

A Comparative Analysis of Protein and Peroxidase Blood Enhancement Reagents Following Laundering and their Impact on DNA Recovery

Gabrielle Hartley and Claire L Glynn*

Department of Forensic Science, Henry C Lee College of Criminal Justice and Forensic Science, University of New Haven, CT, USA

*Corresponding author: Claire L Glynn, University of New Haven, 300 Boston Post Road, West Haven, CT 06516, USA, Tel: +1 2013 508 4846; E-mail: cglynn@newhaven.edu

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Abstract

Blood is a commonly encountered biological fluid in criminal investigations concerning a violent incident, and visual traces of the fluid on a suspect's clothing can be diminished through laundering. This study aims to analyze the effects of laundering and the application of commercially available blood enhancement reagents commonly used to improve visualization of dilute bloodstains and their impact on DNA recovery. Enhancement reagents Hungarian Red, Coomassie Blue, Amido Black, luminol, Bluestar® Forensic Magnum, and aqueous Leuco Crystal Violet (LCV) were used to enhance human blood on cotton, polyester, denim, and wool following laundering. DNA was extracted from these samples using a QIAamp® DNA Investigator Mini Kit and quantified using a NanoDrop™ One C UV-Vis spectrophotometer. This study revealed the peroxidase based reagents to produce the greatest sensitivity on the natural fabrics, reacting positively down to a blood dilution of 1:1000. The protein reagents produced greater sensitivity on the synthetic fabrics, reacting positively down to a blood dilution of 1:10. Peroxidase stains relying on chemiluminescent properties rather than colorimetric results produced positive results on the dark colored fabrics as sufficient color contrast was not achieved with the protein stains. The resulting yields of extracted DNA suggest that quantifiable amounts of DNA originating from bloodstains persist despite laundering and enhancement. Additionally, measurements indicated that the application of some blood enhancement reagents, particularly Amido Black, may affect DNA recovery.

Keywords: blood enhancement; peroxidase; proteins; blood; crime scene

Introduction

In criminal and forensic investigations, the connection between a perpetrator and a victim can often be the most condemnatory piece of evidence an investigator can find. Just as it is the forensic scientist's job to bring this linkage to light, a perpetrator will take the required steps to minimize the chances this connection will be discovered. Blood evidence is common in cases of violent crime, and it is not unlikely that a suspect will try to destroy this evidence as its presence can serve as a link between an offender, a victim, and a crime. Often, these stains can be washed or laundered by perpetrators in hopes to clear away the evidence and diminish their association with a crime. While laundering bloodied clothing may remove the more pronounced visible blood, latent traces may remain and it is imperative that the probative value of this evidence is not overlooked simply because the clothing has been washed. Washing blood off of clothing results in dilute stains that are often difficult to detect. Previous studies have investigated the impact of laundering on bloodstains [1-3], however there is no comprehensive analysis using a wide variety of enhancement reagents. There are several methods at the forensic scientist's disposal which can aid in the enhancement of dilute blood stains. The two main types of reagents that can be used for detection of trace amounts of blood are *peroxidase* stains and protein stains. Each category of staining method exploits different properties found within the composition of blood to produce reactions that can be indicative of the presence of blood.

Peroxidase reagents for blood react with the iron in heme and produce colorimetric results in the presence of an oxidizer. Due to its peroxidase-like activity, heme acts as the catalyst of the reaction between the stain and peroxide in which the dye is oxidized, producing a rapid color change. While some peroxidase-based reagents can produce results in

the form of a colorimetric change, others produce chemiluminescent results. Peroxidase enhancement reagents include presumptive blood tests such as aqueous Leuco Crystal Violet, luminol, and Bluestar® Forensic Magnum. While these methods indicate that blood may be present, they do not come without hindrance and cannot be used to imply that blood was categorically present. Numerous substrates, including some plant materials, cleaning solutions, metals, and other iron sources may yield positive results with these stains even in the absence of blood [4].

Protein reagents, on the other hand, react with amines or other groups that are present within all proteins found in blood. Examples of these stains include Hungarian Red, Coomassie Blue, and Amido Black. When applying a protein enhancement reagent to detect a dilute bloodstain, a three step process is utilized. First, the stain is fixed to the surface. Fixing the bloodstain involves the disruption of secondary and tertiary protein structure, altering the hydrophilic regions and rendering the proteins less soluble. This prevents diffusion of the bloodstain when other reagents are applied. Second, protein-specific staining occurs. Generally, protein reagents consist of colored organic, aromatic molecules that provide both visible color and the ability to bind to a material. These dyes bind to proteins found within blood, and remain present through the final step: de-staining. De-staining involves removing excess dye, allowing for better contrast between an identified blood pattern and a background substrate. While protein stains are not confirmatory for blood, they provide an inexpensive method of locating potential blood patterns on both porous and non-porous surfaces. Protein stains are not typically considered the first method of choice for blood identification/enhancement as a result of their lack of specificity. They are however, frequently used to enhance weak/dilute bloodstains with impression evidence, such as bloody

fingerprints or footwear impressions. Protein stains are the favored choice when dealing with this evidence type as they typically adhere to the residues left behind, while the peroxidase reagents can cause a more dispersive reaction. Many of these reagents, both protein and peroxidase based, are inexpensive, easy to use, and can generally be used on porous or nonporous substrates, including fabrics.

Despite the potential to arouse suspicion and help reconstruct a crime, the mere presence of blood on laundered clothing is not often sufficient enough to make conclusive inferences about the nature and circumstances of a specific person's involvement in a crime. Because of this, it is essential that a method exists that both enhances a forensic scientist's ability to visualize a dilute bloodstain on a piece of laundered fabric while maintaining and preserving the quality of the DNA evidence that may be present. While there are number of blood enhancement reagents available, the effect of the application of these reagents on the ability to recover DNA from within a bloodstain has been under explored. While many studies surrounding the topic put forth the idea that full DNA profiles can still be obtained despite the application of enhancement reagents [5-10], others espouse the idea that DNA recovery may be diminished following the application of such reagents, particularly after prolonged exposure [9]. In order to preserve DNA evidence that may prove critical to an investigation, the effects of each of these reagents on DNA recovery must be carefully scrutinized [11]. This study first aimed to provide a comprehensive analysis of a selection of both protein and peroxidase blood enhancement reagents on laundered bloodstains, on a variety of different fabric types, and at a range of dilutions. The second aim was to investigate the impact these reagents have on the ability to recover quantifiable DNA from the stains.

Materials and Methods

Blood Collection

Following approval from the Institutional Review Board (IRB) at the University of New Haven, venous blood was obtained and collected from volunteers with informed consent. Blood donations were collected and stored in sterile vacutainer EDTA vials and refrigerated at 4°C until required.

Sample preparation

Six commonly used and commercially available blood enhancement reagents were selected, these include; Hungarian Red, Coomassie Blue, Amido Black, aqueous leuco crystal violet, luminol, and Bluestar® Forensic Magnum, (all purchased from Sirchie®, Youngsville, NC, USA.). To test the reagents with neat and diluted blood, five different types of fabrics were selected and purchased from local stores with pure compositions, including white cotton, black cotton, white polyester, black polyester, and blue denim. In order to create a sample accurately representative of that which would be found during a criminal investigation, each fabric type was purchased as a manufactured article of clothing, using the clothing tag as an indication as to the composition of the garment. To minimize the potential of pre-existing blood or DNA located on the fabrics prior to blood sample deposition, each fabric sample was laundered, followed by treatment using a Spectrolinker™ XL-1500 UV Crosslinker for 20 minutes. Swatches (approx. 8 × 6 inch) of each of the 5 fabric types were prepared for each of the 6 enhancement reagents and 100 µL of human blood was deposited onto each swatch in a range of seven dilutions; neat, 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000, and 1:1,000,000. Each swatch was prepared in triplicate, resulting in 90 individual swatches, comprising 630 individual stains. To test the impact of the reagents on DNA recovery, four different types of fabrics were selected and purchased from local stores with pure compositions, including white cotton, white polyester, blue denim and tan wool. 100 µL of neat human blood was deposited onto each fabric

type. Each sample was performed in triplicate and photographed prior to laundering. 100 µL of molecular grade sterile water was deposited onto the control samples. All samples were allowed to dry for twenty-four hours at ambient temperature prior to laundering.

Laundering

All samples were washed under standard laundering conditions at 30°C. Each group of replicates was washed separately. All wash cycle parameters were kept consistent between replicates of each fabric type. After laundering, all samples were removed from the washing machine and allowed to dry for twenty-four hours.

Enhancement

All samples were enhanced following laundering using each of the 6 selected enhancement reagents. Spray techniques were chosen instead of submerging the samples in the reagents due to the porous nature of fabric. Control tests of unwashed blood samples were used prior to application of each reagent.

Amido Black: Amido Black was applied following the instructions provided by the manufacturer. A working solution was created by mixing 1 gram of Amido Black and 10 g of citric acid. 500 mL of dd was added and stirred for 30 minutes. A rinse solution using 100 mL of glacial acetic acid and 900 mL of methanol was used to dilute the working to a 1:4 ratio. Each sample was sprayed with the dilute Amido Black working solution. Upon bloodstain development, the fabric was sprayed with the rinse solution until sufficient contrast was achieved. Samples were allowed to dry for twenty-four hours before DNA extraction.

Hungarian Red: Hungarian Red was applied following the instructions provided by the manufacturer. Each sample was sprayed with the supplied Hungarian Red solution. The dye was allowed to set for one minute and washed by spraying a 1:1 dd to acetic acid mixture on the fabric until sufficient contrast was achieved. Samples were allowed to dry for twenty-four hours before DNA extraction.

Coomassie Blue: Coomassie Blue was applied following the instructions provided by the manufacturer. A working solution was created by combining 4 gram of Coomassie Blue in 200 mL of methanol, 200 mL of ddH₂O, and 40 mL of glacial acetic acid. A rinse solution was created by combining 450 mL methanol, 450 mL of ddH₂O, and 100 mL of glacial acetic acid. Each sample was sprayed with the Coomassie Blue working solution. 60 seconds was allowed for bloodstain development, and the sample was sprayed using the rinse solution until sufficient contrast was achieved. Samples were allowed to dry for twenty-four hours before DNA extraction.

Aqueous Leuco Crystal Violet: Aqueous leuco crystal violet was applied following the instructions provided by the manufacturer. A working solution was created using the supplied components. Each sample was sprayed with the aqueous leuco crystal violet working solution. Samples were allowed to dry for twenty-four hours before DNA extraction.

Luminol: Luminol was applied following the instructions provided by the manufacturer. A working solution was created by combining the supplied components and gently shaking until the powder was completely dissolved. Each sample was sprayed with the working solution in the dark. Samples were allowed to dry for twenty-four hours before DNA extraction.

Bluestar® Forensic Magnum: Bluestar® Forensic Magnum was applied following the instructions provided by the manufacturer. A working solution was created by combining the supplied components and gently swirling until the tablets were completely dissolved. Each sample was sprayed with the working solution in the dark. Samples were allowed to dry for twenty-four hours before DNA extraction.

Photography: Photographs of each sample were taken using a Canon EOS Rebel T3i Digital SLR camera (18.0 MP) at each stage following blood deposition, laundering, and application of enhancement reagents using automatic settings and focused manually. For blood enhancement reagents exhibiting chemiluminescent results, photographs were taken using an aperture of f/8, an ISO value of 400, and a shutter speed of 45 seconds. These photographs were taken in the dark during reagent application, and the room lights were quickly turned on and off during the collection of the photograph in order to allow for visualization of the fabric swatch.

DNA Extraction: Following the required treatment, each bloodstain was cut out using sterile scissors and placed in a sterile 2 mL micro centrifuge tube. DNA extractions were performed using a QIAamp® DNA Investigator Kit following a procedure adapted from the “Isolation of Total DNA from Body Fluid Stains” protocol located in the QIAamp® DNA Investigator Handbook. For the final elution step, 30 µL of the provided Buffer ATE was applied to the center of the column membrane, and the column was allowed to incubate at room temperature for five minutes. The tube was centrifuged at 14,000 rpm for one minute. Following centrifugation, the column was discarded. The eluate was stored at -20°C until required.

DNA Quantitation: A NanoDrop™ One C UV-Vis spectrophotometer was used to quantify DNA present in each of the extracts. Parameters for double stranded DNA quantitation were chosen, and the instrument was blanked using 1 µL of Buffer ATE provided in the QIAamp® DNA Investigator Kit. 1 µL of DNA extract was pipette onto the pedestal, and the concentration of DNA in the sample was recorded. Each DNA extract sample was quantified three times and the concentration was reported as an average of the three measurements.

Results

Enhancement Reagents Sensitivities

The results of the post-laundering enhancement of the neat blood and dilutions on the varying fabric types revealed the peroxidase based reagents (luminol, LCV and Bluestar® Forensic Magnum) to have the greatest sensitivities on the natural fabric types (white cotton, black cotton and denim) as they all reacted positively on these fabrics down to 1:1,000 Table 1. However, when the protein reagents were tested, they revealed the greatest sensitivities (1:10) on the white polyester when compared to the peroxidase reagents, which only produced positive reactions on the laundered neat blood. As the protein based reagents are color reactions and are not based on chemiluminescence, their use on dark fabrics revealed indeterminate results as even if positive reactions were obtained, the results were not visible due to the lack of contrast between the dark color background and the color reaction. Negative results were obtained with all reagents on samples 1:10,000, 1:100,000, and 1,000,000. Sample images of the enhancement reactions on white cotton are displayed in Figure 1. All enhancement reagents reacted as expected with the test control samples.

DNA Recovery

Despite laundering and the application of enhancement reagents, quantifiable amounts of DNA were obtained from each sample. Overall, washed and enhanced blood samples (mean 15.9 ± 8.3 ng/µL) had a lower DNA recovery than unwashed blood (mean 18.0 ± 1.5 ng/µL). Washed blank samples (mean 8.6 ± 3.7 ng/µL) had higher DNA recovery than unwashed blank samples (mean 3.6 ± 3.9 ng/µL), indicative of possible DNA transfer during the laundering process, see Figure 2.

Table 1: Results of enhancements post-laundering showing the sensitivity of each reagent on the various fabric types. ✖ indicates no reaction, while a ✔ represents a positive reaction for each trial. All samples above 1:1,000 were negative.

Keywords: L = Luminol ; BFM = Bluestar® Forensic Magnum ; LCV = Aqueous Leuco Crystal Violet ; HR =Hungarian Red ; CB = Coomassie Blue ; AB = Amido Black.

| | Neat | | | | | | 1:10 | | | | | | 1:100 | | | | | | 1:1000 | | | | | |
|-----------------|------|-----|-----|----|----|----|------|-----|-----|----|----|----|-------|-----|-----|----|----|----|--------|-----|-----|----|-----|----|
| | L | BFM | LCV | HR | CB | AB | L | BFM | LCV | HR | CB | AB | L | BFM | LCV | HR | CB | AB | L | BFM | LCV | HR | C.B | AB |
| White Cotton | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ |
| | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ |
| | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ |
| Black Cotton | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ |
| | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ |
| | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ |
| Denim | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ |
| | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ |
| | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ |
| White Polyester | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ |
| | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ |
| | ✔ | ✔ | ✔ | ✔ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ |
| Black Polyester | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ |
| | ✔ | ✔ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ |
| | ✖ | ✔ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ | ✖ |

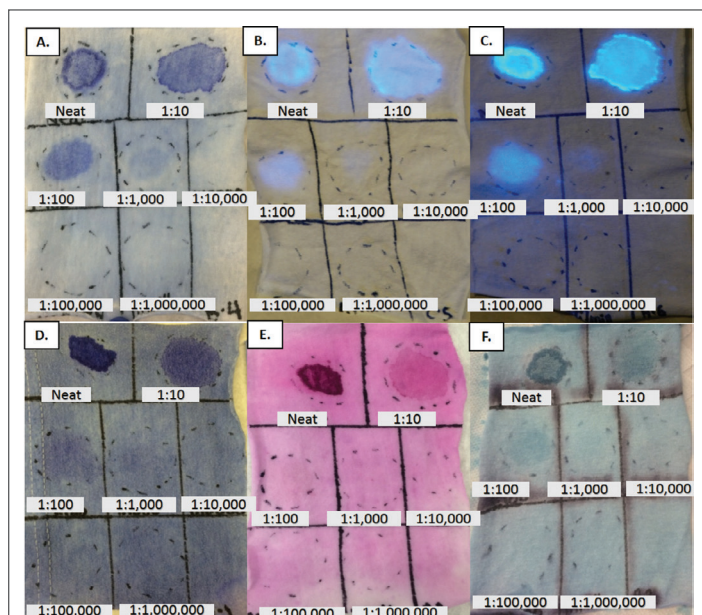


Figure 1. Images of blood enhancement reactions post-laundering on white cotton; (A.) Leuco Crystal Violet, (B.) Luminol, (C.) Bluestar® Forensic Magnum, (D.) Coomassie Blue, (E.) Hungarian Red, (F.) Amido Black.

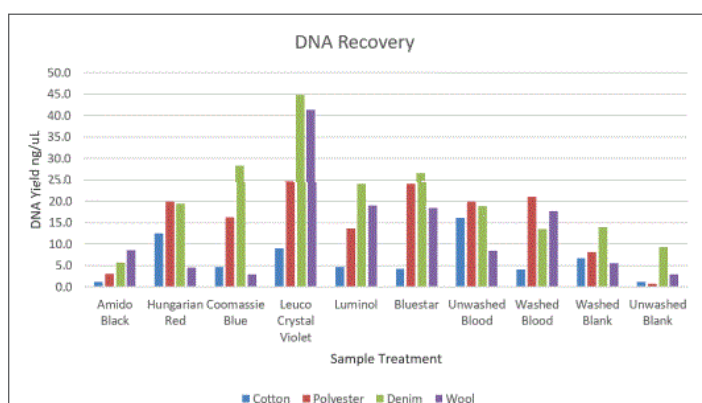


Figure 2: DNA recovery from fabric samples treated with enhancement reagents, including controls.

The DNA yield across all fabric types were; Hungarian Red (mean 14.1 ± 6.2 ng/µL), Coomassie Blue (mean 13.0 ± 10.2 ng/µL), luminol (mean 15.4 ± 7.2 ng/µL), and Bluestar® Forensic Magnum (mean 18.3 ± 8.6 ng/µL), and the washed(untreated) blood samples(mean 14.1 ± 7.3 ng/µL). LCV (mean 29.9 ± 14.3 ng/µL) treated samples had the highest DNA recovery from all enhanced samples. Samples treated with Amido Black (mean 4.6 ± 2.8 ng/µL) had the lowest recovered DNA yield and was comparable to the unwashed blank samples(mean 3.6 ± 3.9 ng/µL), indicating amido black to have an impact on DNA recovery. Cotton had the lowest yield of DNA recovery (mean 6.0 ± 3.6 ng/µL) from all enhanced samples. Denim samples revealed the highest DNA yield (mean 24.8 ± 11.6 ng/µL) following laundering and the application of enhancement reagents. Polyester (mean 16.9 ± 7.3 ng/µL) and Wool (mean 15.8 ± 13.0 ng/µL) samples revealed comparable yields.

Discussion

When bloodied clothing is laundered, the stains become more dilute and can lack visual detection without treatment or enhancement. Several

studies have previously demonstrated the ability to enhance blood stains using both protein and peroxidase enhancement reagents [1-3]. Because protein and peroxidase reagents are formulated to enhance traces of blood that are otherwise undetectable, the reagents work to provide sufficient contrast between dilute bloodstains on laundered fabric substrates [12]. Protein stains often produce colored reactions and frequently used to enhance dilute/weak bloodstains in impression evidence such as bloody fingerprints and footwear impressions. However, they have the distinct disadvantage of low specificity and also requiring fixation prior to protein staining and de-staining after. Throughout this study the fixation and de-staining procedures was time consuming and cumbersome, particularly when compared to the ease of the one step reactions using the peroxidase reagents. Fixation is necessary to precipitate the basic proteins and prevent leaching of the blood. Fixation has been suggested via a number of methods such as cross-linking, dehydrating, precipitation, or disruption of the secondary/tertiary structure [13,14]. Fixation with 5-sulfosalicylic acid is the preferred method as it is safe, effective and convenient [15]. The required fixation and also de-staining steps may have an impact on the recovery of DNA as it introduces extra steps which could encourage loss of what may already be minute levels of DNA. In this study, samples treated with amido black post laundering revealed the lowest recoverable yields of DNA and it is possible the fixation and de-staining procedures contributed to this low yield recovery. The peroxidase reagents that produce chemiluminescence with blood, luminol and Bluestar® Forensic Magnum, have the distinct advantage that the background color of the fabric does not have an impact, as the reaction can be visualized on both light and dark surfaces. This was observed in this study with positive reactions obtained on the dark colored fabrics with the chemiluminescent reagents, while insufficient color contrast was obtained with the color reaction reagents to document a result. Luminol was first described by Albrecht HO [16], with its application in the forensic field introduced by Specht HW [17]. There have been a few formulations suggested over the years; however the Grodsky formulation remains the gold standard [18]. Bluestar® Forensic Magnum is a commercial product based upon the luminol formulation. It has however, overcome some of the difficulties encountered with luminol and produces a longer lasting chemiluminescence [19]. This was observed in this study with Bluestar positive reactions producing bright and long lasting reactions. Indeed, Bluestar® can be visualized in normal light eliminating the need for complete darkness which is required for luminol, offering a distinct advantage for use in the field. A number of substances however can interfere with the luminol/Bluestar® reaction. Some can produce false positive results, such as peroxidases, metal ions and other oxidants such as hypochlorite [4]. While other substances may suppress the luminol reaction, producing false negative results such as chemiluminescence quenchers (e.g. oxygen and tertiary amino acids), and antioxidants [20,21]. Standard household bleach contains hypochlorite and can produce false positive results with luminol. The reaction however produces brighter flashes and is easily discerned by an experienced forensic scientist. There is risk however if the perpetrator has used household bleach in the laundering process to wash clothing. A false positive may be reported when the underlying washed latent stain is indeed blood. This study did not include any bleach containing detergents and therefore requires further investigation in the future. The ability to visualize bloodstains on articles of clothing following laundering with the assistance of enhancement reagents indicates that components of blood remain in the fabric even after this treatment, suggesting that DNA may persist on the clothing as well. Previously, studies have indicated that DNA persists despite attempts to clean up evidence through laundering [5,7, 22,23]. One such study [24], investigated the effects of laundering on the ability to recover DNA from semen stains on various items of

clothing. This study found that DNA could be obtained from items of clothing despite laundering, and found that the DNA yield they obtained did not significantly diminish as the number of wash cycles increased. Additionally, the study found that items of unstained clothing in the wash with stained items also had quantifiable amounts of DNA recovered, indicating the potential for DNA transfer from one article of clothing to another during the wash cycle [24]. This is in agreement with this research study where DNA was recovered from previously unstained samples which had been laundered with stained samples. Other studies have also indicated the ability to obtain full DNA profiles following the application of enhancement reagents [7,9]. Although no studies have been published to date in regards to DNA collection following both laundering and enhancement of blood stains, studies such as this one are indicative of its plausibility. In this study, across all fabric types, laundered samples treated with Amido Black had a low DNA recovery when compared to samples treated with the 5 other blood enhancement reagents. Although it cannot be said that DNA profiles would be unattainable from laundered bloodstains enhanced with this reagent. Amido Black differed from other protein reagents in its composition; unlike Hungarian Red and Coomassie Blue, Amido Black working solution and wash solutions were prepared with a significant amount of methanol, acetic acid, and citric acid in comparison to water. Since DNA is a highly reactive molecule, it is possible that interactions between these compounds led to a higher rate of DNA degradation. Because of these results, caution should be used when choosing Amido Black as an enhancement reagent for blood on laundered clothing. Additionally, water-based working solutions should be chosen over methanol-based working solutions when possible.

In this study, throughout each fabric type, quantifiable amounts of DNA were shown to persist despite the laundering process and the application of blood enhancement reagents. Although washed blood samples often had a lower DNA recovery than unwashed blood samples, washed samples on all fabric types had enough DNA present in order to likely yield a probative DNA profile. Similarly, samples treated with all enhancement reagents except Amido Black had DNA yields higher than the blank samples. Since factors like heat and humidity can aid DNA degradation by speeding up the breakage of bonds holding together DNA molecules, it is to be expected that the process of laundering and enhancement reagent application would lead to a diminished DNA recovery. Across the board, washed blank fabric samples had a higher amount of recovered DNA than the unwashed blank samples; however, these samples were treated exactly the same with the exception of laundering. Because of this, it is suggested that cross transfer of DNA between samples during the laundering process is possible. Because these blank samples were in a shared environment with the other samples submerged during laundering, it is likely that this resulted in the transfer of DNA from the blood samples to the blank samples as the blood was washed from the fabric. This result aligns with previous studies that demonstrated the cross transfer of DNA from semen stained clothing to unstained clothing during the laundering process [24]. When choosing an enhancement reagent, it is important for a forensic examiner to not only choose a method that is least destructive to other potential types of evidence, but to also consider the origin the stains and the possibility of cross-transfer.

Conclusion

The results of this study provide a valuable analysis of the persistence of DNA on various fabrics following laundering and treatment with commercially available blood enhancement reagents. The results suggest that quantifiable amounts of DNA originating from bloodstains persist despite laundering and enhancement with commercially available protein and peroxidase reagents commonly used throughout the course of

forensic investigations. This conclusion supports the idea that laundered clothing should not be overlooked during the course of an investigation, and could potentially yield probative and identifying information about a violent crime that occurred. The results also suggest that the application of blood enhancement reagents, including Amido Black, may affect the ability to recover DNA. Because of this, it is suggested that a forensic examiner's choice of enhancement reagent be made with caution and with the persistence of DNA in mind. This information serves as a valuable resource for forensic professionals in the future when making decisions to both enhance the ability to visualize dilute bloodstains on laundered clothing while maintaining the integrity of the DNA evidence that may be present.

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