*		*145.89*
Bluestar©	*184.17*	*155.28*	*144.46*	*123.91*	*145.89*	

The null hypothesis was that the two samples originate from two populations with the same distributions. Numbers in italics reject the null hypothesis at 95% confidence; KM, Kastle–Meyer; LMG, leuchomalachite green.

Acknowledgments

Thanks are due to WA Products for providing the Hemastix®, Hemident™, and Bluestar© reagents.

We thank the entire Centre for Forensic Science at Strathclyde University for lab space and equipment.

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