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# TMB vs. Phenolphthalein Test-Blood Stain Detection

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**ABSTRACT**

Forensic Biology is the study of biological evidences which found at the crime scene & their study and examination for the purpose of justice. And in regards with the same of serological evidences, is known to be Forensic Serology. Blood is biological evidence which is a common type of evidence found at most of the crime scenes. Blood is a bodily fluid which consists of cellular & non-cellular components. In this review article we will discuss about the two types of preliminary tests for blood detection which may found at the possible crime scenes, here we will analyze through the TMB (i.e., Tetra Methyl Benzidine) and Phenolphthalein Assay (also known as Kastle Mayer's test) and differentiate between those & will come to the conclusion which is the best method or test for the preliminary blood stain detection amongst the both while conducting the assays for blood samples.

**Keywords:** Crime Scene; Blood; TMB Test; Phenolphthalein Assay.

## INTRODUCTION

Blood is the bodily fluid found in the body of human as well as animals (Richard, 2009), is red in colour due to the presence of haemoglobin and iron. Blood is composed of two types of components such as; 1. Cellular component – RBC (Erythrocytes), WBC (Leucocytes) & Platelet (Thrombocytes), 2. Non-cellular component – Plasma. Blood is the most common type and source of evidence found at the crime scenes, it must be handled as physical as well as biological evidence to be tagged and bagged; in addition, everyone entering the scene of crime must take care not to disturb the patterns of blood, which can reveal as much to the trained eyes as the result of the laboratory testing of the blood itself. The interpretation of the bloodstain patterns requires careful planned experiments utilizing surface materials comparable to those found at the crime scene. Whoever is assigned the responsibility of interpreting these stains must be given first access crime scene so that the blood patterns may be photographed before other crime scene processing activities obscure them. Because of the violent nature of the criminal homicide, blood is commonly found at the crime scene.

The investigator may encounter the availability of the blood evidence at mostly four areas; 1) On the victim, 2) At the crime scene, 3) On a weapon, 4) On the assailant. Police, prosecutors and experts all want to know the answer with three things which confronted with the any form of blood found at the crime scene: 1. Is it blood? 2. Is it human blood? 3. How closely related is it to the blood of known or discovered suspects? In answering the questionns, forensic scientist uses variety of chemical test known as presumptive assays. Presumptive blood assays are designed and developed to detect the traces of blood. The chemical reaction employed in this assay is an oxide-tion and reduction reaction catalysed by heme, a haemoglobin component. The heme catalyses various colourless substrates to undergo an oxidation reaction that results in change of colour, whether it may be then produce chemiluminescence or fluorescence. An oxidation -reduction reaction involves changes of the oxidation state. In biochemical reactions, oxidation is often relates and coincides with a loss of hydrogen, which depicts an example of an oxidation-reduction reaction for blood identification. In the presumptive assays, hydrogen peroxide ($H_2O_2$) is usually played as an oxidant. Heme serves as the catalyst for the oxidation-reduction reaction. In the presence of heme, a colourless substrate is oxidized to a product with colour, such to be chemiluminescence or fluorescence.

Presumptive assays also known to be calorimetric assays, consists of various procedure such as; phenolphthalein, leucomalachite green, tetramethylbenzidine, and benzidine derivatives, for the detection of heme in blood sample through the colour reactions (Ronald, 2010). The colour reactions produced by these assays can be observed immediately with the naked eyes. The assays are very sensitive and can detect blood in samples with $10^{-5}$ fold dilutions. A positive reaction indicates the possible presence of blood. This study is aimed to identify the most sensitive and best presumptive assay amongst the Phenolphthalein and Tetramethylbenzidine (TMB) for the blood identification at primary level.

## MATERIAL AND METHODS

Blood sample collection: For the study of this review literature a crime scene (Biology Methods Manual 1978) was considered i.e., a murder. For the collection of blood evidence variety of collection techniques and equipment are used such as; 1. Moist cotton for dried blood stain, 2. Sterile vials for liquid blood in amount, 3. Sterile cloth for pull amount of blood to be soaked on it, etc. and packed it in a paper envelop.

**Sample preparation:**

A sample is prepared by taking a stains on the filter paper or on a slide, and then mixed it with the possible reagents through which they can emit out a colourful appearance for the primarily detection of a blood sample identification.

**Phenolphthalein or Kastle Meyer Test:**

Phenolphthalein test or Kastle Meyer Test is a "Presumptive test" for blood. This test is one of the two main classes of forensic tests commonly employed by forensic labs in chemical identification of blood. Phenolphthalein is used as a chemical indicator in these years for detection of blood. This test was first described in 1903. It was named after the American agricultural chemist Joseph Hoeing Kastle who first invented and tested crude year blood in 1901, and Erich Meyer who modified the test in 1903.

This test relays on the peroxidase like activity of haemoglobin present in the blood to catalyse the oxidation of phenolphthalein reagent in which colourless reduced form of phenolphthalein turns into visible as bright pink colour. It is a form of Catalytic blood test.

**Reagent Preparation:**

Stock Solution-

- Phenolphthalein-2.0g
- Potassium Hydroxide-20g
- Distilled water – 100ml
- Zinc dust- 20g

Mix and add few boiling chips of above reagents and boil under reflux for 2-3 hours until the solution has lost its pink colour. Cool it and decant into container containing few zinc to keep it in reduced form.

**Working solution-**

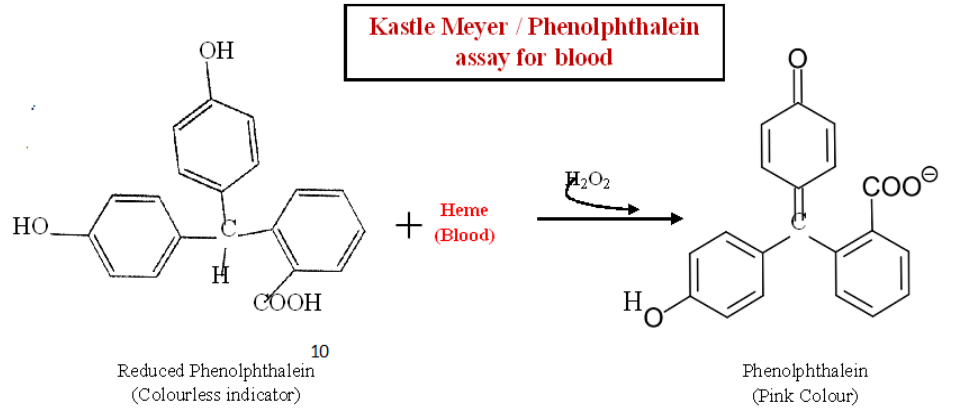
Solution 1- Ethanol – 10ml

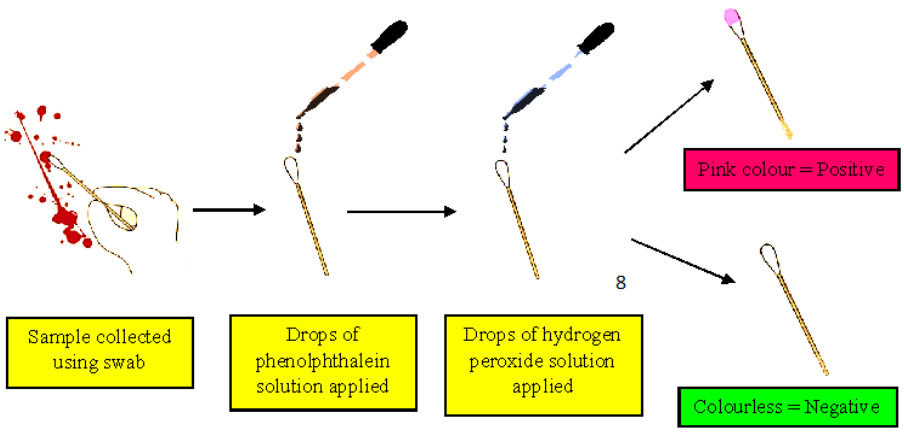
Solution 2- Phenolphthalein stock- 2ml
Distilled water-10ml
Ethanol-2ml

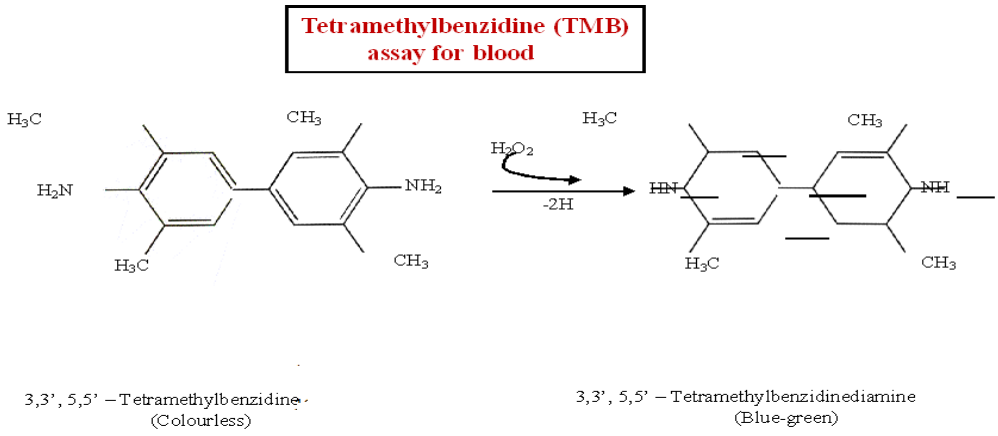
Solution 3- 3% Hydrogen peroxide-10ml

**Process:**

- A small cutting, swabbing or extract of suspected blood stain is place on a filter paper or spot test paper.
- Add 2-3 drops of Ethanol and 2 drops working phenolphthalein solution on the stain. After waiting to insure no colour develops at this stage, add 2-3 drops of 3% Hydrogen Peroxide.
- The development of intense "pink colour" after 30 seconds is positive test for peroxidase activity, indicative of haemoglobin.



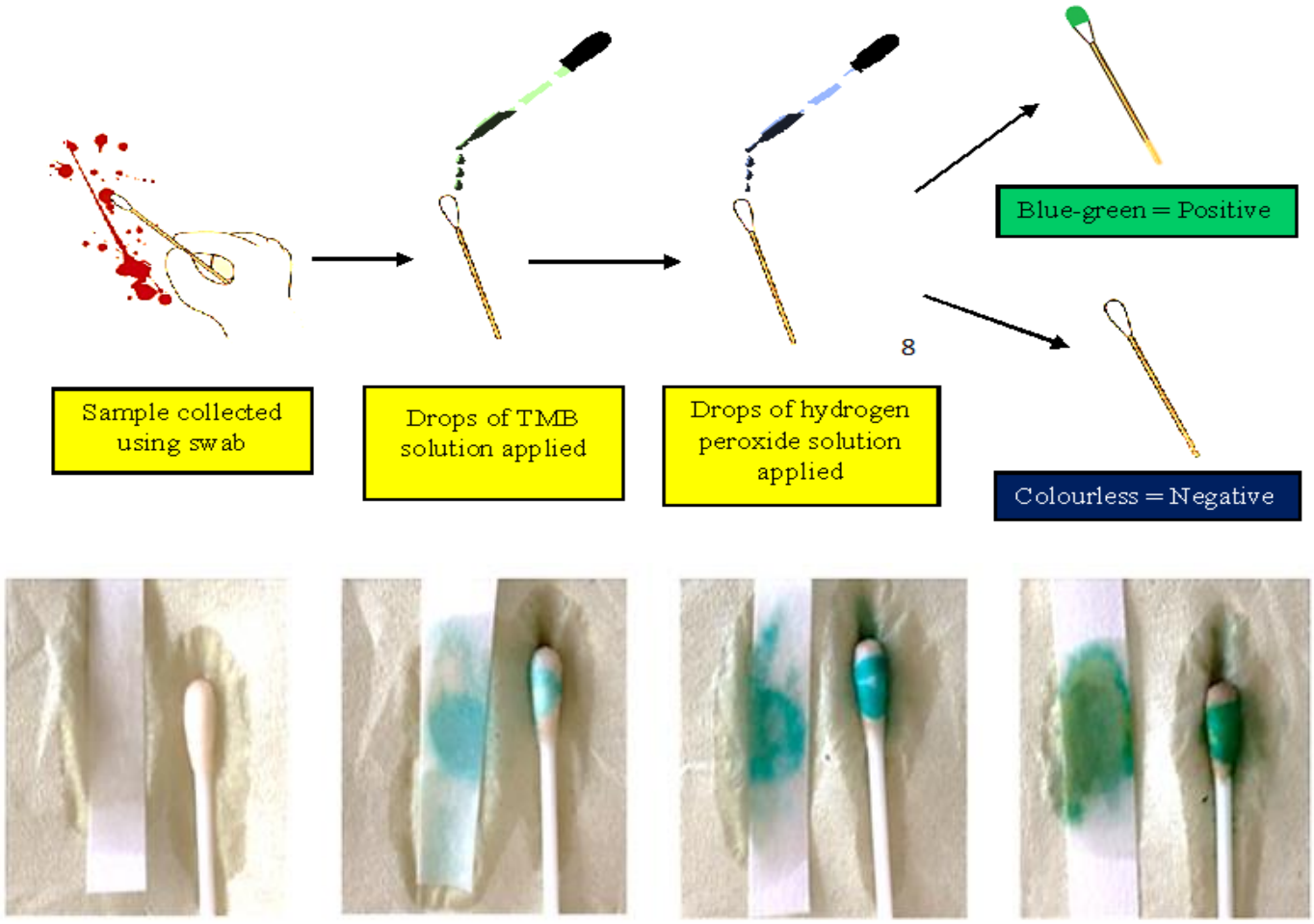



**Figure 1:** The result showing blue-green colour compound formation after the testing of blood sample with TMB solution in presence of hydrogen peroxide.

**Tetramethylbenzidine Assay (TMB):**

Tetramethylbenzidine test is a presumptive test generally apply for the detection of blood. In year 1974, Holand-et-al implemented the TMB and reported that the way of possibility the detection of blood from a sample. Within the same year, it is also reported that the occupational Safety and Health Administration Banned the usage and even manufacturing of benzidine in US as it leads the toxicity which is carcinogenic in nature. The ban leads the finding of alternative method for the detection procedure.

After a year in 1976 Garner-et-al also reported and claimed that the TMB is much more preferable than the benzidine. Its comparable analysis also resulted with sensitivity and specificity that forwarded the evolution of Tetramethylbenzidine as their alternative to the benzidine test.

The tertramethyl benzidine is a tetra methyl derived from the derivatives of benzidine. which is used for the detection of presence of blood with in a sample. The principle of this test is based on reagent reaction of peroxide with heme compound that resulted the appearance of blue green colour. It happens when the compound heme present in the blood exposed with reagent TMB in the presence of oxidizing agent hydrogen peroxide leads to oxidation of TMB which is responsible for forming the blue green appearance of colour.

In certain examination without addition of hydrogen peroxide the blue green colour may appear instantly after the addition of TMB reagent it provides a false positive result due to presence of other oxidants in sample. Mainly at this stage there no such colour changes is observed. The

blue green colour is visible via addition of hydrogen peroxide.

**TMB reagent preparation**

- 0.2 gram of 3,3', 5,5 TMB reagent will put into the 50 ml of beaker.
- Then 10 ml of glacial acetic acid will be added to the TMB reagent.
- The solution become mixed thoroughly.

**Hydrogen peroxide preparation**

- The 10 ml of 30% hydrogen peroxide is taken into the beaker.
- Then 90 ml of distilled water will be added onto it.
- Then the solution will mixed well and get stored into cool condition.

**Procedure**

- Take a suspected blood swab and moisten or moisture is with distilled water or with ethanol if it required.
- Then 1 drop of TMB reagent will be added into the suspected sample.
- Then 1 drop of 3 % hydrogen peroxide will be added.
- Within 20 sec a blue-green colour changes may appear which may indicates the presence of blood and provide a positive result. If there is no colour changes may occur it provide a negative result.

**Difference between Phenolphthalein & TMB assays:**

***Phenolphthalein:***

- Advantages – sensitive, definitive test, low false positive test
- Disadvantages – expensive, time-consuming.

***Tetramethylbenzidine:***

- Advantages – fast showing result, sensitive
- Disadvantages – Carcinogenic in nature, corrosive in nature, some of the false positive results.

**RESULTS & DISCUSSION**

Presumptive assays for blood are used to primarily detect the sample whether it is blood, it is more sensitive test to identify the sample being blood, but it is not all time sure that the solutions which are used for the presumptive analysis to be reacts with the haemoglobin of human or animals to be catalysed the compound which tends to produce the colours. From the overall results after testing and analysis it have been observed that, after trying with both the chemicals i.e., phenolphthalein and tetramethyl-benzidine, the phenolphthalein is find to be favourable presumptive amongst the both assays. After examination and procedure followed for the presumptive blood testing, phenolphthalein shows the primary pink colour at the end of reaction though it indicates that the result or test is positive, in case of tetratmethylbenzidine it shows the catalytic blue-green colour substrate which confirms that the sample analysed is blood and test is positive. Considering the difference between sensitivity, tetramethylbenzidine is more sensitive than the phenolphthalein when it is testing on the dried blood stains, but when it comes to liquid blood stains the phenolphthalein gets more sensitive towards the blood identification. Tetramethylbenzidine shows the fast result within the time lapse of 20 seconds, instead the phenolphthalein took some more time to give the result. Phenolphthalein gives the low false positive results but in case of tetratmethylbenzidine sometimes it shows false positive results as it get reacts with the other molecules other than the blood samples and oxidized hence sometimes it produces the same colour as shown in the blood test. Even the tetramethylbenzidine took shorter duration to detect a blood but it can also reacts with the other compounds at same time since they give the same result as the blood as blue-green colour compound. But phenolphthalein get reacts to other things at very lower chances. The tetramethylbenzidine is also found to be carcinogenic in nature, and in comparison with the phenolphthalein is not found to showing any of the effect as like tetramethylbenzidine.

**CONCLUSION:**

In this review article, we had analysed and considered the phenolphthalein as a best mean for presumptive test of the blood identification. Tetramethylbenzidine is the derivative of benzidine and hence it is carcinogenic in nature, it better works in the acetate buffer solution. Since luminol is the best method to primary detect the blood stains but it would be only used in the dark places or dark sites. Hence in the comparative study of TMB & Phenolphthalein, it is found and concluded that the phenolphthalein is the best presumptive test than the tetramethylbenzidine for blood identification as a primary means of crime scene investigation.

**Conflicts of interest:** The authors stated that no conflicts of interest.

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