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Cross reaction and forensic comparison of blood testing done by private and public sector laboratories

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Resumo

Manchas hematóides consistem em um dos vestígios mais importantes em locais de crime. No entanto, nem sempre é fácil identificar uma mancha como sangue. A melhor maneira, e mais rápida, de fazê-lo é usando métodos de triagem derivados de testes imunocromatográficos para detectar a hemoglobina. No entanto, a reação cruzada entre espécies pode ser um problema, já que a hemoglobina de outros animais pode estar presente, especialmente considerando crimes contra a fauna, locais de crime externos com fácil acesso de animais e casos envolvendo lesões de animais domésticos e mesmo de humanos. De acordo com a estrutura específica em cada país, existem laboratórios privados e/ou públicos que trabalham no campo forense. Métodos e recursos dos setores público e privado são diferentes e os procedimentos também diferem de país para país. O objetivo deste trabalho foi validar a presença de sangue humano utilizando amostras interespecíficas e os critérios definidos em cada laboratório utilizando kits de teste de detecção de hemoglobina (imunocromatográficos) do setor público relacionados à análise forense no Brasil e do setor privado na Alemanha. Como objetivo secundário, avaliamos a sensibilidade, o Efeito Hook e o efeito do pH, em alguns kits comercialmente disponíveis. Os resultados mostraram que os testes analisados são adequados para a detecção de sangue humano em amostras forenses com os métodos comumente empregados nos setores públicos e privados.

Palavras-Chave: Identificação de sangue humano; Hemoglobina; Efeito Hook; Testes imunocromatográficos; Testes rápidos.

Abstract

Blood evidence consists of one of the most important forensic pieces. However, it is not always easy to identify a stain as blood. The best and fastest way of doing so, is by using rapid screening derived from immunochromatographic tests to spot hemoglobin. However, cross reaction between species might be an issue since hemoglobin of other animals could be present, especially considering crimes against fauna, outdoor crime scenes with an easy access for animals, and human and domestic animal trauma cases. According to the particular structure in each country there are private and/or public laboratories working in this field. Methods and resources from private and public sectors are different, and procedures also differ from country to country. The main objective of our work was to validate the presence of human blood using interspecific samples and the criteria defined by each lab using rapid blood detection test kits from the public sector related to forensic analysis in Brazil and from the private sector in Germany. As a secondary goal, we have evaluated sensibility, the Hook Effect and pH effect in some commercially available kits. Our results showed that the tests carried out are suitable for the detection of human blood in forensic samples using methods commonly employed in the public and private sectors.

Keywords: Human blood identification; Hemoglobin; High Dose Hook effect; Immunochromatographic tests; Rapid screening tests.

1. INTRODUCTION

Blood presence on a crime scene always motivates further analysis regarding its relation to the criminal event. But confirming that a blood-looking sample is really blood might be tricky. The best and fastest way of doing forensic human blood screening is by using forensic kits or rapid tests derived from immunochromatographic tests of clinical purposes. Considering the need to validate these tests for the forensic use, studies have been conducted by several local police agencies in South America not only to evaluate their efficiency [1-2], but also to identify

possible false positive results. Most of the kits have the same principle: a polyclonal antibody against human hemoglobin is immobilized at the test region on a membrane; the upstream control region contains other immobilized antibody on the membrane, e.g. polyclonal goat anti-rabbit antibodies; a fiber glass pad downstream of the membrane is used for sample loading and transmission to a second fiber pad that contains the dried and gold-labeled monoclonal anti-human Hemoglobin (anti-hHb antibody that will bind the hemoglobin present in the sample). Additionally, the pad contains gold-labeled polyclonal rabbit antibodies for the control reaction.

The SERATEC HemDirect Hemoglobin test is a chromatographic immunoassay for the rapid detection of human hemoglobin in forensic samples. It contains two monoclonal anti-human-hemoglobin antibodies as active compounds. The OBTI HEXAGON test uses as well Human hemoglobin (hHb) in the sample – it will react with the reagent consisting of blue colored particles marking the monoclonal anti-hHb antibodies. The immunocomplex migrates to the test zone and is captured by the immobilized second antibody directed against hHb. As a consequence, a blue line forms in the test zone which indicates a positive result. The unreactive reagents migrate further and are bound in a second line where the immobilized anti-mouse antibodies are located. This is a control that indicates the proper function and correct handling of the test. The SERATEC HemDirect test uses a decoupled control reaction (different antibodies are used for the control and result line), because having two different reactions increases the reliability of the test, so the control reaction is not affected by the result line. The label pad includes gold labeled monoclonal anti-human hemoglobin antibodies and gold labeled rabbit antibodies. In the result line area monoclonal anti-human hemoglobin antibodies are immobilized and the control area contains anti-rabbit antibodies. A summary from both products is presented in Table 1.

Table 1. Technical comparison between different two available commercial tests.

Test properties	SERATEC HemDirect	Hexagon OBTI
Label pad	Two gold labeled antibodies: monoclonal anti-human hemoglobin antibodies and rabbit antibodies	One labeled antibody: monoclonal anti-human Hb
Result line	monoclonal anti-human Hb	monoclonal anti-human Hb
Result line color	Red	Blue
Control line	Anti-rabbit antibodies	Anti-mouse antibodies
Sensitivity	20 ng/ml human Hemoglobin	100 ng/ml human Hemoglobin
Cross reactivity	Upper primates, ferret	Upper primates, ferret, cat
Plastic pipette	Yes	No
Storage temperature	2 - 30°C	2 - 25°C
Application	Forensic	Forensic
Certification	Yes (ISO 9001 and 13485)	No

Very high human hemoglobin concentrations could lead to false negative results. This Hook effect or prozone effect occurs when all mobile antibodies are ligated to an analyte and excess hHb migrates faster on the membrane and ligates to the immobilized antibodies at the result line. Then the mobile antibody-hHb complex cannot bind to the immobilized antibody, since it's occupied by unmarked hHb. In this case, hemoglobin will bind not only to the mobile labeled antibody, but also, considering the high concentration, to the immobilized antibodies at the result area, competing with the complex (hemoglobin/labeled antibody).

To achieve our aim, we planned the experiments in order to answer the following questions: *i.* Are the results the same when using fresh human blood samples as when they have been frozen for long period?, *ii.* Are the results and work efficiency the same using blood samples from Germany and Brazil? *iii.* Are the results the same using different rapid kits? *iv.* Have the results with the rapid kits been robust in the last 9 years? *v.* How is the sensitivity and is the high hook effect present? and *vi.* What is the buffer pH effect on the test performance?

2. MATERIAL AND METHOD

2.1. Samples and method from aged human blood tests carried out in the public sector (Brazil)

The samples used for these experiments were human blood aged in EDTA tubes from 74 till 88 months (Table 2), collected by a nurse for clinical analysis in VACUETTE® Tubes (Greiner Bio-one International GmbH) or VACUTAINER® Tubes (BD™), and the residual blood fraction. In the period between the collection and the tests, the tubes were maintained at 4°C. A resume of each sample condition is represented

in Table 2. As a positive control, freshly collected human blood was used. The appearance of the control blood was liquid and red.

Considering the blood condition of the samples, a dried piece of the blood with about 0.5 x 0.5mm was used, which would give a dilution of around 10^{-3}. For the liquid sample (D - see Table 2), blood droplets with a size smaller than a satellite drop (less than 1µl in volume) were used for each test to maintain the same approximate dilution of 10^{-3} (this dilution is closer to the field work of crime scene processing in Brazil). Only one analysis for each sample (except for sample D, in which we repeated three times in the same day using the same method) was carried out. The mentioned amount of blood was added to 1,5ml of the buffer, properly mixed and four drops (about 200µl) were added to the membrane. The results were read 10min later.

The batch number of the SERATEC tests used was "LOT 14511", with an expiration date "2016-12". The diluent buffer had the following inscriptions on the label: "Code: 2070002201", "Lot: B14511", "Exp: 2016-12", amongst others. All the kits were valid when tested.

Table 2. Aged human blood tests done in the public sector (Brazil).

	Sample age	Tube Characteristics	Blood condition
A	≈88 months	EDTA, EXP 2010/03,"VACUETTE", "greiner bio-one", "454036 C090842"	dried, dark red
B	≈87 months	EDTA, EXP 2010/03,"VACUETTE", "greiner bio-one", "454036 C090842"	dried, dark red
C	≈86 months	EDTA, EXP 2009/06,"BD Vacutainer", "REF367861", "LOT 8036613"	dried, dark red
D	≈83 months	EDTA, EXP 2010/03,"VACUETTE", "greiner bio-one", "454036 C090842"	liquid, darker red
E	≈74 months	EDTA, EXP 2010/04,"BD Vacutainer", "REF 367856", "LOT 8339125"	dried, dark red

2.2. Experimental designs and conditions used for testing human blood in the private sector (Germany)

The following tests have been used: HemDirect (SERATEC® Germany) (Lot 150053, validity for 2 years) and HEXAGON OBTI (BLUESTAR® Forensic; Monaco, Lot 15001).

Just like in Brazil, in Germany the blood from different donors and species was also received in EDTA vials. The standard dilution for testing was set to 10^{-4}. Most of the experiments were carried out with 2 to 5 repetitions in the same experimental conditions in each laboratory (one laboratory in each country), but in different days. The experiments have been performed according to the user instructions provided by the manufacturer without any modifications for all the cross reactions tests, and in liquid samples.

With the human blood we test the following parameters: *i*. Test sensitivity and the high dose hook effect using human blood (SERATEC and OBTI); *ii*. Performance of the test with fresh and frozen blood samples (SERATEC); *iii*. pH effect.

The sensitivity and high dose Hook Effect were analyzed as following: Pure blood was diluted with the corresponding buffer solution from 10^{-1} to 10^{-8}, diluting a 10^{th} of each value down.

The detection limit for blood was evaluated with three different donors (one male and two female). Initially the fresh blood was tested. Then the blood was frozen at -80°C and -20°C and tested again three years later. As very high concentrated samples may cause weak or negative results due to the high dose hook, the upper limit of the assay was checked as well as the sensitivity limit. The blood was diluted with the appertaining buffer solution. Every concentration was tested three times.

To evaluate the influence of the pH-value a negative and a positive (100 ng/ml hemoglobin) buffer solution were mixed in a 1:1 ratio with solutions of pH-values from 1-14, going one by one steps in pH values.

2.3. Experimental designs and conditions used for different species blood in the private sector (Germany)

We selected a variety of species that are common in Brazil and/or Germany (origin of the Brazilian samples are mentioned on Table 5). Some of the selected species have been reported as problematic for human blood screening, giving false positive, like ferrets and monkeys. The samples were received, handled and used as explained before for the human blood. All the animal samples were donated from Veterinary clinics.

These experiments were performed in the laboratory of the SERATEC GmbH Germany; the numbers of the test batches for fresh blood were: 14332, 14444, 14511, 150015, and 150018, in order to test as well the different batches' performance. In Brazil the Lot 14511 was used (the same as one of the ones used in Germany), validity for a year and 6 months. We additionally evaluated: *i*. Effect of the storage conditions on the blood - fresh and frozen; *ii*. Cross reactivity of blood samples from different animal species (SERATEC and OBTI), *iii*. Test performance from 2009 and 2015. Samples have been stored according to the test manual.

Figure 1. Blood test with different animal species. (A) SERATEC HemDirect, dog, ferret in two different concentrations (10^{-4} and 10^{-5}) and human tests. (B) OBTI HEXAGON test with cat and mule blood from 2009, in 10^{-3} concentration. (C) SERATEC HemDirect, monkey blood (*Callithrix jacchus*) at different concentrations (10^{-2}, 10^{-4} and 10^{-6}). (D) OBTI HEXAGON monkey blood (*Callithrix jacchus*) at different concentrations (10^{-4}, 10^{-5} and 10^{-6}).

2.4. Samples and method for the interspecific tests carried out in the public sector (Brazil)

All the tested samples consisted of fresh venal blood, collected by a veterinarian, in sterile EDTA tubes (VACUPLAST® GesmbH, lot 1504507, exp. 2017-04). The tubes were maintained at 4ºC until the tests were carried out (not more than 24h). As a positive control, freshly collected human blood was used.

Figure 2. High Dose Hock Effect. (A) SERATEC HemDirect in different concentrations (10^{-1}, 20^{-1}, 50^{-1} and 10^{-2}); (B) OBTI HEXAGON in different concentrations (10^{-1}, 20^{-1}, 50^{-1}, 10^{-2} and 10^{-3}), a faint line appeared after 3h.

Considering the need to approximate the test to the real evidence analysis, an amount of visible blood - as small as it can be seen - was considered. In practical matter, blood droplets with a size smaller than a satellite drop were used (less than 1mm in diameter, so less than 1µl in volume) for each test. Considering the SERATEC´s buffer volume in each test tube, the dilution of the sample on this scenario was calculated to about 10^{-3}.

The tests for each species were repeated three times using the same method. The mentioned amount of blood was added to the buffer, properly mixed and four drops (about 200µl) were added to the membrane. The results were read 10 min later.

The batch number of the tests used was "LOT 14511", with an expiration date "2016-12". The diluent buffer had the following inscriptions on the label: "Code: 2070002201", "Lot: B14511", "Exp: 2016-12", amongst others. All the kits where valid when tested.

3. RESULTS AND DISCUSSION

3.1. Test Range: Sensitivity and High Dose Hook Effect (HDHE)

HemDirect SERATEC was able to detect hHb in a dilution down to 10^{-7} (Table 3) and there was no high dose Hook Effect with it, being the positive result line visible but weak even at 10^{-1} (Table 3). The best reading results were obtained at dilutions between 10^{-4} and 10^{-5} for HemDirect SERATEC (Table 3). Hexagon OBTI showed the best reading result line at 10^{-4} and 10^{-5} dilutions (Table 3) in a test ranging from 10^{-1} to 10^{-7} (Table 3), and the minimum sensitivity was observed at 10^{-6}. OBTI starts presenting a high dose Hook Effect that leads to negative results for blood concentrations of 10^{-2} and above. As shown on Fig. 2B, at 10^{-2} blood concentration no line was observed in the test. But 3 hours after the test started a faint line appeared.

The HDHE is well known for causing false negative results for immunoassays that are run with too high antigen concentration, as explained earlier. This may lead the inexperienced interpreter to deceive himself in the face of a human bloodstain. Even specialists that well know the possibility of false negative due to HDHE may encounter problems of interpretation if the maximum blood concentration for positive results is not known. These studies give a warranty for a better performance of the evaluated test and robustness of protocols [3-4].

In case of the SERATEC HemDirect, the HDHE did not lead to false negative results, but the line intensity at a dilution of 10^{-1} had been clearly fainter compared to less concentrated samples (Table 3). It might show a HDHE for concentrations higher than 10^{-1}. Additionally the background of the test got reddish, which might be

an issue when reading a faint line of gold labeled antibodies.

The Hexagon OBTI leads to false negative results for dilutions of 10^{-1} and 10^{-2}. This test is used by many forensic laboratories and has been tested previously [5]. For both tests we suggest to dilute the suspected blood samples at least 1:100 using the provided buffer.

Table 3. Sensitivity and Hook Effect reading obtained with the SERATEC HemDirect and OBTI after 10 minutes.

Dilution	SERATEC			Card description	OBTI	Card description
	Fresh blood	-20°C	-80°C		Fresh blood	
10^{-1}	+	+	+	weak positive, red background	-	negative, red background
10^{-2}	++	++	++	positive, red background	*	negative, red background
10^{-3}	++	++	++	positive	++	positive
10^{-4}	+++	+++	+++	strong positive	+++	strong positive
10^{-5}	+++	+++	+++	strong positive	+++	strong positive
10^{-6}	++	++	++	positive	++	positive
10^{-7}	+	+	+	weak positive	-	negative
10^{-8}	-	-	-	negative	n.a.	n.a.

Weak positive (+), positive (++), strong positive (+++) description are represented on Fig. 2A. *a line could be seen after more than two hours (Fig. 2B).

3.2. Influence of the pH-value

All of our results with the pH come from years of internal validation using the SERATEC HemDirect Test in Germany. The pH-values below 3 or above 12 may lead as well to false or invalid results caused by either nonspecific binding or lowering the affinity of the antibodies [6]. Therefore and among other issues a buffer solution is provided with the tests by many products (SERATEC HemDirect product instructions, unpublished internal studies). Extreme use of pH has been shown with other fluids and other type of tests to have several negative results [7].

Our results shows that the use of a buffer solution with a pH value of 1, 13 and 14 leads to invalid results with the absence of the control line. This means the test is invalidated because it was destroyed by the extreme acidity or alkalinity (a line is only formed partially or no line is formed). With the buffer alone, the test result is negative, showing that our control was working. When human blood was diluted with the different pH buffers to the 10^{-4} concentration, with pH 2 and pH 12 the positive lines are not as strong colored as expected for a positive result, because these were not the ideal pH values used for the buffer to dilute the blood. It might be that pH influences the binding of antigen and antibody or that the extreme acidity or alkalinity is degrading either hemoglobin or the anti-hHb.

Table 4. Cross reaction performance results from different batches.

Species	SERATEC 2004[1]	SERATEC 2009[1]	OBTI 2009	SERATEC 2015[2]	SERATEC 2016[3]	OBTI 2016[4]
Apaalosa and Domestic Horse (*Equus ferus caballus*)	n.a.	-	-	-	-	-
Cat (*Felis domesticus*)	-	-	+	-	-	-
Chicken (*Gallus gallus docmesticus*)	n.a.	n.a.	n.a.	n.a.	-	-
Cow (*Bos taurus*)	-	-	-	-	n.a.	n.a.
Dog (*Canis lupus familiaris*)	n.a.	-	-	-	-	-
Ferret (*Mustela putorius furo*)[5]	n.a.	+	+	+	-	+
Goat (*Capra aegagrus hircus*)	n.a.	-	-	-	-	-
Mouse (*Mus musculus*)	n.a.	n.a.	-	n.a.	-	n.a.
Monkey (*Callithrix jacchus*)[5]	n.a.	n.a.	n.a	n.a.	+	+
Mule (*Equus mulus*)	-	-	-	-	n.a.	-
Rabbit (*Oryctolagus cuniculus*)	-	-	-	-	-	-
Red Dear (*Cervus elaphus hippelaphus*)	-	-	-	n.a.	-	-
Sheep (*Ovis aries*)	-	-	-	-	n.a.	n.a.
Controls						
Human (Positive)	+	+	+	+	+	+
Buffer (Negative)	-	-	-	-	-	-

"n.a." means "not available"

[1]Done in three repetitions, diluted at 10^{-4}. [2]Reading done with SERATEC HemDirect in frozen blood since 22/01/2009 at -20 °C. Diluted at 10^{-4}. Blood kept in EDTA. [3]Fresh received blood samples. Kept in EDTA at 4 °C, diluted at 10^{-4}. [4]Blood from frozen or from fresh received samples, diluted at 10^{-4}. [5]Aditional tests with cassettes from five different lots was done, but not reported on this table. We used human and ferret frozen blood at a 10^{-5} concentration. The positive was very clear for human and very clear negative for ferret and monkey.

3.3. Test performance in the last 9 years and cross reaction testing

The results for dried and aged human blood tests were positive with the 74 to 88 months old samples, although sample "D" showed a negative result. A brief description of the sample can be found in Table 2. The methodology of the public sector (Brazil) was used with samples nearest to the forensic situation as earlier

described. Sample D was the only liquid sample and showed characteristics of degraded blood (like dark color and strong putrefaction odor). It is likely that, since the sample was kept liquid, the hemoglobin got degraded after sometime, even maintained refrigerated. On the other hand, all the other samples were dry when tested; suggesting that removing moisture of blood samples will keep them viable for future immunoassays. Our results are accordingly to other publications were blood samples stored at room temperature for 33 years have given positive results [3].

Comparing the results of experiments carried out in 2009 with experiments carried out in 2015, we noticed that the OBTI Hexagon resolved some of the 'problematic' results seen in 2009 (Table 3), although it was not done for all the blood samples (Table 4). The 2015 performance for the cross reaction test was better than in 2009. The human blood test was negative at a concentration of 10^{-7} (next section), the ferret's blood was negative at 10^{-5} (and below) and the monkey was negative at 10^{-5} (Fig. 1C). The interspecific blood test done in the public sector in Brazil had consistent results with the tests done in Germany for SERATEC HemDirect, and the observed results are expressed in the Table 5. The same 10^{-4} blood concentration was used to evaluate different samples from different origins, sources and maintenance conditions in Germany and Brazil. The performance of the SERATEC HemDirect test in 2009 and 2015 were the same in either tries (Table 4).

Table 5. Results for the interspecific tests done in the public sector (Brazil), with SERATEC HemDirect.

Species	Sample origin	Result
dog (*Canis lupus familiaris*)	Jaguariúna/SP	-
goat (*Capra aegagrus hircus*)	Jaguariúna/SP	-
sheep (*Ovis aries*)	Jaguariúna/SP	-
horse (*Equus ferus caballus*)	Jaguariúna/SP	-
rabbit (*Oryctolagus cuniculus*)	Piracicaba/SP	-
rat (*Rattus norvegicus*)	Piracicaba/SP	-
guinea pig (*Cavia porcellus*)	Paulínia/SP	-
red-faced spider monkey *(Ateles paniscus)*	Americana/SP	-
positive control (human blood)	Campinas/SP	+
negative control (buffer)	SERATEC	-

Based on the calculation considering the average hemoglobin found in human blood, the hemoglobin concentrations were estimated at the dilutions shown in Table 3 as about 15-25 ng/ml. This implies a minimum sensitivity of 20 ng/ml of the SERATEC HemDirect test.

The results with SERATEC HemDirect were all negative for non-human, with exception of ferret and monkey (Table 4, Fig. 1A and C), and equal comparable to the fresh blood test results (Table 4). An extra test was done with fresh ferret and monkey blood at a lower concentration (10^{-5}) and was negative in both concentrations for both species (Fig. 1A).

4. CONCLUSIONS

The SERATEC HemDirect test and HEXAGON OBTI are very suitable for the detection of human blood in forensic samples. But it matters how both tests performed in the HDHE test and in the sensitivity test, considering cross-reaction as possible false positives. The results here showed that, in order to avoid HDHE we should make dilutions higher than 10^{-2}. On the other hand, the sensitivity of the tests was consistent on concentrations above 10^{-7} for SERATEC HemDirect and 10^{-6} for the HEXAGON OBTI. Interspecific tests suggested that, if it is realistic that the blood sample is monkey or ferret origin, the working concentration should be around 10^{-5} aiming to avoid false positive results. For the forensic field practitioner, it might be difficult to control the sample concentration with that sort of precision. Thus, the field practitioner, knowing the possibility of false results, positive or negative, should carry out further tests at the lab, either from the private or the public sector.

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