CHAPTER II

LITERATURE REVIEWS

Reduced phenolphthalein test or Kastle- Meyers test

Principle and Mechanism of the reduced phenolphthalein test

Reduced phenolphthalein test is a method used for blood detections of the legal science. This method depends on the oxidation reaction between iron (generated from heme using hydrogen peroxide) which is acted like peroxidase enzyme and reacted with reduced phenolphthalein solution caused the color changes to pink. The reaction is showed below. [6, 8]

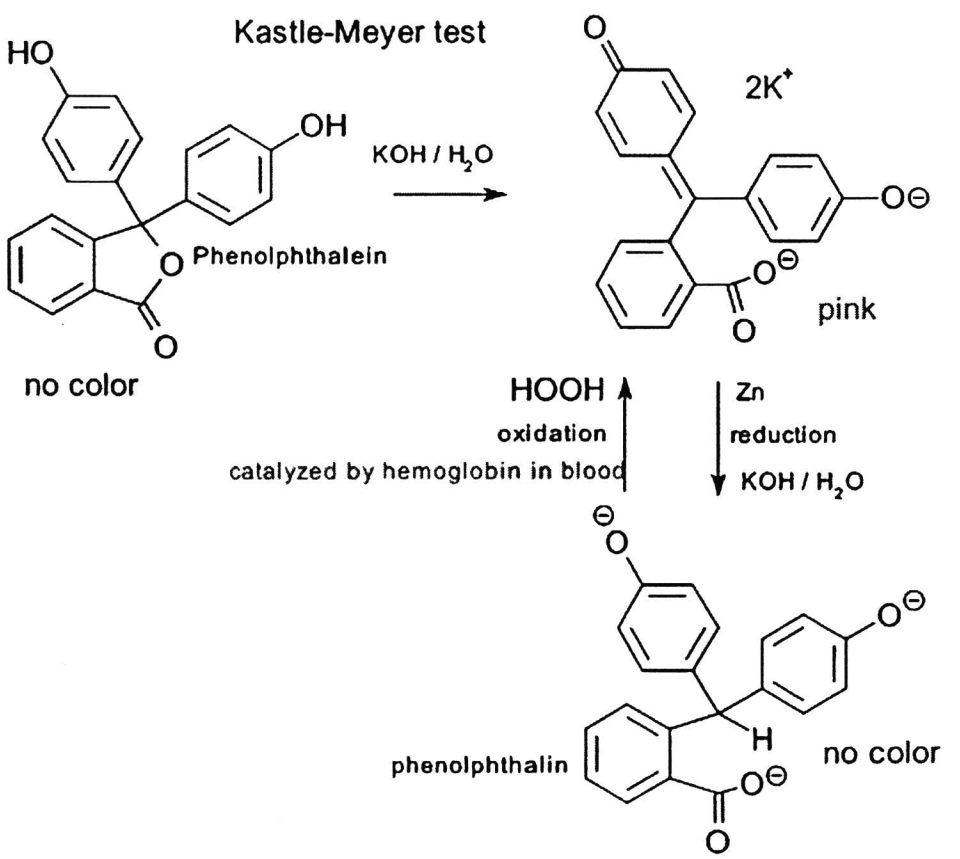

Figure 1 The mechanism of the reduced phenolphthalein

The phenolphthalein has been reduced by two electrons and is pre-dissolved in alkaline solution. This is typically achieved by boiling an alkaline solution of phenolphthalein with powdered zinc, which reduces the phenolphthalein into phenolphthalein. Upon reduction, the very intense pink color of the cationic form of phenolphthalein fades to a faint yellow color. In order to generate the intense pink color indicative of a positive test, the reduced phenolphthalein must be oxidized back to its normal, colored form. [9]

In the relevant reaction, hydrogen peroxide reacts with the hemoglobin in the blood. Phenolphthalein does not participate in this first process. In its reaction with hydrogen peroxide, the heme center of hemoglobin undergoes the following O-O bond hemolysis reaction:

$$HOOH + Fe^{3+}[heme] \rightarrow Fe^{4+}=O[heme] + OH\cdot + H^{+}$$

Reduced phenolphthalein (Kastle–Meyer/original method) reagent preparation

Stock Solution:

1. Phenolphthalein	2.0 g.
2. Potassium Hydroxide	20 g
3. Distilled Water	100 ml.
4. Zinc dust	20 g
5. Ethanol to bring the total volume up to	120 ml.

Weight and mixed, thoroughly, then heat about 2-3 hours or until the pink color of the solution is disappeared then stored in the amber bottle and keep in refrigerate at 4° C [6]

Interpretation of results

The intense pink color indicative of a positive test, the reduced phenolphthalein must be oxidized back to its normal, colored form. This method has sensitive to 1:10,000 and highly specificity. [10]

There were several preparations could be scaled –up and the low-concentration solution with ethanol created in one step or adjust substances ratios. [4]

1. Phenolphthalin 4.0 g.
2. Sodium Hydroxide pellets 40.0 g
3. Zinc dust 20.0 g
4. Distilled Water 1000 ml.
5. Ethanol to bring the total volume up to 1200 ml.

Reflux in a 5000 ml. round –bottomed flask. After reflux, restore to 1200 ml. with ethanol. [5]

1. Phenolphthalein 1.0 g.
2. Sodium Hydroxide pellets 10.0 g
3. Zinc dust 5.0 g
4. Distilled Water 250 ml.

Using a stirring hot plate, mix and heat until the solution loses its pink colour. Do not boil. This process may take 2 to 3 hours. [4, 9]

Procedure [4, 10]

Using a piece of filter paper or paper towel, rub the stain suspected to be blood to collect a sample. If the stain is dry, you may moisten the paper slightly with distilled water. You will not see a visible blood stain on your filter paper.

1. Lay the paper out so that the spot that you rubbed is exposed.
2. Two or three drops of Ethanol (solution 1) are placed on the stain.
3. Two drops of working phenolphthalein solution (solution 2) are added to the stain.
4. After waiting to ensure that no colour develops at this stage, two or three drops of 3% Hydrogen Peroxide (solution 3) are added.

Interpretations

Positive: an intense pink colour is a positive test for peroxides activity that indicative of hemoglobin (Listed in the index C.). This is not a confirmatory test for blood. A positive reaction is indicated by the development of a pink colour within 5 seconds.

Weakly positive: weakly positive: pink light is a positive test for peroxides activity that indicative of very small amounts hemoglobin (Listed in the index C.).

Negative: no change of colour (Listed in the index C.).

Inconclusive: Reactions occurring after 5 seconds or before the addition of the hydrogen peroxide are inconclusive.

False positive: A pink colour after phenolphthalein has been applied but before hydrogen peroxide has been applied normally indicates a false positive due to an oxidizing agent being present. Rust could cause a false reading of this type.

Other tests for blood detections

Guaiac tests

Guaiac is leuko-dye and the test principle is based on the peroxidase-like activities of hemoglobin or non specific oxidant. The catalyze reaction between peroxide and the chromogen, orthotolidine, is generated to form blue oxidized orthotolidine. [11, 18]

$$Hb + 2\,H_2O_2 \longrightarrow 2\,H_2O + O_2$$

$$O_2 + \text{Guaiac} \longrightarrow \text{Oxidized Guaiac}$$

(Colorless) (Blue)

Luminol test [6, 8]

Luminol test reaction can detect blood from the reaction of hemoglobin with hydrogen peroxide and 3 - Aminophenolpthalhydrazide. Luminol can be oxidized by heme to a product that can be luminesced under darkened condition. This test is very useful in location "latent" blood stains but should only be preformed after a visual search has failed to reveal suspected blood. The degree of luminescence is dependent on the substrate and will fade with time, but can be restored with an addition application of reagent mist. This may be particularly useful for weak stains that require prolonged exposure times to photograph, but care must be taken to avoid diluting of the stains with unnecessary repeat spraying if "latent" blood is suspected on a vertical surface, be prepared to photograph immediately as the spraying may cause the blood to "run" down the surface.

Luminol reagent preparation

Stock Solution

1 Solution A:

1.1 Sodium per borate	0.7 gm.
1.2 Distilled water	50.0 ml.

Mix above ingredients until thoroughly dissolved. (Do not store)

2. Solutions B:

2.1 Aminophthalhydrazide (luminol)	0.1 gm.
2.2 Sodium carbonates	5.0 gm.
2.3 Distilled water	50.0 ml.

Mix above in gradients until thoroughly dissolved. Use immediately (Do not store)

Interpretation : Positive reaction = Immediate luminescence

Negative reaction = No luminescence

Inconclusive Reaction =Slow and / or weak luminescence

Interpretation of results [6, ,8, 9]

Reaction produces light instead of color. It had sensitivity 1:1,000,000. However specificity is low due to Luminol's propensity to react with many substances other than blood. [10]

Confirmatory Tests [16]

Many different tests have been used to confirm that a stain contains blood. The oldest is chemical confirmation of the presence of hemoglobin or its derivatives by the formation of specific crystals. For example, the Takayama or hemochromogen test, in which ferrous iron from hemoglobin reacts with pyridine to produce red feathery crystals of pyridine ferroprotoporphyrin [12]

Another confirmatory test uses was the Teichman reagent, consisting of a solution of potassium bromide, potassium chloride and potassium iodide in glacial acetic acid, and is heated to react with hemoglobin. [13] The reaction first converts the hemoglobin to heme, and then the halides react with the heme to form characteristic brownish-yellow rhomboid crystals. Blood can be identified as being of human origin by precipitin reactions with antisera specific for components of human blood.

Cross-over Electrophoresis.

Cross-over electrophoresis for species identification is conducted using agar at a pH of 8.6. Stain extracts are loaded into wells arranged in a line at the cathode end of the plate and the antiserum is loaded into wells at the anode end. During electrophoresis, the electric field drives the serum proteins towards the anode, but the IgG molecules, which are essentially neutral at this pH, are driven to the cathode by the process of electro endosmosis. The antigen-antibody precipitation occurs at the interface between the two rows of wells. Electro endosmosis occurs because the supporting medium acquires a net negative charge. If free, the negatively charged molecules would migrate to the anode, but this is not possible because the agar is immobilized on the plate. Instead, the effect is countered by positively charged water molecules migrating to the cathode. The migrating water molecules carry any dissolved neutral molecules (such as IgG) with them [13, 14, 15, 16]

ABA card [16]

The method of choice today is the ABA card HemaTrace test strips manufactured by Abacus Diagnostics, Inc. Stain extract is applied to the bottom of the test strip, where any human hemoglobin present in the extract will combine with a monoclonal antihuman hemoglobin antibody. The antibody is labeled with a dye. Any antibody-antigen formed then migrates through an absorbent membrane to the test area of the strip. The test area has immobilized polyclonal antihuman hemoglobin that will capture the Ag-Ab complex to form an Ab-Ag-Ab sandwich. The pink dye becomes visible as a band in the test region at concentrations of human hemoglobin above 0.05 μg/ml. An internal control consisting of human hemoglobin antibody–dye conjugate cannot bind to the antibody in the test area but is captured by an antibody in the control area. A correctly functioning positive test will therefore show two pink bands, one in the test area and one in the control area. A correctly functioning negative test will show only one pink band, in the control area. If there is any problem with the test there will be no visible bands.

The ABA card test has been extensively validated and shown to be sensitive, specific and rapid.

Polymerase chain reaction (PCR) [17]

The polymerase chain reaction (PCR) is a technique to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating millions or more copies of a particular DNA sequence

Principles of PCR

An older model three-temperature thermal cycle for PCR is used to amplify specific regions of a DNA strand (the DNA target). This can be a single gene, a part of a gene, or a non-coding sequence. Most PCR methods typically amplify DNA fragments of up to 10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size. A basic PCR set up requires several components and reagents. These components included template that contains the DNA region (target) to be amplified. Two primers that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target. Polymerase or another DNA polymerase with a temperature optimum at around 70 °C, the Deoxynucleoside triphosphates (dNTPs; also very commonly and erroneously called deoxynucleotide triphosphates), the building blocks from which the DNA polymerases synthesizes a new DNA strand, buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase. Divalent cations, magnesium or manganese ions; generally Mg^{2+} is used, but Mn^{2+} can be utilized for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis.[13, 14, 15, 17]

Blood contamination detections

Lowe and co worker [18] studied of blood contamination of siqveland matrix bands using Kastle – Meyer. The reagent test was prepared by phenolphthalein 2 g, potassium hydroxide 20 g, powdered zinc 30 g and distilled water 100 ml. in a conical flask. This mixture was boiled until colorless. In this form the solution is stable for several weeks. They found that Kastle – Meyer could test both fresh and dry blood and found that the concentration of hydrogen peroxide didn't have the effect with the reagent but horse blood dilution and sheep blood dilution have positive to Kastle – Meyer reagent at dilution of 1:6400 lower than the blood contamination test in dental

surgical room. and test with Kastle – Meyer reagent was test with cow and sheep blood sampling by smearing on slide, then took it in autoclave at 134 ° C. It was found that sterile by using steam pressure have no effect to blood detect

Gettler and Kaye [19] used reduced phenolphthalein test for the detection of "occult" blood in feces, urine, spinal fluid and stomach fluid and compared to Guaiac, benzedrine and orthotolidin method. The phenolphthalein reagent test was prepared by phenolphthalein 1 g, sodium a reflux hydroxide 20 g, powdered zinc 20-30 g and distilled water 200 ml. Using condenser to prevent evaporation, the mixture is now slowly boiled until the red color of the alkaline phenolphthalein disappears leaving a colorless solution. The result of study found that reduced phenolphthalein was sensitivity to $1:10^7$ for new blood and $1:10^6$ in old and dry blood while Guaiac, benzedrine and orthotolidin have sensitivity to blood at concentration of $1:10^4$, $1:10^6$ and $1: 10^7$ respectively. The study found that they were copper sulfate interfere with the reaction of the four test.

Lee, et al. [20] used a Kastle- Meyer in commercial test kit (KM) the following equipment from the emergency departments of six UK hospitals (four trusts) and three regional ambulance services was tested for blood contamination: extrication ("spinal") boards, cervical collars, straps, box splints, head blocks, and headboards. Only equipment ready for patient use was tested. Over half of trauma equipment (57%) tested positive for blood, including 15% of equipment that was visibly stained with blood. There have been no recorded cases of infection from contaminated trauma equipment but their study has identified the potential risk. Disposable covers for boards, disposable straps, and disposable radiolucent head blocks which are currently available provide a solution but have resource implications

Louie et al. [21] studied blood contamination on glucose Meters using a Kastle - Meyer as commercial test kit (KM).They studied in 12 hospitals and divided the hospitals 9 hospitals in town , 2 suburb hospitals and one countryside hospital. They used reduced phenolphthalein to test blood stain. The result found that reduced phenolphthalein can detected correctly 100%, The sensitivity of blood dilution between $1: 10^3$ and $1:10^4$ or between 0.0786 and 0.786 microgram of hemoglobin, they also found that departments of hospital having the differential blood contamination

rate such as emergency department (ICU) were high blood contamination more than other department. Apart from this they can detect blood both wet and dry form.

Louise M.E. and Andrew, R. [22] studied the cleaning on blood contamination in the dental surgery following periodontal procedures using Kastle - Meyer reagent test The reagent was prepared by phenolphthalein 2 g, potassium hydroxide 20 g, powdered zinc 30 g and distilled water 100 ml. and stored over zinc dust. This reagent was validated using dilutions blood of horse and sheep. It can be detected at a 1:6400 dilution or less. The blood of horse or sheep gave positive reactions to the Kastle-Meyer reagent within 10 seconds.

Nowadays, in the work of forensic science, the police, making a decision to find blood clue as evidence, in fresh blood or dry blood which passed freeze and used chemical reaction to help analysis, the chemical reaction of screening test always uses procedure of reduced phenolphthalein (Kastle – Meyer test) and Luminol test. Both procedures were sensitive for high blood clued detection. It could only find small blood spot, although the spot can not see both procedures of test used the same principle for oxidation reaction of heme with chemical agents.

Glucose meter is a medical device that used to measure sugar level in the blood. It had usability easy step is simple. Generally glucose meter will exist 2 the principle be, Electrochemical and, Optical Reflectance. Glucose meters have 2 formats, on meter system (add strip testing before drop blood on strip) and off meter system (drop blood sample on strip testing then add to meter) [21] Glucose meters are widely used because of a small size and easy to carry. Nurses in various departments of the hospital as well as primary unit care and the patients themselves have this device for blood checking. Unless mentioned, users pay no attention in properly cleaning lead to the high contaminate. For this reason, the spreading of infection disease will occur.

Hematology analyzer, was used for complete blood count (CBC) and differential leukocyte count (DLC). Now hematology analyzer, have developed with high efficiently, precision, accuracy, convenient, and, fastness. There are two principle of this machine. [3]

1. Electrical impedance method or Electronic gating or Aperture impedance is the principle that blood cells are flowed through narrow aperture from the cathode goes to the anode of the direct current, make the electric current jerks. The jerking amount was count the amount, cells and change in electrical resistance regarded capacity of cells. [3]

2. Light scattering is the principle refracts in the light when blood cells flow through and can cause spread in the light. Blood cells are counted while changing the beam of light. The spreading of the light is caught a signal by the low angle of the photo diode results in the capacity of blood cells and the high angle photo diode results in hemoglobin concentrations.[3]

From reviewing literary work showed that reduced phenolphthalein reagent could be used for blood contamination detection. Although there was a few blood quantity. So the qualification and modified reduced phenolphthalein in sensitivity, selectivity was studied and also easy to use product was developed, Then the developed kit was applied to detect blood contamination on medical devices and laboratory device of medical service place including private sector and government sector in Uttaradit and Pichit Provinces. This work would be useful for user to aware of disease infection transfer from the medical devices.

Having blood contaminated on medical equipments may cause from many factors related to blood contamination on medical devices cleaning methods, the cleaning time reagent potency, and user knowledge of user.

Infectious agents found on medical devices and in hospital settings

Kadi Z., Saint-Laurent, et.al [24] studied the retrospective investigation of patients exposed to possible transmission of hepatitis C virus by a capillary blood glucose meter. A 75-year-old female with no known risk factors for hepatitis C virus (HCV) infection was hospitalized and a diagnosis of HCV sero-conversion was established (HCV immunoblot and a positive quantitative viral load). An epidemiological investigation revealed that, during a previous hospitalization resulting in a diagnosis of diabetes, she had shared the Glucotrend capillary blood glucose meter (CBGM; Roche Diagnostics, France) with a known HCV-positive diabetic patient. Poor hygiene practices were observed when using this device. Since the Glucotrend

CBGM had been purchased, the suspected source patient had been hospitalized eight times and another 19 diabetic patients with known anti-HCV antibodies also regularly attended the same hospital. Consequently, 35 diabetic patients who had been hospitalized at the same time as the suspected source patient and 1305 patients who had used the Glucotrend CBGM were invited to undergo serum anti-hepatitis B virus, anti-HCV and anti-human immunodeficiency virus testing. Among the 35 diabetic patients, none of the 24 subjects tested were positive. Among the 1305 other patients, 995 were tested and 19 (2%) were anti-HCV positive. Although this prevalence is higher than that reported in the general French population, this excess risk cannot be attributed to use of the CBGM. Furthermore, molecular analysis showed that the two HCV strains isolated did not belong to the same phylogenetic cluster. However, as a result of this incident, measures were taken to minimize the transmission of blood borne viruses in the hospital concerned. Other French hospitals were informed by a national alert message from the French Agency for the Safety of Health Products.

Danchaivijitr, and co workers [1, 2] studied the infection control and drug resistance in hospital. The factor of their studies was agent, host and environment. In Thailand agent caused infection in hospitals. The agent was maximal found such as gram-negative bacilli. These bacilli were found in hospital. So it was Antibiotic resistant, as this bacillus has ever touched with antimicrobial drug. In Thailand used more antimicrobial drug. So, the resistance drug rate was high level. Human and environment were factors to- cause infection in hospital. Person who was low immunity, it would be risk to infect more than usual immunity. Environment such as building, place including agent-contamination medical devices, as took then to use for patient, it wound was high risk infection in hospital. The factor that affect spreading the micro-organism that, be from contamination blood on the medical device, which the user was careless to maintain, or did not clean in a correct method., Besides the ability of sterilizes have no meet with contamination efficiency of the blood on medical device implies that cleaning has no the efficiency of who relate induce which, bring about to from spread of micro-organism.