Milton Cox,[1] *Ed.D.*

A Study of the Sensitivity and Specificity of Four Presumptive Tests for Blood

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ABSTRACT: The purpose of this work was to conduct a comparative study of the sensitivity and specificity of phenolphthalein, tetramethylbenzidine, leucomalachite green, and orthotolidine as presumptive tests for blood. The findings of this study indicate that the phenolphthalein and the leucomalachite green tests are the most specific and that the tetramethylbenzidine and orthotolidine tests are the most sensitive of the group. The author concludes that the phenolphthalein test is the best single test for evaluating suspected bloodstains.

KEYWORDS: criminalistics, blood, serology, forensic serologists, bloodstains, blood dilutions, presumptive tests, vegetables, fruits, phenolphthalein, tetramethylbenzidine, leucomalachite green, orthotolidine

Could the stain be blood? Forensic serologists have been asked this question innumerable times. One of the most invaluable tools in a forensic science laboratory is the test used to screen for blood. It is the first of several procedures that are conducted on bloodstains by the forensic serologist. In many cases involving suspected blood, there is an insufficient amount of stain to proceed beyond the screening test. Such situations place an increased importance on the sensitivity and specificity of the presumptive test employed. The chosen test must be sensitive enough to detect low concentrations of blood, and at the same time, it should possess a relatively high degree of specificity. In addition to these important features, the test should be safe, be simple to use, and provide rapid results.

For many years, forensic science laboratories relied heavily upon the benzidine test to screen for blood. Because of the carcinogenic effects of benzidine, other suitable replacements have been sought by laboratories.

The purpose of this research was to conduct a comparative study of the sensitivity and specificity of phenolphthalein, tetramethylbenzidine, leucomalachite green, and orthotolidine as presumptive tests for blood.

In the past 40 years, several studies have been conducted on the sensitivity and specificity of presumptive tests for blood [*1–4*].

Grodsky, Wright, and Kirk [*1*] in 1951 presented a comparative study of benzidine, leucomalachite green, and phenolphthalein. These researchers used blood solutions to test the sensitivity of the reagents rather than dry bloodstains, which are the usual form in which blood is tested in forensic science laboratories.

In 1960, Hunt, Corby, and Dodd [*2*] conducted a survey of the orthotolidine, leuco-

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[1]Assistant professor, Criminal Justice and Forensic Science Department, University of Southern Mississippi, Long Beach, MS.

malachite green, phenolphthalein, and luminol tests. These workers determined that phenolphthalein was sensitive for blood in the solution form at dilutions of 1:10 000 000, but they also reported negative results with this test when applied directly to small spots of blood. The findings of the author of this paper are not in agreement with those results.

An evaluation of the tetramethylbenzidine test as a presumptive test for blood was reported by Garner, Cano, Peimer, and Yeshion in 1976 [*3*]. These authors observed no significant differences between the benzidine and tetramethylbenzidine tests in sensitivity or specificity.

A study of the sensitivity and specificity of the phenolphthalein test as an indicator test for blood was reported by Higaki and Philip, also in 1976 [*4*]. That study compared the phenolphthalein and benzidine tests as presumptive tests. The results of that work indicated that plant peroxidases contribute to false positive results in the benzidine test but not in the three-stage phenolphthalein test.

Material and Methods

This study focused on the four presumptive tests for blood, using phenolphthalein, tetramethylbenzidine, leucomalachite green, and orthotolidine.

Preparation of Reagents

The reagents were prepared according to the following formulas:

1. Phenolphthalein solution

(*a*) Stock solution

Phenolphthalein (Aldrich Co.), 2 g
Potassium hydroxide, 20 g
Distilled water, 100 mL

The mixture was refluxed with 20 g of powdered zinc for 2 h until the solution became colorless. The stock solution was stored in a dark bottle and refrigerated, with some zinc added to keep it in the reduced form.

(*b*) Working solution

Phenolphthalein stock solution, 20 mL
Ethanol, 80 mL
Hydrogen peroxide, 3% solution in a dark bottle

A pink-rose color indicates a positive reaction.

2. Tetramethylbenzidine solution

3,3',5,5'-Tetramethylbenzidine (Aldrich Co.), 2 g
Glacial acetic acid, 100 mL
Hydrogen peroxide, 3% solution in a dark bottle

A green-blue color indicates a positive reaction.

3. Leucomalachite green solution

Leucomalachite green (Aldrich Co.), 0.1 g
Glacial acetic acid, 66 mL
Distilled water, 33 mL

Hydrogen peroxide, 3% solution in a dark bottle

A bright green color indicates a positive reaction.

4. Orthotolidine solution

o-Tolidine (Kodak), 1.5 g
Ethanol, 40 mL
Glacial acetic acid, 30 mL
Distilled water, 30 mL

Hydrogen peroxide, 3% solution in a dark bottle

A green-blue color indicates a positive reaction.

Sensitivity Tests

Fresh blood was used to prepare test solutions in dilutions of 1:50, 1:100, 1:500, 1:1000, 1:5000, 1:10 000, 1:50 000, 1:100 000, 1:500 000, 1:1 000 000, and 1:2 000 000 parts of blood in distilled water.

Pieces of filter paper (approximately 1 cm^2 each) were placed in each blood dilution, removed, and allowed to air dry at room temperature for 24 h. The same procedure was repeated using 100% cotton cloth.

A piece of dried filter paper stained with a blood dilution was tested for each dilution with each of the four reagents. The time in seconds required for a positive color reaction was recorded for each test. Each square of stained filter paper or stained cotton cloth was placed on a 1 by 3-in. (2.5 by 7.6-cm) glass slide when tested. The blood solutions were tested in a white spot plate. The results are given in Table 1.

Two drops of the reagent being tested were added to each specimen tested (filter paper, cloth, or blood dilution solution) and observed for a color change, which would indicate a positive reaction. If no color change occurred, two drops of 3% hydrogen peroxide were added to the sample. The reaction time was recorded. A maximum time of 20 s was allowed for a color change.

Specificity of Reagents

The specificity of each of the four presumptive tests was evaluated with various vegetables and fruits. Each vegetable or fruit was ground into a fine paste with a mortar and pestle and allowed to air dry at room temperature for 24 h. The dried (D) vegetables and fruits were each tested with the four tests being considered. A fresh (F) sample of each vegetable and fruit was likewise tested. These procedures were conducted in white spot plates. Readings for each specimen were recorded at 20 s and at 60 s. The findings are presented in Tables 2 and 3.

Results and Discussion

Phenolphthalein Test

This test gave positive reactions for blood on filter paper and cotton cloth at dilutions of 1:10 000. The findings of the author of this paper were that positive reactions were obtained with blood solutions at dilutions of 1:1 000 000. Higaki and Philip [*4*] reported sensitivities of 1:500 to 1:5000 for stained thread and 1:50 000 to 1:500 000 for blood solutions. On the other hand, Hunt, Corby, and Dodd [*2*] reported that this test was sensitive to a dilution of 1:10 000 000 for a solution of blood. Grodsky, Wright, and Kirk [*1*] also found the phenolphthalein test to be very sensitive to blood solutions (1:100 000 to 1:5 000 000).

No interference color change was observed with the phenolphthalein test when testing the fresh (F) and dried (D) vegetables and fruits (Tables 2 and 3). Higaki and Philip [*4*] obtained weak false positive results with fresh green bean, potato, and horseradish.

Tetramethylbenzidine

Blood-stained filter paper, cloth, and blood solutions with dilutions of 1:10 000 gave an immediate (1-s) positive reaction. Stained filter paper, cotton cloth, and blood solutions responded positively within 20 s at a dilution of 1:1 000 000. However, it was discovered that unstained cotton cloth and cotton swabs will produce an interference color, which may appear to be a positive result, within a period of 15 to 20 s when tetramethylbenzidine solution and hydrogen peroxide are added to the test material. The stained filter paper

TABLE 1—*Sensitivity results, showing the time required for a result or no reaction, in seconds.*[a]

Blood Dilution	Specimen Form	P	TMB	LMG	OT
1:50	Filter paper	+1	+1	+1	+1
	Cotton cloth	+1	+1	+1	+1
	Solution	+1	+1	+1	+1
1:100	Filter paper	+1	+1	+1	+1
	Cotton cloth	+1	+1	+1	+1
	Solution	+1	+1	+1	+1
1:500	Filter paper	+1	+1	+2	+1
	Cotton cloth	+1	+1	+2	+1
	Solution	+1	+1	+2	+1
1:1000	Filter paper	+1	+1	+4	+1
	Cotton cloth	+1	+1	+3	+1
	Solution	+1	+1	+2	+1
1:5000	Filter paper	+3	+1	+11	+1
	Cotton cloth	+2	+1	+10	+1
	Solution	+1	+1	+12	+1
1:10 000	Filter paper	+5	+1	−20	+1
	Cotton cloth	+4	+1	−20	+1
	Solution	+1	+1	−20	+1
1:50 000	Filter paper	−20	+8	−20	+3
	Cotton cloth	−20	+9	−20	+2
	Solution	+1	+10	−20	+5
1:100 000	Filter paper	−20	+14	−20	+13
	Cotton cloth	−20	+10	−20	+8
	Solution	+1	+14	−20	+18
1:500 000	Filter paper	−20	+18	−20	−20
	Cotton cloth	−20	+15	−20	+18
	Solution	+1	+19	−20	−20
1:1 000 000	Filter paper	−20	+18	−20	−20
	Cotton cloth	−20	+15	−20	−20
	Solution	+2	+19	−20	−20
1:2 000 000	Filter paper	−20	−20	−20	−20
	Cotton cloth	−20	+(15–20)	−20	−20
	Solution	−20	−20	−20	−20
Unstained controls	Filter paper	−20	−20	−20	−20
	Cotton cloth	−20	+(15–20)	−20	−20
	Cotton swab	−20	+(15–20)	−20	−20
	Distilled water	−20	−20	−20	−20

[a]Key to abbreviations:
P = phenolphthalein test.
TMB = tetramethylbenzidine test.
LMG = leucomalachite green test.
OT = orthotolidine test.
− = negative result.
+ = positive result.

TABLE 2—*Specificity results for vegetables.*[a]

Vegetables	P		TMB		LMG		OT	
	20 s	60 s	20 s	60 s	20 s	60 s	20 s	60 s
Asparagus (F)	–	–	+	+	–	–	+	+
Asparagus (D)	–	–	+	+	–	–	+	+
Avocado (F)	–	–	+	+	–	–	+	+
Avocado (D)	–	–	+	+	–	–	+	+
Bean, green (F)	–	–	+	+	–	–	+	+
Bean, green (D)	–	–	+	+	–	–	+	+
Broccoli (F)	–	–	+	+	–	–	+	+
Broccoli (D)	–	–	+	+	–	–	+	+
Brussel sprout (F)	–	–	+	+	–	–	+	+
Brussel sprout (D)	–	–	+	+	–	–	+	+
Cabbage (F)	–	–	+	+	–	–	+	+
Cabbage (D)	–	–	+	+	–	–	+	+
Carrot (F)	–	–	+	+	–	–	+	+
Carrot (D)	–	–	+	+	–	–	+	+
Cauliflower (F)	–	–	+	+	–	–	+	+
Cauliflower (D)	–	–	+	+	–	–	+	+
Celery (F)	–	–	+	+	–	–	+	+
Celery (D)	–	–	+	+	–	–	+	+
Corn, yellow (F)	–	–	+	+	–	–	+	+
Corn, yellow (D)	–	–	+	+	–	–	+	+
Cucumber (F)	–	–	+	+	–	–	+	+
Cucumber (D)	–	–	+	+	–	–	+	+
Eggplant (F)	–	–	+	+	–	–	+	+
Eggplant (D)	–	–	+	+	–	–	+	+
Garlic (F)	–	–	+	+	–	–	+	+
Garlic (D)	–	–	+	+	–	–	+	+
Lettuce (F)	–	–	+	+	–	–	+	+
Lettuce (D)	–	–	+	+	–	–	+	+
Mushroom (F)	–	–	+	+	–	–	–	–
Mushroom (D)	–	–	+	+	–	–	–	+ (weak)
Okra (F)	–	–	+	+	–	–	+	+
Okra (D)	–	–	+	+	–	–	+	+
Onion, white (F)	–	–	+	+	–	–	+	+
Onion, white (D)	–	–	+	+	–	–	+	+
Onion, green, top (F)	–	–	+	+	–	–	+	+
Onion, green, top (D)	–	–	+	+	–	–	+	+
Pepper, bell (F)	–	–	+	+	–	–	+	+
Pepper, bell (D)	–	–	+	+	–	–	+	+

TABLE 2—*Continued*

Vegetables	P		TMB		LMG		OT	
	20 s	60 s	20 s	60 s	20 s	60 s	20 s	60 s
Potato, Irish (F)	–	–	+	+	–	–	+	+
Potato, Irish (D)	–	–	+	+	–	–	+	+
Potato, sweet (F)	–	–	+	+	–	–	+	+
Potato, sweet (D)	–	–	+	+	–	–	+	+
Radish (F)	–	–	+	+	–	–	+	+
Radish (D)	–	–	+	+	–	–	+	+
Spinach (F)	–	–	+	+	–	–	+	+
Spinach (D)	–	–	+	+	–	–	+	+
Squash, yellow (F)	–	–	+	+	–	–	+	+
Squash, yellow (D)	–	–	+	+	–	–	+	+
Tomato (F)	–	–	+	+	–	–	+	+
Tomato (D)	–	–	+	+	–	–	+	+
Turnip root (F)	–	–	+	+	–	–	+	+
Turnip root (D)	–	–	+	+	–	–	+	+

[a]Key to abbreviations:
P = phenolphthalein test.
TMB = tetramethylbenzidine test.
LMG = leucomalachite green test.
OT = orthotolidine test.
20 s = within 20 s.
60 s = within 60 s.
F = fresh.
D = dried.
– = negative result.
+ = color reaction which may be considered positive.

and blood solution at the dilution level of 1:2 000 000 failed to give a positive reaction during the 60-s testing period. In this study, unstained filter paper did not give a false positive reaction with the tetramethylbenzidine reagent, as was found by Garner, Cano, Peimer, and Yeshion [*3*].

All the vegetables and many of the fruits listed in Tables 2 and 3 produced an interference color which may be viewed as positive during the 20-s testing period. It was noted that citrus fruit, including grapefruit, lemon, and orange, did not react with this reagent. Strawberry also failed to react. A difference was observed between the reaction of red grape and green grape. A false positive was obtained within 20 s with the green grape. The red grape did not react within 20 s, but did give a false positive within 60 s. Interference color reactions with certain vegetables were noted by Garner, Cano, Peimer, and Yeshion [*3*].

Leucomalachite Green Test

This test was found to be the least sensitive of the four investigated. The filter paper, cotton cloth, and blood dilution solutions gave positive reactions at a dilution of 1:5000

TABLE 3—*Specificity results for fruits.*[a,b]

Fruits	P		TMB		LMG		OT	
	20 s	60 s	20 s	60 s	20 s	60 s	20 s	60 s
Apple, red (F)	–	–	+	+	–	–	–	–
Apple, red (D)	–	–	+	+	–	–	–	–
Apricot (F)	–	–	+	+	–	–	+	+
Apricot (D)	–	–	+	+	–	–	+	+
Banana (F)	–	–	+	+	–	–	+	+
Banana (D)	–	–	+	+	–	–	+	+
Cantaloupe (F)	–	–	+	+	–	–	+	+
Cantaloupe (D)	–	–	+	+	–	–	+	+
Cherry (F)	–	–	+	+	–	–	–	–
Cherry (D)	–	–	+	+	–	–	–	–
Grape, green (F)	–	–	+	+	–	–	–	–
Grape, green (D)	–	–	+	+	–	–	–	–
Grape, red (F)	–	–	–	+	–	–	–	–
Grape, red (D)	–	–	–	+	–	–	–	–
Grapefruit, pink (F)	–	–	–	–	–	–	–	–
Grapefruit, pink (D)	–	–	–	–	–	–	–	–
Lemon (F)	–	–	–	–	–	–	–	–
Lemon (D)	–	–	–	–	–	–	–	–
Nectarine (F)	–	–	+	+	–	–	+	+
Nectarine (D)	–	–	+	+	–	–	+	+
Orange, navel (F)	–	–	–	–	–	–	–	–
Orange, navel (D)	–	–	–	–	–	–	–	–
Peach (F)	–	–	+	+	–	–	+	+
Peach (D)	–	–	+	+	–	–	+	+
Pear, D'Anjio (F)	–	–	+	+	–	–	+	+
Pear, D'Anjio (D)	–	–	+	+	–	–	+	+
Pineapple (F)	–	–	+	+	–	–	+	+
Pineapple (D)	–	–	+	+	–	–	+	+
Plum, red (F)	–	–	+	+	–	–	–	–
Plum, red (D)	–	–	+	+	–	–	–	–
Strawberry (F)	–	–	–	–	–	–	–	–
Strawberry (D)	–	–	–	–	–	–	–	–
Tangerine (F)	–	–	+	+	–	–	+	+
Tangerine (D)	–	–	+	+	–	–	+	+
Watermelon (F)	–	–	+	+	–	–	+	+
Watermelon (D)	–	–	+	+	–	–	+	+

[a]The key to the abbreviations used is found in the footnote to Table 2.

[b]Note that there was a variation in the color changes and in the time required for different fruits marked positive (+). Some color reactions may be distinguished under certain conditions from a positive reaction for blood. However, the author is of the opinion that the noted color change may also be considered by some workers to be positive for blood.

within 20 s. This sensitivity is far less than the 1:100 000 reported by Grodsky, Wright, and Kirk [*1*].

Orthotolidine Test

The sensitivity of this test approximated the sensitivity of the tetramethylbenzidine test. Positive reactions were observed with dilutions of 1:100 000 on filter paper, cotton cloth, and blood solutions.

This test also paralleled the results of the tetramethylbenzidine test with the vegetables tested, with the exception of mushroom, which gave a weak positive within 60 s with dried pulp. This test did not react with apple, cherry, green grape, red grape, or red plum, as the tetramethylbenzidine did.

This author found orthotolidine to be more sensitive than phenolphthalein in an earlier study evaluating presumptive identification of washed bloodstains [*5*]. It was also noted in that study that cotton had a greater retention of bloodstains than the other fabrics tested. Cotton's strong affinity for blood is a possible explanation for the greater sensitivity of the bloodstained cotton cloth over that of the stained filter paper, as indicated in Table 1.

Conclusions

From the results of this study of four presumptive tests for blood, the following can be concluded:

1. The phenolphthalein test is the best single test of the four investigated, based on its sensitivity of 1:10 000 with stained filter paper and cotton cloth and on the failure of this reagent to react with plant peroxidases.
2. The phenolphthalein and the leucomalachite green tests are the most specific of the four examined.
3. The tetramethylbenzidine and the orthotolidine tests are the most sensitive of the group.
4. The tetramethylbenzidine and the orthotolidine tests are the most likely to produce interference color reactions with plant peroxidases.
5. The leucomalachite green test is the least sensitive of the four tests.
6. To obtain reliable sensitivity, a maximum of 10 s should be allowed for the color reaction to occur. In high blood concentrations, the color change will occur almost immediately (1 to 5 s).
7. The various differences in the sensitivities reported by different researchers of presumptive tests for blood are undoubtedly caused by differences in the reagent concentrations, in the methods of preparing the samples and of expressing the results, and in the type of material containing the blood.

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Address requests for reprints or additional information to
Milton Cox, Ed.D.
Assistant Professor
Criminal Justice and Forensic Science
University of Southern Mississippi
East Beach Blvd.
Long Beach, MS 39560