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RESEARCH PAPER

Comparative study of presumptive and confirmatory tests for detection of blood on serial dilutions and washed stains

***Gupta Shivangi*[1], *Garg Apoorva*[2], *Babu Suresh*[3], *Yadav DS*[4]**

[1,2]*Forensic Professional,*
[3]*Deputy Director,*
[4]*Jr. Scientific Officer*
(Corresponding author)
***Email**: dsyadav12@gmail.com*
***Mobile**: +918357000056*
Department of DNA/Biology Division
Central Forensic Science Laboratory,
Barkheda Bondar, Bhopal-30
Madhya Pradesh, India

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***Background and aims**: Detection of blood from blood stains is the first crucial step for forensic analysis, such as DNA profiling. After committing the crime, the criminal tries to destroy evidence such as blood stains by washing their clothes or other circumstances found at the crime scene make the blood or blood stains diluted due to washing by water or detergent. This study aimed to identify the most sensitive presumptive test among phenolphthalein, tetramethylbenzidine (TMB), benzidine, leuco-malachite green (LMG), and luminol for the washed and serially diluted blood and bloodstains and confirmatory test of blood among Takayama, Teichmann, and Wagenaar. **Materials and methods**: In this study, serially diluted blood, stains of the serially diluted blood, and blood-stained clothes were prepared and subjected to different kinds of washings. Blood was detected using reagents of phenolphthalein, TMB, benzidine, LMG, and luminol with variable protocols reported in various literatures. The samples were further tested for the confirmatory tests of blood using Takayama, Teichmann, and Wagenaar tests. **Results**: It is observed that in presumptive testing of serially diluted bloodstains, luminol shows positive results in all (1:10 to 1:100,00,000) followed by TMB (1:10000). TMB in acetate buffer solution works better for liquid blood; however, solution of TMB in glacial acetic acid works better for stains. LMG solution with zinc has higher sensitivity as compared to a solution without zinc. **Conclusion**: Luminol is the most sensitive presumptive test for detecting blood on washed and diluted bloodstains, and sensitivity decreases with an increase in wash cycles. Among confirmatory tests, the Takayama test is more promising than Teichmann and Wagenaar test.*

***Keywords**: Presumptive test of blood; confirmatory test of blood; washed bloodstains; tetramethylbenzidine; luminol; takayama.*

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INTRODUCTION

Blood is one of the most essential and common physical evidence associated with crimes. In many cases, the detection of blood is the first step for further forensic analysis, such as DNA profiling, which provides crucial evidence of individualization in most cases. In many cases, the suspected bloodstains may have been washed either with water or with detergent. In some cases, evidence found in water bodies or gets wet due to rain or accidental spill of water leads to dilution of blood. There is limited literature showing comparative analysis of the sensitivity of various presumptive and confirmatory tests of blood. This study aimed to identify the most suitable and sensitive method for serially diluted blood and washed bloodstain fabrics by comparing commonly used methods for detecting blood in forensic laboratories.

In 1991, Cox M. reported that TMB is the most sensitive test out of the phenolphthalein, TMB, o-toluidine, and Leucomalachite green test.[1] In 2007, Tobe et al., reported that luminol and phenolphthalein gave positive results till 1:100,000 and leucomalachite green till 1:10,000.[2] Rebecca Andersson, in 2017, studied the sensitivity of leucomalachite green along with other reagents and reported that it gave a positive result of blood diluted till 1:2048.[3]

The basic principle of presumptive tests of blood is based on oxidation-reduction or redox reaction. In the presence of a catalyst, i.e., peroxidase, e.g., hydrogen peroxide, the heme part of hemoglobin will undergo oxidation, reducing reagent and giving color or fluorescence/luminescence.[4] During the detection of blood, phenolphthalein test gives bright pink colour,[1,5] while green-blue colour appeared when testing with tetramethylbenzidine (TMB),[5] and benzidine.[6] A bright-green colour appeared with leucomalachite green,[7] and the luminol test gives bluish-white fluorescence.[8] The confirmatory test involves the formation of crystals in the presence of hemoglobin. In the takayama test, the hemoglobin converts into the haemochromogen (ferroprotoporphyrin) crystal in the presence of pyridine and glucose. In the Teichmann test, the hemoglobin is converted into the haemin crystal in halogens and glacial acetic acid.[5] In the Wagenaar test, acetone chlorhaemin crystals are formed in the presence of acetone and HCl.[9]

This study aimed to identify the most sensitive presumptive test among phenolphthalein, tetramethylbenzidine (TMB), benzidine, leuco-malachite green, and luminol for the serially diluted blood, bloodstains prepared by diluted blood and washed bloodstains; and confirmatory test among Takayama, Teichmann, and Wagenaar.

MATERIAL AND METHODS

Blood collection: The blood samples were collected from the blood bank of Hamidia Hospital, Bhopal, M.P., with all ethics.

Sample preparation of serially diluted blood and diluted blood-stained cloth: The serial dilution of freshly collected blood was prepared in distilled water in the ratio of 1:10; 1:100; 1:1,000; 1:10,000; 1:1,00,000; 1:10,00,000 and 1:1,00,00,000. Part of all the serially diluted samples was carefully spread on the cotton fabric cloth and kept for drying for ten days.

Sample preparation of washed bloodstains: The fresh blood was also poured uniformly on the cotton fabric to make bloodstain and kept for drying for ten days. After the cloth dried, it was washed using various washing steps:

A1: Dip in water: Stained cloth was dipped in water for 2 hours.

B1: Rinse with water: Stained cloth was washed with water for 60 minutes.

C1: Dip in detergent water: Stained cloth was dipped in detergent water for 2 hours.

D1: Rinse with detergent water with soft cycle: Stained cloth was washed with detergent water for 10 minutes.

E1: Rinse with detergent water with moderate cycle: Stained cloth was washed with detergent water for 30 minutes.

F1: Rinse with detergent water with hard cycle: Stained cloth was washed with detergent water for 60 minutes.

Thus all the above-washed cloth fabrics were again air-dried. After that, all the above three kinds of samples, i.e., serially diluted blood samples, stained fabrics prepared by serially diluted blood samples, and washed blood-stained fabrics, were tested for the presence of blood to know the detection efficiency and sensitivity of different methods.

Reagent preparation and test procedure: The samples were tested for presumptive and confirmatory tests with different protocols reported earlier.

1. Phenolphthalein Test: The stock solution for the phenolphthalein test was prepared by adding 2g phenolphthalein and 20g potassium hydroxide in 100ml of distilled water. The mixture was refluxed with 20g of powdered zinc for two hour until the solution became colourless. The stock solution was stored in a dark bottle and refrigerated, with some zinc added to keep it in the reduced form.

Working Solution-1 of phenolphthalein: To prepare the working solution, 20ml of stock solution was added in 80 ml of ethanol and was stored in an amber bottle. The samples were placed on filter paper, and 2-3 drops of the working solution were added, followed by 2-3 drops of 3% hydrogen peroxide. The development of pink colour indicates a positive result.[1]

Working Solution-2 of phenolphthalein: To prepare working solution, 2ml of stock solution was added in 10ml of distilled water and 2ml of ethanol.[5] The samples were

placed on filter paper, and the first few drops of ethanol were added, followed by few drops of working solution and few drops of 3% of hydrogen peroxide. The development of the pink colour indicates a positive result.

2. Benzidine Test: It was prepared by taking 13 ml of glacial acetic acid in a beaker and heated on a water bath at 50° C for 8 to 10 minutes; 1.5 g of benzidine was added and dissolved in glacial acetic acid. The beaker was removed from the water bath, and 57 ml of double-distilled water was added.[6] The samples were placed on filter paper, and 2-3 drops of the working solution were added, followed by 2-3 drops of 3% of hydrogen peroxide. The development of blue colour indicates the presence of blood.

3. Tetramethylbenzidine Test:

Solution-1: Prepared by adding 0.4 g of TMB in 20 ml of acetate buffer (prepared by adding 5 g of sodium acetate in 43 ml of glacial acetic acid and 50 ml of deionized water).

Solution-2: Prepared by adding 2 g of TMB in 100 ml of glacial acetic acid.

For both of the above TMB solutions, the samples were kept on filter paper, and 2-3 drops of the working solution were added, followed by 2-3 drops of 3% hydrogen peroxide. The development of green-blue colour indicates the presence of blood.

4. Leucomalachite green Test:

Solution 1: 0.25 g of leucomalachite green was added in 100 ml of glacial acetic acid and 150 ml of distilled water. Then, 5g of zinc powder was added to the solution and kept for boiling under reflux for 2-3 hours until the solution had lost its colour.[7]

Solution 2: 0.1 g of leucomalachite green was added in 66ml glacial acetic acid and 33 ml distilled water to prepare the working solution.[1]

For both of the above solutions, the samples were placed on filter paper, and 2-3 drops of the working solution were added, followed by 2-3 drops of 3% of hydrogen peroxide. The development of green colour indicates the presence of blood.

5. Luminol Test: A solution of luminol was prepared by dissolving 5.0 g of sodium carbonate in 100 ml of distilled water. 0.1 g of luminol reagent was added and was stirred until dissolved completely. The solution was transferred into a spray bottle. Before the analysis, 0.7 g of sodium perborate

Table 1 Results of various presumptive test on serially diluted blood stains

Serial dilution	Phenolphthalein Working solution 1 (Ref.1)	Phenolphthalein Working solution 2 (Ref.5)	Benzidine (Ref.6)	TMB Solution 1 (Ref.5)	TMB Solution 2 (Ref.1)	LMG Solution 1 (Ref.7)	LMG Solution 2 (Ref.1)	Luminol (Ref.8)
Positive Control	++++ve	++++ve	++++ve	++++ve	+++++ve	++++ve	+++ve	+++ve
Negative Control	- ve	- ve	- ve	- ve	-ve	- ve	-ve	-ve
A1(1:10)	+++ve	+++ve	+++ve	+++ve	++++ve	+++ve	+++ve	+++ve
A2(1:100)	++ve	++ve	++ve	++ve	+++ve	++ve	++ve	++ve
A3(1:1000)	- ve	- ve	+ve	+ve	++ve	+ve	-ve	+ve
A4 (1:10,000)	- ve	- ve	- ve	- ve	+ve	- ve	-ve	+ve
A5 (1:1,00,000)	- ve	- ve	- ve	- ve	-ve	- ve	-ve	+ve
A6 (1:10,00,000)	- ve	- ve	- ve	- ve	-ve	- ve	-ve	+ve
A7 (1:1,00,00,000)	- ve	- ve	- ve	- ve	-ve	- ve	-ve	+ve

Note: ++++ Very strong positive, +++ Strong positive, ++ Mild positive, + Weak positive, - Negative

Table 2 Results of various presumptive tests on serially diluted blood (liquid) samples

Serial dilution	Phenolphthalein Working solution 1 (Ref.1)	Phenolphthalein Working solution 2 (Ref.5)	Benzidine (Ref.6)	TMB Solution 1 (Ref.5)	TMB Solution 2 (Ref.1)	LMG Solution 1 (Ref.7)	LMG Solution 2 (Ref.1)	Luminol (Ref.8)
Positive Control	++++ve	+++ve	++++ve	+++++ve	+++++ve	++++ve	+++ve	+++ve
Negative Control	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
A1 (1:10)	++++ve	+++ve	++++ve	+++++ve	++++ve	++++ve	+++ve	+++ve
A2 (1:100)	+++ve	++ve	+++ve	++++ve	+++ve	+++ve	++ve	++ve
A3 (1:1000)	++ve	+ve	++ve	+++ve	++ve	++ve	-ve	+ve
A4 (1:10,000)	-ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve
A5 (1:1,00,000)	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Note: ++++ Very strong positive, +++ Strong positive, ++ Mild positive, + Weak positive, - Negative

Table 3 Results of various presumptive test on washed blood stain

Serial dilution	Phenolphthalein Working solution 1 (Ref.1)	Phenolphthalein Working solution 2 (Ref.5)	Benzidine (Ref.6)	TMB Solution 1 (Ref.5)	TMB Solution 2 (Ref.1)	LMG Solution 1 (Ref.7)	LMG Solution 2 (Ref.1)	Luminol (Ref.8)
Positive Control	++ve	+++ve	++++ve	++++ve	+++++ve	+++ve	+++ve	+++ve
Negative Control	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
A1 (Dip in water)	++ve	++ve	+++ve	+++ve	++++ve	+++ve	++ve	+++ve
B1 (Rinse with water)	+ve	+ve	+++ve	+++ve	++++ve	++ve	++ve	+++ve
C1 (Dip in detergent water)	-ve	+ve	++ve	++ve	++ve	+ve	+ve	+++ve
D1 (Rinse with detergent water with soft cycle)	-ve	+ve	++ve	++ve	++ve	+ve	+ve	+++ve
E1 (Rinse with detergent water with moderate cycle)	-ve	+ve	++ve	++ve	++ve	+ve	+ve	+++ve
F1 (Rinse with detergent water with hard cycle)	-ve	++ve	++ve	++ve	++ve	+ve	+ve	+++ve

Note: ++++ Very strong positive, +++ Strong positive, ++ Mild positive, + Weak positive, - Negative

Table 4 Results of various confirmatory test on serially diluted blood stains

Serial dilution	Takayama Test	Teichmann Test	Wagenaar Test
Positive Control	**++++ve**	**++++ve**	**+++ve**
Negative Control	- ve	- ve	- ve
A1(1:10)	**+++ve**	**+++ve**	**+ ve**
A2(1:100)	**++ve**	**++ve**	- ve
A3(1:1000)	**+ ve**	**+ ve**	- ve
A4(1:10,000)	- ve	- ve	- ve

Note: ++++ Very strong positive, +++ Strong positive, ++ Mild positive, + Weak positive, - Negative

Table 5 Results of various confirmatory test on washed bloodstains

Washing level	Takayama Test	Teichmann Test	Wagenaar Test
Positive Control	**++++ve**	**++++ve**	**+++ve**
Negative Control	**- ve**	**- ve**	**- ve**
A1 (Dip in water)	**+++ve**	**++ve**	**+ ve**
B1 (Rinse with water)	**++ve**	**+ve**	**- ve**
C1 (Dip in detergent water)	**+ ve**	**- ve**	**- ve**
D1 (Rinse with detergent water with soft cycle)	**- ve**	**- ve**	**- ve**
E1 (Rinse with detergent water with moderate cycle)	**- ve**	**- ve**	**- ve**
F1 (Rinse with detergent water with hard cycle)	**- ve**	**- ve**	**- ve**

Note: ++++ Very strong positive, +++ Strong positive, ++ Mild positive, + Weak positive, - Negative

DISCUSSION

Presumptive tests are usually sensitive but not specific as it reacts with the haemoglobin of all blood (human and animal) to catalyze the oxidation of a chromogenic compound, which produces a color change.[10] The degree of presence of haemoglobin increases the rate of positive results. A positive reaction will result in identifying the sample as possible blood but not necessarily human blood. Confirmatory tests should always follow presumptive positive tests. Various presumptive and confirmatory tests with different compositions are in use to detect blood on crime scene exhibits. In this study, a comparative analysis of various tests was performed to determine the most effective tests for particular conditions, i.e., serially diluted blood, stains prepared from serially diluted blood, and washed bloodstains.

For serially diluted blood stains, the luminol test is the most effective as showed results in all serially diluted samples (1:10 to 1:1,00,00,000), followed by 3,3,5,5-tetramethylbenzidine (solution made in glacial acetic acid), which showed positive result till 1:10,000 (**Table 1**). When serially diluted blood (liquid) was tested for the presence of blood by various presumptive tests, it was observed that benzidine and TMB

(solution made with acetate buffer) gave results up to 1:10,000. In contrast, others gave positive results up to 1:100 or 1:1,000 (**Table 2**). We could infer from the above results that TMB composition with glacial acetic acid is effective on cloth stain, while composition with acetic buffer is more effective on liquid blood. It could also be inferred that the sensitivity of benzidine and TMB with acetate buffer solution is the same. The two working solutions of the phenolphthalein test did not differ in results, although it gives better results with liquid blood (1:1000) compared to stains (1:100). LMG solution with zinc is more sensitive than a solution without zinc, as the former gave results up to 1:1,000 and later up to 1:100 in both serially diluted blood and bloodstains (**Table 2**). The presumptive test of serially diluted bloodstain samples showed positive results up to 1:10,000, involving all the presumptive tests performed. When confirmatory (microcrystal) tests were performed, Takayama and Teichmann showed similar results up to 1:100, while the Wagenaar test showed results up to 1:10 (**Table 4**).

The washed bloodstain samples showed a positive result for all the presumptive tests except for samples washed with detergent and tested with phenolphthalein test with the composition of 20:80 of stock solution and ethanol (**Table 3**). The confirmatory test on washed blood-stained fabrics showed that the Takayama test showed results for blood-stained fabrics, washed as a dip in the water, rinse in water, and washed with detergent. Teichmann test showed results for a dip as well as a rinse in water. Wagenaar test showed the result in a dip in water only (**Table 5**). Therefore for washed bloodstains on fabrics, the Takayama test is the most suitable confirmatory test. There are few previous reports available, which also showed that sensitivity of detection of blood decrease when blood-stained fabrics were washed with detergent.[11, 12]

CONCLUSION

Benzidine and TMB in acetate buffer solution have similar sensitivity for blood detection. TMB in acetate buffer solution works better for liquid blood; however, solution of TMB in glacial acetic acid works better for stains. LMG solution with zinc has higher sensitivity as compared to a solution without zinc. Luminol is the most sensitive presumptive test for detecting blood on washed and diluted bloodstains, and sensitivity decreases with an increase in wash cycles. Among confirmatory tests, the Takayama test is more promising than Teichmann and Wagenaar test.

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Ethics considerations: Blood samples were collected from the blood bank of Hamidia Hospital, and the study was conducted following the Declaration of Helsinki.

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REFERENCES

1. Cox M. A study of the sensitivity and specificity of four presumptive tests for blood. Journal of Forensic Sciences, JFSCA 1991;36(5):1503-1511.
2. Shanan S Tobe, Nigel Watson, and Niamh Nic Daeid. Evaluation of six presumptive tests for blood, their specificity, sensitivity, and effect on high molecular-weight DNA. J Forensic Sci 2007 Jan;52(1):102-109.
3. Andersson Rebecca. An evaluation of two presumptive blood tests and three methods to visualise blood; 2017; Linköping University, Swedish; Available from: URL: http://www.diva-portal.org/smash/record.jsf?pid=diva2%3A1131528&dswid=5818
4. Li Richard. Forensic Biology. 2nd ed. New York: CRC Press; 2015.
5. Biology Manual, Directorate of Forensic Science, Ministry of Home Affairs, GOI. 2005.
6. Modi JP. A textbook of medical jurisprudence and toxicology. 6th ed. Bombay. Butterworth & Co. (India) Ltd.; 1940.
7. National Forensic Science Technology Center. DNA analyst training laboratory manual. Available from: URL: https://static.training.nij.gov/labmanual/Linked%20Documents/Protocols/pdi_lab_pro_2.18.pdf
8. O'Hara P.; Engelson C.; St. Peter WJ. Light and molecules, secondary level use of luminol in forensic analysis. Chem. Educ. 2005, 82, 49. Available from: URL: https://www.amherst.edu/system/files/media/0005/Day%25206%2520Luminol%2520Forensics.doc
9. Identification of blood. Sourcebook in forensic serology, immunology, and biochemistry. Available from: URL: https://www.ncjrs.gov/pdffiles1/pr/160880_unit_2.pdf
10. Kobilinsky L. Liotti TF, Oeser-Sweat J. DNA: Forensic and legal applications. New York: Wiley; 2004.
11. UKEssays. Comparison of Washing Powders: Blood on Clothes [Internet]. November 2018; Available from: URL: https://www.ukessays.com/essays/biology/dna-tracing-in-forensics-biology-essay.php?vref=1.
12. Castelló A. Francés F. Verdú F. An alternative to the human hemoglobin test in investigating bloodstains treated with active oxygen: the human glycophorin A test. Scientific World Journal. 2011 Apr 19;11:907–16.