TECHNICAL NOTE

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Validation Studies of an Immunochromatographic 1-Step Test for the Forensic Identification of Human Blood*

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ABSTRACT: An immunochromatographic 1-step test for the detection of fecal occult blood was evaluated for applicability for the forensic identification of human blood in stained material. The following experiments were conducted: 1) determination of the sensitivity and specificity of the assay; 2) evaluation of different extraction media for bloodstains (sterile water, Tris buffer pH 7.5 provided in the test kit, 5% ammonia); 3) analysis of biological samples subjected to a variety of environmental insults; and 4) evaluation of casework samples.

This immunochromatographic 1-step occult blood test is specific for human (primate) hemoglobin and is at least an order of magnitude more sensitive than previous methods for detecting human hemoglobin in bloodstains. The antigen is insensitive to a variety of environmental insults, except for exposure to certain detergents and household bleaches and prolonged exposure to certain preparations of luminol.

The entire assay can be conducted in field testing conditions within minutes. When in the laboratory the supernatant from a DNA extraction is used for the assay, there is essentially no consumption of DNA for determining the presence of human hemoglobin in a forensic sample. The data demonstrate that this test is robust and suitable for forensic analyses.

KEYWORDS: forensic science, hemoglobin, identification, human blood, bloodstains, luminol, species identification, DNA typing, validation studies

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When biological samples are obtained from a crime scene, at times it may be important to determine if the material is blood and if so, if it is human in origin. The two most often used confirmatory tests for the presence of hemoglobin are the Takayama test (1) and spectrophotometric methods (2). However, these methods can not determine whether or not bloodstains are human in origin and additional tests must be performed. Human specificity assays include immunological tests (2) or DNA-based hybridization assays (3). Therefore, it would be desirable to implement a single simple assay for the detection of human hemoglobin.

An anti-human hemoglobin antibody based system can provide information both on the presence of hemoglobin and human specificity. An overview of immunological tests is provided by Gaensslen (2). In 1994, Spear and Binkley applied an immunochemical fecal occult blood test (HemeSelect™; SmithKline Diagnostics, San Jose, CA) to detect the presence of human hemoglobin in forensic samples (4). The test was able to detect hemoglobin in bloodstain extracts diluted to 1:100 000 and was primate specific. Although effective and scientifically sound, this assay has not gained wide use in the forensic community.

Recently, the Hexagon OBTI Test (5), a test for the detection of fecal occult blood, has been introduced to a few police laboratories in Germany and Switzerland. The test is designed to detect the presence of human hemoglobin in stool samples through the use of an anti-human hemoglobin antibody in an immunochromatographic procedure. The assay offers several advantages compared with the test described by Spear and Binkley. These are: 1) the test is a 1-step immunographic assay, 2) there is no manipulation of the reagents, 3) the test does not rely on an antibody coated cell reagent, 4) the assay takes no more than 10 min, and 5) the assay is at least an order of magnitude more sensitive. Thus, this anti-human Hb assay may prove useful as a confirmatory test for the presence of human hemoglobin in forensic casework analyses. This paper presents the results from a forensic validation study of the Hexagon OBTI test.

Materials and Methods

Immunochromatographic 1-Step Test for the Detection of Fecal Occult Blood

The Hexagon OBTI test was purchased from Human Gesellschaft fuer Biochemica und Diagnostica mbH, Silberbach-

strasse 9, D-65232 Taunusstein, Germany, (Tel. ++49 6128 8750, Fax ++49 6128 875 100). The test kit consists of an all inclusive single test device, a sample tube with Tris-buffer pH 7.5 (containing a mild detergent) as a transport medium for the stool sample and an applicator stick for manipulating the stool sample. The test utilizes mobile monoclonal anti-human hemoglobin antibodies, which are conjugated to blue dye particles and can bind hemoglobin (Fig. 1). The resulting antigen-antibody complexes migrate on the membrane of the test device to a reaction zone, where immobilized polyclonal antihuman Hb antibodies reside. An antigen-antibody-antigen sandwich concentrates the dye particles resulting in the formation of a blue line, indicating the presence of human hemoglobin. Unbound mobile monoclonal antihuman hemoglobin antibodies migrate on the membrane to a control zone, where immobilized anti-Ig antibodies reside (Fig. 1). A complex is formed concentrating dye particles also resulting in the formation of a blue line. The test is considered valid when one blue line in the control zone is observed. With a positive test result, two blue lines will appear; with a negative result, only the control line will be visible (Fig. 1). From all samples used in this study, 100 μL of an extract were placed in the round opening at the end of the device. The test results were read after 10 min.

Determination of Sensitivity and Specificity

Whole blood from 10 individuals and whole blood from Chimpanzee (*Pan troglodytes*), Gorilla (*Gorilla gorilla*), Orangutan (*Pongo pygmaeus*); Celebes black ape (*Cynopithecus niger*) and Spider monkey (*Ateles sp*) were serial diluted to 1:10 000 000 with either sterile water or the Tris-buffer pH 7.5, provided in the kit. Ten μL of whole blood from 10 individuals were placed on cotton cloth, air dried for 24 h, extracted with either sterile water or the Tris-buffer, pH 7.5, provided in the kit, for 2 h at room temperature on a shaker and then serially diluted to 1:10 000 000. Negative controls (sterile water and Tris-buffer pH 7.5) also were prepared and analyzed.

Saliva, perspiration, urine, stool, vaginal secretions, and semen samples were recovered on cotton swabs from 5 male and 5 female volunteers. Each swab was cut in half and extracted in 0.5 mL of the Tris-buffer, pH 7.5, provided in the kit, as well as in sterile water for 2 h at room temperature on a shaker. After centrifugation for 3 min at 13 000 rpm the supernatant was removed and serial diluted to 1:10 000.

The pH-dependency of the test was evaluated using whole blood diluted to 1:1000 with sterile water and the pH adjusted to 1–14 using either acetic acid or NaOH.

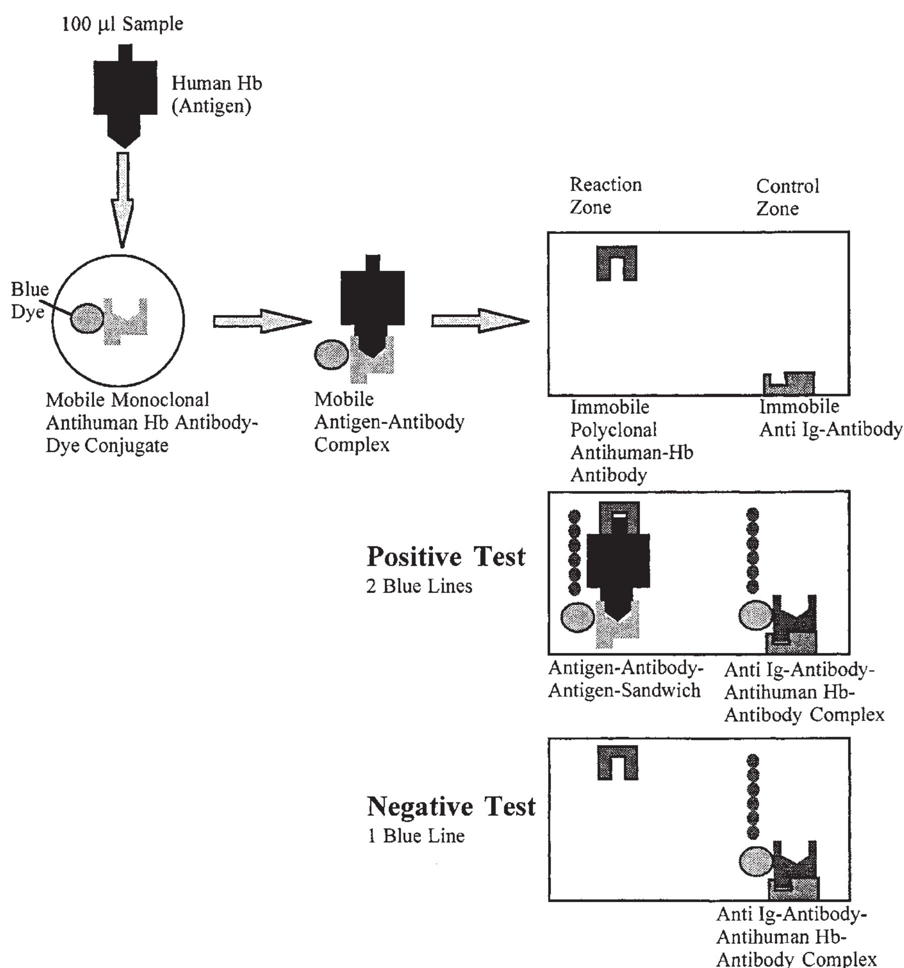

FIG. 1—*Unbound mobile monoclonal anti-human hemoglobin antibodies migrate on the membrane to a control zone, where immobilized anti-Ig antibodies reside.*

Microorganisms (*Bacillus Clostridium, Pseudomonas, Escherichia coli, Streptococcus, Staphylococcus, Candida albicans*) and 5 × 5 mm bloodstains from different animal species: Herring (*Clupea harengus*), Sea trout (*Salmo trutta*); Pigeon (*Columba livia*), Goose (*Anser anser*), Chicken (*Gallus gallus*); Toad (*Xenopus laevis*), Rat (*Rattus rattus*), Guinea pig (*Cavia cutleri*), Rabbit (*Oryctolagus cuniculus*), Cat (*Felis Catus*), Dog (*Canis familiaris*), Badger (*Meles meles*), Lamb (*Ovis aries*), Pig (*Sus scrofa*), Sika deer (*Cervus sika*), Red deer (*Cervus elaphus*), Horse (*Equus caballus*), Cow (*Bos taurus*), Elephant (*Loxodonta africana*); Chimpanzee (*Pan troglodytes*), Gorilla (*Gorilla gorilla*), Orangutan (*Pongo pygmaeus*); Celebes black ape (*Cynopithecus niger*) and Spider monkey (*Ateles sp*) prepared on Schleicher & Schuell paper were extracted in 0.5 mL of the Tris-buffer, pH 7.5, provided in the kit, as well as in sterile water for 2 h at room temperature on a shaker. After centrifugation for 3 min at 13 000 rpm the supernatant was removed and serial diluted to 1:10 000.

Extraction of Aged Blood Stains from Forensic Casework Samples

Eight stains of human blood on clothing from different forensic cases that have been stored at room temperature under ambient conditions for 2–15 years were tested. Extraction was performed using either 0.5 mL of the Tris-buffer, pH 7.5, provided in the kit, or 0.5 mL sterile water. From each stain two 5 × 5 mm pieces were removed, each placed into a separate 2 mL plastic tube and extracted for 2 h at room temperature on a shaker. After centrifugation for 3 min at 13 000 rpm, 100 μL of each supernatant were recovered and tested.

In five of the eight aged blood stains the hemoglobin was insoluble, when sterile water or Tris-buffer was used for extraction. Therefore, from all stains an additional 5 × 5 mm cutting was placed in a microtiter plate and extracted with 0.5 mL of 5% (v/v) ammonia (6,7). The ammonia was allowed to evaporate completely (approx. 30 min) and then 100 μL of the Tris buffer provided in the kit was used to resolubilize the extract.

In addition, 100 human blood stains from recent forensic cases, which tested positive for the presence of hemoglobin using the Sangur® presumptive test for blood (8) and a spectrophotometric method (2) were extracted using 0.5 mL of the Tris-buffer supplied in the kit. In addition, a human (*primate*) specific DNA hybridization assay (3) was performed. The age of the stains was between 1 day and 6 months. All stains were removed from a variety of substrates either by scraping or swabing and ranged from very small particles or flakes to large reddish brown stains. Nine stains, that would not dissolve in the Tris-buffer, were extracted in 5% ammonia as described above.

Degradation Study

Fifty μL aliquots of human blood from 10 individuals were placed on cotton cloth and air dried for 24 h. Samples of deep muscle tissue (50 g) from 10 recently deceased individuals were obtained. All samples were subjected to different environmental conditions for 1 month: (a) frozen at −20°C (control samples), (b) exposed to ambient outdoor conditions during the summer months (rain, sunlight, an average temperature of 25°C), (c) wrapped in plastic wrap and stored under the same conditions as (b), (d) immersed in water taken from a river and maintained at a temperature of 25°C, and (e) buried in soil at a depth of 50 cm.

From each stain two 5 × 5 mm pieces were removed and placed into separate 2 mL plastic tubes and each extracted with 0.5 mL Tris-buffer, supplied in the kit, or sterile water for 2 h at room temperature on a shaker. In addition, from each stain a 5 × 5 mm piece was placed in a microtiter plate and extracted with 0.5 mL of 5% (v/v) ammonia as described above. 100 mg of muscle tissue were extracted using 0.5 mL of the Tris-buffer supplied in the kit, sterile water, or 5% ammonia as described above and the supernatant was used for the assay.

DNA Compatible Assay

Ten μL of whole blood from 10 individuals were placed on cotton cloth, air dried for 24 h and extracted with stain extraction buffer (9), that did not contain detergent or Proteinase K, or extracted with 5% Chelex solution (10), and the supernatant was tested as described above. An additional 100 μL of supernatant was removed to verify the absence of DNA in the supernatant using a DNA hybridization assay specific for human DNA (3). Then detergent and proteinase K were added to the original extract and DNA extraction was continued (9).

Contamination with Detergents

Whole blood from 10 individuals was serially diluted to 1:1000 with sterile water and mixed with a variety of detergents including SDS, sarcosyl, stain extraction buffer used for organic DNA extraction (9) with and without SDS or sarcosyl, Triton X-100, and different brands of soaps and household-bleaches (final concentration of all contaminants in the samples 2%) and kept at room temperature for 1 h. Before testing, the pH of all extracts was determined using litmus paper and adjusted to approximately pH 7.

Contamination with Luminol

Three replicates of 50 μL aliquots of human blood from 10 individuals were placed on cotton cloth and air dried for 24 h. Each aliquot was exposed to a different preparation of luminol. Preparation one and two both used 0.1 g of luminol and 5 g of sodium carbonate dissolved in 100 and 90 mL, respectively, of distilled water (final luminol concentration, 0.1%). In the first protocol, 0.7 g of sodium perborate was added immediately prior to use, while in the second, 10 mL of 3% hydrogen peroxide were added immediately prior to use (2,11). In preparation three, 70 mL distilled water was mixed with a 10 mL solution of 8 g of sodium hydroxide dissolved in 500 mL of distilled water, 10 mL solution of 10 mL of 30% hydrogen peroxide in 490 mL of distilled water, and 10 mL solution of 0.354 g luminol dissolved in 62.5 mL of 0.4 N sodium hydroxide to a final volume of 500 mL distilled water (12). Using an aerosol sprayer each test reagent was added to the stains to produce a strong positive result. Special care was taken to ensure that the entire surface of the stains was covered with the reagent. From each luminol-exposed stain 5 × 5 mm pieces were removed and extracted as described above.

In addition, 50 μL of whole blood from 10 individuals diluted 1:1000 and 1:10 000 were mixed with 50 μL aliquots of the three different preparations of luminol and kept at room temperature for 1, 12 and 72 h. Before testing the pH of all extracts was determined using litmus paper and adjusted to approx. pH 7.

TABLE 1—*Results with various samples.*

Samples	Result
Liquid human blood/Fresh human blood stains	Diluted with water: pos. to 1:100 000 Diluted with Kit-Buffer: pos. to 1:1 000 000
Microorganisms/Different animal species	Neg.
Various human body fluids	Some body fluids tested pos.
pH-dependency of test	Pos. pH 1- 12, Neg. pH >12
High dose hook effect (false negative test)	Not observed when >1:100 fold dilution of whole blood was used
Aged bloodstains from forensic casework	Pos. (up to 15 years of room temperature storage)
Human bloodstains/Human muscle tissue exposed to various outdoor conditions	Pos.
DNA compatible assay	Pos.
Fresh human bloodstains contaminated with soaps, detergents, and household bleaches	Pos./Neg. (depending on brand, concentration, time)
Liquid human blood/Fresh human blood stains contaminated with luminol (3 preparations)	Pos./Neg. (depending on preparation, dilution of blood, time)

Results and Discussion

A summary of the results is presented in Table 1.

Determination of the Sensitivity and Specificity

All whole blood samples from human donors and blood samples from Chimpanzee (*Pan troglodytes*), Gorilla (*Gorilla gorilla*), Orangutan (*Pongo pygmaeus*); Celebes black ape (*Cynopithecus niger*) and Spider monkey (*Ateles sp*) tested positive for human hemoglobin to a dilution of 1: 100 000 when sterile water was used to dilute the sample. However, when Tris-buffer provided in the kit was used to dilute the whole blood, dilutions up to 1:1 000 000 tested positive. Therefore, as little as 500 total erythrocytes are required for a positive result. Additional components, in the Tris buffer provided in the kit, such as a mild detergent, may enhance the sensitivity of the assay. Only a few positive reactions were observed for blood samples diluted more than 1: 1 000 000. Similiar results were obtained from fresh bloodstains. For the negative control samples only the control line was visible.

In the species specificity experiments only human and primate blood tested positive with the assay. These data suggest that the assay is primate specific. Serial dilutions of human and primate blood revealed no difference in the reactivity.

Similiar to the findings of Spera and Binkley some of the extracts of saliva, urine, stool, vaginal secretions, and semen yielded a positive reaction with the assay. Perspiration tested negative in all cases. These results were anticipated since hemoglobin may be present at low concentration in various body fluids.

When the pH-dependency of the test was evaluated, a positive test result was obtained when the pH of the supernatant ranged from 1–12. At a pH $>$ 12 the test was always negative, but if the pH was adjusted to approximately pH 7 the very same samples tested positive.

High Dose Hook Effect

When performing immunoassays the concentration of the antigen and the titer of the antibody have to be considered. Obviously, an antigen concentration in a sample below the sensitivity of detection of the assay will yield a negative result. A negative result also may be obtained when too high a concentration of antigen is assayed. The latter situation is known as high dose hook effect. In the Hexagon OBTI test excess hemoglobin will overwhelm the mobile monoclonal antihuman Hb antibodies and unbound hemoglobin will migrate towards the reaction zone, where the immobilized polyclonal antihuman Hb antibodies reside. Free unbound hemoglobin binds to the immobilized antibodies and prevents these antibodies from reacting with the antigen-mobile antibody complexes (Figs. 2, 3). The result will be a false negative. The manufacturer of the Hexagon OBTI test states a high dose hook effect has been observed at concentrations of hemoglobin of 2 μg/μL. We confirmed this observation and recommend that a dilution of blood at least 1:100 should be used to avoid the high dose hook effect. This can be achieved either by extracting a smaller portion of a sample, by increasing the extraction volume, or by diluting the extract prior to assay. Hemoglobin loses its visible color at whole blood dilutions greater than about 1:1000 (approximately 0.2 μg Hb/μL). This may be used as a guide for avoiding the high dosis hook effect.

Extraction of Aged Blood Stains from Forensic Casework

Out of 108 human blood stains, 14 were insoluble in either Tris buffer or water. These stains were extracted using 5% ammonia (6,7). All aged blood stains yielded positive results for human hemoglobin and a positive signal when using a human (*primate*) specific DNA hybridization assay (3). These results demonstrate, that ammonia does not appear to have a deleterious effect on the immunological reaction of this assay.

Degradation Study

All samples of the degradation study, even heavily putrified human muscle tissue, yielded positive results. Thus hemoglobin appears to persist in samples exposed to extreme conditions. This demonstrates the usefulness of the test for the identification of the origin of recovered remains, such as putrified soft tissue.

DNA Compatible Assay

All human blood samples yielded positive results. In the aliquots that were removed to determine the quantity of DNA present in the portion of the extract used for the detection of human hemoglobin there was no detectable DNA. Therefore, no DNA is consumed when assaying for human hemoglobin during a DNA extraction.

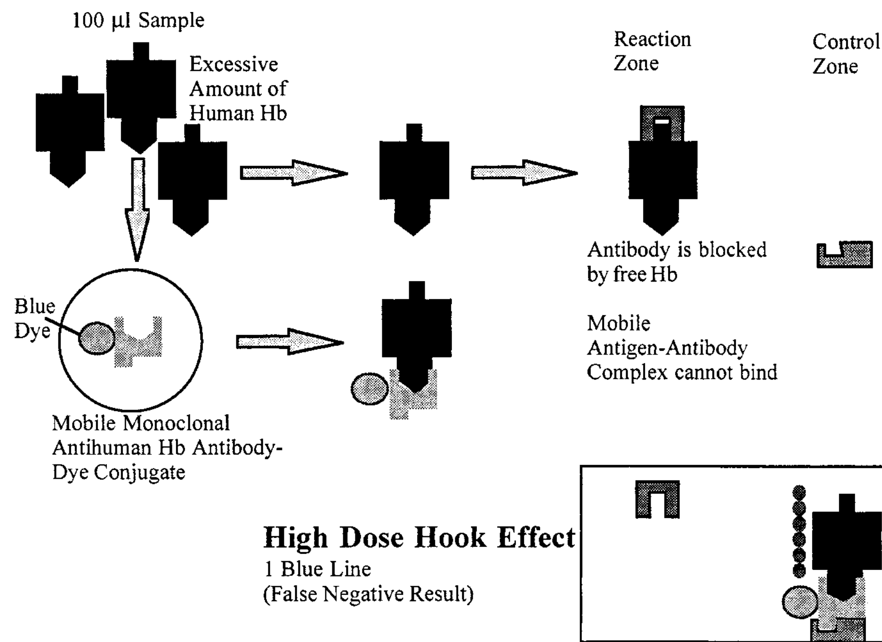

FIG. 2—*Free unbound hemoglobin binds to the immobilized antibodies and prevents these antibodies from reacting with the antigen-mobile antibody complexes.*

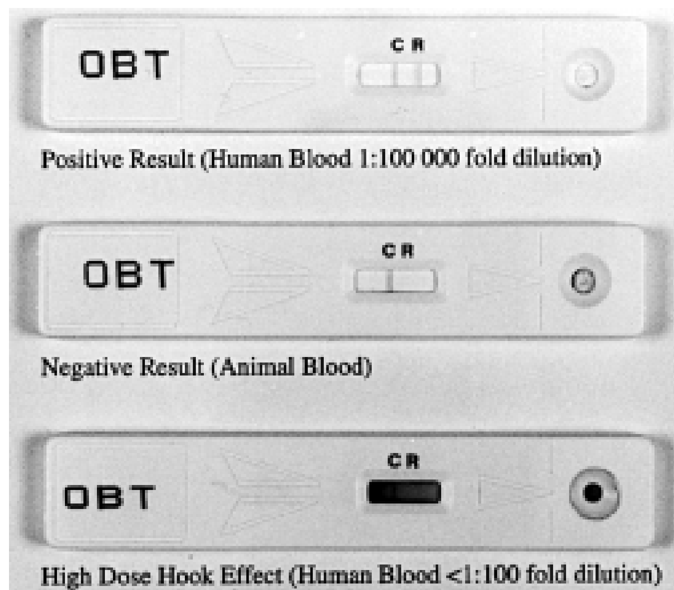

FIG. 3—*Immunochromatographic 1-step test for the identification of human blood. One hundred µL of the extract were placed in the round opening at the end of the device. A positive result is indicated by the formation of a blue line in the reaction zone (R) and in the control zone (C). A negative result is indicated by the formation of a blue line in the control zone. The high dose hook effect is a false negative result due to excess hemoglobin in the sample and indicated by the formation of a blue line in the control zone only.*

Contamination with Detergents

When human blood was mixed with SDS or sarcosyl (ionic detergents), at a final concentration of 2%, no human hemoglobin was detected in the assay, presumably due to denaturation of proteins and/or interference with protein-protein interactions. Triton X-100 (a non-ionic detergent) at the same concentration yielded a weak positive signal. The mixture of blood with stain extraction buffer without SDS or sarcosyl yielded a strong positive result, whereas stain extraction buffer with SDS or sarcosyl yielded a negative result. Therefore the use of a detergent to improve the DNA extraction should be avoided, if an anti-Hb assay is intended to be performed.

It could be anticipated that an individual might attempt to clean bloodstained items. Contamination of blood with different brands of soap at a final concentration of 2% yielded positive results. However, contamination with various household bleaches in the same concentration yielded either an extremely weak positive signal or a negative result, presumably due to the denaturation of proteins and/or inhibition of the immunoassay (data not shown). When the pH of the supernatant was adjusted to pH 7 before testing, the samples still yielded a negative result.

Contamination with Luminol

Luminol is often used in crime scene investigations to presumptively detect blood. Therefore, stains that arrive at the forensic laboratory for analysis may be contaminated with luminol and related reagents. Of the luminol preparations tested, only preparation one and two yielded detection of hemoglobin in all contaminated blood samples throughout a 72 h period. However, it was necessary to adjust the pH of the supernatant to <12 in order to obtain a positive result. Blood mixed with luminol preparation three tested positive only at a 1:1000 fold dilution of blood up to 1 h of exposure. Presumably certain components of the third luminol preparation, such as sodium hydroxide have deleterious effects on the immunoassay, depending on the dilution of blood and time of exposure. Therefore, preparation one and two may be considered the most desirable of the three preparations for the detection of blood at crime scenes. Furthermore, if samples were exposed to luminol, it is necessary to check and adjust the pH of the extract prior to testing to <12.

TABLE 2—*Test procedure. Test of suspected blood stains for the presence of human hemoglobin (field testing/laboratory testing).*

Apparently Fresh Stains	Aged Stains	Stains Exposed to Luminol
Extract a small portion of the stain in a microfuge tube or a microtiter plate with an appropriate amount of the Tris-buffer provided in kit, water, 5% Chelex solution, or any other suitable buffer that does not contain SDS/Sarcosyl or Proteinase K	If insoluble, extract with an appropriate amount of 5% ammonia. Let ammonia evaporate. Resolubilize with buffer provided in kit, water, or any other suitable buffer that does not contain SDS/Sarcosyl/Proteinase K	Extract with one of the described methods. Check and adjust pH before testing.
Use 100 μL of supernatant for confirmatory test		

TABLE 3—*Confirmatory testing. Confirmatory test for the presence of human hemoglobin during DNA-extraction.*

Depending on age and condition of the stain use one of the methods described above to resolubilize the stain
Centrifuge briefly
Use 100 μL of supernatant for confirmatory test
Continue DNA extraction

Conclusions

The Hexagon OBTI test has been demonstrated to be a powerful and robust tool as a confirmatory test for human blood, because it is human specific (or at least primate specific) and is suitable for use with both aged and degraded material. It can be a useful tool for either field testing or laboratory testing (Table 2). The antigen is insensitive to a variety of environmental insults, except for exposure to certain detergents and household bleaches and prolonged exposure to certain preparations of luminol. This immunoassay is compatible with highly solubilizing extraction procedures, such as those that use ammonia, that may be needed for the extraction of aged blood stains.

While this assay is specific for primate hemoglobin, it is not specific solely for blood. Body fluids other than blood may contain trace amounts of hemoglobin and yield a positive result. However, this observation should not be a problem for the detection of human blood in the large bulk of cases.

Most forensic laboratories characterize human blood stains using DNA-based procedures. This assay is compatible with the current DNA extraction procedures with slight modifications (Table 3). It enables the analysis of human hemoglobin and DNA comparable to results that would be obtained if each test was done individually and has the ancillary benefit of not consuming any DNA in the sample.

This test is simple to perform, requires a minimum amount of equipment, produces results that are easy to interpret and is relatively rapid to perform. Furthermore, the test was found to be at least one order of magnitude more sensitive than current presumptive or confirmatory tests described in the literature. The procedure can be easily implemented in the forensic laboratory.

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