COMPARATIVE STUDY OF DIFFERENT PRESUMPTIVE BLOOD TEST USED IN FORENSIC INVESTIGATION

[1]* Sangle S.G., Ghuge A.D.[2], Game P.[3], Suryawanshi S.B.[3]
[1]* Government Institute of Forensic Science, Chhatrapati Sambhajinagar, M.S. India,
[2] Government Institute of Science, Nagpur, M.S. India,
[3] Government Institute of Forensic Science, Chhatrapati Sambhajinagar, M.S. India
sgsangle@gmail.com , arunghuge12@gmail.com , sagar.suryawanshi8030@gmail.com

Abstract— Blood is the most common body fluid found on crime scenes. Several presumptive tests are available for the detection of blood based on the peroxidase-like activity of the heme group, like Phenolphthalein (PHP) Test, Leucomalachite green (LMG) Test, Ortho-Tolidine (OT) Test, Benzidine (BZ) Test, Tetra-Methylene Benzidine (TMB) test, Bluestar, Luminol, Fluorescein, Hexagon OBTI, Hematix, and Alternate Light Sources etc. Amongst the available methods, the investigating officer has to choose the most suitable method for the identification of the blood, particularly at the scene of a crime, the sensitivity and specificity of the reagent are also crucial factors to be considered before it is used on forensic exhibits.

Although, few studies have been published on the comparison of a couple of blood identification tests, no article was found comparing six presumptive tests based on their sensitivity and specificity. In the present study, we considered six reagents (PHP, LMG, OT, BZ, Bluestar, and Luminol) routinely used in six presumptive tests for blood identification and compared and evaluated them based on their sensitivity, using blood dilution from 1:10 to 1:1,00,000 dilution and specificity, using 22 blood and blood alike red liquid, semi-liquid materials. All tests were performed in triplicate on white tissue paper with fresh samples, dilution, and substrates.

Amongst the evaluated reagents, results suggest PHP, LMG, Bluestar, and Luminol have greater specificity than sensitivity. However, PHP, OT, BZ, and Bluestar show higher sensitivity up to 1:1,00,000 compared to LMG and Luminol. Among the six reagents studied, the LMG and Luminol test, which needs a dark environment, were found to be a much more reliable presumptive test for the detection of blood at the scene of a crime.

Key words— Blood identification, Bluestar, Forensic Serology, LMG, Luminol, Presumptive test.

I. INTRODUCTION

Forensic science helps crucially to the investigating agencies, helps in the speedy disposal of judicial matters thus supports law enforcement. Forensic biology deals with the study of biological evidences, a sub-branch of it, particularly forensic serology deals with different blood fluids such as blood, semen, saliva, urine, vaginal fluids, sweat, hairs, bone, and teeth, etc. These evidences play a vital role in criminal investigation and individualization.
Most criminal cases are violent, and the study of body fluids gives deep insights into the investigation process. Blood is the most abundant and inevitable source of biological evidence found at the crime scene. Blood provides vital information about the victim and suspects, that aids in the investigation of criminal cases, linking suspect, victim, and crime scene to person identification, etc. [1]

Blood is a liquid connective tissue that contains hemoglobin (Hb) as the prime component (heme and globin protein) along with red blood corpuscles (RBCs), white blood cells (WBCs), and platelets. Hb is a tetrameric protein, consisting of 2α and 2β subunits, each subunit is linked to a central heme molecule. Hemoglobin gives red color to our blood, and serves an important function in the transport of oxygen and carbon dioxide to different body parts[2,3] Blood contains coagulating factors, thus gets coagulated rapidly, liquid or dried blood can be recovered from the crime scene or spatters from the dried bloodstain [4,5]

Some criminals, wipe out the blood stains to escape themselves or deliberately add red color blood-like liquid at the crime scene to deviate or mislead the investigation. Starting with non-blood material can hamper the success of the investigation which also can waste time and resources. To avoid any future discrepancies during the court trials it is expected to preliminary identify the sample then send or proceed for further investigation.

Another important issue at the crime scene is, in most investigations, police officials are the ones who first visit the scene of crime collect the specimens, and send them to forensic labs. Only in selected cases if needed and decided by the investigating officer, the forensic team is requested to visit the crime scene and collect the exhibits.

Several questions need to be raised by the Investigating officer while observing the suspected stain at the scene of the crime. Whether the obtained stain is blood or not? [6] If yes, then the obtained blood is of human origin? Thus, detecting traces of blood from wiped-surface and preliminary identification of suspected blood samples is the prime task of forensic serologists.

Thus, a presumptive test should be easy to be performed by any police official. A simple, inexpensive, safe, and reliable test to be performed even at the scene of crime is always demanded. Accordingly, many tests are known today, but choosing the best amongst them is crucial. The majority of the known presumptive tests used in blood identification focus on the detection of hemoglobin molecules.

In order to examine suspected stains there are several catalytic tests which are commonly used for presumptive identification of blood based on peroxidase-like activity of the heme group [7–13] such as Phenolphthalein test (PHP), Kastle Meyer (KM) [14–25] Leucomalachite green (LMG) [6,7,15,22,24], Benzidine test (BT) [7] TMB [6,17,22] Ortho tolidine (OT) Luminol Test (LT) [2,3,23,25–30,7,8,12–15,21,22], and alternate light source (ALS) [13]

The major problem associated with most of the above-mentioned presumptive tests also shows false positive results due to lack of specificity, also OT and Benzidine are highly carcinogenic, thus care has to be taken while handling these reagents [6,9,31].

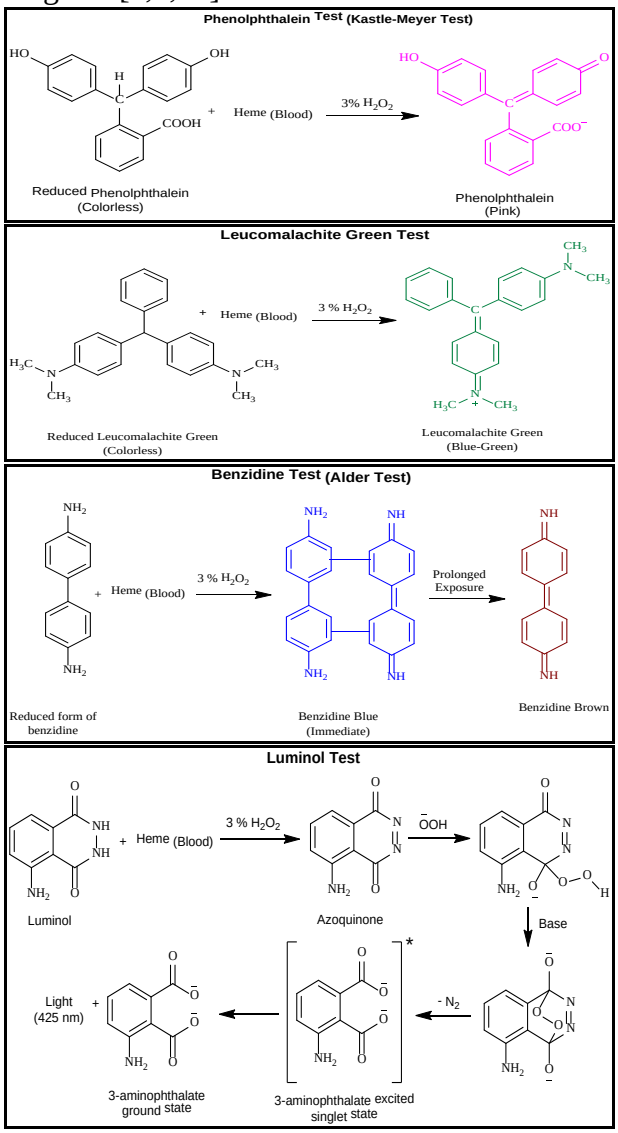

Figure 1 Showing reactions of different reagents with blood: a) Phenolphthalein test, b) Leucomalachite Green test, c) Benzidine test and d) Luminol test.

The aim of our current study is to find out the most reliable, sensitive, and specific presumptive test for blood identification, which is either devoid of or overcomes most of the lacunas arising out of false results or compromising the test result. We tested different presumptive tests and compared them on blood and other selected substances like red paint, vegetable juice, tomato, beetroot, cherry, root nodules, etc. for their specificity and also diluted them up to one lakh fold and checked for sensitivity.

As always demanded, the presumptive test should be rapid and user friendly, so that any investigating officer could use these spot tests on crime sites to differentiate red-color spots from blood, and identify the traces of blood even from wiped-out or washed surfaces.

Two important characteristics, specificity, and sensitivity must to be crucial to collect the evidences presumptively identify them at the scene of the crime and following chain-of-custody send to forensic laboratory for further analysis.

The sensitivity of the test is the ability to detect blood or any fluid, like semen or saliva with minimum availability of sample. The better the ability to identify the highly diluted samples, the better will be the test to detect the exhibits at the crime scene. The various differences are observed in sensitivities of presumptive blood tests which were reported by different researchers. The reason for variation probably caused by differences in reagent concentration, methods of preparation of samples and reagents, substrate[21] testing conditions i.e., wet stains versus dry stains [7], etc.

Earlier studies conducted on the sensitivity of blood detection limits ranging from, PHP (1:10,000 [3,6]- 1:1,00,000,000 [32]), LMG (1:1000 [13]- 1:1,00,000 [24]), OT (1:1,00,000 [6]), BZ (1:1,00,000 [7]), Luminol (1:1,00,000 - 1:5,000,000 [13]). Grodsky et. al. (1951) studied four presumptive tests PHP, LMG, BZ, and luminol for the sensitivity of blood detection. He reported the sensitivity of PHP (1:50,00,000), LMG (1:1,00,000), BZ (1:300,000) and Luminol (1:5,000,000)[7]

Webb et.al. (2006) compared five presumptive tests of blood for sensitivity purposes. The study indicates the luminol test is more sensitive than PHP, LMG, Hematix, and Forensic light sources [13]. Tobe et.al. (2007) conducted a study on a comparison of six presumptive tests for blood. He reported sensitivity of LMG at 1:10,000 and PHP, Luminol, Bluestar, Hematix, and Hemident™ at 1: 1,00,000 [3].

Johnston et.al. (2008) compared four presumptive blood tests, PHP, LMG, Hexagon OBTI, and Hemastix® for their sensitivity to detect dried blood stains [24]. Vennemann (2014) compared the PHP and LMG tests for their sensitivity and specificity for blood detection. The study confirmed the PHP test shows higher sensitivity than the LMG test [32].

The specificity of presumptive blood tests indicates that the test is indicating positive results only for blood components. Several studies reported false positive results of presumptive tests of blood detection for tea, fruit juices, vegetables, detergents, sauce, bleach solution, common household chemicals, etc. [3,6,7,18,32]. Though benzidine is the most commonly used test which shows false positive results for fruits and vegetables.

Higaki and Philips (1976) studied the sensitivity, stability, and specificity of the phenolphthalein test as an indicator for blood. The study indicated that plant peroxidases contribute to false positive results in the benzidine test but not with the phenolphthalein test [10]. Cox (1991) reported PHP and LMG are most specific than TMB and OT in his comparative study, where TMB and OT gives results with plant peroxidase [6]. Peterson (2014) reported false-positive results from root nodules of leguminous plants with PHP blood detection test [19]. Gomes et.al. (2017) evaluated the most effective presumptive test for blood in consideration of specificity. The study indicated that LMG is most suitable than TMB, OT and Bluestar [22].

Later on, many groups have undertaken the studies to Compare the sensitivity, specificity, easy use and safety of PHP, LMG, OT, BZ and luminol [3,13,31]. Luminol has greatest sensitivity and specificity for detection of blood [3,13,14,28,33] Luminol showed different detection capabilities depending upon the substrate[21]. Luminol is best on fabric after

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treated with sodium perborate at different conditions it performed better than TMB and Bluestar [23]. Luminol shows least false positive results [27].

PHP is higher sensitive and specific than LMG [32]. While reported that LMG is most suitable or sensitive and gives less false positive results [22]. PHP, OT, TMB, are sensitive tests with high specificity while LMG test is least sensitive but more specific [6,13].

II. MATERIALS AND METHODS

Samples were tested on a two-square centimeters white tissue paper placed in glass petri dish. Blood samples were collected from different pathological laboratories of Chhatrapati Sambhajinagar, Maharashtra from healthy volunteers with due consent. Sample dilution was prepared, ranging from 1:50 to 1:100000 and subsequently used for presumptive test.

Along with blood, other red-color liquids or blood-like molecules like, Kum-Kum, Beet-root, edible food color, tomato sauce, Pomegranate seeds, paint, Catechu (*Katha*, in Hindi), routinely used in making of edible leaves red, consumed in most part of India after dinner), root nodules, red wine, Fountain-pen ink, Strawberries, $KmNO_4$, Jam, Lipstick, Tomato, Safranin and Orange fruits, all these were collected from local market and some available in the lab. In addition to biological fluids such as sweat, saliva, semen and urine samples were also collected freshly from healthy male volunteers

A. Reagents Preparation

PHP and OT were prepared as per Cox Milton [6], LMG was prepared according to DNA Analyst training, Laboratory training manual BZ according to [7], Luminol by [13] luminol formulation protocol mentioned under panel B of study and Bluestar from commercially available kits [26].

Phenolphthalein (PHP) Test: The reagent stock solution was prepared by combining 2g of phenolphthalein (fisher), 20g potassium hydroxide and 100ml distilled water. The solution was refluxed until it turned colorless, was cool and stored in amber color glass bottle. 20ml stock was mixed in 80ml absolute ethanol and used as working solution with 3% H_2O_2 [6].

Leucomalachite green (LMG) Test: The LMG reagent was prepared by combining 0.25 Leucomalachite green (Himedia), 100ml glacial Acetic Acid (Qualigens), 150ml distilled water with 5gm zinc dust powder and refluxed until green color solution turn into clear solution and used with 3% H_2O_2

Ortho-Tolidine (OT) Test: The OT reagent is prepared by mixing 1.5g Ortho tolidine (), in 40ml ethanol, 30ml glacial acetic acid and 30ml distilled water. Solution mixed well and used with 3% H_2O_2 [6].

Benzidine (BZ) Test: The benzidine reagent were prepared by combining 0.1g benzidine powder and 0.2g sodium perborate with 10ml glacial acetic acid [8]

Luminol Test: The luminol stock A solution was prepared by dissolving 8g sodium hydroxide in 500ml distilled water, Stock B was prepared by adding 10ml 30% H_2O_2 in 490ml of distilled water and Stock C by adding 62.5ml stock A in 0.354g Luminol (Loba Chemie). Modified method used for preparation of working solution; 10 ml of each stock were added in 70ml of distilled water [26].

Bluestar test: The bluestar reagent was prepared by mixing two tablets of kit in 125ml of distilled water. The durability of solution is only for 1 day or it is reactive for few hours for higher dilutions.

B. Test/ Methodology

All reagents were firstly tested on positive control which prepared by applying reagent on blood-stained tissue paper and Negative control by applying reagent on tissue paper containing drop of distilled water with/ followed by 3% hydrogen peroxide (H_2O_2.)

The tissue paper was placed in sterile glass Petri-dish and new micropipette tips were used for addition of reagents and for each blood dilution. To test blood dilutions, 40 µl of each dilution was pipetted on tissue paper. This was then tested by immediately adding 40 µl of reagent PHP, LMG, OT followed by a drop of 3% H_2O_2. A positive reaction was indicated by color change to Pink, Green and Blue-Green respectively.

In such manner Benzidine reagent were tested on stained tissue paper, this test does not require

H_2O_2, the positive reaction will show color change to Green-blue-Yellow. Luminol and Bluestar with 3% H_2O_2. reagent was tested in Dark Chamber for each dilution.

Each of the above listed substrates, which looks like blood, were tested with six selected presumptive tests. The solutions of substrates were prepared and added one drop on tissue paper, were then tested against reagent to observed whether substance cause reaction. The time taken for reaction was recorded. The test was considered positive if there was any color change, and negative if there was no color change within two minutes after addition of reagent. All tests were run in triplicate and confirmed for their repetition efficiency.

I. OBSERVATION TABLE

Table 1 Indicates test sensitivity on selected blood dilutions of six presumptive tests viz., Phenolphthalein (PHP), Leucomalachite Green (LMG), Ortho-Tolidine (OT), Benzidine (BZ), Bluestar and Luminol.

Sr. No.	Blood Dilutions	Reagents					
		PHP	LMG	OT	BZ	Bluestar	Luminol
1	1:50 #	+	+	+	+	+	+
	RT	1	1	1	1	1	1
2	1:100 #	+	+	+	+	+	+
	RT	1	1	1	1	1	1
3	1:500 #	+	+	+	+	+	+
	RT	1	1	2	1	1	1
4	1:1000	+	+	+	+	+	+
	RT	1	2	2	1	1	1
5	1:5000	+	+	+	+	+	+
	RT	3	2	10	1	1	1
6	1:10,000	+	+	+	+	+	+
	RT	4	5	12	5	1	1
7	1:20,000	+	+	+	+	+	+
	RT	6	10	12	30	1	1
8	1:30,000	+	+	+	+	+	+
	RT	7	14	12	30	1	1
9	1:40,000	+	+	+	+	+	+
	RT	9	20	11	30	1	1
10	1:50,000	+	+	+	+	+	+
	RT	12	24	15	30	1	1
11	1:1,00,000	+	-	+	+	+	-
	RT	19	30	23	30	2	30

(+ indicates positive results, - Indicates negative results, # indicates positive results within a second, RT indicates reaction time in seconds)

Blood dilution showing results within 30 seconds were only considered as positive and indicated by plus (+) sign, while negatives were represented by negative (-) sign. Fresh human blood was considered as positive control and distilled water as negative control. RT indicates reaction time of reagent in seconds.

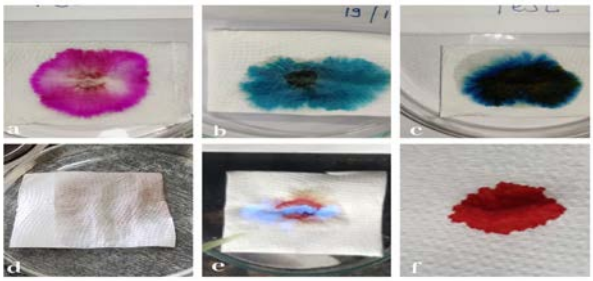

Figure 2 Showing different reagents interacting with the blood. a) Phenolphthalein test, b) Leucomalachite test, c) Ortho tolidine test, d) Benzidine test, e) Bluestar test and f) Luminol test.

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Table 2 Indicates test specificity on selected substrates of six presumptive tests viz., Phenolphthalein (PHP), Leucomalachite Green (LMG), Ortho-Tolidine (OT), Benzidine (BZ), Bluestar and Luminol.

S.N.	Substrate	PHP	LMG	OT	BZ	Bluestar	Luminol
1	Orange	-	-	-	-	-	-
2	Kum-kum	-	-	-	-	-	-
3	Beet root	-	-	+	-	-	-
4	Food color	-	-	-	-	-	-
5	Tomato sauce	-	-	-	-	-	-
6	Pomegranate	-	-	-	-	-	-
7	Paint/water	-	-	-	-	-	-
8	Catechu	-	-	-	-	-	-
9	Root nodules[#]	+	+	+	+	+	+
10	Red wine (pinkish)	-	-	-	-	-	-
11	Fountain pen ink	-	-	-	-	-	-
12	Strawberry	-	-	+	-	-	-
13	$KmNO_4$ [#]	+	+	+	+	+	+
14	Jam	-	+	+	+	-	-
15	Lipstick	-	-	+	-	-	-
16	Sweat	-	-	-	-	-	-
17	Saliva	-	-	+	-	-	-
18	Semen	-	-	+	-	-	-
19	Urine	-	-	-	-	-	-
20	Tomato	-	-	+	-	-	-
21	Safranin	-	-	-	-	-	-

(Substrate showing results within 30 seconds were only considered as positive and indicated by plus (+) sign, while negatives were represented by negative (-) sign. Fresh human blood was considered as positive control and distilled water as negative control.

I. RESULTS

Sensitivity: Amongst the studied test Bluestar and luminol were found to be highly sensitive even up to 1:100000 times diluted sample, followed by PHP, OT and LMG which showed positive results up to 1:40000, 1:20000 and 1:5000 times diluted samples respectively. Benzidine on the other hand was least sensitive towards higher dilutions of blood and took comparatively longer time amongst the studied tests.

Specificity: Amongst the studied test PHP, Bluestar and Luminol found to show only two false positive results with KmNO4 and Jam. While LMG and BZ shows false positive results with three different substrates i.e., root nodules, KmNO4 and Jam. OT was found to be the least specific amongst them, showing false positive results with 9 different blood like molecules viz., beet-root, root nodules, Strawberry, KmNO4, Jam, Lipstick, Saliva, Semen and tomato.

II. DISCUSSION AND CONCLUSION

Advancements in technology is assisting investigation agencies in solving cases with increased accuracy and confidence, which is evident from number of cases successfully convicted at an alarming speed, but even with increase in conviction rate, many incidences are reported where the suspects are freed because of lack of convincing evidences in the court of law.

Success of any forensic investigation relies much fold on the primary task of collection of samples from the scene of crime, identifying the clues and maintaining the chain of custody while handling the sample, packaging and transporting to the forensic lab for further investigation.

At the scene of crime many-a-times the sample gets degraded/ contaminated because of lack of information or awareness. Several studies have been conducted on the sensitivity and specificity of presumptive tests for blood detection [3,9,18,34,35] are reported by many authors, but

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literature exclusively on the comparison of selected six presumptive tests for blood identification is not available to much extent.

Amongst the studied test Bluestar and luminol were found to be highly sensitive even up to 1:100000 times diluted sample, these studies are consistent with earlier studies carried out by Butler et.al. (2019) showing Luminol has greatest sensitivity and specificity for detection of blood [13,14,28,32,33]. Luminol showed different detection capabilities depending upon the substrate [21]. Luminol is best on fabric after treated with sodium perborate at different conditions it performed better than TMB and Bluestar [23]. Luminol shows least false positive results [27].

While Johnston et.al. (2008) reported sensitivity of PHP and LMG up to 1:1,00,000 [24]. PHP is better spot test for identification of human and animal blood. Which detect blood up to dilution of 1:10,000 for dog and up to 1:10,00,000 for feline (cat) blood [20], our study found PHP, OT and LMG to showed positive results up to 1:40000, 1:20000 and 1:5000 times diluted samples respectively. Benzidine on the other hand was least sensitive towards higher dilutions of blood and took comparatively longer time amongst the studied tests.

Amongst the studied test PHP, Bluestar and Luminol found to show only two false positive results with KmNO4 and Jam, while LMG and BZ shows false positive results with three different substrates i.e., root nodules, KmNO4 and Jam. Similar findings were reported by Grodsky et. al., (1951) studied four presumptive tests PHP, LMG, BZ and luminol for the sensitivity of blood detection. He reported the sensitivity of PHP (1:50,00,000), LMG (1:1,00,000), BZ (1:300,000) and Luminol (1:5,000,000) [7].

OT was found to be the least specific amongst them, showing false positive results with 9 different blood like molecules viz., beet-root, root nodules, Strawberry, KmNO4, Jam, Lipstick, Saliva, Semen and tomato. These findings are consistent with showing PHP, OT, TMB, are sensitive tests with high specificity while LMG test is least sensitive but more specific [6,13].

In conclusion, Bluestar and luminol seems to be highly specific and shows greater sensitive amongst the studied test.

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