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Comparison of methods for visualizing blood on dark surfaces

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ABSTRACT

Difficulties can arise when screening dark casework items for blood, a poor contrast between blood and the background can mean stains are not always evident. Typical indirect searching methods can be time consuming and may result in potentially important bloodstains being missed. Luminol, fluorescein, hydrogen peroxide, ultraviolet light and infrared photography were tested in an effort to find a rapid and efficient blood search tool for direct application to dark surfaces. Methods were compared in their sensitivity, specificity, ability to work on various surface types and their effect on DNA extraction and typing. Along with experimental results, the ease of use, costs and the health and safety considerations were also compared. Hydrogen peroxide was determined to be the most effective method. However, where blood was likely to be dilute, luminol was proposed due its greater sensitivity.

1. Introduction

Blood is one of the body fluids most commonly encountered by forensic scientists, particularly in association with violent crime. It is a good source of deoxyribonucleic acid (DNA) and blood pattern analysis can assist in assessing the likelihood of prosecution and defence scenarios.

Locating blood on light coloured items is often a relatively easy process. However, on dark items the lack of contrast makes visualizing bloodstains much harder, particularly with older stains, due to the darkening of blood over time [1].

The detection of bloodstains, both at the crime scene and at the laboratory is often vital in many investigations. Occasionally it has later emerged that bloodstains were missed. The Damilola Taylor case demonstrates the high profile consequences of failing to find blood during forensic examination [2]. Whilst it is accepted as inevitable that some bloodstains may be missed, reasonable efforts must be made to locate all significant blood evidence.

If no stain is found after a visual and low power microscopic examination of a dark item then often an indirect search is undertaken. This can involve rubbing filter papers over the entire items surface and the application of reagent, such as Kastle–Meyer (KM) or leucomalachite green (LMG), to the filter paper followed by hydrogen peroxide. A colour change indicates a presumptive positive result for blood. This method is time consuming, and unless diligently undertaken, may result in missed blood evidence. There is also a risk, on some surfaces, of removing bloodstains or disrupting marks.

If blood is not found on dark items after the initial blood search then, depending on the item, alleged case circumstances and expectations, a second examination may be undertaken to confirm the negative result. This research was aimed at finding a quick and effective method for use, alongside current procedures, to confirm the negative result and to aid in the detection of any missed blood.

1.1. Selection of methods

Haemoglobin possesses peroxidase-like activity; it is involved in the catabolism of peroxides, into water and oxygen, and the oxidation of various substrates [3]. Many of the chemical presumptive tests for blood exploit this property. They are applied in a reduced, mostly colourless, form and become oxidised in the presence of haemoglobin and an oxidising agent (typically hydrogen peroxide), becoming coloured, fluorescent or luminescent. However, many of these tests can affect subsequent DNA extraction and/or typing [4–6,32], or have unacceptable health risks [4], therefore cannot be directly applied. Other tests, for example leucocrystal violet (LCV), become dark in colour, so are unlikely to improve the contrast on a dark background. Luminol, fluorescein and hydrogen peroxide were identified as chemicals that could be used to visualize bloodstains on dark items, and still allow for DNA profiling [5,7,31].

Spectroscopic methods, such as those involving ultraviolet (UV) or infrared (IR) light are also viable options for visualizing blood. These have an advantage over chemical methods because they do not physically interact with the bloodstain; therefore they do not affect the blood morphology.

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Table 1
Production of tested fluorescin formulations.

Fluorescin formulation	Xanthan gum concentration	Fluorescin concentration	Applied using
1	2.5 g dissolved in 500 ml distilled water (0.5%)	25 ml in 475 ml 0.5% xanthan gum (1 in 20)	Spray bottle
2	1 g dissolved in 500 ml distilled water (0.2%)	25 ml in 475 ml 0.2% xanthan gum (1 in 20)	TLC sprayer
3	1 g dissolved in 500 ml distilled water (0.2%)	25 ml in 225 ml 0.2% xanthan gum (1 in 10)	TLC sprayer
4	0%	25 ml in 475 ml distilled water (1 in 20)	EcoSpray

A vast array of methods exist for the presumptive identification of blood and have been examined thoroughly in the literature [*inter alia:* 4, 5, 22]. This paper investigates just six of the many available.

1.1.1. Luminol

Luminol has been used in the detection of latent blood for more than 40 years. A single reagent is applied in an aqueous, alkaline form, containing an oxidising agent (hydrogen peroxide or sodium perborate). Oxygen released from the breakdown of this agent reacts with luminol to form unstable 3-aminophthalate which emits light at 454 nm as it decays [8]. When applied to blood a blue chemiluminescence is produced. The reagent must be applied under total darkness because this chemiluminescence is not bright enough to be seen under normal lighting [7].

Several formulations exist, the most common being those described by Grodsky [9] and Weber [10]. In 2006 an optimized formulation was created by Blum et al. [11]. This became Bluestar® Forensic (referred to as Bluestar throughout this paper), an easy to create commercial luminol formulation with improved chemiluminescence [12]. Bluestar was chosen for experimentation.

1.1.2. Fluorescein and Hemascein™

Fluorescein is applied in its reduced form, fluorescin, followed by hydrogen peroxide. Oxygen, released from hydrogen peroxide oxidises fluorescin, forming fluorescein. Application of a blue light (425–485 nm) results in green/yellow fluorescence (peak at 521 nm) being emitted where fluorescein is present [7]. The reagent application is performed under normal lighting, the blue light visualisation requires darkness. Several authors have noted background fluorescence which can interfere with the visualization of blood [7,13]. This is likely to be due to the instability of the reduced fluorescin, which naturally oxidises, at a relatively fast rate, to regenerate fluorescein [14]. To compensate for the double reagent application Cheeseman and DiMeo [18] suggested the addition of paint thickener (xanthan gum/Keltrol RD) to the fluorescin solution. Three different concentrations of xanthan gum were chosen for testing.

Hemascein™ (referred to as Hemascein throughout this paper) is a commercial fluorescein preparation that simply requires the addition of distilled water. Information provided with the kit states that it is stable for 28 days. Hemascein was selected for comparison, alongside fluorescein, to determine if the improved reagent stability removes, or reduces, background fluorescence.

1.1.3. Hydrogen peroxide

Hydrogen peroxide was used as a test for blood in the late 19th century, known as the catalase test, but was superseded by tests such as KM, benzidine and leucomalachite green (LMG) due to their greater sensitivity [15]. However, a 6% hydrogen peroxide solution has been used to successfully screen dark items for blood at the Scottish Police Services Authority Forensic Services, Aberdeen, Scotland (personal communication). This method also relies on the peroxidase-like activity of haemoglobin as well as enzymes within blood, such as catalase. Producing oxygen bubbles at the site of the bloodstain, resulting in the appearance of white foam. Hydrogen peroxide is applied under normal lighting and requires no special light sources or photography.

1.1.4. Ultraviolet absorbance

The absorbance spectrum of the haemoglobin in blood generally differs when compared to that of the background surface. Blood absorbs UVA light (300–400 nm) strongly, whereas many backgrounds will transmit some of this light at a longer wavelength, appearing to fluoresce violet. Using a light source to generate UVA light, a bloodstain can be made to appear dark in contrast to a lighter background [16].

1.1.5. Infrared photography

Similar to UV light, near infrared light (700–1500 nm) is absorbed strongly by blood, many backgrounds will reflect it. Using a device that can record infrared wavelengths produces an image in which blood appears dark on a light background [17].

If bloodstains can be visualized *in situ*, then DNA testing can be focused onto specific areas, saving time and money. The morphology of bloodstain patterns can be determined and interpreted. DNA profiles can be attributed to a single stain rather than a surface and the risk of generating a mixed profile can be reduced.

Each of the methods identified can be used to visualize blood on dark items and each has specific advantages and disadvantages associated with it. The aim of this work is to test methods across a number of areas and find the best for use alongside current screening procedures.

2. Experimental

2.1. Materials

2.1.1. Biological fluids

Human blood was obtained from one male volunteer via venipuncture into ethylenediaminetetraacetic acid (EDTA) vacutainers. To determine method specificity to blood a variety of other body fluids were also collected. Seminal fluid was donated by three male volunteers. Vaginal fluid was obtained from three female volunteers who had not participated in sexual intercourse for at least five days. Saliva, urine and faeces were provided by three volunteers, both male and female, who had not

Table 2
Sensitivity results for blood on cotton.
(+ Indicates at least one positive result; — Indicates no positive result obtained, ( ) Indicates the number of samples testing positive).

Visualization method	Blood dilution							
	Neat	1 in10	1 in 100	1 in 1000	1 in 10,000	1 in 100,000	1 in 1,000,000	Negative control
Bluestar	+ (10)	+ (10)	+ (10)	+ (10)	—	—	—	—
Fluorescein (1 in 10 & 1 in 20 dilution)	+ (10)	+ (10)	+ (10)	+ (10)	—	—	—	—
Hemascein	—	—	+ (1)	—	—	—	—	—
Hydrogen Peroxide (6–30%)	+ (10)	+ (10)	—	—	—	—	—	—
UV	+ (10)	+ (10)	+ (10)	—	—	—	—	—
IR Photography	+ (10)	+ (10)	—	—	—	—	—	—

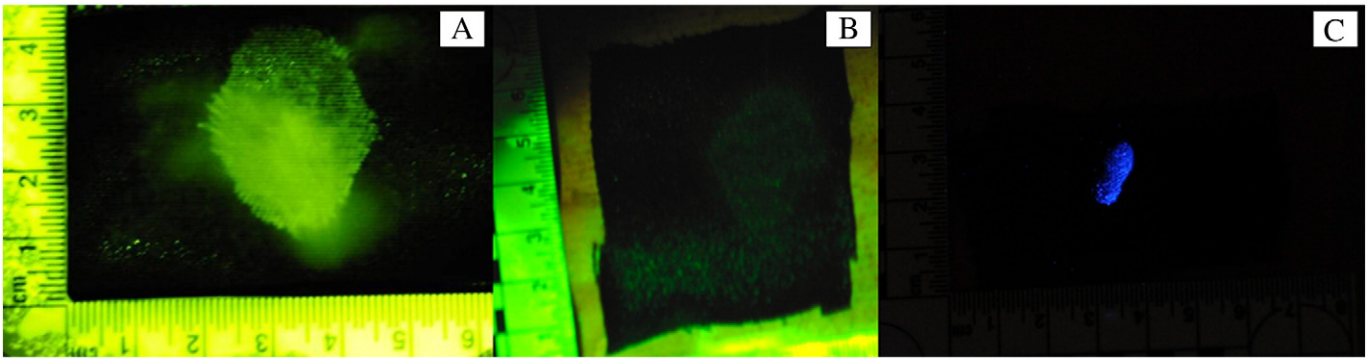

Fig. 1. Fluorescin, Hemascein and Bluestar applied to dilute blood on black cotton. (A: 1 in 20 fluorescin, 0.2% xanthan gum applied to 1 in 100 blood, B: Hemascein applied to 1 in 100 blood, C: Bluestar applied to 1 in 1000 blood).

participated in sexual activity for at least five days. Three samples of dog faeces from gender unknown dogs were also collected.

2.1.2. Substrates

A variety of substrates were collected including rolls of black and white cotton, a leather jacket, vinyl floor tiles, carpet (80% wool; 20% polyester), a hammer with rubber grip and a pair of trainers made of dark suede material.

2.1.3. Reagents, chemicals and equipment (set up and use)

2.1.3.1. Bluestar. As per the manufacturer's instructions one white tablet and one yellow tablet were added to 125 ml of distilled water. The solution was gently mixed until both tablets were fully dissolved. The Bluestar solution was sprayed using an EcoSpray atomiser under darkness. The reagent was used for approximately 4 h [11], after which point a new solution was prepared.

Photographs were taken immediately using a Nikon D300 DSLR camera fixed to a tripod, using 1600 ISO, f/2.8 and an exposure time of 30 s.

2.1.3.2. Fluorescein/fluorescin. Fluorescein was produced using the Cheeseman and DiMeo formulation (0.5% xanthan gum; 1:20 fluorescin dilution) [18]. Slightly modified formulations, containing 0.2% and 0% xanthan gum, were also tested, along with a 1:10 fluorescin dilution).

For reagent preparation, 10 g sodium hydroxide was dissolved in 100 ml distilled water. 1 g fluorescein was added, followed by 10 g zinc powder; this was heated and stirred to reduce the fluorescein. Table 1 describes the formulations prepared with this solution and their method of application.

The reagent was used for 2 h, after which point a new solution was prepared. 10% hydrogen peroxide was applied after fluorescin using an EcoSpray atomiser. A Crime-Lite® with blue filter was used to illuminate fluorescin coated items, and yellow barrier goggles were worn to block out the blue light.

Photographs were taken using a Nikon D300 DSLR camera. The camera was handheld, using 1600 ISO, f/5.1 and an exposure time of 1/30th s. An orange Hoya filter (G) was used to block out blue light.

2.1.3.3. Hemascein. As per the manufacturer's instructions, 5 ml distilled water was added to the Hemascein powder to produce a stock solution. 2 ml stock solution was added to 198 ml distilled water in an ABASpray™ bottle. The second ABASpray™ bottle was filled with 200 ml 3% hydrogen peroxide which was applied after the Hemascein.

Abacus Diagnostics® claim the Hemascein working solution to have a shelf life of 28 days, when refrigerated; accordingly only one working solution was produced for use in all experiments. A Crime-Lite® with blue filter was used to illuminate Hemascein coated items, and yellow barrier goggles were worn to block out blue light.

Photographs were taken using a Nikon D300 DSLR camera as described for fluorescein/fluorescin.

2.1.3.4. Hydrogen peroxide. 6% hydrogen peroxide was applied using the Merck TLC sprayer, EcoSpray atomizer or ABASpray™ bottle. Stronger concentrations were also tested, created through dilution of 30% hydrogen peroxide, producing 10%, 15% and 20% concentrations.

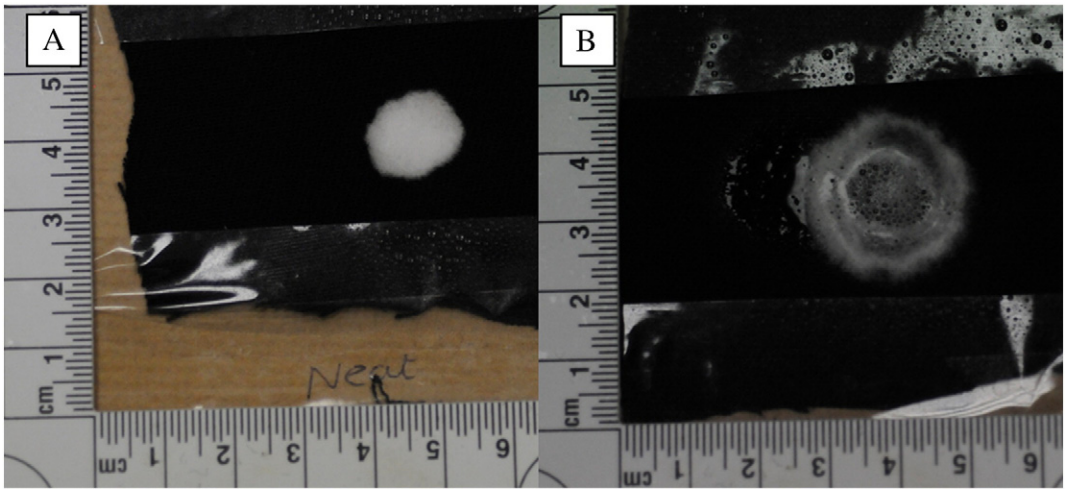

Fig. 2. Hydrogen peroxide applied to blood on black cotton. (A: 6% hydrogen peroxide applied to neat blood, B: 6% hydrogen peroxide applied to 1:10 blood).

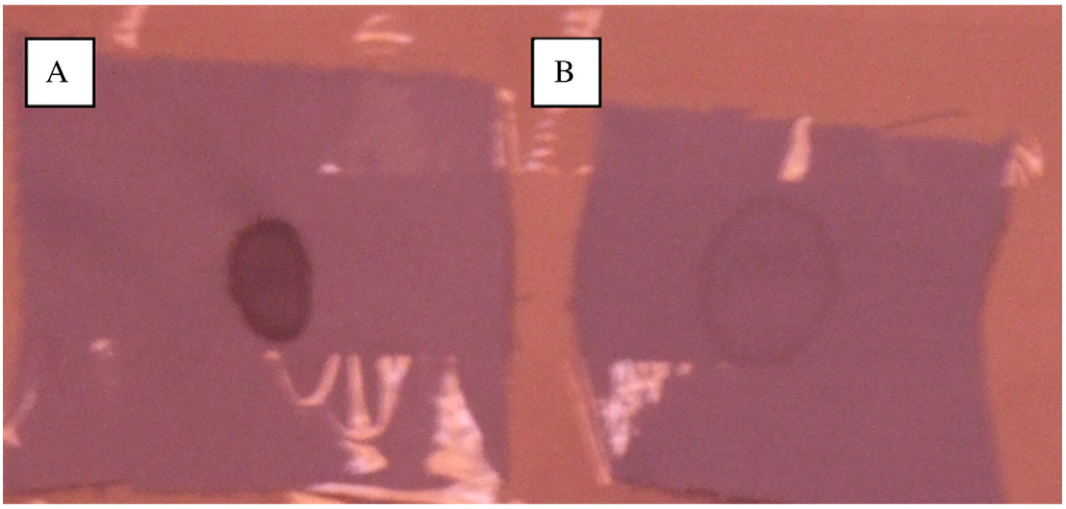

Fig. 3. IR photography of blood on black cotton. (A: neat blood, B: 1:10 blood).

Photographs were taken immediately using a Nikon D300 DSLR camera. The camera was handheld, set on 1600 ISO, f/9.0 with an exposure time of 1/30 s.

2.1.3.5. Ultraviolet light. A Streamlight UV torch was used to produce light at 395 nm. Clear UV protective goggles were worn during use.

Photographs were taken using a Nikon D300 DSLR camera. The camera was handheld using a 1600 ISO, f/5.1 and an exposure of 1/30 s.

2.1.3.6. Infrared photography. Photographs were taken using a Fujifilm IS-1 camera with a charge coupled device (CCD) unit, sensitive up to 900 nm. The camera was handheld while examining items. Camera settings for photographs were 400 ISO, f/9.0 and an exposure time of ½ s. An infrared Hoya filter (R72) was used to block out all light below a wavelength of 720 nm.

2.2. Methods

2.2.1. Sensitivity

A tenfold dilution series of human blood mixed with distilled water was produced, ranging from neat to 1 part blood in 1,000,000 parts water. 50 μl of neat or diluted blood was applied to ten pieces of black cotton per method and allowed to dry overnight, prior to testing. Distilled water was applied to an additional piece of black cotton per method to act as a negative control.

2.2.2. Surfaces

Drops of blood (approximately 30 μl) and fine blood spatter (<=1 mm diameter) were applied to carpet, leather, vinyl, rubber, suede and cotton. These were allowed to dry overnight, prior to testing, with each method.

2.2.3. Specificity

Fifty microlitres of seminal fluid, saliva and urine were applied to black cotton. A wipe of vaginal material, human faeces and dog faeces from a swab were also applied to black cotton. Biological fluids were allowed to dry overnight, prior to testing.

Fifty microlitres of a variety of household and laboratory products were applied to two areas on ten pieces of black cotton per method. These were allowed to dry for 2 h. Ten stains were tested with each method after drying, the second set of ten were tested 24 h later.

2.2.4. DNA quantification and typing

A tenfold dilution series of human blood mixed with distilled water was produced, ranging from neat to 1 part blood in 100 parts water. 50 μl of neat or diluted blood was applied to UV treated white cotton and left to dry overnight, prior to application of hydrogen peroxide or Bluestar. An untreated control was produced for comparison. Each bloodstain was created in triplicate and a single distilled water negative control was used per method.

Approximate 3 mm^2 areas of bloodstaining were cut out within 30 min of method application. DNA was extracted using an organic

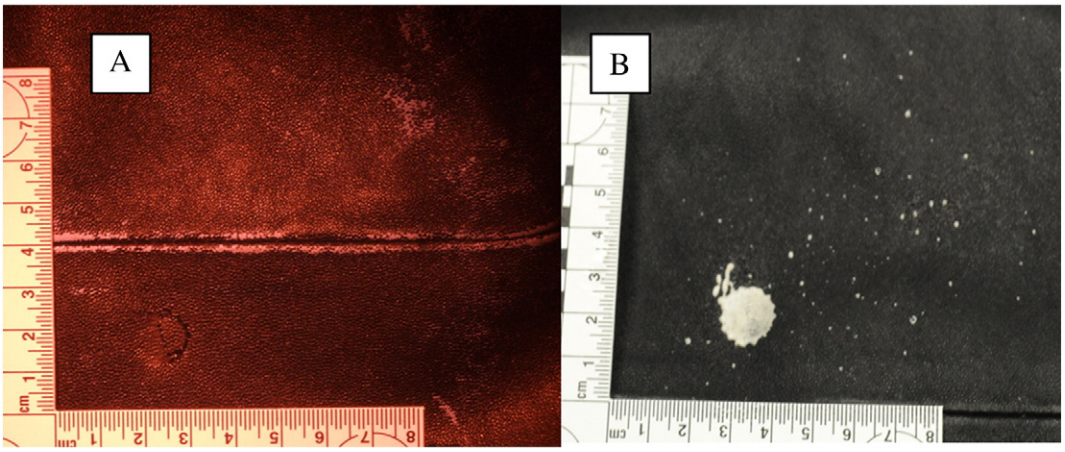

Fig. 4. Blood on leather. (A: IR photograph of blood on leather, B: hydrogen peroxide applied to A).

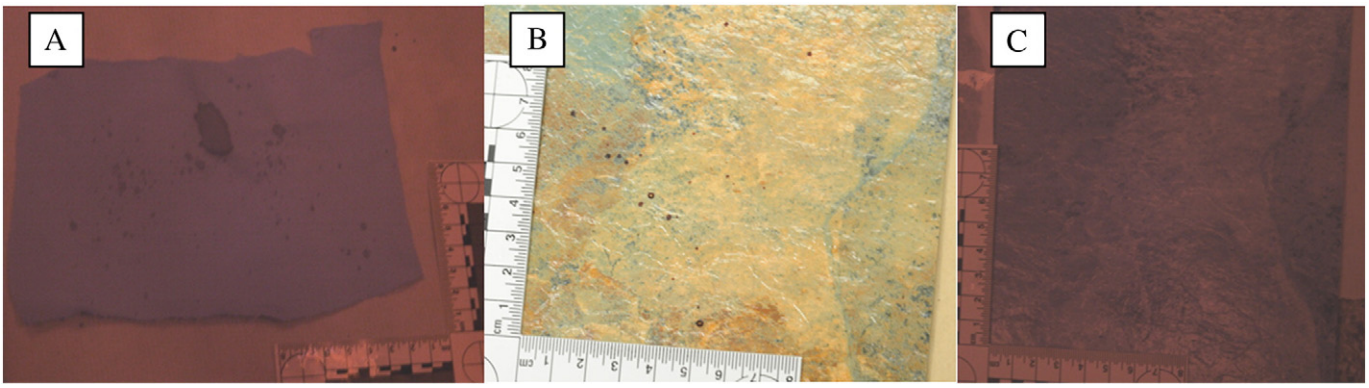

Fig. 5. Blood on black cotton and vinyl. (A: IR photograph of blood on cotton, B: blood on vinyl, C: IR photograph of B).

method, involving phenyl/chloroform/isoamyl alcohol and concentration through a Microcon® tube. Quantification, amplification, separation and analysis were performed using in-house methods [19]. Quant negative samples were amplified twice, once with 2 μl and again with 10 μl of extract. PCR amplification involved AmpF⁄STR® SGM Plus® and 28 thermocycles only.

DNA quantification results were compared between treated and untreated bloodstains. Resulting DNA profiles were also compared between controls and treated stains.

A further experiment was performed using neat blood, applied to nine pieces of UV treated white cotton. Hydrogen peroxide and Bluestar were applied to three stains each, three were left untreated. Areas of blood staining, approximately 3 mm^2, were cut from three blood stains treated with Bluestar and hydrogen peroxide and three non-treated stains within 30 min of method application, and again after 30 days. Blood stains were left at room temperature during the experiment. DNA profiling was performed as above.

3. Results and discussion

Experiments were performed to highlight important characteristics when visualizing blood *in situ* on casework items. After each experiment the results were considered critically in the context of the ultimate goal of the project. Those methods not performing adequately were not considered further.

3.1. Sensitivity

Whilst it is generally neat blood that is present on casework items, there is the possibility blood may be washed from surfaces, either deliberately or through the action of rain, prior to examination; any blood remaining will be dilute.

Luminol, in its various formulations, has been thoroughly tested by many authors and a wide range of sensitivities have been reported [7,13,20,21]. This has been attributed to slight variations in the experimental conditions and the method of preparation and use [22].

Fluorescein is generally acknowledged as having a similar sensitivity as luminol based formulations [7,13,18]. Abacus Diagnostics® suggest Hemascein is reactive with much greater dilutions than luminol based products. However, at the time of writing, there were no peer reviewed papers confirming this.

As stated previously, a 6% hydrogen peroxide solution is used to screen dark items for blood in a laboratory in the UK. There is little data about the sensitivity of hydrogen peroxide in the literature; therefore to determine if sensitivity is proportional to the concentration used, a range of hydrogen peroxide concentrations were tested. These were 6, 10%, 15%, 20% and 30%.

The literature regarding UV and IR sensitivities for blood is limited when compared to chemical methods. However, detection of 1 in 8 diluted blood by IR photography [24] and 1 in 100 by a 400 nm light source [20] give an indication of sensitivities previously obtained.

Experiments were performed in an effort to determine the relative sensitivities of methods to each other. The results of sensitivity testing are presented in Table 2.

Bluestar and fluorescein both achieved a sensitivity of 1 in 1000. The Bluestar reaction at this dilution produced very weak chemiluminescence when viewed with the naked eye, but was recorded strongly by the camera due to the long exposure time. This result was reproducible on all ten samples of the 1 in 1000 bloodstain. The fluorescein reaction with the 1 in 1000 dilution was also faintly visible to the naked eye. However, there was excessive background fluorescence on many of the cotton samples used, an example of which is shown in Fig. 1A.

The inclusion of 0.5% xanthan gum in the creation of fluorescin, as suggested by Cheeseman and DiMeo [18], produced a viscous reagent. Application using a conventional spray bottle resulted in an uncontrollable, thick coating with strong background fluorescence. Reagents created using 0.2% and 0% xanthan gum were applied in a controlled manner as they could be applied with finer sprayers. However, these too produced background fluorescence on the cotton, interfering with blood visualization. The stronger fluorescin solution (1 in 10) had the same maximum sensitivity as the 1 in 20 version.

Hemascein application, like fluorescein, resulted in some background staining of the cotton substrate. The only visible reaction occurred with one of the 1 in 100 diluted bloodstains tested. This was

Table 3
Specificity results for selected methods on various laboratory and household products. (+ Indicates at least one positive result; − Indicates no result, +/− Indicates a positive that becomes negative over time, ( ) Indicates the number of samples testing positive).

Product	Bluestar	Hydrogen peroxide	KM (indirect)
Presept bleach	+/− (10)	−	−
Distilled water	−	−	−
Tap water	−	−	−
Mediwipe®	−	−	−
Ethanol	−	−	−
Tomato ketchup	−	−	−
Perfume	−	−	−
Marmite	−	−	−
Vinegar	−	−	−
Banana (pulp)	+ (10)	−	−
Potato	+ (10)	−	−
Soil	−	−	−
Vegetable oil	−	−	−
Motor oil	−	−	−
Chocolate	−	−	−
Turnip (pulp)	+ (10)	−	−
Cider	−	−	−
Lager	−	−	−
Positive control	+	+	+

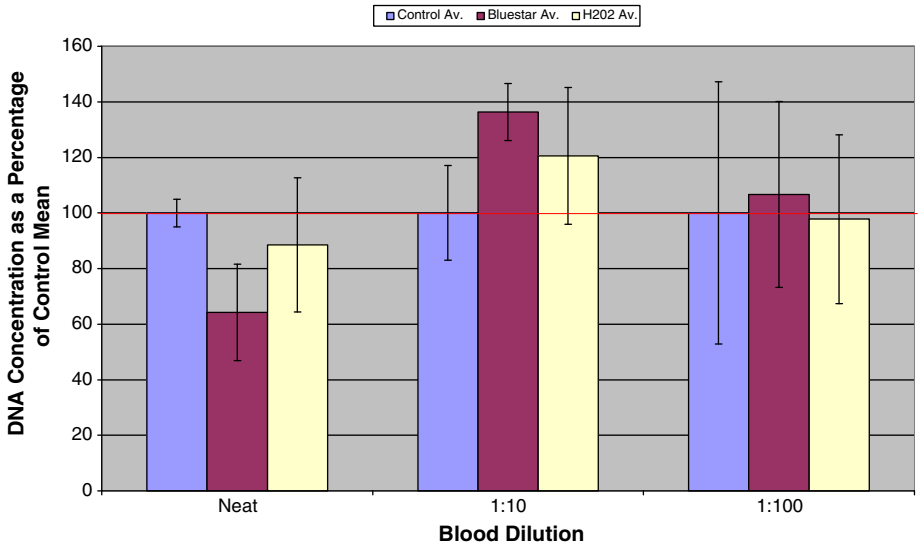

Fig. 6. Mean concentration of DNA recovered from bloodstains after method application; standard deviations included.

very weak and is shown in Fig. 1B. When applied to other blood dilutions no fluorescence was noted as a direct result of reaction with blood, only background fluorescence of the cotton.

In one online resource, Hemascein was shown to produce background fluorescence and also appears to record negative results with neat, 1 in 10, and 1 in 100 bloodstains [23].

The lack of reaction with neat blood and 1 in 10 diluted blood is concerning and is expected to lead to missing blood evidence on dark surfaces. For this reason Hemascein was no longer considered as a viable option and no further tests performed using this reagent.

The fact that background fluorescence was present at similar levels in Hemascein and all fluorescein formulations tested suggests that this is an inherent problem of fluorescein based blood detection methods. This can be contrasted against the Bluestar reaction shown in Fig. 1C, where no background staining occurred. Fluorescein was removed from further testing.

All hydrogen peroxide concentrations tested had a maximum sensitivity of 1 in 10. The strength and longevity of the hydrogen peroxide reaction was, however, very noticeably diminished with diluted blood as shown in Fig. 2. There were fewer bubbles produced, these disappeared after 15 s, whereas the reaction with neat blood lasted for around 30 s. Despite the relatively low sensitivity compared to other methods, the contrast between white reaction and black background was excellent.

As the concentration of the hydrogen peroxide had no effect on sensitivity all further experiments were performed using 6% hydrogen peroxide due to the reduced health and safety risks.

Infrared photography also had a maximum sensitivity of 1 in 10 diluted blood. Neat blood photographed on black cotton had a very clear contrast compared to the background, shown in Fig. 3A. Dilute blood appeared as a halo (Fig. 3B). This may be due to the blood being dilute before it was applied and the way in which it dried. However, dilute blood had a reduced contrast with the background.

Ultraviolet light could be used to visualize neat and dilute blood down to 1 in 100. The more dilute the blood, the poorer the contrast between blood and background.

3.2. Surfaces

Blood can be present on a variety of surfaces, some porous, others non-porous. To this end, fine blood spatter (<= 1 mm diameter) as

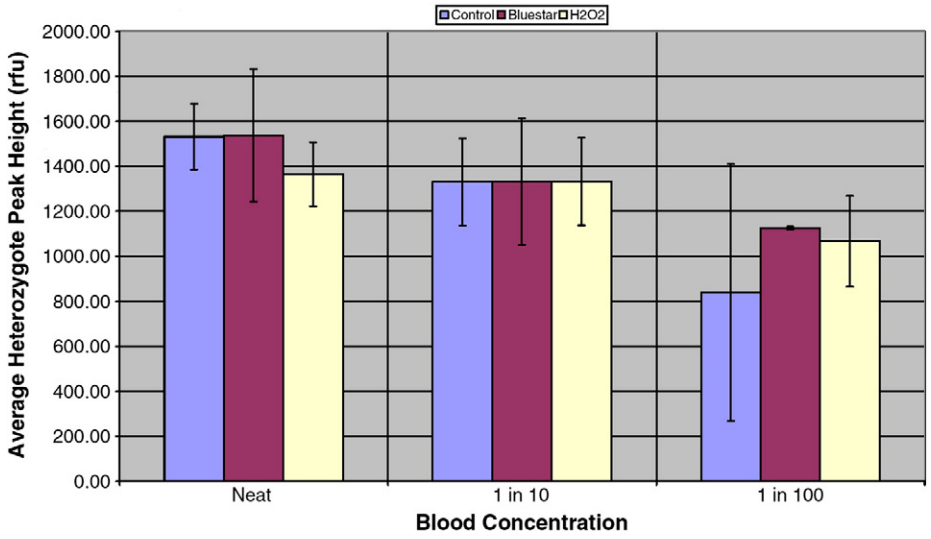

Fig. 7. Average heterozygous peak heights from profiles generated from bloodstains after method application; standard deviations included.

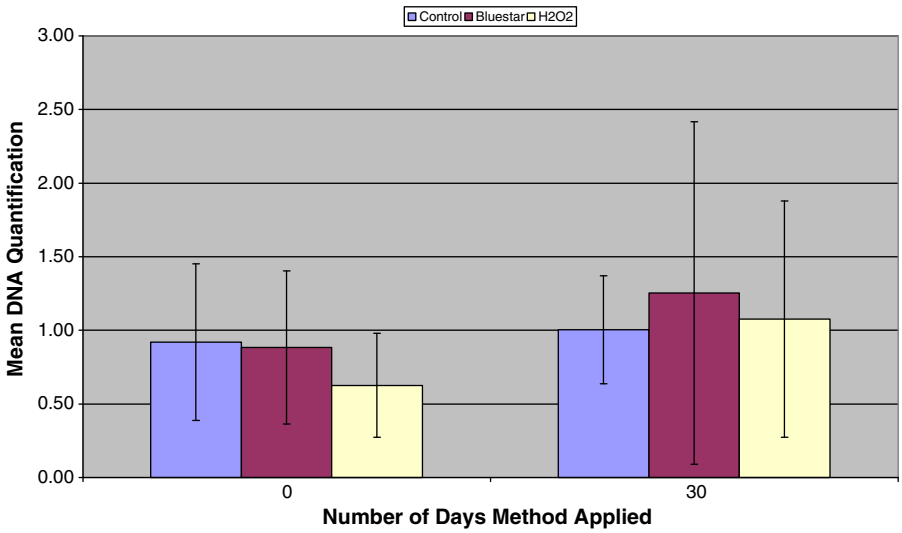

Fig. 8. Mean concentration of DNA recovered from neat bloodstains, 0 and 30 days after method application; standard deviations included.

well as larger bloodstains (>= 5 mm diameter) were applied to a range of surface types (cotton, rubber, suede, leather, vinyl and carpet).

Both hydrogen peroxide and Bluestar allowed visualization of larger blood stains and spatter on all the surfaces tested. UV light allowed visualization of larger stains on all surfaces, but did not produce a visible contrast between small spattered blood spots and the background. IR photography was adequate for detecting larger stains on porous substrates (cotton, suede and carpet). However, with small spattered stains and on the non-porous surfaces (leather, rubber and vinyl), it was less successful.

In IR photographs of non-porous items the background appeared dark and the contrast with blood was reduced. Fine blood spatter was also impossible to detect, demonstrated on leather in Fig. 4A when compared with hydrogen peroxide in Fig. 4B. On both leather and rubber the only *stains* visible under IR were those that were already visible under white light.

The conclusion from the IR photography results is that it works well when blood has been absorbed into a surface i.e. where stains are large and the item is porous. When blood is on the surface, i.e. when in small volumes, or on a non-porous item, it is not readily detected. This can be shown clearly when comparing IR photographs of blood on cotton against blood on vinyl (Fig. 5). Blood on the vinyl surface, easily visible to the naked eye, became invisible under infrared.

UV light and IR photography were no longer considered for further experimentation at this stage; the inability to work on all surfaces, or to locate small bloodstains, could lead to the missing of blood evidence.

3.3. Specificity

A range of body fluids and household products can be present on items being examined for blood. Luminol in its various formulations has been the subject of a large number of specificity studies [5,25,26], several substances shown to cause false positive reactions have been identified, including iron, copper, a number of paints, vegetables and chemicals. There is no published literature on the specificity of hydrogen peroxide.

Bluestar reacted very faintly with all samples of dog faeces but no other body fluids. This is not necessarily a false positive as dog faeces may contain small traces of blood. Hydrogen peroxide did not produce a reaction with any of the biological fluids except for the control blood. The lack of reaction with dog faeces was potentially a result of hydrogen peroxides sensitivity.

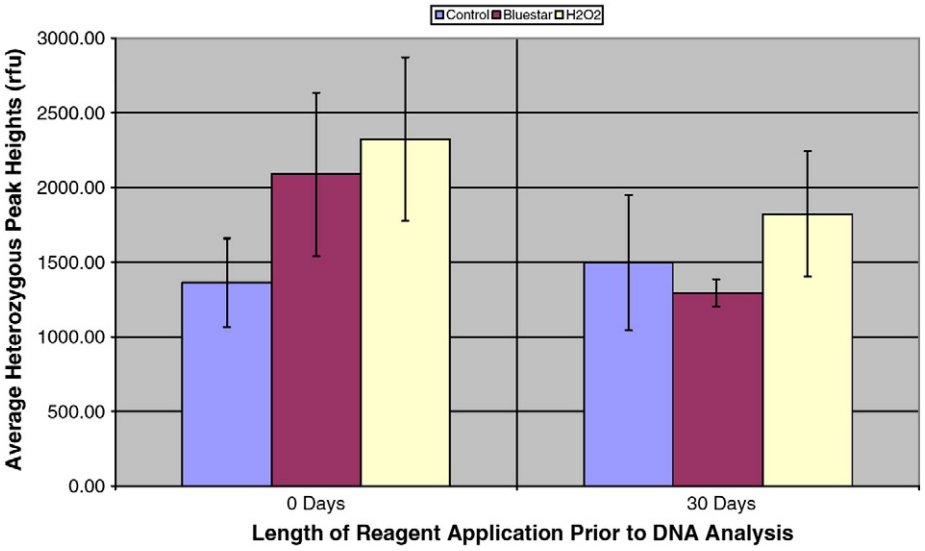

Fig. 9. Average heterozygous peak heights from profiles generated from neat bloodstains, 0 and 30 days after method application; standard deviations included.

Fig. 10. Dried bloodstains after hydrogen peroxide application on leather.

Items that can produce a red or brown stain and others that are commonly found in the home or the laboratory were also tested. Several substances known to produce Bluestar false positives were also used for comparison with hydrogen peroxide. The results of this experiment are shown in Table 3.

Bluestar was expected to react with bleach, banana, turnip and potato, as shown in another study [26]. These reactions were short lived compared to blood and could be distinguished from it. Bleach produced a "firework" effect, with short bursts of bright chemiluminescence, potato, turnip and banana produced a dull chemiluminescent glow, which disappeared after approximately 20 s. With experience these reactions can be distinguished from that of blood. Stains testing positive would also be tested using the KM reagent to confirm the positive result. Banana, bleach, turnip and potato did not react positively with this reagent, therefore would not be misreported as bloodstains.

Application of Bluestar to bleach 24 h after drying produced no chemiluminescence, therefore time is a factor as to whether bleach is a false positive or not. It has been shown that bleach interference is negligible after 8 h when using luminol [27].

Hydrogen peroxide produced no false positive reactions with the household products tested; again this may be a result of its low sensitivity.

3.4. DNA quantification and typing

Once a bloodstain has been located it will likely undergo DNA profiling. Application of a visualization method should therefore have no detrimental effect on the DNA extraction and typing process. Previous studies into DNA typing after Bluestar application to blood indicate that it has no effect on the profiles produced [5,7,28]. One study into the application of 30% hydrogen peroxide to blood suggests that it actually improves the efficiency of PCR by helping to dissolve heme which can cause inhibition [31]. Another study, involving fluorescein, used a 10% hydrogen peroxide solution as the second reagent and demonstrated there was no effect on the DNA profiles generated [7].

Whilst it is advisable to profile treated bloodstains as soon after chemical application as possible, an experiment was performed, leaving treated stains for up to 30 days before extraction, PCR and typing to determine any long term effects.

In both experiments extraction was evaluated by the concentration of DNA detected using real time PCR and typing was assessed by numbers of alleles present and the profile quality.

3.5. Short term application to neat and dilute blood (up to one day)

DNA concentration results for each method were expressed as a percentage of a control mean due to the range yielded. This is presented graphically in Fig. 6.

A one way ANOVA test showed no significant difference between the DNA concentrations recovered by any of the methods tested ($F = 0.53$; d.f. 2; $P = 0.59$), indicating that there was no effect on the extraction process.

Full profiles were generated from all samples, heterozygote peaks were balanced and high molecular weight alleles were not degraded, demonstrating that Bluestar and hydrogen peroxide application did not inhibit PCR or degrade the DNA. Fig. 7 shows the average heterozygous peak heights for each method and controls.

3.6. Long term application to neat blood (up to thirty days)

To determine if chemicals left on blood over a longer period of time could cause degradation, samples were taken immediately from treated and untreated bloodstains, a second set of samples were taken from the same stains 30 days later. The DNA quantification results are presented in Fig. 8.

A one way ANOVA test showed no significant difference between the DNA concentrations yielded at day 0 or day 30 after method application ($F = 0.28$; d.f. 5; $P = 0.91$), indicating that there was no effect on the extraction process.

Full SGM Plus® profiles were generated from all samples, again, heterozygote peaks were balanced and high molecular weight alleles were not degraded. Long term exposure to these chemicals did not appear to have a detrimental effect on DNA. Fig. 9 shows the average heterozygous peak heights for each method and controls.

3.7. Additional considerations

Hydrogen peroxide is cheap to purchase. It comes ready to use at 6% concentration. Application is performed under normal lighting and the results can be recorded on any camera without training or specialist knowledge of photography. Bloodstains treated with hydrogen peroxide can change colour, becoming more readily visible when they have dried, an example of this is shown in Fig. 10. Hydrogen peroxide can cause irritation of the skin, eyes, throat or lungs [29], but following good laboratory practices and wearing appropriate safety equipment reduces any health and safety risks to an acceptable level. Hydrogen peroxide is not flammable and is easily disposed of.

Luminol is more expensive than hydrogen peroxide. Several formulations are available, Bluestar is the easiest to prepare, simply requiring the addition of two tablets to water and allowing them to dissolve, this took approximately 10 min. Application is performed under darkened conditions; a degree of training is needed to produce good photographs and interpret the results accurately. Luminol is considered relatively safe, as long as good laboratory practices (GLP) are maintained [30].

4. Conclusions

The aim of this piece of work was to identify a method of visualizing blood on dark items that could be used alongside the current blood screening procedure to maximize the chances of detecting blood on dark items. All of the methods tested were chosen because they allow blood to be immediately visualized *in situ*. Each has its own merits and had the potential to be used as a replacement. Experiments were performed to find the most effective.

Both fluorescein and Hemascein were disregarded as potential methods due to the excessive background staining, and the inability of Hemascein to react with neat blood. The difficulties of identifying small blood spots using the tested UV light source and IR camera removed these methods from consideration.

The remaining methods, luminol and hydrogen peroxide, produced good results in all experiments. Hydrogen peroxide was less sensitive but had results comparable to an indirect KM test. Taking both the experimental results and method practicalities into consideration luminol is deemed to be the most useful option where blood has been washed or has become dilute. This is due to the greater sensitivity over other methods, allowing latent bloodstains to be easily detected. Although this method can be used on any surface and achieved similar experimental results to hydrogen peroxide, the requirement for almost complete darkness is a significant disadvantage in a working laboratory environment.

Apart from having a relatively low sensitivity, hydrogen peroxide achieved good results for such a simple and inexpensive test. With no false positive substances identified, no effect on DNA extraction or typing and the option to use on any surface, in normally lit conditions; hydrogen peroxide is a viable option on all dark casework items.

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