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Review Article

Bloodstain Evidence: From Human Blood Identification to DNA Profiling

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Abstract

Stains observed on a crime scene may correspond to biological fluids and therefore contain DNA which can identify a suspect or a victim as well as exonerate an innocent individual. However, fluids at a crime scene may be difficult to detect with the naked eye and may require specific identification procedures including alternative light sources. After a bloodstain has been evidenced, specific techniques (mainly chemical which, in contact with blood, change color or induce fluorescence or chemiluminescence) are used to proof the presence of human blood. Since blood evidence can help to solve the case, it is essential to adequately collect and preserve biological specimens. Continuous technological progress in biology has led to major developments in the field of forensic DNA profiling in the past 10 years. Restriction fragment length polymorphism (RFLP) profiling may be considered as the first generation of DNA analysis methods. It is no longer used as it requires large amounts of DNA, and because degraded samples cannot be analyzed with accuracy. Technological approach was then based on Polymerase Chain Reaction (PCR) techniques and PCR-RFLP. Modern DNA analysis methods now include Short Tandem Repeat (STR), Single Nucleotide Polymorphism (SNP), mitochondrial DNA (mtDNA) analyses and DNA methylation studies. In this review we analyze some of the recent progresses made in the forensic analysis of DNA. Among these, Next Generation Sequencing (NGS) now has the potential to expand the capabilities of information on STR alleles including DNA database construction, ancestry and phenotype prediction.

ABBREVIATIONS

ALS: Alternative Light Sources; BPA: Bloodstain Pattern Analysis; CAS: Chemical Abstract Service; CODIS: Combined DNA Index System (USA); ELISA: Enzyme-Linked Immunoabsorbent Assay; EMPOP: European DNA Profiling Group mtDNA Population Database; ENFSI: European Network of Forensic Science Institutes; ESS: European Standart Set; FBI: Federal Bureau of Investigation; HPLC: High-Performance Liquid Chromatography; HV: Hypervariable; MtDNA: Mitochondrial DNA; mRNA: Messenger RNA; NDIS: National DNA Index System (USA); NDNAD: National DNA Database (UK); NGS: Next Generation Sequencing; PCR: Polymerase Chain Reaction; RFLP: Restriction Fragment Length Polymorphism; SNP: Single Nucleotide Polymorphism; STR: Short Tandem Repeat.

INTRODUCTION

Stains observed on crime scenes may correspond to biological fluids such as saliva, sperm, urine or blood and may therefore contain DNA evidence which can identify a suspect or a victim as well as exonerate an innocent individual (for review, see Virkler and Lednev, 2009 [1]). However, biological fluids at a crime scene may be difficult to see with the naked eye and may require specific identification procedures.

The presence of blood on a crime scene is one of the most common, and naturally one of the most informative findings in death investigations. After a bloodstain has been detected, specific techniques (mainly chemical which, in contact with blood, change color or induce fluorescence or luminescence) are used to proof the presence of human blood. Biological fluids often require particular light or chemical additions to reveal their presence: methods used to achieve this goal are called "presumptive tests". Such methods are by no means conclusive by themselves, and further analysis remains essential.

When formally identified, the blood sample must be collected and stored appropriately to preserve its integrity as best as possible for further genetic analyses. Factors that can lead to DNA degradation include time, temperature, humidity, ultra-

violet light and exposure to various chemical substances, some of which being used for presumptive evidence of blood. However, the strength of DNA lies in the fact that even though it can be partially degraded, testing remains possible for months or even years afterwards.

Locating and interpreting bloodstains on various surfaces of the crime scene give useful information on the sequence of events that might allow crime scene reconstruction. This area of forensic investigation, known as bloodstain pattern analysis (BPA) is currently federated by the International Association of Bloodstain Pattern Analysts (IABPA), founded in 1983. BPA attempts 1) to collect the size, shape, location and distribution of bloodstains and 2) to determine the most probable sequence of actions that have created the bloodstain pattern at the crime scene. Numerous publications have been devoted to BPA: for review, see [2,3].

The present review, however, will not address BPA. It will only focus on the currently available blood identification methods, including age estimation of bloodstains. It will also address methods to confirm the sample as human blood prior further testing, with special emphasis on the potential adverse effects of these tests on biomarker analysis and DNA profiling.

Collection and preservation of blood evidence

Documentation, collection and preservation of the samples associated with a crime scene is essential in order to study the case. Examination of the scene should lead to note, record and/or photograph any stain using, for suspected bloodstains, color print films and/or infrared films to document traces on dark surfaces. Once the scene crime has been thoroughly documented, the collection process can begin. Every collected bloodstain must be stored separately to avoid contamination. Samples of wet bloodstains must be collected using a swab, later sealed in an airtight container. Dried bloodstains should be scraped onto a sheet of clean paper, or into a paper bag (for review, see G. Schiro. Collection and Preservation of Blood Evidence from Crime Scenes. Crime Scene Investigator Network).

Blood visualization enhancing methods (presumptive blood identification tests)

Examination of bloodstains under various light sources: Crime scene analysts first document visible stains and patterns on site. In some cases, bloodstains can be evident to detect. However on some media, vizualization of bloodstains can be difficult because of lack of contrast and/or absorption of the fluid. Stains are sometimes almost invisible under natural, polychromatic light because they are easily confused with the support. Therefore, alternative light sources (ALS) are useful to make stains fluorescent or to improve their contrast.

Bloodstains have a very high absorption rate, ranging between 300 and 900 nm, which covers wave lenghts from ultra-violet to infra-red. This explains the growing utilization of ALS, particularly of electro-luminescent diodes with high intensity such as CrimeLite® (distributed by Foster + Freeman Ltd., Evesham, Worcestershire, United Kingdom), CrimeScope® and Mini-CrimeScope® (distributed by SPEX Forensics Headquarters, Edison, NJ, USA, and by Horiba, Ltd., Kyoto, Japan), TracER™ Laser (Arrowhead Forensics, Lenexa, KS, USA) and Ultra-Lite ALS® (distributed by SPEX Forensics Headquarters, Edison, NJ, USA, and by CAO Group, Inc., West Jordan, UT, USA). Light beams of different wavelengths allow detecting traces of different fluids such as blood, saliva, and sperm on various materials (cotton, metal, glass, wood, carpet, brick, clothes or other fabrics). For the CrimeLite® device, the range includes one white polychromatic and eight narrow band light sources with wavelengths in ultraviolet, violet, blue, blue-green, green, orange, and red spectra for detecting fluorescent stains.

Both techniques allow to detect red or brown bloodstains (following decay process), indicating the existence of crime scenes. These methods also allow determining if the scene has been cleaned after the crime, which would require further chemical techniques [4,5].

Commonly used chemical techniques: In order to obtain as much information as possible, investigators search for latent bloodstains and patterns. Latent bloodstains may be the result of old scenes, scenes altered by weather conditions or fire or cleaned in an attempt to destroy evidence. As a consequence, invisible or cleaned bloodstains need specific forensic detection methods. Presumptive tests with chemiluminescent reagents are the most useful, revealing latent bloodstains invisible to the naked eye.

Several chemical products are diversely used to visualize latent bloodstains. Some have a direct and known negative effect on DNA such as ortho-tolidine, benzidine and tetramethylbenzidine (the latter two being recognized as carcinogen and no longer used), while some others can improve both visualization and DNA analysis after sampling (Amido Black®, luminol).

Amido Black (CAS Number 1064-48-8, distributed by Merck KGaA, Darmstadt, Germany) is a biological dye that stains the hemoglobin and produces a dark blue-black image. It is useful in developing latent fingerprints contaminated with blood (Figure 1, Right). This method has no influence on DNA analysis [6]. Amido Black can be used on almost all surfaces, porous and non-porous. However, both the water and methanol-based formulations have issues associated with their use: in particular, the methanol-based formula reveals highly flammable and toxic, possibly damaging some surfaces. It is therefore preferably used in the laboratory area on non-porous surfaces that will give no adverse reaction. Experiments have shown that an ethanol/water-based formulation was as effective as the water-based formulation and could be used on all surfaces [7].

Acid yellow 7 (CAS Number 2391-30-2, distributed by BVDA America Inc., New Bedford, MA, USA) is a dye used to point out fingerprints and shoeprints made in blood (Figure 1, left). Prints become yellow after treatment and fluoresce under blue/blue-green light (385-509 nm). This solution is not suitable for absorbent surfaces such as paper, cardboard, sheets or carpets. It is also very useful on non-absorbent substrates such as linoleum, glass, bricks, or painted surfaces. Before staining, blood prints should be fixed to prevent fading when the working solution is applied. Prints must be fixed with a 2% solution of 5-sulphosalicylic acid [8]. This method needs complete darkness and utilization of specific light sources (Crime Lite®, CrimeScope®).

Figure 1 Bloody fingerprints revealed with Acid Yellow 7 (left) and Amido Black (right). Fingerprints are stained yellow after treatment with Acid Yellow 7 and then fluoresce under blue/blue-green light (385-509 nm). Amido Black stains the proteins of blood in a blue-black color. Amido Black can be either methanol or water based as it readily dissolves in both. Images obtained from an exercise made by the Gendarmerie Nationale in Orléans, France.

With time, bloodstains undergo alterations and their color changes from red to more or less dark brow [4]. In such cases, the luminol (CAS Number 521-31-3, distributed by Merck KGaA, Darmstadt, Germany) is a frequently used reagent, particularly if the crime scene has been cleaned. This chemiluminescence method is used for a long time to detect latent blood traces by police investigators because it is easy to handle on site. It does not pose health concerns, while providing trace evidence for DNA analysis, contrary to other reagents that have harmful effects on DNA profile determination [9].

When sprayed on bloodstains luminol reacts with the iron of the hemoglobin, which causes a blue chemiluminescence in the dark (Figure 2). Luminol is water-based. It should be used immediately on site after mixing its components since its sensitivity will diminish within two hours. Porous surfaces, retaining blood, give better results than non-porous surfaces.

Luminol has several drawbacks and it should not be used as a first choice. Investigator should first use ALS to search for traces of blood before applying any reagent. Luminol may give false reactions: it reacts with copper ions, copper compounds, iron compounds, and cobalt ions. It also reacts with potassium permanganate found in some dyes and with hydrated sodium hypochlorite (bleach). Ferricyanide and plant peroxidases also may give false positive results. It has also been shown that luminol may cause the loss of several genetic markers. However, DNA can be successfully extracted from samples treated with luminol reagent [10].

Luminol is the method of choice for examining clothing. Clothes worn during the offense are often destroyed or washed afterwards by the offenders [11]. A recent study showed that luminol could detect bloodstains even when concealed by several layers of paint (intensity of chemiluminescence depending on the nature of paint and the number of layers) and on various surfaces such as cement, wood, metal [12].

Luminol being sensitive to dilute bloodstains, it is sometimes used to enhance bloody impressions (shoeprints, fingerprints). However luminol is not the best reagent for this purpose because of its water base. Better methods for enhancing bloody impressions use either rapidly evaporating organic solvents (such as merbromin and ortho-tolidine, but this latter technique is nowadays avoided as derivative of benzidine are carcinogens) or an alcohol-based medium which acts as a fixative, such as in the ethanol/water-based Amido Black technique.

Similar to the luminol reagent, the Bluestar® (Bluestar® Forensic kit, distributed by Bluestar USA, Huntersville, NC, by Arrowhead Forensics, Lenexa, KS, USA, and other suppliers) utilizes hemoglobin's peroxidase-like activity. The extreme sensitivity of Bluestar® allows the detection of bloodstains down to 1:10,000 dilutions, including minute traces that have been washed out, with or without detergent. Unlike some other blood reagents, total darkness is not required. BlueStar® reacts as well as luminol on all substrates. Overall, BlueStar® is found to give longer reactions than other luminol kits, because of its ability to detect more dilute stains and of longer operational applicability than noted in the manufacturer's instructions [13].

Other chemical techniques: Most can be grouped under the term "catalytic tests". These methods depend on the fact that the hem group of hemoglobin possesses a peroxidase-like activity which catalyzes the breakdown of hydrogen peroxide. The oxidizing derivatives formed can then react with a variety of substrates to produce a visible color change. Among substrates/tests commonly used, one can find:

The Kastle-Meyer test (distributed by WA Products Ltd, Burnham on Crouch, Essex, UK) in which the chemical indicator

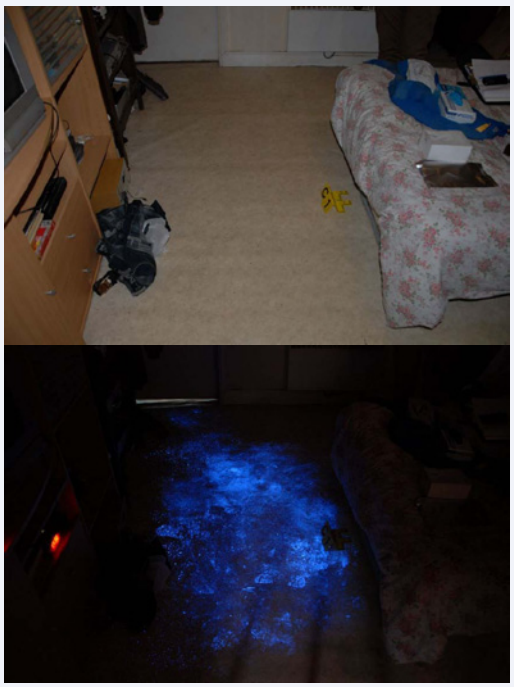

Figure 2 Luminol is a chemical that exhibits chemiluminescence, with a blue glow, when mixed with an appropriate oxidizing agent (here, the iron of hemoglobin). Bloodstain patterns can be enhanced using luminol. In this case, an attempt to cleanup the victim's blood and shoeprints made in blood can be seen on the carpet where nothing was visible prior to enhancement.

phenolphthalein is used to detect the possible presence of hemoglobin: in this test, the reduced phenolphthalein is kept in alkaline solution in the presence of zinc. The solution is colorless. Oxidation with hemoglobin and peroxide causes an immediate color change to bright pink. [14]. However, there are several possible interferences: thus, the reagent is known to react with some plant materials, cleaning agents, metals, metal salts, and other sources of iron [15]. In its original, one-step formula, a small amount of the Kastle-Meyer reagent is mixed with equal volumes of 95% ethanol and 10% hydrogen peroxide solution. The suspected bloodstain is rubbed gently with a small piece of filter paper and a drop of the mixed reagent is added to the paper. The development of a pink color indicates the presence of hemoglobin. However, the test may give false-positive result with other oxidizing materials.

In the two-step version of the test, the Kastle-Meyer reagent is mixed only with an equal volume of 95% ethanol. The solution is first added to the filter paper on which the suspected bloodstain has been rubbed. If a pink or red color develops at this point (without the addition of hydrogen peroxide), the stain in question is not blood. If there is no reaction at this point, a drop of hydrogen peroxide solution is added, and the presence of a pink color will then indicate the likely presence of blood.

The reaction is time sensitive, and the results only reliable within 10-15 seconds (in most laboratory protocols) [16]. After 10-15 seconds a false positivity will result. Phenolphthalein is considered as one of the most specific reagents with sensitivity for the detection of blood of 1/10,000 ppm [17,18]. However, the Kastle-Meyer test gives the same reaction with human blood as with any other non-human hemoglobin-based blood, so a confirmatory test such as the Ouchterlony test (see infra) must be performed to definitively conclude if the blood found on the crime scene is of human origin.

Sangur sticks (Hemastix®, originally designed for testing for blood in urine, owned by Bayer AG, Leverkusen, Germany), are quick and easy disposable materials applicable as a presumptive test for the presence of blood. The detecting reagent only requires to be rubbed on the suspected bloodstain, and moistened. The strips contain diisopropylbenzenedihydroperoxide and 3,3',5,5' tetramethylbenzidine. The test is based on the peroxidase-like activity of hemoglobin which has the ability a) to cleave oxygen molecules from H_2O_2 and b) to catalyze the reaction from the reduced colorless form of 3,3',5,5' tetramethylbenzidine to the oxidized colored form. An immediate color change from pale yellow to orange indicates the probable presence of blood. High concentrations of blood may cause the color development to continue to an intense greenish blue.

Hemastix® has several drawbacks: it does not detect the difference between human and animal blood and it can severely reduce the ability to recover DNA from any suspected stain [19]. It has also been shown that Hemastix® may return false-positive results from negative controls [20].

Leucomalachite green (CAS Number 129-73-7, distributed by Merck KGaA, Darmstadt, Germany) is used as a detection method for suspected bloodstains, since hemoglobin catalyzes the reaction between leucomalachite green and hydrogen peroxide, converting the colorless leucomalachite green into malachite green. This colorimetric test is often used. It is performed by rubbing a cotton swab that has been moistened in a sterile solution on the suspected bloodstain [16]. If a color reaction occurs before the hydrogen peroxide is added, it indicates the presence of a chemical oxidant and the test result should be considered inconclusive [15]. It is considered by some authors as the most specific but least sensitive of the presumptive blood detection reagents [18]. Leucomalachite green is as specific of blood as luminol, but its sensitivity is 10 times lower than luminol and it may alter the DNA in some situations [21]. Like luminol, leucomalachite green is preferably used when suspected bloodstains have been cleaned up from a surface, or to develop faint bloody prints on a lightly colored carpet or floor.

Leucocrystal violet (CAS Number 603-48-5, distributed by Merck KGaA, Darmstadt, Germany, and by Tritech Forensics, Leland, NC, USA for the Leucocrystal Violet Presumptive Blood Test Kit) reacts with the heme group of hemoglobin to give a purple color. It is used to identify bloodstains that are not visible and to enhance contrast to visible bloodstain patterns. This reagent contains hydrogen peroxide, so if used on heavy bloodstains may cause loss of details due to foaming. Leucocrystal violet is typically used on porous surfaces. Leucocrystal violet needs to be studied with ALS, any of the multiple forensic light sources readily available including, but not limited to the CrimeScope®, Mini-CrimeScope®, TracER™ Laser and Ultra-Lite ALS® as already described[22].

All these tests are extremely sensitive but are subject to a number of interferences. They are therefore not totally specific for blood. Results must be interpreted with caution particularly when testing outdoors, where many types of plant materials can be present, or when testing in vehicles where metal surfaces can interfere. The general principle is that if the test is negative, blood is absent, but if the test is positive, blood is probably, but not definitely identified. For this reason these tests are often described, as already said, as "presumptive" tests.

Species determination of bloodstains

It is not always apparent whether the blood found on a crime scene is human or non-human. The traditional standard in forensic science laboratory is to apply presumptive tests for blood and to confirm the sample as human before performing further DNA analyses. To determine if the bloodstain is of human origin, a simple "precipitin" test can be used: blood contains proteins which vary between species, meaning that the blood of a given species may precipitate when it is in contact with circulating antibodies of other species. Serums for such tests are commonly obtained from rabbits after human blood has been injected to the rodent. The resulting anti-human serum is therefore added to the suspected bloodstain. If the blood is of human origin, the rabbit anti-human serum will precipitate its proteins.

Species determination of bloodstains can be tested by various commercial methods including the Ouchterlony and the OneStepABAcard® Hematrace™ methods.

The Ouchterlony double diffusion method (also known as agar gel immunodiffusion) was first described in 1949. It is a classic and simple technique that permits evaluation and comparison of antibodies in animal or human sera against proteins or complex carbohydrate antigens. It is a low-technical procedure involving the use of agar gel plates with wells for both antibodies and antigens. The soluble antigens and antibodies

diffuse into the gel where they form an insoluble complex and precipitate. The Ouchterlony method allows both qualitative and semi-quantitative evaluation of the reactants [23].

The ABAcard® Hematrace™ method (distributed by Abacus Diagnostics, West Hills, CA, USA) consists of a plastic card with two windows, one of which allowing the sample to be applied to the test membrane and another Interpretation window allowing the analyst to view the antigen-antibody reaction [24]. If human hemoglobin is present in the suspected bloodstain, it will react with the monoclonal anti-human antibody present in the test membrane and a mobile antigen-antibody complex will form. This complex can migrate through the HemaTrace™ absorbent membrane towards the test area. In this area, a polyclonal anti-human hemoglobin antibody is located. This antibody captures the above-mentioned complex so that an antibody-antigen-antibody sandwich is formed. When the human hemoglobin concentration is above a minimum detection limit (0.05 µg/ml) the purple dye particles present in the device form a purple band at the interpretation area which is indicative of a positive result for the presence of human blood.

The test shows a high specificity for human blood and it seems that positive results are unaffected by a variety of contaminants and under a range of conditions, with the exception of stains subjected to a tumble-dryer cycle or prolonged exposure to soil [25]. The test shows a good specificity for human, higher primate, and ferret blood. Known animal blood samples from deer, cow, pig, horse, dog and cat were also tested negative with the HemaTrace™ method. Human saliva and urine give negative results [18]. The detection threshold of the kit was determined to be 0.07µg of hemoglobin/mL.

Non-human DNA may also be identified by modern genome profiling methods. Very high sensitivity can be obtained with DNA testing due to amplification of target regions with the polymerase chain reaction (PCR). DNA results can be obtained from as little material as a single cell [26]. However, when such high-sensitivity techniques are used, a possibility exists for contamination from DNA coming from a technician on the crime scene under investigation. If consumables such as swabs or tubes are not DNA-free, then a foreign, non informative DNA may be detected. Examination of negative controls is therefore necessary in order to prevent drawing incorrect conclusions if DNA contamination occurs [27,28].

Age estimation of bloodstains

No reliable methods are currently available to accurately establish the age of a bloodstain on a crime scene. The risk of inaccuracy increases with the age of the bloodstain and with the conditions of blood deposition since temperature, humidity and exposure to sunlight may lead to oxidation and denaturation of hemoglobin.

Non genomic methods: Correct assessment of the age of a bloodstain is difficult and it is necessary to associate several techniques. Some authors have used a) high performance liquid chromatography (HPLC), which is able to identify and quantify the hemoglobin derivatives, b) reflectance spectroscopy (a white light source, spectral range 450-700 nm and a spectrometer can detect the variation of bloodstains from red to brown), c) oxygen electrodes (determine the amount of HbO_2), d) electron paramagnetic resonance (measures the change of the iron ion after denaturation of hemoglobin), and e) atomic force microscopy (determines the elasticity of extra-corporeal red blood cells) [29].

Reflectance spectroscopy with microspectrophotometry may determine the age of a bloodstain up to 19 days old with an average error of less than one day, whereas in bloodstains from 23 to 37 days, the average error in age determination would be about 3 days [30]. In a study using infra-red spectroscopy, the age of bloodstains up to one month old was estimated successfully with a mean error of prediction of 8.9% [31].

RNA- and DNA-based methods: In dried bloodstains, the relative ratios of two different RNAs, using real-time reverse transcriptase PCR (RT-PCR) may allow to estimate the age of a bloodstain. 18S-actin and β-actin (microfilaments of the cell cytoskeleton) are expressed in all cell types at high levels, allowing detecting their RNA products. With time, the ratio increases as the value of 18 S-actin does not appreciably change whereas the value for β -actin significantly reduces. This method is advantageous as it is independent of the sample size, allowing handling relatively small samples. Additionally, this method allows isolating both RNA and DNA, which is useful to identify the perpetrator [32]. Hara et al. (2016), pointed out that short tandem repeats (STRs: see infra) present in the DNA as well as messenger RNA (mRNA) markers could be detected up to 20 years because of great stability when stored at -20˚C to -80˚C in darkness. On the contrary, DNA from bloodstains stored at room temperature or at +4°C were significantly degraded compared to DNA from all other samples [33]. Storage temperature and dryness are therefore important factors, influencing mRNA and DNA stability. Therefore, to prevent both RNA and DNA degradation during long-term storage, it is recommended that bloodstains and blood be stored below -20˚C.

Another method for estimating the age of a bloodstain is to quantify the degree of RNA fragmentation, which is significantly correlated with the post-mortem interval [34,35]. RT-PCR allows quantification of RNA degradation level. But this method cannot be used as a rapid procedure, since RNA degradation does not occur before 3 to 4 days post-mortem.

ABO blood group recognition and genotyping

In the early seventies and until the 90s, most crime laboratories relied upon the ABO blood grouping system to characterize a bloodstain. However, blood type alone usually cannot positively identify a suspect because many people share the same blood type. For example, blood group A is shared by one-third of the world population, mainly Caucasians and persons of European origin. Blood Type O is common among Native Americans and Latin Americans. Type B is seen commonly among African Americans and AB is most frequent in Japanese individuals. This simple type of classification can be useful when analyzing a case; however ABO grouping has a low discrimination power. Several serological techniques exist, including absorption-elution or absorption-inhibition assays, mixed cell agglutination reaction and enzyme-linked immunoabsorbent assay (ELISA). A recent study showed interest of an indirect competitive ELISA technique, as conventional ELISA needs immobilization of ABO blood group antigens located on the membrane surface, which are difficult to extract from dried stains, except if one uses solubilization with detergents or organic solvents [36].

Genetically, differences in nucleotide sequences of ABO alleles may lead to powerful ABO genotyping methods. Multiplex systems simultaneously targeting ABO and STR genotypes in a rapid procedure have been developed: the one described by Jiang et al. (2012) allows for the simultaneous detection of 15 autosomal STR loci containing all CODIS STR loci (see infra: DNA databases) as well as Penta D and Penta E, six ABO genotypes (O/O, B/B, A/A, A/O, A/B, and B/O) and the gender-determining locus Amelogenin [37], Such multiplex systems may have important applicability not only to improve screening efficiency of the suspected individuals, but also to reduce test costs.

Forensic DNA technology developments in recent years

Continuous technological progress in biology has led to major developments in the field of forensic DNA profiling in the past 10 years. Restriction fragment length polymorphism (RFLP) profiling may be considered as the first generation of DNA analysis methods (1985-95). It is no longer used as it requires large amounts of DNA, and because degraded samples cannot be analyzed with accuracy. Technological improvements then allowed very high sensitivity to be achieved with PCR, a technique that allows amplification of target regions. PCR- RFLP analysis has been a popular technique for genotyping. As stressed by Rasmussen (2012) [38], a search in PubMed/Medline as of October 30, 2011, using the search "RFLP and PCR" produced 15,725 hits. PCR-RFLP consists of several technical steps including the choice of primers, the identification of an appropriate restriction enzyme, an amplification step, and restriction enzyme treatment of amplified products and electrophoresis to resolve the restriction fragments. Advantages of the PCR-RFLP technique included low expensiveness and unnecessity of advanced technology. Disadvantages included the necessity of a large sample, the need of specific endonucleases and difficulties in identifying the exact variation in cases where several SNPs affected the same restriction enzyme recognition site. Additionally, the technique was not suitable for the simultaneous analysis of a large number of different SNPs due to the requirement for a specific primer pair and restriction enzyme for every SNP [38]. Current DNA typing involves the use of fluorescent dyes to label PCR products and capillary electrophoresis (CE) to separate and analyze these labelled PCR products. Modern DNA analysis includes STR analysis and other DNA testing methods described below.

STR analysis: Short tandem repeats (STRs) are microsatellites, consisting of DNA regions with repeat units of 2-6 base pairs in length repeated hundreds of times on the DNA strand surrounding the chromosomal centromere (the structural center of the chromosomes). STR analysis measures the number of repeating units. It represents the most commonly used form of genetic information in forensic identification, although it reveals unsuitable for highly degraded or low copy number DNA samples.

Forensic DNA profiles have historically been generated by PCR and capillary CE-based methods to detect length variation in STR loci. In STR analysis, the PCR technique can be used to amplify STR with highly polymorphic DNA sequences of repeated 2-7 base pairs. These STR loci are considered to being unique to each individual. In particular, 5-10 alleles of particular STRs are often the focus of forensic profiling [39-41].

STRs are classified according to the lengths of their repeat, eg. mono-, bi-, tri... or hexa-nucleotides. Tetranucleotides are most often used in STR analysis due to the fact that they have a smaller probability of stutter products (band that has the wrong number of repeats) or amplicons (piece of DNA that is the source and/or product of natural or artificial amplification or replication: repeat and not true allele).The amplification of STR, via PCR, starts with targeting loci by sequence-specific primers. Electrophoresis is therefore used to separate the DNA fragments [42]. The method differs from PCR-RFLP since STR analysis does not cut the DNA with restriction enzymes.

In cases where DNA is highly degraded when exposed to environmental injurious agents or inhibitors, standard STR testing may reveal inadequate. Analysis of these low-quality DNA samples often result in dropout of the larger STR loci from the sample and only a reduced DNA profile can be obtained. A solution to this problem can be found with mini-STR. Mini-STR primers focalize on the STR locus. Thereafter, the resulting DNA product is smaller, thus increasing the chances of successful amplification of the larger loci. This technology increases the sensitivity of DNA detection and allows obtaining more informative DNA profiles from low template or compromised samples.

Development of DNA databases using STR analysis: National DNA databases are maintained by the governments for storing DNA profiles of the population based on PCR, and using STR analysis. Their purpose is to search and match DNA profiles of potential criminal suspects [28].

In the 95-05, national databases of DNA profiles based on PCR and STR analysis have be launched for the UK (1995), the USA (1998) and many European countries, as well as standardization of multiplex STR systems and CE.

The United Kingdom National DNA Database (NDNAD) and the European Network of Forensic Science Institutes (ENFSI) were founded in 1995 with the purpose of improving the mutual exchange of information in the field of forensic science. By 2006, the NDNAD contained 2.7 million DNA profiles (about 5% of the UK population), which increased to 5.7 million profiles in 2015. In the USA, the National DNA Index System (NDIS: national level of CODIS, Combined DNA Index System) contains the DNA profiles contributed by federal, state, and local participating forensic laboratories. NDIS was implemented in 1998.

The Interpol DNA Gateway contains DNA profiles submitted by member countries collected from crime scenes, missing persons, and unidentified bodies. The DNA Gateway was established in 2002, and at the end of 2013, it had more than 140,000 DNA profiles from 69 member countries.

Technically, a first-generation quadruplex system consisting of TH01, vWA, FES/FPS and F13A1 was released in 1994. A second-generation multiplex followed a few years later and examined six STR loci (TH01, vWA, FGA, D8S1179, D18S51 and D21S11) and the sex-typing marker Amelogenin. The NDIS was launched in 1998 with 13 core STR loci (TH01, vWA, FGA, D8S1179, D18S51, D21S11, CSF1PO, TPOX, D3S1358, D5S818, D7S820, D13S317 and D16S539), and commercial kits began to be used for autosomal STR and Y-chromosome STR markers [40] (Table 1).

Beginning in 1996, the FBI laboratory launched a nationwide effort to establish core STR loci for inclusion within the CODIS.

Table 1: Overview of some of the most commonly used (autosomal and Y-) STR kits in forensic science.

Name of the kit	Provider	Loci	References
Multiplex autosomal STR kits			
MiniFiler™ kit	Applied Biosystems, Inc., Foster City, CA, USA	D13S317, D7S820, Amelogenin, D2S1338, D21S11, D16S539, D18S51, CSF1PO, and FGA	DNA Diagnostics Center, Fairfield, UK
PowerPlex® 16 System and PowerPlex® 6C Fusion System	Promega Corporation, Madison, WI, USA	16-27 loci including the CODIS core loci, and ESS loci (for the Fusion 6C System: all markers of the expanded CODIS core loci + Amelogenin and DYS391 + optional markers and 2 Y-STR loci)	[57-60]
Other PowerPlex® STR amplification kits (ESX-16/17)	Promega Corporation, Madison, WI, USA	CODIS, D6, ESS, SE33, and Y-STR loci	[58]
AmpFlSTRGlobalFiler	Thermo Fisher Scientific Inc., Waltham, MA, USA	13 original CODIS loci, 7 from the expanded ESS loci + SE33 locus, 1 Y-STR (DYS391), 1 insertion/deletion polymorphic marker on the Y chromosome (Y indel), and Amelogenin	[59,61]
AmpFlSTR® NGM™ PCR Amplification Kit	Thermo Fisher Scientific Inc., Waltham, MA, USA	14 tetranucleotide repeat loci and one trinucleotiderepeat locus, D22S1045	[62]
Investigator® 24plex QS Kit, Investigator® STRGO! and Investigator®® 24plex Go! Kit	Qiagen GmbH, Hilden, Germany	CODIS core loci, ESS markers, SE33, DYS391, D2S1338, D19S433, and Amelogenin	[58,63]
Investigator® HDplex	Qiagen GmbH, Hilden, Germany	13 loci (D2S1360, D3S1744, D4S2366, D5S2500, D6S474, D7S1517, D8S1132, D10S2325, D12S391, D18S51, D21S2055, SE33, and Amelogenin	[59]
Y-STR kits			
Yfiler™ Plus PCR Amplification Kit	Thermo Fisher Scientific Inc., Waltham, MA, USA	27 Y-STR markers	[64]
PowerPlex® Y23 System	Promega Corporation, Madison, WI, USA	23 male-specific STR loci: DYS576, DYS389I/II, DYS448, DYS19, DYS391, DYS481, DYS549, DYS533, DYS438 (penta), DYS437, DYS570, DYS635, DYS390, DYS439, DYS392, DYS643 (penta), DYS393, DYS458, DYS385a/b, DYS456 and Y-GATA-H4.	[65]

The 13 CODIS loci are CSF1PO, FGA, TH01, TPOX, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51 and D21S11. These loci are now nationally and internationally recognized as the standard for human identification.

In 1999, the DNA working group of the ENFSI decided on a European Standard Set (ESS), which included seven loci: TH01, vWA, FGA, D21S11, D3S1358, D8S1179 and D18S51. These loci have been confirmed by a resolution of the European Council in 2001. The ESS of STR loci has been increased from 7 to 12 in 2009 (expanded ESS loci).

Chromosome Y and X studies (Y-STRs and X-STRs): The Y chromosome is widely used in forensic DNA analysis, particularly in cases where standard autosomal STR analysis is not informative. Haplotypes of Y-STRs are used to characterize paternal lineages of unknown male suspects, especially when males and females have contributed to the same event, such as in sexual assault. Y-STR can therefore a) exclude a male suspect from involvement in a crime scene, b) identify the paternal lineage of a male perpetrator, c) evidence multiple male contributors to a crime scene, and d) help to find unknown male perpetrators.

In recent years, commercial kits (Y-STRs only, or included in multiplex kits with autosomal STRs: see Table 1) have been developed, enabling increased paternal lineage resolution. X-chromosome STR markers also have a real interest in forensic science due to their advantage over autosomal and Y chromosome markers in cases where the alleged father is absent and the child is female.

Single Nucleotide Polymorphism (SNP): About 90% of human genetic variations arise by substitution of one nucleotide for another. Single nucleotide polymorphism (SNP) analysis focuses on these differences in nucleotide sequence. As SNP indicates, they consist of a single base change: an “A” (for the nucleotide Adenine) may be replaced in another individual by a “G” for Guanine. While STR analysis requires duplication of DNA fragments containing about 200 to 400 nucleotides, SNP analysis requires DNA fragments in the 50 to 90 nucleotide range. Consequently, the SNP technique can be used to analyze DNA that may be too degraded for STR analysis [43].

In recent years, advances in the information on SNPs have raised the possibility that these markers could replace the forensically established STRs. Furthermore, STRs have been shown to have a relatively high mutation rate, estimated to be in the order of 10-3, compared to 10-8 in SNPs [44]. This fact, added to easier laboratory processing has increased the possibility that SNP markers be used instead of STRs in solving criminal cases and for paternity testing. Contrary to STR loci that have multiple alleles, SNPs possess only two possible alleles and more SNP loci are therefore needed in order to obtain higher sensitivity. It has been shown that 25-50 SNP loci might be needed to obtain

equivalent performance as the 13 CODIS loci [45]. However the number of STR loci may fluctuate according to the considered population groups and it is mot likely that a panel of 50-100 SNPs would be required to obtain equivalent sensitivity and discrimination power to the currently used sets of STR loci.

Mitochondrial DNA (mtDNA): Human remains exposed to adverse environment for extended periods can be challenging for analysis. DNA may be highly degraded or present in very small quantities. Mitochondrial DNA (MtDNA) is found in the mitochondria, which are tiny organelles present in every cell, not associated with the nuclear chromosomes. MtDNA remains as a valuable source of DNA because of its quantity: mtDNA is often used due to the high proportional amount of mtDNA versus nuclear DNA and its ability to beless sensitive to degradation. MtDNA being present in hundreds of copies per cell, it can survive environments where nuclear DNA does not, and could be a powerful tool for human identification [46].

The hypervariable (HV) regions of mtDNA are used for analysis due to their polymorphic characteristics and are considered as a valuable source of DNA. More specifically, the HV regions I and II (HVI and HVII) have been found to exhibit the more variations [47-49]. Sequencing of mtDNA has proven very useful for criminal investigations where the traces were found in very small amounts or highly degraded, such as in hair shafts and bones. In routine mtDNA analysis, HVI and HVII, the two most hypervariable regions of the control region (aka the D-loop region), are sequenced. The method has the potential to amplify and sequence as few as 30 fg DNA or approximately 10 mtDNA molecules [50]. MtDNA analysis based on the pyrosequencing technology may provide fast and accurate results from the human mtDNA present in many types of evidence materials.

The European DNA profiling group mtDNA population database (EMPOP) is an international collaborative project between DNA laboratories performing mtDNA analysis and the DNA laboratory of the Institute of Legal Medicine in Innsbruck, Austria. It aims at collecting and ensuring quality control of mtDNA population database, which can be used in routine forensic casework [51].

DNA methylation studies: DNA methylation is emerging as a potentially very useful marker in forensic genetics. The age estimation based on DNA methylation is expected to reduce the number of potential suspects, when the DNA profile from the sampled tissue does not match with any person in the crime scene environment, including those stored in the forensic database [52]. Moreover, DNA methylation process implicates environmental factors such as cigarette smoking and alcohol consumption, thereby suggesting the possibility to appreciate the potential lifestyle of a suspect. More and more DNA methylation markers are recognized as age-specific, smoking-specific, and alcohol consumption-specific markers. Such findings require further validation with various types of tissues and cells [53-55].

Next Generation Sequencing (NGS): In forensic daily practice, DNA samples are often limited and cannot fulfill the requirements of simultaneously analyzing multiple loci on different chromosomes. This may result in difficulties in providing useable information and can limit their use as legal evidence. In addition, mixed bloodstain analysis and complex paternity cases cannot be solved with traditional STR genotyping. NGS technology not only meets these requirements but can also be applied in many areas of research [56].

CONCLUSIONS

Non-destructive identification, proper collection, age estimation and further DNA profiling of bloodstains found on a crime scene remain significant steps in the forensic daily investigations. The standard practice at a crime scene is to perform a minimum of presumptive blood identification tests and to confirm the sample as human prior to continuing on to DNA testing. Relevant information can now be extracted from limited quantities of biological samples, which offers unprecedented opportunities for DNA analysis.

There is a growing consensus that better efficiency could be achieved by adding more standard STR loci to the existing national DNA databases, in order to promote data sharing across a wide number of jurisdictions over the world. Challenges can come through poor-quality (degraded or containing inhibitors) or low template DNA samples that fail to produce usable data.

With the development of NGS technology, it is likely that costs will rapidly decrease and NGS kits for forensic application will become available. This will allow the simultaneous detection of multiple autosomal and Y- or X-STR loci, analysis of mtDNA polymorphism and analysis of SNPs related to ancestry and physical and psychological characteristics, thus providing important information for forensic investigations.

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