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Review

Immunochromatographic Detection of Human Blood: A Forensic Review

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Abstract: Body fluid identification is fundamental in forensic science as it links a specific biological source to a genetic profile, thus providing critical clues for crime scene reconstruction. Blood is one of the most common body fluids found on the crime scene, and several strategies have been developed for its identification in recent decades. Usually, after a preliminary (or presumptive) test to determine the presence of blood (both human and non-human), a confirmatory test is needed to prove that the sample is human blood. Out of the confirmatory tests, immunochromatographic (IC) assays are the most commonly and widely used. This work gives a review of the use of commercial kits specifically developed to detect human hemoglobin or glycophorin A (a surface protein of human red cells) in forensics. Claimed sensitivity varies broadly (ranging from 0.06 to 75 nanoliters of fresh blood), but different values (as low as 0.002 nL) were found during validation procedures. Specificities are high, and the possibility of cross-reaction (with the risk of false-positive results) is so low that it can be considered negligible. False-negative results, however, can be found due to the so-called "*hook effect*" as well as to the target degradation/modification, which interferes with the Ag-Ab binding. In addition, the chemical compositions of the presumptive test, detergents, and washing can also promote false negative outcomes in peculiar situations. Although IC assays are rapid, inexpensive, specific, and easy to use even on the crime scene, their major limitation is represented by the destructive approach required by this kind of confirmatory test. Since the final goal of the forensic investigation is the genetic typing of a bloodstain, we will describe the strategies developed for IC assays of faint stains as well as the strategies adopted to ensure that exactly the same sample undergoes human blood identification and DNA typing.

Keywords: blood; blood stain; immunochromatography; forensics

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1. Introduction

The identification of biological fluids at the crime scene, on the victim's body or the perpetrator's body, is crucial to forensic investigations. Blood is the most frequently found biological fluid of forensic interest [1–5]. If the quantity is sufficient, blood is a reddish-colored biological fluid that can be detected by the naked eye. Nevertheless, it is also true that there are many other reddish substances (tomatoes or paint, for example), making it necessary to use chemical-physical methods to identify the actual presence of blood [5]. One of the most widely used is the Combur test [6], which provides good results in a few seconds. The Combur test—initially developed for detecting blood in urine—together with other chemical tests based on the detection of the peroxidase activity of hemoglobin is both fast and highly sensitive. On the other hand, it is important to keep in mind some critical issues when using these kinds of tests: (1) they are non-specific for human blood as they cannot distinguish human from non-human blood; (2) some vegetables show a high

peroxidase activity, just like hemoglobin; (3) some reagents with a strong oxidizing power (such as bleach, that is, sodium hypochlorite) can produce a false-positive [1,6]. The same limitations also apply to the widely used Luminol test [7]. For the above reasons, all these tests are usually considered to be preliminary (presumptive, screening, or field) tests.

As we have seen, before proceeding with DNA typing, it is mandatory to verify if the sample is human blood or not by performing a confirmatory test since the DNA analysis does not provide any information on the type of body fluid from which the DNA profile originated [3]. For this purpose, several methods have been developed over the last decades based on different chemical-physical principles: immunological [7] and immunochromatographic assays [8], high-performance liquid chromatography (HPLC) [9], capillary electrophoresis (CE) [10], mass spectrometry (MS) [11], Raman spectrometry [12], ATR FT-IR (attenuated total reflection Fourier transform-infrared) [13], NIR (near infrared) [14], RNA analysis [3,15], and DNA methylation [3]. The choice of the most appropriate confirmation method depends on various factors, first and foremost, simplicity along with the robustness of the method but also the length and cost of analysis and the possibility of performing the analysis directly on the crime scene. In addition, except for Raman spectrometry, FTIR, and NIR, all the methods mentioned above are destructive. Therefore, even the size of the stain becomes crucial in driving the choices of the operator who might choose to omit, in some situations, the basic confirmatory step in favor of DNA typing (thus giving rise to possible claims regarding the real human blood origin of the trace).

Immunochromatography (IC) combines two basic techniques: (i) the separation of molecules based on their ability to migrate on solid supports by capillary flow; (ii) the identification of the target molecules based on the antigen–antibody reaction. Since IC assays are easy to use, they are widely employed, such as self-tests for monitoring health status (pregnancy or SARS-CoV-2 infection, for example). In Forensics, IC is used in several fields, such as body fluid (blood, semen, saliva, illicit drugs (methamphetamine and amphetamine)) and biotoxins (*B. antracis*, botulinum neurotoxin type B, recin) identification [8]. This review shows the advantages (and the limits) of the IC assays, which are routinely employed as confirmatory tests for human blood [16–43] (see Table 1). The identification of menstrual blood through IC testing is treated separately in Section 6.

Table 1. Main features of the six commercial kits specifically developed for forensic purposes. 1: ABAcard® Hematrace®; 2: Hexagon OBTI; 3: SERATEC® HemDirect; 4: RSID™ blood; 5: Bluestar® OBTI; 6: BLUESTAR® Identi-HEM®. Target: target of the assay (hHb: human hemoglobin; Gly-A: glycophorin A); test: antibodies used for the identification of the target (mono: monoclonal; poly: polyclonal); ctrl: antibodies fixed in the "C" (control) line of the cassette (*: Seratec uses internal control; see text for details); ref.: references. n.p.: not provided by the manufacturer.

Kit	Target	Test	Ctrl	Ref.
1	hHb	mono + poly	n.p.	[16–22,40,43]
2	hHb	mono + mono	n.p.	[23–32,38]
3	hHb	mono+ mono	anti-rabbit Ab *	[32–35]
4	Gly-A	mono + mono	anti-mouse Ab	[22,31,36–40]
5	hHb	mono + poly	n.p.	[23]
6	hHb	mono + poly	n.p.	[42]

2. Operative Principles

The most commonly used method to confirm the presence of human blood is immunochromatography (IC). Most of the tests available were initially developed to identify human hemoglobin in feces as a screening tool for colon cancer and were used for forensic purposes only later [24].

Table 1 lists the kits specifically developed for forensic identification of human blood. Besides the high specificity of the results (see below), the first advantage of IC testing is the commercial availability of the devices used for the analyses at a cost of less than 10 EUR per test. In addition, even if IC assays are usually carried out in a laboratory, the tests can

also be performed on the crime scene directly using easily available equipment. The test is straightforward and requires—in standard conditions—less than one/two hours.

The first step is to incubate a fragment of the sample to be tested in a tube prefilled with the liquid extraction medium (from 0.1 to 2.0 mL). As shown in Table 2, each kit works at its' specific conditions.

Table 2. Analytical conditions and sensitivity (data from the kit's brochures). 1: ABAcard® Hematrace®; 2: Hexagon OBTI; 3: SERATEC® HemDirect; 4: RSID™ blood; 5: Bluestar® OBTI; 6: BLUESTAR® Identi-HEM®. Buffer (volume): volume of extraction buffer (n.p.: no provided); incubation (time): length of incubation in the extraction buffer at room temperature (* up to 8–12 h to avoid false negatives; **: up 2 h with shaking); load (volume): volume loaded into the cassette; LOD [hHb]: limit of detection (given as the amount of hHb/mL of extraction buffer); LOD (blood): volume of fresh blood needed to assure the hHb concentration shown in the LOD [hHb] column (for this calculation, the mean value of the physiological concentration of hHb (15 g/dL of blood) was used [44]).

Kit	Buffer (Volume)	Incubation (Time)	Load (Volume)	LOD [hHb]	LOD (Blood)
1	0.3 mL	1–5 min	150 µL	50 ng/mL	0.3 nL
2	1.7 mL	briefly	80 µL	100 ng/mL	0.6 nL
3	1.5 mL	Briefly **	100 µL	40 ng/mL	0.25 nL
4	0.1–0.3 mL	1–2 h	≤20 µL	12.5 µg/mL	75 nL
5	n.p.	5–20 min *	120 µL	50 ng/mL	0.3 nL
6	2.0 mL	10 s	240 µL	10 ng/mL	0.06 nL

Following the manufacturer's instructions, 1 to 120 min are needed to extract the target antigen successfully. After that, a few drops of the solution (40 to 240 µL) are loaded onto the test cassette and absorbed through migration (see Figure 1). During this phase, a chromophore-labelled monoclonal specific antibody (Ab), conjugated to the cassette's pad, binds with the antigen (human hemoglobin (hHb) or glycophorin A (Gly-A) to form an antibody-antigen complex. Then, the immunocomplex (if any) migrates to the test ("T") zone, where it is captured by a second (mono or polyclonal) immobilized Ab directed against the human target. The positive reaction is, therefore, shown by the colorimetric reaction in the "T" zone, and the intensity of the band is proportional to the antigenic charge. A second reaction, in the control line ("C" line), assures the reliability of the reaction by binding the excess of the free chromophore-labelled antibody (in the case of the SERATEC® HemDirect, the internal control is done by rabbit Ab plus anti-rabbit chromophore-labelled Ab). Note that the colorimetric reaction in the "T" zone must be visible within a well-defined time from the sample loading (three to ten minutes, depending on the kit).

Clearly, the operative principles of the test imply that the IC assay is destructive of the biological stain sampled for the test. For this reason, several strategies were developed to recover the extraction solution's residual volume and use it for genetic typing [24,27]. Briefly, only a small part (less than 10%) of the extraction solution is used with IC testing, while the remaining volume is submitted to DNA typing. Importantly, this approach ensures that biological tissue identification and genetic typing are performed exactly from the same sample [3]. More recently, Basset et al. described the use of a single extraction buffer (RSID™-universal buffer) to load three fluid-specific cassettes (for the identification of blood, saliva, and semen) and to perform the subsequent DNA typing [31]. In another case, the eluted body fluid sample was used to load simultaneously up to four different cassettes, allowing the identification of five biological fluids, whereas the cassette pads were then used for successful DNA typing [36]. Wallis et al. [20] described the successful recovery of the swab from the extraction buffer and its reuse for DNA typing. As is shown, many are the strategies available to ensure that exactly the same sample is used for human blood identification and DNA typing.

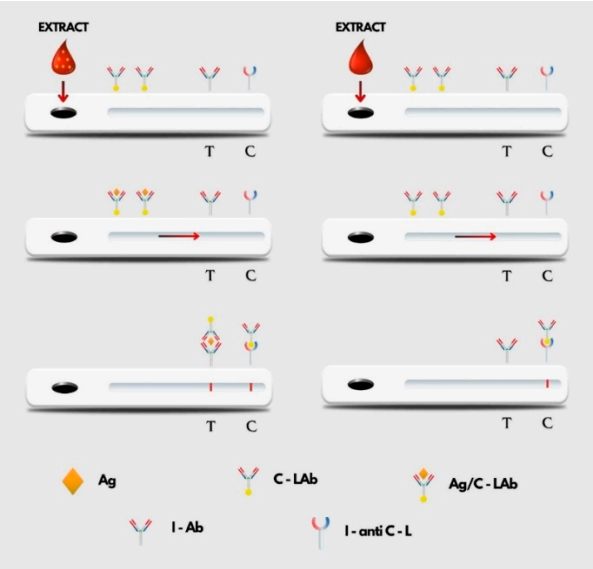

Figure 1. Schematic representation of the IC assay in case of positive results (**left**) and negative results (**right**). Ag: free hemoglobin or glycophorin A; C-Lab: chromophore-labelled monoclonal specific antibody conjugated to the cassette's pad; Ag/C-Lab: immunocomplex; I-Ab: immobilized Ab directed against the antigenic target; I-anti C-L: immobilized Ab directed against the chromophore. T: test line; C: control line. The arrow indicates the flow direction.

3. Sensitivity of the IC Assays

The brochures in the kits contain detailed operative protocols, and different kits claim different sensibilities in the IC assays. On average, the sensitivity is always higher in kits enabling the detection of hHb (with values ranging from 10 to 50 ng/mL) than in the RSID™ blood kit, which targets glycophorin A, a surface protein of the red cells (for this kit, a sensitivity of 75 nL of blood is claimed). It has to be noted, however, that the methodology used to establish the sensitivity (that is, the limit of detection, LOD) is not fully described because scarce/null details are provided of the blood samples used in those internal validation procedures. In addition, the LOD value is not provided at all in some kits developed for clinical purposes only (this applies, for example, to the OC-Hemocatch tests, which were developed to detect occult blood in feces but are widely employed in East Asia even for forensic purposes [45–47]. Certified hHb was used only in the SERATEC® HemDirect kit [33], whereas other kits seem to have used fresh blood from unknown donors. Lastly, the LOD is given in nanograms of hHb/mL (of solution) in five out of six kits, whereas in the RSID™ blood kit, the LOD is given in the volume of blood needed to achieve a positive reaction. In other words, comparing the LODs can be tricky because the volume of buffer used for extraction and the volume loaded into the cassette vary greatly. Table 2 reports the sensitivity claimed for the commercial kits together with the volume of blood needed to obtain such LODs. Less than one nanoliter of fresh blood should be enough to provide a positive reaction in kits assessing the presence of hHb, whereas the sensitivity of the RSID™ blood kit is at least 100 times lower. In any case, however, the volume of blood needed is small, often undetectable to the naked eye. It has to be noted, however, that several validation studies reported LODs that were different from those declared in the manufacturer's brochure. For example, Holtkötter et al. [36] reported sensitivity as low as 0.002 nL of blood for Seratec technology, which is about 100-fold higher than the one claimed by the manufacturer. Furthermore, the minimum hHb concentration detectable by the ABAcard® Hematrace® kit is claimed to be 50 ng/mL [16], whereas it ranged from 8 ng/mL to 260 ng/mL in different validation studies [17,21]. These discrepancies could be due to hHb concentration estimations, differences in extraction protocols, and differences in dilution protocols [19]. It has to be noted that the composition of the extraction solutions provided by the manufacturer, as well as all other components of

the kit, are usually unknown; for this reason, any modification to the original protocol can be expected to lead to different sensibility values (likely at the expense of accuracy) [19]. Thus, for example, Seratec® recommends using the extraction buffer to dilute the samples because improper use of water leads to lower sensibility [33]. Finally, the number of human blood samples tested in some studies is not stated at all, whereas, in other studies, it seems too small to produce final conclusions. In summary, a lack of standardization in performing validation studies can lead to uncertain LOD values, even in fresh samples. Anyway, the sensitivity of these assays is undoubtedly very high, with less than one nanoliter of fresh blood needed to produce a positive outcome (about 50–75 nanoliters are needed for the less sensitive RSID™). It must be noted, however, that even the working temperature can affect the sensibility of IC assays. For example, the sensitivity of the SERATEC® HemDirect Hemoglobin assay is reduced by ten times at the temperature of 8 °C [33]; furthermore, also in the case of the ABAcard® Hematrace® kit exposure to elevated temperature (above 55 °C) the sensitivity of the assay was reduced, likely because of antibody denaturation [19]. Therefore, analytical parameters, as well as working conditions, should always be monitored carefully.

In forensic routine, finding fresh or un-clotted blood is unusual and bloodstains occur much more frequently. From an operative point of view, when a bloodstain lays on a porous substrate (as cotton wear, for example), a small part of it (one to two square millimeters or less) is used for IC assay directly. Conversely, if the bloodstain lays on an un-porous substrate (glass, for example), it is usually collected with a swab, and only part of it is processed for IC testing. In both circumstances, the amount (i.e., the volume) of blood needed for a positive outcome could be higher than that required from fresh blood samples because a complete extraction of the antigenic target from a stain cannot be assured. The hHB/Gly-A extraction could be inhibited by several factors such as heat, which can modify the solubility of the antigenic target [47]; in other cases, the intrinsic properties of the swab used for sampling the stain can interfere with the accuracy of the assay [28]. Validation studies performed on different sets of bloodstains usually evaluate and report on the aging of the samples, the storage conditions, and, sometimes, the size (in square millimeters) of the sample used for IC testing, whereas the outcome is indicated as "positive" (with +++, ++ and + arbitrary scores) in only a few cases or "negative"; as a result, it is impossible to establish any LOD for the selected IC assay. Of great importance in forensics, bloodstains are often aged and/or exposed to environmental factors that can potentially modify/degrade the antigenic properties of the proteins [24,47]. In such cases, much higher LODs are expected, and, in some instances, false-negative results can be found (see Section 5).

Strategies Developed for Tiny/Aged Bloodstains

Confirmatory tests are recommended even on tiny, faint, or non-visible stains, which provide positive results to a presumptive test (Combur test, for example). In such cases, however, it is also clear that the stain cannot be entirely consumed for confirmatory testing, as DNA typing remains the final goal. To this aim, several strategies have been developed in recent years. The first and simplest strategy is to reduce the volume of the extraction buffer for the IC test [27], for example, from 2 mL (i.e., the volume of the prefilled commercial tube) to 0.2 mL or less. In this way, the stain will be eluted in a small volume, which can be used for both confirmatory tests and DNA typing. In addition, also the incubation times can be increased up to 12 h, leading to a higher sensitivity [23]. Also, a shacking apparatus can be introduced to increase the extraction of the target from the stain [24,33] as well as incubation at moderate temperatures (37 °C). It should be noted, however, that these experiments were performed using only one (or a tiny part) of the infinite set of substrates on which the blood trace can be found in the real case (jeans, glass, wood, etc.) and that any improvement of the sensitivity observed in porous substrates cannot be extended to the non-porous ones (and vice-versa) [27,35].

As stated above, since the final goal is DNA typing, residual extraction volumes can be successfully used for DNA analysis [27,31,36]. However, since the amount of blood needed for a positive IC outcome is lower than that required for genetic typing, partial or no DNA profiles could be observed in the case of tiny blood samples [36].

The use of chemicals for improving the solubility of aged samples is described in Section 5.2.

4. Specificity and False Positive Results

Since hemoglobin is a protein present only in the red cell line, only blood and no other body fluids such as saliva, semen, etc. are expected to provide positive results. However, few studies reported a positivity for human blood even in saliva [24,35] and semen [17] samples. These unwelcome outcomes are likely due to the presence of a minimal amount of blood in those samples coupled with the extreme sensibility of the IC assays [24] and should be taken into account in real casework analysis.

A few IC assays based on the immunological detection of hHb have shown cross-reaction with primate blood samples [16,18,23], whereas the RSID™ blood kit does not. Importantly, no cross-reaction has been observed with the Hb of many animals (bovine, dog, rabbit, cat, pig, wild boar, chicken, sheep, mule, goat, red deer, etc.), whereas a weak cross-reaction has been observed only for BLUESTAR® OBTI [23] with high amounts of horse blood. Furthermore, weak positivity was reported for ferrets, weasels, and badgers as well, but only in unusually high volumes of blood [19,23,33]. Lastly, even low pH values (less than 5.0) are known to have produced false positive results in SERATEC® HemDirect assays [33]. On the other hand, these presumably rare cases are not a real concern, even if they are time-consuming, because DNA testing will indirectly reveal the non-human origin of those samples through unsuccessful human DNA qPCR quantification. In addition, even following PCR amplifications with human-specific STR primers would have yielded negative outcomes. Of course, the final demonstration of the non-human origin of a sample will be provided only by molecular tests that can positively identify non-human targets, such as ribosomal RNA typing or cytochrome (cytb) molecular analysis [3].

5. False-Negative Results

The occurrence of false-negative results can potentially drive the operator not to consider human bloodstains for DNA testing. Therefore, this occurrence needs to be monitored carefully. The three main factors causing false-negative results are listed and discussed hereafter.

5.1. The "Hook Effect"

The use of unknown amounts of blood exposes the operator to the risk of false negative results due to the high dose of blood, the so-called "*hook effect*", which can be observed when too many free antigens (not bound to the chromophore-labelled antibody) reach the "T" region of the cassette. In this case, the antibody fixed in the "T" area will be saturated, preventing the binging of the Ag-Ab complex. The result will be negative despite the presence of hHb in the sample. The "*hook effect*" has been observed in all IC tests, particularly in the most sensitive ones [16,23,33,37,42]. If there is the suspect that too much stain has been eluted and the negative results can be ascribed to the "*hook effect*", the solution can be diluted till 1:100 or even 1:1000, and the IC test has to be repeated into a new cassette (see Table 3). In these cases, the use of the extraction buffer is recommended for dilute samples because water leads to lower sensibilities [33].

Table 3. IC assay troubleshooting: false negative outcomes of the IC assay. Ag.: antigen.

Possible Cause	Molecular Reason	Suggested Action
hook effect	High Ag. concentration	Dilution of the extract
Ageing	Ag. denaturation	-
	Ag. insolubility	Extraction with 5% ammonia
	Ag. washing out	-
Chemicals/washing	Ag. denaturation	-
	Ag. insolubility	Extraction with 5% ammonia
	High/low pH	Adjustment of the pH to 7
	Detergents (SDS)	-

5.2. Ageing of the Sample

As already stated, also the aging of the stains is a potential source of false-negative results because environmental factors can promote the modification/denaturation of the antigenic properties of the targets or make the targets insoluble and therefore un-accessible to the assay [17,24,26,35,45,47]. In line with this, a lower sensitivity leading to false negative results was observed in the analysis of year-aged bloodstains stored at room temperature [26]. Although the number of samples tested in some validation studies was limited, the RSID™ blood kit provided higher rates of false-negative results in agreement with its lower intrinsic sensibility [22]. In other studies, human bloodstains that had been buried [24] or exposed to heat [27] or even to fire [17] also gave negative results. In some cases, although scarce information is sometimes given on the stain size used for the assays, the evidence suggests that the degradation/modification of the antigenic epitopes could prevent them from being recognized by the antibodies for which they are targets [24]. In other cases, such as heating, the reduced solubility of the antigenic targets seems to be involved in the negative outcome of the assay [47].

As shown in Table 3, in the case of aged [24] and heated [27] samples which are not, or scarcely, soluble in the usual buffers, the employment of 5% ammonia has been described to increase the rate of positive outcomes [24,47]. The use of ammonia for treating aged bloodstains was described in the 70s [48] and was recently evaluated even for immunofluorescence-based assays [49]. Protein solubility is lower in acidic pH than in alkaline pH; therefore, the addition of ammonia by a pH increment boosts protein solubilization. In addition, acidic peptides can be solubilized better by the addition of ammonium hydroxide solutions than alkaline Tris buffers [24,47,49].

In the end, from a practical point of view, caution must always be used in front of a negative outcome from an aged bloodstain, even if stored at room temperature.

5.3. Chemical Compounds and Washing

Chemical compounds can compromise the performance of the IC tests on the real case bloodstains, involving several mechanisms (modification of the antigenic epitope, reduced solubility of the antigenic target, interference with the Ag-Ab binding, and inhibition of the colorimetric reaction). Therefore, for example, detergents (such as Na_2-dodecyl-sulphate or Sarcosyl) decrease the sensibility of the SERATEC® HemDirect Hemoglobin Assay [33] and OBTI assay [24], likely interfering with the Ag-Ab binding. Similarly, even pH values higher than 9.0 reduce the sensibility of the tests, leading to unreliable results with pH values higher than 12.0. Therefore, pH values lower than 12.0 are recommended for the reliable use of the Hexagon OBTI in validation studies [24,25]. Thus, from a practical point of view, it is fundamental to exclude that the chemical composition of the presumptive tests performed previously (for example, Luminol or Bluestar spry testing) interferes with the IC assay. The performance of two commercial kits (ABAcard® Hematrace® and RSID™-Blood) was compared in the analysis of bloodstains treated with Luminol (Grodsky formulation) and Leuco Cristal Violet (LCV) [21]. The study showed that RSID™-Blood produced false-negative results for all bloodstains treated with Luminol and almost half of those treated with LCV. The presence of Na-perborate (as oxidizer) in the Grodsky formulation (instead

of the H_2O_2 of the Weber formulation) was suggested as a possible cause of this failure, together with the pH of the Luminol formulation. The reasons for the loss after treatment with LCV are still unclear. Lastly, RSID™-Blood was proved to give false negative results in bloodstains treated with Bluestar spry [21]. Even Hochmeister et al. described the interferences of some Luminal formulations (containing HaOH) in the performance of the OBTI assay [24]; in those cases, however, the pH adjustment below 12 returned positive results [24]. Thus, from a practical point of view, it is recommended to evaluate, during intra-laboratory validation procedures, the possible interference of the chemicals used for presumptive testing.

Detecting blood on laundered clothes is also tricky, as assessed in several studies [19,22,24,35,40,50]. Overall, the critical factor is the size of the bloodstain against the launder strength. The chemical composition and volumes of the washing solutions, temperature, length of the treatments, and chemical-physical properties of the clothes can vary wildly. Therefore, it is not possible to provide general considerations. However, most treatments are expected to promote false-negative results, having removed the body fluid from the substrate and interfering with the accuracy of the IC assay. In a pivotal study [24], it was observed that several household bleaches gave no or very weak results; in addition, since the adjustment of the pH to 7 before testing still yielded a negative result, the denaturation of the antigen was the most likely cause of the misleading result. Howard et al. [22] focused on the detection of blood on clothing laundered with Na-percarbonate, showing that ABAcard® Hematrace® produced false negative results on all samples tested, irrespective of the washing temperature adopted in that validation study (24 °C and 40 °C, respectively). Notably, when tested with the RSID™, the same samples gave positive outcomes [22], thus supporting previous findings that indicated that the kit (based on the detection of glycophorin A) is less sensitive to some detergents containing active oxygen [51]. In a more recent study, Kulstain et al. [40] showed that laundered blood-stained pieces of cloth were negatively assayed by IC testes, whereas DNA typing continued to provide positive outcomes. Similarly, Nakanishi et al. showed that the reliability of IC tests was affected even by washing the stains in water at room temperature [50].

Even the nylon swabs used to collect blood samples have proven to potentially cause false negative results in the Hexagon OBTI assay [28]. More specifically, the sensitivity of the kit was reduced by a factor of at least 100 by sampling the blood with ethylene oxide-treated flocked swabs. The sensitivity of the kit is also reduced by quebracho, a chemical used for treating leather [29].

In conclusion, the possibility of false negative results in the IC assays must be considered, particularly when tiny amounts of aged or washed bloodstains are tested. At the same time, the influence of the chemical composition of presumptive tests used in particular cases must also be evaluated carefully. With these considerations in mind, the risk of classifying human blood samples as non-human ones should be reduced to the minimum.

6. Identification of Menstrual Blood

Since blood flows from the body even after minimal skin or mucosal lesions, blood marks can also appear in a crime where the victim or the perpetrator (or both) have reported an injury. It is also true, however, that a bloodstain can occasionally be composed of menstrual blood. Therefore, there are circumstances in which it is essential to differentiate between peripheral and menstrual blood, especially in sexual assault casework. In the last decade, a few IC-based tests have been developed for this aim [52–54]. In detail, IC assays specific to the D-dimer (a terminal degradation product of fibrinolysis) have been commercialized. The sensitivity of these tests, as recently reviewed by Bruijns et al. [8], is high, and the test comes out positive with as little as three nanoliters of menstrual blood. The D-dimers, however, are not human-specific, and positivity was proved also in rabbits [52]. More importantly, recent studies highlighted the risk of using the Seratec® PMB test in mock samples because false-positive results can lead to misleading conclusions [54].

Thus, other methods, such as tissue-specific mRNA profiling, should be preferred to positively identify human menstrual blood [3].

7. Discussion

Body fluid identification is one of the crucial points of the criminal investigation [3,55] as it adds weight to the evidence. In fact, the link between the donor identity (assured by DNA typing) and the activity that occurred is provided just from the cell/fluid typing. Thus, the positive identification of human blood is a fundamental step in forensic investigation.

Positive identification of human blood was of interest in forensics even before the DNA typing era. Blood group typing, performed with immunological-based approaches, coupled the positive identification of human blood with personal identification [56]. Similarly, the identification of human plasmatic proteins allowed the positive identification of bloodstains [57]. In the last decades, thanks to extraordinary technological development, several methods based on different chemical-physical principles [7–15], such as HPLC [9], CE [10], and MS, have been proposed for forensic purposes. Unfortunately, the instrumentation required and the laboratory setup make it impossible to perform the analyses required, at least in the routine. Instead, there are other methods, such as Raman Spectrometry [12], ATR FT-IR [13], and NIR [14], that look promising but are still not fully validated for forensic employment in casework. On the contrary, IC assays are the ideal tool to confirm the presence of human blood, and the commercialization of several kits boosts this trend, allowing rapid and conclusive results at acceptable costs. The sensitivity of these assays is very high, and infinitesimal volumes of fresh human blood (less than one nanoliter) are required to yield positive results. Furthermore, even specificity is high as the cross-reaction with the blood of primates or other rare animals (ferrets, weasels, and badgers) does not seem to be a real concern. The positive identification of tiny, aged, or washed bloodstains instead appears to be more problematic; at the same time, both the chemicals employed in presumptive tests and other chemicals present in the substrate that can be co-eluted [19,21,22,37,39,47,50] have been found to cause false negative results. Therefore, it is precisely the occurrence of false negatives that needs to be carefully monitored and for which appropriate solutions have been developed. As shown in Table 3, a rational approach to the main causes of false negative outcomes can help manage those cases.

Considering that the IC test is destructive of the sample stain, several authors suggest using the extraction solution's residual volume for genetic typing [27,31,36]. In this way, only a small part of the sample (no more than 10%) is submitted to IC assay, whereas the remaining sample is used for DNA typing. In some cases, when the stain is very small, other strategies should be considered. RNA/DNA co-extraction seems to be the method promising the best chances [58]. Following this approach, DNA and RNA are extracted simultaneously, and, whereas the DNA sample is processed through the DNA typing workflow, the RNA sample is analyzed separately to provide information on the nature of the body fluid. To this aim, messenger RNA (mRNA), circular RNA (circRNA), and other non-coding RNA have been explored as potentially useful candidates by the employment of different technologies, such as reverse transcriptase (RT)-end-point PCR [59], RT-massive parallel sequencing [60], nanostring analysis [61] and loop-mediated isothermal amplification (LAMP) [62].

It must be noted that the study of RNA markers can be problematic in tiny mock samples because of RNA degradation [46,63,64]. In such cases, microRNA (miRNA) markers are to be considered the ideal candidate [65], although their tissue-specificity has to be evaluated carefully. However, no general rule can be applied to real casework analysis because too many factors are involved both in RNA typing and protein detection through IC in aged/damaged samples. For example, blood samples containing 250–500 μg/μL of methamphetamine gave false-negative results for the IC assay, whereas a successful RNA typing through end-point PCR could be achieved [45]. On the other hand, 20-year-old blood samples gave positive results for the IC assay but not for RNA typing [46]. It is certain, however, that the bloodstain size remains an irresolvable issue in many cases.

Even the non-destructive approach of Raman spectrometry [12], ATR FT-IR [13], NIR [14], or ion mobility spectrometry [66] could be useful, but, regrettably, size, aging, and the possible interference of the item on which the bloodstain is dropped remain crucial points; in addition, there are only a few validation studies available on these topics at present.

Recently, an interesting study by Samie et al. [67] described the use of Bayesian Networks (BNs) to investigate the nature of body fluids. In this challenging approach, three main features of the bloodstain were evaluated within the BN. As a result, the evaluation of the color of the stain (red, light red, and other), the IC-based results (positive, weakly positive, and negative), and the DNA yields recovered from the stain (given in eight classes from 0 to 0.02 ng/μL) should provide a probabilistic answer to this crucial point of the forensic investigation. Further studies are, however, recommended to avoid the occurrence of misleading conclusions.

8. Concluding Remarks

The present review represents a critical evaluation of the use of IC assays as the most common tool for the forensic identification of human blood. The sensitivity and specificity offered by commercial kits reflect their use for forensic purposes. In addition, the test is straightforward, can be performed on the crime scene in less than two hours, and requires no special equipment. Thus, IC assays represent the ideal tool for the positive identification of blood in most cases.

Since the IC identification of blood is a procedure that destroys the biological stain, it could be questioned that DNA typing is not performed from exactly the same stain used for IC blood testing [3]. This is likely the crucial weakness of the IC assay, but several solutions have been developed and are now available to any laboratory. Most simply, to use the residual extraction solution for DNA typing [27,31,36].

In some cases, the intrinsic properties (size, aging, and preservation) of the stain make the IC assay infeasible, very problematic, or error-prone, making false-negative outcomes possible [19,21,22,37,40,47,50]. Although several approaches have been developed to overcome these situations in a well-defined set of bloodstains, other methods—based on molecular biology techniques—are available nowadays even if those innovative techniques do not belong to the "routine workflow", at least in most laboratories. Introducing such techniques in a forensic laboratory requires extensive validation and implementation of interpretation and reporting, with additional unaffordable costs that can only be justified by a large number of real case casework.

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