Review: Biological Evidence Collection and Forensic Blood Identification

Authors:
Diana M. Castro, M.S. and Heather Miller Coyle, Ph.D.
Forensic Science Department
Henry C Lee College of Criminal Justice & Forensic Science
University of New Haven
300 Boston Post Road
West Haven, CT 06516 USA

Contact information for authors:

Diana M. Castro   M.S. Forensic Science – Advanced Investigation
Tel: 347-829-9279   Email: dcastro501@gmail.com

Heather Miller Coyle   Associate Professor – Forensic Science
Tel 203-479-4595   Email: Hcoyle@newhaven.edu   Fax 203-931-6073

Abstract

Blood is one of the most important forms of biological evidence that can be collected for forensic analysis. Proper documentation, collection, preservation and testing of blood and DNA are critical for the overall outcome of casework. Recent audits of laboratories have called for better science and improved quality control in forensic testing. This review article speaks to the current issues under discussion in the forensic community and illustrates circumstances for when care in interpretation of blood evidence is needed. The need for human blood to be identified conclusively is essential in many cases due to the possibility of combinations of body fluids or blood epithelial mixtures on items. DNA degradation must be avoided at all costs by employing proper collection and laboratory processing techniques in the field and at the forensic science laboratory, respectively. Appropriate preservation of sample after initial testing is necessary to avoid contamination and other factors that can affect retesting of samples in the future.

Keywords: blood, evidence collection, DNA, standard procedures

1. Introduction
Blood is one of the most invaluable types of physical evidence that can be found at any crime scene. As individualizing as a fingerprint, blood cannot only link an individual to a scene, but it may also provide the sequence of events that could have occurred. Therefore, it is with no question that proper collection and preservation of blood is a technique that every forensic investigator and scientist must learn to master [1-15]. Too often we are faced with cases where the improper collection or testing of a blood sample can make the interpretation of the blood evidence difficult or invalid [16-19]. We, as forensic investigators and scientist, must be able to provide accurate and complete results of the evidence at hand and be certain the integrity of the evidence has not been compromised in order to allow justice to prevail.

The standard forensic science practice at the crime scene is for the investigator to perform a minimum of a presumptive blood identification test during the evidentiary collection process. The traditional standard forensic science practice in the forensic science laboratory is for the scientist to repeat the presumptive test for blood; then confirm the sample as human blood prior to continuing on to a DNA test [2-15]. Recently, some forensic science laboratories have created a short cut in their procedures by eliminating the human blood confirmation test. In some case circumstances, this can create interpretational and legal difficulties and is not a favored approach by the authors [16-19]. Current advances in human blood identification allow for investigators and laboratories to confirm the human specificity of the blood sample within minutes [20].

Blood is a complex liquid that is composed of a mixture of cells, enzymes, proteins and inorganic substances. The composition of blood is made up of two components; plasma and formed elements, which make up 55% and 45% of the blood, respectively [1]. The formed elements are comprised of erythrocytes (red blood cells, RBCs), leukocytes (white blood cells), and thrombocytes (platelets). The red blood cells have two important properties; they contain hemoglobin and do not contain a nucleus. Hemoglobin is the protein that is targeted by many forensic blood detection products and is present in RBCs; a lack of a nucleus signifies that the red blood cells are not the target for DNA detection. The platelets are also very important as they provide the clotting factor in blood, but much like the red blood cells they do not contain any nucleus, and therefore have no forensic value for DNA tests. However, it is the small amount of white blood cells (approximately 1% of a bloodstain) that contains the genetic material of DNA [1].

After biological samples are collected, it is important to avoid DNA degradation. Factors that can lead to DNA degradation include time, temperature, humidity, ultra-violet light and exposure to various chemical substances [2]. The strength of DNA lies in the fact that even though it can be partially degraded, testing of it does not become impossible [2]. DNA is also more stable than the conventional protein markers used in the past (ABO system, isoenzymes). One study showed bloodstains placed in direct sunlight over a 20 week period could still type

with the DNA-based D1S80 marker [3]. However many of the conventional protein enzyme markers (e.g. ADH, PGM) degrade in 2 to 3 months (with the exception of protein systems like ABO); DNA under cool, dry preservation conditions remains stable and typable for years which is why this technology has replaced isoenzyme typing systems of the past [2]. The blood identification reagents, presumptive (e.g. phenolthalein, luminol) and confirmatory (e.g. Ouchterlony, HemaTrace), have remained stable and inexpensive technology from the conception of forensic serology.

2. History of Blood Testing

Prior to the 1900's the examination of blood was not an easy task. In 1901, Karl Landsteiner first recognized that all human blood was not the same; instead blood was distinguishable by its type. This resulted in the ABO typing system [4, 5]. For ABO typing, the following methods can be performed; absorption inhibition method, mixed agglutination method, and absorption-elution method all of which have a specific antibody reaction for the detection of human blood [6]. Historically, the discovery of blood antigens was important to the medical community as it assisted in conducting blood transfusions without negative health effects. Blood typing was used for many years in forensic testing as a useful exclusionary tool but was ultimately discontinued due to the greater discrimination power of the DNA tests introduced in the 1990's.

The first form of DNA testing was developed by Alec Jeffreys and was called VNTR analysis [7]. It was an enzymatic test for the digestion of DNA into fragments which were then separated by gel electrophoresis to generate a complex band pattern to uniquely identify a sample. The next advent in technology was RFLP testing, which had a very high power of discrimination but took a long time to complete and required a substantial amount of starting sample (e.g. a bloodstain approximately the size of a quarter) [8]. With the development of PCR technology in 1986, DNA fragments could be copied and fluorescently tagged for easier detection and required significantly smaller starting sample (e.g. a droplet of blood) [2]. D1S80 silver stain tests and DQ alpha/polymarker strips were the first forensic PCR tests for DNA, but these methods lacked the ability to uniquely individualize a stain to a particular source, although they were much better than blood typing by ABO [2, 8]. Now, short tandem repeat (STR) testing has become the standard method for DNA testing in forensic science. STR analysis has been implemented in almost all forensic science laboratories for the past decade [7]. The STR markers have been standardized so that all laboratories in the United States use the same ones, to allow for data entry and information sharing in the federal DNA CODIS database and they have significantly better individualization power than the early PCR tests [9].

Case Example

One cannot begin to discuss the important role that DNA has played in the forensic investigation of cases without briefly discussing the first landmark DNA case. In 1983, in the small Leicestershire Village of Narborough England, the body of Lynda Mann was found raped and murdered on a frosty lawn by the Black Pad footpath. Three years later a young girl by the name of Dawn Ashworth met the same fate [10]. Around this time Alec Jeffrey's was researching disease markers and created the breakthrough application of VNTR technology to

change the science of personal identification. Jeffrey's referred to this as "DNA fingerprinting" or "VNTR analysis", which was modified to become RFLP analysis to detect loci sequentially rather than in a combined manner [2].

In this English case, blood samples were collected from local men in a DNA dragnet and it was through DNA fingerprinting that the suspect was linked to the case. The original suspect was Richard Buckland, who was arrested for the murders, as it appeared that he knew some information unknown to the public but he was later released. Roughly 4,500 men had DNA extracted and tested to see if a match to the evidence could be found. Colin Pitchfork had persuaded a friend, Ian Kelly, to provide the DNA sample in his place. One of the female patrons in a local pub had overheard the conversation and contacted the police. Pitchfork's DNA was then taken and it matched to the DNA found at the scene [10]. This first case illustrated the importance of DNA testing of blood and how it can be used to uniquely identify suspects.

Case Example
As with all scientific testing, care must be taken in the testing process or errors will occur. In 1993, Gregory F. Taylor was convicted in Wake County Criminal Superior Court of the first-degree murder of Jaquetta Thomas and sentenced to life in prison [19]. On September 3 and 4th, 2009 Taylor's case was accepted for formal evaluation before the Innocence Inquiry Commission. In this case, the testimony of a laboratory analyst stated there were "chemical indications for the presence of blood" on parts of Taylor's vehicle. When questioned by Wake Country Assistant District Attorney, the analyst stated in addition to conducting less sensitive presumptive tests for possible blood on items associated with Taylor's vehicle, he had also conducted more sensitive confirmatory tests on those same items [11].

The analyst then disclosed his lab notes, which indicated the results of the confirmatory tests for blood were actually negative, a fact never mentioned in the final official laboratory report or at trial testimony. The misrepresentation of blood presumptive test results as being confirmatory for human blood identification led to a substantial independent audit of the laboratory practices and policy. This case illustrates the absolute need for constant training in blood identification methods as well as the value of auditing laboratories and their staff for the justice system.

3. Blood Collection and Handling Procedures
Documentation of the crime scene is imperative for the successful outcome of any case analysis and a must for later crime scene reconstruction [11]. Case notes at the scene and at the laboratory document the condition of the packaging and the evidence, describe the stains on the evidence, present the results of the presumptive and confirmatory tests, support the conclusions of the report, and refresh the analyst's memory when he or she has to testify in court [12]. One of the first people on the scene is the first responder, although they are not trained investigators, they should be trained in methods for proper note documentation. First responders are at times the only ones who observe the scene in its original condition [13]. Aside from note taking, there are other methods of documentation that are used, which are:

videotaping, still photography, sketching and audio taping [14]. Good photography is important for bloodstain evidence documentation; however, this method alone does not demonstrate relative distances between objects and other details, therefore it is also important to include sketches with measurements in one's report. Each stain should have a unique identification number, and its location on the evidence or at the scene should be documented by attaching a tag with the unique number directly to the evidence or area [12]. Samples that contain blood should never be collected until after all the bloodstains have been properly documented for potential crime scene reconstruction later on.

After documentation, the investigator can begin the collection of the blood evidence. The most easily lost blood must be collected first, as these are bloodstains that are in high volume traffic areas of the scene and are susceptible to being contaminated or easily lost by traffic through the scene [14]. Any tool that is used in the collection of blood evidence must be sterilized, this is commonly done by using a 10% bleach solution followed by a rinse in 100% ethanol to remove trace amount of bleach or by use of pre-sterilized swabs, etc. [15]. Typically, when the blood at the scene is liquid, it is collected and air dried, and if the blood-stained object is movable, then the entire object is to be collected and transported to the laboratory [14]. When collecting samples it is also a good idea to collect one or more unstained samples from an area adjacent to the bloodstain; this is to determine what was on the substrate before the evidence was deposited and serves as a substrate control [2]. A good example for how DNA mixtures can occur from two cell sources is a blood stain collected from a couch. Epithelial (skin) cells are expected to be on a couch where people have been sitting, the bloodstain that is overlaid on that area could easily result in a DNA profile from both blood and skin cells.

Blood and bloodstained evidence are never to be placed in airtight containers; this will cause samples to accumulate moisture. Moisture will lead to the formation of bacteria, fungi, and the enzymatic breakdown of blood components to produce inconclusive or lesser quality results [14, 21]. For collection of dried samples, the following methods are used: swabbing, scraping, tape lifting, elution, and recovery of the entire item [11, 13, and 15]. For swabbing, the sterile cotton swab should be moistened before use with a sterile saline solution (made by dissolving 8.5g of sodium chloride in 1 liter distilled sterile water) or with pure distilled sterile water [13]. Sterile cotton applicator swabs work well as they can be placed into a small box with pre-cut drying holes to avoid contamination while drying [22]. When labeling the swabs, it is important to maintain a sequence of the order in which the swabs were collected. This is an important step because if the stain happened to be a mixture of two blood sources, and the swabs were collected from different areas of the stain, it is possible that DNA typing could give different results and still be scientifically accurate [12].

For scraping, a clean sterile sharp instrument is used to scrape the blood from the surface and evidence is placed in a paper packet (druggist fold) and placed in a secondary package (e.g. coin envelope). The scraped samples should not be placed into a plastic container as the static charge from the plastic will cause the blood flakes to disperse and stick to the sides of the container [12]. One advantage to scraping compared with swabbing is that it avoids using a sterile solution, which may lead to the potential dilution of the sample. This technique can also

be used with the tape lift method, as static charge will cause the blood flakes to stick to the tape. However, the investigator should take note of the tape used and check with the laboratory that this method will not hinder later DNA tests [11, 14]. Most tapes and fingerprinting procedures do not affect the ability to later test for DNA.

For tape lifting bloodstains, fingerprint tape is placed over the bloodstain. The non-sticky side of the tape is rubbed with a pencil eraser to ensure good contact is made between the stain and the tape [11]. Do not place the sample on backing paper, as this will make it difficult to analyze the sample. Instead the sample is to be placed on vinyl acetate [11, 14]. Investigators must know when to use the tape lift method as not all samples lift well and the success of the technique is highly dependent on the surface. Elution is to be considered the last resort in the collection of blood samples. This is done using a small amount of saline solution to dissolve the stain and transfer it to a sterile tube [13]. Wet pooled blood samples are collected by placing an absorbent piece of material (e.g. filter paper, cotton-tipped applicators, and cotton gauze) into the liquid pool of blood [23]. An exception to the collection techniques described above is when blood samples need to be collected from snow. In this case a clean spoon-like utensil should be used to collect the blood specimen with as little surrounding snow as possible and placed into a clean vial for immediate transport to the laboratory [13].

Known sample comparisons must be collected from victims and defendants in the case. These samples were traditionally collected in purple top EDTA tubes, which are specifically made for blood collection [13]. For known sample processing from blood, recommended sample size is 3 uL of whole blood, a 2-3 millimeter cutting from a bloodstain or swab, and sufficient dried blood scrapings to give a light straw-colored aqueous extracts [12]. Alternatively and more commonly used today, a buccal swab of the interior of the cheek can be collected as a known DNA reference sample for later comparison with the evidence.

One of the most important steps for biological evidence is the record keeping or the chain of custody. This is very important to ensure that there is a clear and concise log of all parties that have handled the sample and where it was located last. Maintaining a proper "chain of custody" involves producing and maintaining written documentation which accompanies the evidence and provides an uninterrupted timeline showing the secure location of the evidence from the time it was discovered and recovered at the scene until the present time [24]. Good records and appropriate packaging after the original trial should also be maintained by the storage facility (i.e. court clerk's office, police department, attorney's office or laboratory) which can become critical for retrials and retesting in the post-conviction judicial process [23, 24].

4. Presumptive Testing
Due to the high cost of DNA analysis and for scientific integrity, prior to sending any sample to the lab for DNA testing, it is important to determine if what appears to be blood is in fact human blood. This is achieved by performing a presumptive and then a confirmatory test on the samples in question. Presumptive tests react with the hemoglobin of all blood (human and animal) to catalyze the oxidation of a chromogenic compound, which produces a color change

[25]. A positive reaction will result in the identification of the sample as *possibly* blood but not necessarily human blood. A presumptive test is used to describe these types of tests because they will react with substances other than blood, including certain metals and plant peroxidases resulting in false positives [5]. For this reason, anti-human hemoglobin tests (e.g. Ouchterlony, HemaTrace) to confirm species of origin are performed as a subsequent confirmatory test on any samples suspected to be blood [26]. A blood presumptive test should never be taken as a final measure, and should always be followed by confirmatory testing at the forensic science laboratory before making the statement in court that a sample is human blood.

 A presumptive test should be sensitive enough to detect low concentrations of blood, and at the same time it should possess a relatively high degree of specificity, must meet the Frye and Daubert standards for court admissibility, and not damage the DNA [4, 27]. Various differences in sensitivity are reported by researchers for presumptive tests for blood and are undoubtedly caused by differences in the reagent concentration, methods of sample preparation, and the type of material containing the blood [27].

5. Presumptive Blood Test Reagents
There are various types of blood testing reagents that are currently used for the identification of blood. For purposes of this paper, only the most commonly used reagents will be discussed.

Luminol
In 1937, Walter Sprech introduced luminol (3-aminophthalhydrazide) and since then it has been used for forensic analysis [28]. When luminol comes in contact with the hemoglobin in blood it produces light with a blue green color [29]. Due to television portrayal, it is considered by most of the public as the most popular reagent used for blood detection. As a presumptive test for blood it has several drawbacks and should only be used under specific circumstances for investigators [11]. Luminol is often used only for latent bloody prints or stains (especially those of greater age, 10-20 years); it is relatively non-toxic and due to the fluorescence, is highly sensitive for detection [30]. It is a chemical reagent that when applied to a bloodstain, even if it is a very dilute amount, will cause the reaction to glow in the dark. It must be viewed in complete darkness in order to visualize the luminescence, which limits its use for outdoor daylight scenes [12, 23]. Unlike many other presumptive tests for blood, luminol is the only one that creates a reaction in light rather than in color.

Luminol is a very practical reagent because the components that are needed to create the reagent can be kept in a kit that would fit easily in a crime scene vehicle. However, once these reagents are mixed together, the traditional luminol solution has a short shelf life and should be used within 24 hours [31]. The sensitivity of luminol depends on the concentration of reagents that are used to create the initial solution [3]. The luminol reaction, like other presumptive tests for blood, can result in false positives (i.e. it is not 100% specific to human hemoglobin). Luminol has been found to react with copper ions, copper compounds, iron compounds, cobalt ions, potassium permanganate, sodium hypochlorite (bleach), plant peroxidases (such as fresh potato juice), metals, some cleaners, soil, and cigarette smoke [11, 32, 33]. It is also possible for metallic items (faucet fixtures) to give a quick fluorescence, often called twinkling [12].

Certain body materials such as bone marrow, brain tissue, spinal fluid, leukocytes, mucous, and intestinal and lung tissue can also produce false positives and fluorescence though they are not strictly liquid blood [31]. Luminol is fairly specific to blood and tissues, however, since it does not react with any other biological fluids such as pure semen, saliva, or urine [12].

Absorbent materials are easy to analyze, and the bloodstains on materials such as these can be easily re-sprayed [33]. A study on denim at a dilution of 1:1000 demonstrated that luminol could easily detect dilute blood [35]. For nonabsorbent materials such as glass, vinyl, and porcelain, bloodstains will 'run' when sprayed; only a minimum amount should be sprayed and the area should be photographed quickly [33]. It is recommended that luminol be used on older stains, the older the bloodstain the stronger the luminescence [31]. In one study, stains older than 17 years stored at room temperature still produced luminescence [33]. The covering up of blood at a crime scene by paint is one factor that investigators must be aware of and be prepared to examine. One study found that luminol was able to penetrate up to 8 layers of paint [34]. It is advised that if a positive reaction with luminol is achieved, the stained area should be checked again with another reagent such as tetramethylbenzidine, phenolphthalein, or orthotolidine and always confirmed with a human-specific confirmatory test for blood [23].

Another study confirmed that luminol does not have an adverse effect on PCR testing, but that it did show that the substrate (varnished wood) did cause DNA degradation [36]. The sensitivity of luminol is dependent on the concentration of its reagents; this also affected the ability to detect genetic markers; the stronger the concentration of the reagents, the greater the chance of affecting DNA genotyping [37]. The use of a cotton swab versus a sponge tipped swab has also been shown to affect the amount of DNA recovered in a blood stain. In a study testing a cotton swab versus the Puritan Sponge, the Puritan Sponge was more effective in yielding DNA results [38]. Weber's method of preparation seems to be the one most favorable due to the lower concentration of reagents since it does not degrade the DNA [33, 39]. One study considered luminol to be the best presumptive test, as it had the greatest sensitivity, specificity, it did not destroy the DNA and it could be reapplied for presumptive blood analysis [40].

Leuco-malachite Green (LMG)
Leuco-malachite green is a colorimetric test used to screen for possible blood. It is often used to test for the presence of patent blood samples and is prepared by heating in an acidic solution until it is colorless [30]. This test is performed by rubbing a cotton swab that has been moistened in a sterile solution on the suspected bloodstain [15]. Like luminol, LMG is used when suspected blood has been cleaned up from a surface, or to develop faint bloody prints on a light colored carpet or floor [41]. The LMG reaction is an oxidation reaction, which is catalyzed by heme to produce a green color, and it is carried out in an acetic acid medium with hydrogen peroxide as the oxidizer [25]. If a color reaction occurs before the hydrogen peroxide is added, this will indicate the presence of a chemical oxidant and the test result should be considered inconclusive [30]. It is considered by one study as the most specific but least sensitive of the presumptive blood detection reagents [23, 27]. It has a sensitivity of 1 in 10,000 ppm [42]. LMG is as specific to blood as luminol, but its sensitivity was 10 times less

than luminol and it destroyed the DNA in the sample in some situations [40]. Blood samples that have been contaminated with ascorbic acid have been found to produce false positives [25].

Phenolphthalein

Phenolphthalein, which is also referred to as the Kastle-Meyer reagent, is a simple acid-base indicator and it is used primarily on patent blood prints [25, 30]. In the presence of blood, phenolphthalein will cause an alkaline solution to turn pink after its oxidation by peroxidase [25]. The reaction is time sensitive, and the results are only reliable within 10-15 seconds (by most lab protocols) of application, after which the results are no longer reliable [43]. After 10-15 seconds a false positive will result. The reagent consists of reduced phenolphthalein in alkaline solution, which is oxidized by peroxidase in the presence of hemoglobin in the blood [25]. Like LMG, phenolphthalein is considered one of the most specific reagents with a sensitivity of detection of 1 in 10,000 ppm [23, 27, 39]. Various studies on sensitivity and specificity have been performed on blood detection presumptive reagents. One study compared phenolphthalein to that of benzidine [44]. That study demonstrated that plant peroxidases contribute to false positive results in the benzidine test but not in the three-stage phenolphthalein test. This reagent has also been known to react with some plant materials, cleaning agents, metals, metal salts, and other sources of iron [30]. One author's experience has proved that phenolphthalein, along with benzidine and tetramethylbenzidine (TMB) have given positive results with blood crusts or stains up to 56 years old [25]. The color of the evidence being tested also affects the result obtained; if the evidence happens to be red or any shade of pink and leaches that color into the swab, the result will be very difficult to interpret since the swab will end up being fuchsia regardless of the presence or absence of blood [45]. This test result would then be considered inconclusive and a second presumptive test for blood should be performed with an alternate color detection reagent.

Orthotolidine

Orthotolidine is the 3, 3'-dimethyl derivative of benzidine [30] and as such must be handled with care as it is considered to be a carcinogen. One study showed that orthotolidine induced neoplasm formation [46]. The test is performed by rubbing a moistened sterile cotton swab on a suspected bloodstain followed by the addition of 2-3 drops of orthotolidine; waiting for 5-10 seconds to be sure no color change occurs and then a drop of 3% hydrogen peroxide. After 15 seconds, a positive result is an intense blue color [15]. Color changes noticed after 15 seconds are not valid. It must be handled wearing nitrile gloves and is never to be sprayed [25]. Out of the reagents discussed in this paper, one study considered orthotolidine the most sensitive [23]. With this reagent, positive reactions were observed with dilutions of 1 in 100,000 ppm on filter paper, cotton cloth, and bloodstains [27]. It has also been considered to be more sensitive than phenolphthalein on dried blood stains that have been washed [47]. Orthotolidine will produce false color reactions with plant peroxidases so it is not 100% specific for blood [27]. One study also showed that the addition of ascorbic acid could create false negatives under certain conditions [48].

Tetramethylbenzidine (TMB)

Benzidine had been used as a presumptive test until the Occupational Safety and Health Organization banned its use in 1974 [49]. Due to the cancer causing effects of benzidine, a new reagent had to be synthesized. A safer reagent was created from benzidine, known as tetramethylbenzidine (TMB) [25]. Studies compared the specificity and sensitivity of benzidine to that of TMB and found that they both served well as a presumptive blood test reagent, and more importantly TMB did not produce any carcinogenic mutations [50]. TMB is used in an acid medium when it is issued as a solution and the resultant positive color is green to blue-green [25]. One has to be careful not to add too much of the reagent to the bloodstain pattern as this will cause the reaction to turn a dark blue color and can mask ridge patterns [43]. A false positive can be seen with substances that have been pretreated with some cosmetics [25]. This reagent is also considered to be very sensitive but not very specific [27, 40].

One of the most common simple tests used in the field is the Hemastix test [30]. The test consists of a plastic strip with a reagent-treated paper filter tab containing TMB, diisopropylbenzene dihydroperoxide, buffering materials and non-reactants at one end. Testing of the bloodstain may be accomplished by moistening a cotton swab with distilled water, sampling the stain, and touching the swab to the reagent tab on the strip [25]. It is important not to expose the entire bloodstain sample to the TMB as this can prove to be destructive to the DNA; one study found that the TMB in the Hemastix strip degraded the DNA [51].

6. Human Blood Confirmatory Test Techniques
After evidentiary samples have tested positive for possible blood, the next step is to confirm the presence of human blood. German Professor Paul Uhlenhuth developed the antigen-antibody precipitin test for species, to identify the human or animal source [52]. Confirmatory tests are species-specific but use additional sample for testing. Some traditional forensic tests are the Teichmann or Takayama crystal tests which were microscopy based methods for blood identification but lacked species-specificity and were difficult to use. The crystal tests are no longer used by most forensic laboratories; current Ouchterlony and HemaTrace tests use antibodies to specifically detect human hemoglobin and are rapid and simple to use [53].

Ouchterlony Double Diffusion Technique
The Ouchterlony method is an immunological species test that involves analyzing extracts of the bloodstain with a specific antiserum to human hemoglobin [53]. This technique depends upon the passive diffusion of antigen and antibody through a gel matrix followed by a precipitation of cross-linked proteins which is considered positive and is visible as a white line [54]. These methods of testing were also recognized as "precipitin tests" due to the precipitate that is visible in the test gel from the species-specific antibodies binding to their homologous antigens [53]. This test requires only a small amount of blood for testing, and even blood stains that have been dried as long as 15 years may still give a positive precipitin reaction [53]. It has been shown that human bloodstains diluted with water that have only a faint color may also yield a positive precipitin reaction [53]. One study showed that even when the majority of the

blood proteins are denatured or washed away, residual antigen activity remains trapped on the substrate, allowing one to determine the species of origin [55].

HemaTrace

The ABAcard HemaTrace, the method of choice today, is an immunochromographic one-step test for the detection of human blood [56]. It consists of a plastic card with two windows; one of which allows the sample to be applied to the test membrane and the other window permits the analyst to view the antigen-antibody reactions [57]. HemaTrace uses anti-human hemoglobin (Hb) antibodies to provide a means of detection for the presence of human (primate) Hb [58], which is coated on test strip and has a detection limit of 0.07 ug hemoglobin/mL [59]. One of the advantages of the ABAcard HemaTrace is that it does not give false-positive results with other peroxidases and is primate specific [58]. Compared to the Ouchterlony technique discussed above, the ABAcard HemaTrace is far more sensitive, rapid and tolerant to different evidence conditions taking only ten minutes to perform and read the results [56, 59]. One study showed a positive result in a liquid blood sample diluted 1 in 1,000,000 ppm [57]. However, if performed incorrectly, false negative reactions can occur if the sample is too concentrated. This causes the antigen to saturate the antibody and prevent the "antibody-antigen-antibody" sandwich formation required for positive color detection [57]. This is referred to in forensic science as the "high dose hook effect".

7. Quality Control of Blood Detection Reagents

Positive and Negative Controls

An everyday task that should be conducted by any analyst prior to using test reagents on evidence is to test a known blood sample (positive control) and reagent blank (negative control) [32]. Quality control checks are important in order to guarantee that the reagents are working properly. One method for doing this is to serially dilute whole blood with distilled water and then apply samples of the dilution to cardstock or cotton swatches that can be dried and stored [32]. These portable standards can be used in the laboratory or transported to the crime scene. The testing of positive and negative controls should always be performed prior to testing of the evidence; this is critical to maintaining the integrity of the test results. Test reagents should be prepared according to standard written protocols, should be stored at the appropriate temperature, and should be discarded after their expiration date. Lot numbers of chemicals and documentation of who made the test reagents should be maintained on a daily basis. An easy positive control for the presumptive reagent luminol is a copper penny; thus illustrating that the luminol is a functioning reagent but also is not human blood specific as it also reacts with the copper metal.

DNA Inhibitors

Different reagents and different substrates can have an effect on blood confirmatory tests and many factors affect the recovery of high quality DNA. The dilution of an already dilute stain may push the stain beyond the genetic marker analysis detection limit [11]. Not only may addition of reagents dilute samples and possibly cause issues with PCR analysis, but additional substances within the collected sample may also cause inhibition [8]. It is possible to detect

human blood using standard serological techniques but not be able to genotype the sample due to the presence of PCR inhibitors. Excess hemoglobin from red blood cells, which are known to remain with the DNA throughout sample preparation, especially on FTA cards can be a PCR inhibitor [49]. Additional factors that can cause inhibition are sand, leaf litter, textile dyes, chemicals, leather and wood surfaces [60]. Most analysts will perform standard laboratory cleanup procedures on the sample in order to obtain a DNA profile. These consist of filters to which the DNA will adhere; the DNA sample is then rinsed with a buffer to remove the inhibitors [8].

In summary, good communication and excellent training for the forensic investigator on the scene and the forensic analyst at the laboratory is of utmost importance to have proper collection and evaluation of biological samples for the presence or absence of human blood. An investigator should be aware of the capabilities of the forensic science laboratory, the proper methods for blood collection and preservation to meet accepted forensic science standards, the investigative information relevant to the forensic scientist, and the type of reference samples required by the forensic science laboratory [11]. The importance of high-quality training for both the investigator and scientific laboratory personnel and the careful use of science are keys to providing the optimal processing for forensic casework samples and for providing best value to the judicial system.

References

[1] Tortora, G. J., and Anagnostakos, N. P., *Principles of Anatomy and Physiology*, fifth edition. Harper and Row, New York: NY, 1987.

[2] Rudkin, N., Inman, K., *An Introduction to Forensic DNA Analysis*, second edition. CRC Press, Boca Raton: FL, 2001.

[3] Baechtel, E S., Presley, K. W., and Smerick, J. B., D1S80 Typing of DNA from Simulated Forensic Specimens*, Journal of Forensic Sciences*, JFSCA, July 1995 Vol. 40, No. 4, pp. 536-545.

[4] Saferstein, R., *Criminalistics: An Introduction to Forensic Science,* third edition. Prentice-Hall, Inc., Englewood Cliffs: NJ, 1987.

[5] Eckert, W.G., *Introduction to Forensic Sciences*, second edition. CRC Press Boca Raton: FL, 1997.

[6] Lee, H.C., Harris, H.A., *Physical Evidence in Forensic Science,* Lawyers and Judges Publishing Co, Inc., Tucson: AZ, 2000.

[7] Goodwin, W., Hadi, S., DNA. In: Black, S., Thompson, T., Forensic Human Identification - An Introduction. CRC Press, Boca Raton: FL, 2007.

[8] Anonymous, Personal Communication, Medical Examiner Office.

[9] Walsh, S., Buckleton, J., DNA Intelligence Databases In: Buckleton, J., Triggs, C. M., Walsh, S., J., Forensic DNA Evidence Interpretation. CRC Press, Boca Raton: FL, 2005.

[10] Wambaugh, J., *The Blooding*. Perigord Press, New York: NY, 1989.

[11] Schiro, G., *Collection and Preservation of Blood Evidence from Crime Scenes.* Website: http://www.crime-scene-investigator.net/blood.html (accessed Oct 15, 2010).

[12] Shaler, R. C., *Modern Forensic Biology, In: Saferstein, R. Ed., Handbook of Forensic Science,* 2001.

[13] Gaensslen, R. E., Harris, H.A., Lee, H.C., *Introduction to Forensic Science and Criminalistics.* McGraw Hill, New York: NY, 2008.

[14] Lee. H. C., Palmbach, T., Miller, M. T., *Henry Lee's Crime Scene Handbook.* Elsevier, London: UK, 2001.

[15] Geberth, V. J., *Practical Homicide Investigation: Tactics, Procedures, and Forensic Techniques,* fourth edition. CRC Press, Boca Raton: FL 2006.

[16] Kolata, G. 1994. The Code; DNA and O. J. Simpson: Testing Science and Justice. Website: http://www.nytimes.com/1994/06/26/weekinreview/the-code-dna-and-o-j-simpson-testing-science-and-justice.html (accessed May 2011).

[17] Committee on Identifying the Needs of the Forensic Sciences Community, National Research Council. 2009. Strengthening Forensic Science in the United States: A Path Forward. Website: www.ncjrs.gov (accessed May 2011).

[18] North Carolina Department of Justice. SBI Changes: Checklist and Status. Jan. 2011. Website: http://content.news14.com/pdf/SBI2.pdf (accessed May 2011).

[19] Swecker, C. and Wolf, M. 2010. An Independent Review of the SBI Forensic Laboratory. Website: http://dig.abclocal.go.com/wtvd/docs/081810_SBI_Lab_Review.pdf (accessed May 2011).

[20] Swander, C. and Stites, J. Evaluation of the ABAcard HemaTrace for the Forensic Identification of Human Blood. Website: http://www.4n6shop.cz/static_pages_files/file/Hematrace_Michigan State Police_Connie Swander_Jennifer Stites.pdf (accessed May 2011).

[21] James, S. H., Kish, P.E., Sutton, T. P., Documentation and Examination of Bloodstain Evidence. In: *Principles of Bloodstain Pattern Analysis Theory and Practice* CRC Press, Boca Raton: FL, 2005.

[22] Sirchie Forensics. Website: http://www.sirchie.com/Assets/Cat_10_11/forensicEvidence12.pdf. (accessed December 19,2010).

[23] Fisher, B. A., Blood and Other Biological Evidenc*e* In: *Techniques of Crime Scene Investigation*, seventh edition. CRC Press, Boca Raton: FL CRC Press 2004.

[24] Prahlow, J., *Forensic Pathology for Forensic Scientist, Police and Death Investigators*. Springer Science + Business Media, New York: NY, 2010.

[25] Spalding, R. P., Presumptive Testing and Species Determination in Blood and Bloodstain*s* In: James, S. H., Kish, P.E., Sutton, T. P., *Principles of Bloodstain Pattern Analysis Theory and Practice.* CRC Press, Boca Raton: FL CRC Press, 2005 (pp. 349-368).

[26] Coyle, H.M., Ladd, C., Lee, H.C., Biological Evidence On The Human Body In: Spitz, W., Fisher, R., *Medicolegal Investigation Of Death: Guidelines for the application of Pathology to Crime Investigation,* fourth edition*.* Charles C. Thomas Publisher, Springfield: IL, 2006 (pp. 45-59).

[27] Cox, M., A Study of the Sensitivity and Specificity of Four Presumptive Tests for Blood, *Journal of Forensic Sciences*, JFSCA, Vol. 36, No. 5, Sept. 1991, pp. 1503-1511.

[28] Quickenden,T. I., and Copper, P. D., *Increasing the Specificity of the Forensic Luminol Test for Blood*. Luminescence, 2001,16: 251 – 253.

[29] Nilsson, A., *The Forensic Luminol Test For Blood: Unwanted Interference and the Effect on Subsequent Analysis*, Swedish National Laboratory Forensic Science 2006.

[30] Hochmeister, M. N., Budowle, B., and Baechtel, F. S., Effects of Presumptive Test Reagents on the Ability to Obtain Restriction Fragment Length Polymorphism (RFLP)Patterns from Human Blood and Semen Stains, *Journal of Forensic Sciences,* JFSCA, Vol. 36, No. 3, May 1991, pp. 656-661.

[31] Englert, R., Luminol In: Walton, R.H., *Cold Case Homicides Practical* Investigation Techniques. CRC Press, Boca Raton: FL, 2006.

[32] Marie, C., Presumptive Testing and Enhancement of Blood. In: Bevel, T. and Gardner, R., M., (Eds). *Bloodstain Pattern Analysis with an Introduction to Crime Scene Reconstruction*, second edition. (pp. 275-296), CRC Press, Boca Raton: FL 2008.

[33] Laux, D., L., (2005) The Detection of Blood Using Luminol In: Bevel, T. and Gardner, Ross M.. *Bloodstain Pattern Analysis with an Introduction to Crime Scene Reconstruction*, second edition. CRC Press, Boca Raton: FL, 2005.

[34] Bily, C., Maldonado, H., The Application of Luminol to Bloodstains Concealed by Multiple Layers of Paint*. J. For Identification,* 2006*,* Vol. 56 (6) pp 896-905.

[35] Middlestead, C., Thornton, J., Sensitivity of the Luminol Test with Blue Denim*, J Forensic Sci*, September 2010, Vol. 55, No. 5.

[36] Gross AM, Harris KA, Kaldun GL. The Effect of Luminol on Presumptive Tests and DNA Analysis Using the Polymerase Chain Reaction*, J Forensic Sci*, 1999;44(4):837–840.

[37] Laux, D. L., Effects of Luminol on the Subsequent Analysis of Bloodstains*, J Forensic Sci*, JFSCA, Vol. 36, No. 5, Sept. 1991, pp. 1512-1520.

[38] Della Manna A, Montpetit S. A Novel Approach to Obtaining Reliable PCR Results from Luminol Treated Bloodstains, *J Forensic Sci* 2000:45;(4):886–890.

[39] J.P. de Almeida, et al., Effect of Presumptive Test Reagents on Human Blood Confirmatory Tests and DNA Analysis Using Real Time Polymerase Chain Reaction, *Forensic Sci. Int.* (2010)

[40] Tobe, S., Watson, N., Daeid, N.N., Evaluation of Six Presumptive Test for Blood, Their Specificity, Sensitivity, and Effect on High Molecular-Weight DNA, *J Forensic Sci*, January 2007, Vol. 52, No. 1

[41] Grubb, M.J., From Serology to DNA in Cold Case Investigations. In: Walton, R.H., *Cold Case Homicides Practical Investigation Techniques*. CRC Press, Boca Raton: FL, 2006.

[42] E. Johnston, C.E. Ames, K.E. Dagnall, J. Foster, B.E. Daniel, Comparison of Presumptive Blood Test Kits Including Hexagon OBTI, *J. Forensic Sci*. 53 (2008) 687–689.

[43] Geberth, V. J., *Collection of Evidence* In: *Practical Homicide Investigation: Tactics, Procedures, and Forensic Techniques,* fourth edition. CRC Press, Boca Raton: FL, 2006.

[44] Higaki, R. S. and Philip, W. M. S., A Study of the Sensitivity, Stability, and Specificity of Phenolphthalein as an Indicator Test for Blood, *Canadian Society of Forensic Science Journal*, Vol. 9, No. 3, 1976, pp. 97-102.

[45] Anonymous, Personal communication, Medical Examiner Office.

[46] Holland, V. R., Saunders, B. C., Rose, F. L., and Walpole, A. L., A Safer Substitute for Benzidine in the Detection of Blood, *Tetrahedron*, Vol. 30, 1974, pp. 3299-3302.

[47] Cox, M., Effect of Fabric Washing on the Presumptive Identification of Bloodstains, *J Forensic Sci,* Vol. 35, No. 6, Nov. 1990, pp. 1335-1341.

[48] Ponce, A., Pascua, F., Critical Revision of Presumptive Tests for Bloodstains, *Forensic Science Communications* July 1999 Volume 1 No. 2.

[49] Occupational Safety and Health Administration, Regulation H002, Washington, D.C., 29 Jan. 1974.

[50] Garner, D.D., Cano., Peimer, R.S., Yeshion, T.E., An Evaluation of Tetramethylbenzidine as a Presumptive Test for Blood, *J Forensic Sci,* Oct. 1976, Vol. 21, No. 4.

[51] Poon. H., Elliot, J., Modler, J., Fregeau, C., The Use of Hemastix and the Subsequent Lack of DNA Recovery Using the Promega DNA IQ System, *J Forensic Sci*, November 2009, Vol. 54, No. 6.

[52] Inman, K., Rudin, N., The Evolution of Forensic Science In: *Principles and Practice of Criminalistics*. CRC Press, Boca Raton: FL, 2001.

[53] Lee, H.C., Gaensslen, R.E., Pagliaro, E.M., Mills, R.J., Zercie, K.B., *Physical Evidence In Criminal Investigation*, Narcotic Enforcement Officers Association, Westbrook: CT, 1991.

[54] Hyland,D.C., Tersak, J.M., Adovasio,J.M., Siegel,M.I., Identification of the Species of Origin of Residual Blood on Lithic Material, *Am. Antiquity* 55 (1) (1990) 104–112.

[55] Lee, H.C., DeForest, P.R., A Precipitin-Inhibition Test on Denatured Bloodstains for the Determination of Human Origin, *J Forensic Sci*, Oct. 1976, Vol 21, No.4, 804-810.

[56] Swander, C.J., Sites, J.G., Evaluation of the ABAcard HemaTrace for the Forensic Identification of Human Blood, Michigan State Police, Forensic Laboratory, Midwestern Association of Forensic Scientist 1998; 1-5.

[57] Spear, T.F., Khoskebari, N., The Evaluation of ABAcard HemTrace, *CAC News*, 3rd Quarter 1999, 24-25.

[58] Johnston, E., Ames, C.E., Dagnall, K.E., et al, Comparison of Presumptive Blood Test Kits Including Hexagon OBTI, *J Forensic Sci*, May 2008, Vol 53, No. 3, 687-689.

[59] *S*. Johnston, J.C. Newman, R. Frappier, Validation of the One Step ABAcard Hema-Trace Test for the Forensic Identification of Human Blood, *Can. Soc. Forensic Sci.* 36 (3) (2003) 173–183.

[60] Butler, J.M., *Fundamentals of Forensic DNA Typing*, Academic Press, London: UK, 2009.