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Preliminary examinations aiming at confirming presence of human blood in biological traces by means of mRNA analysis

Summary

The aim of the project was to develop a specific and at the same time economical method for detecting human blood in biological traces based on analysis of haemoglobin mRNA with use of PCR reaction in real time and non-specific SYBR Green detector. The test, which has eventually been developed enabled simultaneous analysis of melting curves for three fragments of various lengths: HBB61, HBA197 and HBB503, as well as an additional reference gene: mRNA β-actin. A definite identification was possible already for 0,1 µl of blood. The method is tissue and species specific. The analysed mRNA markers are characterized by high stability, as compared to haemoglobin detected by standard methods. The result of mRNA profiling shows the predictive value as regards quality of genetic material and occurrence of mixture of liquids. Results of analyses performed during the project indicate potential usefulness of HBB and HBA1 markers in routine forensic genetic examinations. However, it is necessary to carry out a broader spectrum of validation experiments, and particularly to analyse a larger number of actual biological casework and precisely determining an optimal quantity of RNA and identifying ontogenetic differences in the levels of expression.

Key words: human blood identification, mRNA profiling, haemoglobin

Introduction:

In forensic DNA laboratories identifications of various biological traces basing on DNA profiles are performed on regular basis. However, in most cases it is equally important to determine the source of stains, i.e. the kind of tissue or body fluid.

The most frequent biological traces recovered at crime scene are blood stains. Their examination provides investigators with extremely valuable information. For example, bloodstain pattern analysis may be helpful in reconstructing the course of events and by analysing DNA isolated from nuclear blood cells unambiguous individualisation of trace's origin is possible. In the majority of cases blood traces, particularly fresh ones, can be easily recognised. However, on various substrates and with passing time dried blood stains may fade or significantly change their colour or crumble off. Due to that they may be taken for another substance or simply overlooked during crime scene investigation. What is more, recovered traces do not always turn out to be human blood. It should also be emphasised that a subjective assessment based solely on observation does not constitute a reliable proof. Therefore additional tests are needed in order to impartially and credibly confirm the presence of blood. Detection and/or confirmation of blood presence is effected by means of methods based on redox, enzymatic, immunological and molecular reactions or with use of a, so called, alternative light source. The review of these methods and their detailed characteristics was made by Virkler and Lednev (2009).

Generally, tests for detecting various biological stains, including blood, are divided into two groups according to their specificity: screening and confirming tests. Screening tests is based on the ability of hem molecules to oxidise, as a rule are not invasive and, in addition to that, they are easy to use and very sensitive, they enable detecting both human and animal blood. Unfortunately, they often give a false positive result with various substances demonstrating oxidising properties, such as fruit juice, detergents, tea. On the other hand, human blood specific tests are based on immunological detection of haemoglobin. These methods are sensitive but they require a comparatively large quantity of material for examination, so they cannot be always used. Besides, they are useless in case of degraded traces.

In recent years, with the development of new technologies, molecular methods have become more widely used in identification of tissue or body fluid types. The reasons for the growth of their popularity include the lowering costs of the analyses. Until this date, among others methods based on analysis of methylation patterns for a particular fluid, determining the level of expression of mRNA and miRNA, as well as microbiome characteristics. More information on advantages and disadvantages of specific methods can be found in the survey work of Sijem (2015). For example, if the laboratory only has DNA extract, the source of trace origin can be determined solely by analysing DNA methylation markers. Difficulties in interpretation of results of this method may occur in case of stains being mixtures of molecules characterised by different methylation patterns. On the other hand, based on microbiome it is possible to determine the origin of a trace not just as a given tissue or fluid but also body region, e.g. oral cavity or vagina. Doubts as to the use of those methods are evoked by the fact that diet, physiological state, climate, ethnic origin or sex influence both the level of methylation and the composition of microbiome (Sijen, 2015). The highest number of works on the subject of determining types of body fluids concern mRNA and miRNA analyses, which demonstrate a specific pattern of expression depending on tissue of origin. Similarly to the case of DNA RNA can be analysed in quantities of the order of nanograms and even picograms. Due to the possibility of simultaneous extraction from a stain both DNA and RNA, even from a small trace it is possible to obtain all necessary information, i.e. a unique genetic profile and tissue of origin (Park, Park, Lee, 2013; Lindbergh, de Pagter, Ramdayal, Visser, Zubakov, Kayser, Sijen, 2012; Wang, Zhang, Luo, Ye, Yan Hou, 2013). Until this day it has been thought that the low stability of RNA molecules as compared to DNA prevents RNA analysis from degraded biological traces. The half-life of nucleic acids is limited by various endogenous factors, e.g. molecule structure, and exogenous factors, such as presence of light, enzymes, water and pH. In relation to that, a view has been established that the double strand DNA molecule additionally bonded with proteins is much more stable than the single-stranded RNA. However, more detailed research of RNA has demonstrated that its molecules also form various double-stranded structures and be bonded with proteins. Lower stability of RNA results first of all from the fact that its degradation occurs mainly by the activity of ribonucleases, which are present in great numbers in higher species cells, in bacteria and external environment. This statement derives from research of *post mortem* RNA degradation, whose outcome shows that in the tissues containing various levels of ribonucleases RNA decomposition occurs in various times ranging from a few minutes to a few weeks (Fordyce, Kampmann, van Doorn, Gilbert, 2013). Biological traces are most often found as dried stains, where the activity of enzymes dramatically drops. Degradation occurs mainly by the effect of physical and chemical factors, similarly as in case of DNA. A detailed description of RNA degradation mechanism is presented in a publication by Fordyce et al. (2013).

There are many scientific works on identification of biological fluids and tissues based on mRNA and miRNA detected in biological material that had been stored for longer periods (Setzer, Juusola, Ballantyne – 2008; Hanson, Lubenow, Ballantyne, 2009; Hanson, Haas, Jucker, Ballantyne, 2012, Sakurada, Ikegaya, Fukushima, Akutsu, Watanabe, Yoshino, 2009; Zubakov, Kokshoorn, Kloosterman, Kayser, 2009; Zubakov, Boersma, Choi, van Kuijk, Wiemer, Kayser, 2010; Courts, Medea, 2011; Haas, Hanson, Bär, Johansen, Lindenbergh et al, 2012; Richard, Harper, Craig, Onorato, Robertson, Donfack, 2012; Jakubowska, Maciejewska, Bielawski, Pawłowski, 2014). Lindenbergh et al. (2012) succeeded in determining mRNA markers even from blood stains as old as over 28 years. The elaborated methods of RNA analysis CE and/or RT-QPCR techniques are used. In case of miRNA markers levels of expression are compared between tissues, while for mRNA markers – presence or lack of expression product. Basing on the review of the above literature it can be determined that the most sensitive and most often used blood marker is haemoglobin mRNA. In addition to that among other blood specific mRNA markers the following should be mentioned: GlycoA, PBGD, CD93, AMICA1, PPBP, NKG7, CCL5, NRGN, GZMH, PRF1. Jakubowska et al (2014) demonstrated that mRNA, MMP7 and MMP11 expression is specific for menstruation blood.

The aim of the hereby project was elaboration of a specific and, at the same time, cheap method for detection of human blood in biological traces based on haemoglobin mRNA analysis with use of PCR reaction in real time.

Materials

For the optimisation and validation the Authors examined human blood recovered during post mortem examinations performed in the Forensic Medicine Department of Collecgium Medicum in Bydgoszcz. In laboratory assessment of tissue specificity cDNA from 10 µl of blood, 10 µl of semen and 10 µl of saliva was used recovered on cotton fabric and RNA obtained from a mixture of tissues with no blood content (Control RNA Human of 50 ng/µl concentration [Applied Biosystems]). Evaluation of reaction sensitivity was achieved by analysing one-day-old dry stains made from 10 µl, 5 µl, 1 µl, 01 µl and 0,01 µl of blood on cotton fabric. Evaluation of species specificity of selected markers was made by analysing blood originating from four animal species: domestic cattle (*Bos Taurus),* domestic pig (*Sus scrofa f. domestica*), dog (*Canis lupus familiaris*) and domestic hen (*Gallus gallus domesticus)*. The stains were made by pouring out 10 µl of every collected blood sample on cotton textile, which was subsequently dried and stored in the dark. The influence of time on RNA stability was evaluated by analysing material that had been stored for periods from 0 to 28 days in room temperature exposed to sunlight in a natural 24 hour scheme, from which full RNA was extracted at week intervals. The evaluation of the influence of type of substrate that the body fluid had been found on was made by examining cDNA obtained from full RNA extracted from one-day-old blood stains made on denim and Whatman blotting paper stored in a laboratory in room temperature and in the dark.

Also real biological stains in form of clippings from garments and fragments of swabs from stains of substances as to which there had been a suspicion of human blood content were tested by means of HemCheck (Hydrex) specific immunochromatographic test.

Controls were made of control RNA Human solution of 50 ng/µL concentration (Applied Biosystems) and RNA extracted from fresh human blood.

Methods

Based on available literature and databases the Authors selected HBB61 and HBB503 markers for identification of human blood mRNA beta haemoglobin (GenBank ref no.: NM_000518.4) and HBA197 marker for human blood alpha haemoglobin (GenBank ref no.: NM_000558.3). As referential gene β-actin mRNA was used. Information on the sequence of starters, their lengths, melting temperature (Tm), percentage of GC pairs and data concerning lengths of all the markers amplifications products formed in reaction with those starters is given in Table no. 1. Sequences of starters for HBB61 fragment originate from literature (Lindenghberg et al., 2012). The remaining ones were designed with use of Primer3 software available on-line.

Full RNA was extracted from dry blood stains with use of TRI Reagent Solution (Ambion), according to Manufacturer's Instruction.

The reverse transcription reaction was carried out with use of High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and random hexamers in the final volume of 10 µl, adding to the reaction 0,5 µg RNA.

Determining mRNA markers was performed with use of PCR in real time with use of the non-specific detector of SYBR Green type. The reaction mixture in volume of 12,5 µl eventually contained 0,5 µl cDNA matrix, starters in respective concentrations: HBB503 – 30 pM; HBB61 – 30 pM; HBA197 – 10 pM; β-actin – 15 pM, 300 nM ROC solution (EURx) – 0,25 U UNG solution (uracil-N glycosides) (EURx), 1 x concentrated SG qPCR Master Mix (EURx). Reaction temperature profile: initial denaturation at 94°C for 5 minutes, 32 cycles: denaturation at 94°C for 30 seconds, starter bonding at 67°C for 30 seconds, elongation of strand at 72°C for 1 minute and final elongation at 72°C for 10 minutes. All the amplification reactions were performed at least twice. For analysis of the melting curves the following reaction conditions were applied: 95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds. The reactions were performed on ViiA 7 Real-Time PCR System. The analysis of results was carried out with use of ViiA 7 Ruo Software appended to the instrument.

Table 1. Characteristics of starters used for mRNA profiling.

Marker	Starter sequence	Starter length (bp)	Product length (bp)	Tm [°C]	GC [%]	Source
HBB	F: 5`GCACGTGGATCCTGAGAAC	19	61	53,2	57,9	(Lindenbergh et al.. 2012)
	R: 5` ATGGGCCAGCACACAGAC	18		52,6	61,1	
HBB	F: 5`CATGGTGCATCTGACTCCTG	20	503	53,8	55,0	own work
	R:5`AGTTGGACTTAGGGAACAAAGG	22		53,0	45,5	
HBA	F: 5` TTCCCCACCACCAAGACCTA	20	197	53,8	55,0	own work
	R: 5`AGGAGCTTGAAGTTGACCGG	20		53,8	55,0	
β-actin	F: 5` CTTCAACACCCCAGCCATGT	20	301	53,8	55,0	own work
	R: 5` CTCTTGCTCGAAGTCCAGGG	20		55,9	60,0	

Results

The created test for identification of human blood enables simultaneous analysis of melting curves of three markers: HBB61, HBA197 and HBB503, as well as, additionally, a reference: β-actin mRNA gene. In such designed reaction, in case of human blood, instead of expected four, three products were obtained of Tm value approx: 80,5°C, 85,3°C and 89°C, respectively, while in case of human biological material without blood content only one product of Tm value approx. 86,8°C was obtained. This was due to the too small difference between Tm values for HNN503 and the reference gene, which causes that in the multiplex reaction melting curves for these two markers overlap. An exemplary plot of multiplex reaction products melting curves for a blood sample is presented in figure 1.

Based on analysis of homologous sequences in haemoglobin gene by means of BLAST tool it was determined that HBB61, HBB503 and HBA197 starters are specific for primates. The comparatively low rate of homology in other mammals (approx. 83%) indicates that selected markers should not amplify at all or, possibly, non-specific products may be formed. Fr the sake of experiment the Authors checked specificity of starters for blood coming from four species of animals: domestic cattle (*Bos Taurus),* domestic pig (*Sus scrofa f. domestica*), dog (*Canis lupus familiaris*) and domestic hen (*Gallus gallus domesticus)*. Among fours markers selected for multiplex reaction: HBB61, HBA197, HBB503 and β-actin, only HBB197 product was obtained from animal blood samples. This amplicon had the same length as in case of human blood and a similar Tm value. In case of canine and pig blood the difference in melting times amounted to 1°C, and as regards standard deviation there is practically no difference between human blood and hen or cattle blood. For human blood used as the positive control all mRNA fragments were detected (Table no. 2.)

In order to determine the sensitivity of the method, several stains made of 10 µl, 5 µl, 1 µl 0,1 µl and 0,01 µl of blood were analysed. It was assumed that the result of reaction was positive if all the products of multiplex reaction were obtained at least in two repetitions. With such a criterion the threshold of method sensitivity was 0,1 µl of blood.

For evaluation of mRNA markers stability in time complete RNA was extracted from bloodstains on the day of their submission (point 0), as well as after 7, 14, 21 and 28 days of storage. The analyses were performed on samples coming from 8 persons. Blood identification from all the stains was successful regardless their age. The analysis of mean Ct values for samples extracted in subsequent weeks demonstrated a small decrease in the quantity of amplification product as compared to the sample extracted at "zero" time. The mean Ct value obtained after amplification of RNA extracted from blood stored for 28 years was lower by approx. 2 cycles (Table no. 3).

To assess the influence of different types of substrates, being the backgrounds for body fluid stains, on the effectiveness of human blood identification method, the Authors used cDNA originating from complete RNA extracted from stains made on denim and Whatman blotting paper. Biological samples from two individuals were analysed. In case of both substrates all specific products enabling unambiguous blood identification were formed.

The test based on analysis of presence of haemoglobin mRNA markers was used for identification of human blood in 22 real biological stains as to which

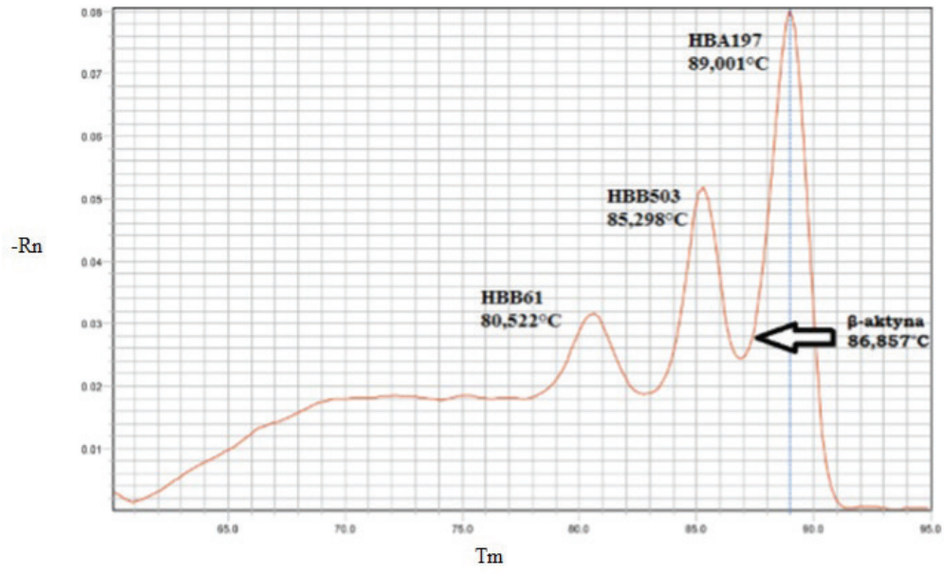

Fig. 1. Melting curve diagram for multiplex reaction products.

Table 2. Mean melting temperatures for animal blood samples and control containing human material.

	Mean Tm [°C]			
Species	**HBB197**	**HBA503**	**HBB61**	**β-actin**
Bos taurus	88,778	-	-	-
Sus scrofa f. domestica	87,995	-	-	-
Canis lupus familiaris	87,995	-	-	-
Gallus gallus domesticus	88,338	-	-	-
Homo sapiens	88,898	85,195	80,615	86,857
Rother tissues*	-	-	-	86,857

* RNA originated from solid tissues was used, no blood content –Control RNA Human, 50 ng/μL concentration (Applied Biosystems).

there was a suspicion of blood content. Selected traces first underwent preliminary examinations aiming at determining presence of blood with use of non-specific HemoPhan test (Pliva-Lachema), as well as a test specific for human haemoglobin, HemCheck (Hydrex). For all these traces genotyping reactions were performed with use of commercial HIS kits: NGMSelect (Applied Biosystems) and/or PowerPlex ESI 17 (Promega). Depending on results of specific tests samples were divided into 7 groups (table 4). Group 1 comprised 13 samples which showed positive results of HemCheck test and for whom full RNA and DNA profiles were obtained, while HemCheck gave negative results. Within this category three traces showed symptoms of degradation on the level of nucleic acids. This was demonstrated by characteristic lowering of peaks of the longest STR sequences (DNA profile) and a significantly larder increase of the shortest product of HBB61 amplification (RNA profile). However, one of the samples was characterized by a much higher increase of HBB503 fragment as compared to the remaining mRNA markers and DNA profile indicated presence of genetic material mixture. Group 2 was made up of samples, for which both conventional blood tests gave positive results and full DNA and RNA profiles of donors were obtained. The third category consisted of one sample, which differed from group 2 by an ambiguous result of HemCheck test. Two traces were included in group 4 characterised by a positive result of HemoPhan test, negative result for human haemoglobin content, negative result of genotyping as regards DNA-STR and incomplete RNA profiles (two out of three mRNA markers were identified). In addition to that, in mRNA profiling reaction non-specific amplification products were obtained. Group 5 consisted of one sample, which gave a positive result of non-specific HemoPhan test and negative results of HemCheck and DNA genotyping, while mRNA analysis showed only HBB61, i.e. the shortest fragment. Two samples were qualified to group 6, where the criteria included: positive result of HemoPhan test, negative result of HemCheck test and degraded DNA and mRNA profiles. Group 7 includes a trace, for which HemoPhan tests gave a positive result, HemCheck was not conducted due to insufficient quantity of material for examination and the results of DNA and RNA profiling were negative.

Table 3. Analysis of time influence on RNA stability. Ct – mean Ct values obtained in multiplex mRNA profiling reaction for individual samples coming from 8 persons, SD – standard deviation.

	Days of storing the stain before extraction									
	0		7		14		21		28	
Ordinal no.	**Ct**	**SD**	**Ct**	**SD**	**Ct**	**SD**	**Ct**	**SD**	**Ct**	**SD**
1	21,792	0,821	23,421	0,745	24,393	0,618	22,064	0,821	23,611	0,760
2	21,324	0,655	22,643	0,227	21,466	0,476	23,786	0,402	23,808	0,431
3	22,734	0,098	22,457	0,879	23,568	0,581	23,419	0,981	23,776	0,881
4	20,345	0,124	21,615	0,145	23,590	0,090	23,987	0,034	24,987	0,095
5	22,000	0,568	21,484	0,098	24,003	0,478	22,998	0,412	24,158	0514
6	21,768	0,987	21,324	0,675	23,948	0,589	22,531	0,634	23,911	0,617
7	21,989	0,099	23,142	0,763	23,098	0,613	24,561	0,752	24,122	0,732
8	20,732	0,878	22,444	0,221	24,108	0,717	24,178	0,248	22,785	0,342
Mean	**21,586**	**0,529**	**22,316**	**0,469**	**23,522**	**0,520**	**23,440**	**0,535**	**23,895**	**0,547**

Table 4. Results of tests for presence of blond and human DNA carried out on real biological traces.

Group.	Number of samples	HemoPhan	HemCheck	RNA profile	DNA profile	Comments
1	13	+	-	+ (3)	+	In case of 3 samples much higher amplification of HBB61 product as compared to other markers was observed. For those samples DNA profile showed symptoms of degradation. In one sample HBB503 peak was significantly higher than peaks for the remaining specific mRNA products and DNA profile was a mixture.
2	3	+	+	+ (3)	+	–
3	1	+	+/-	+ (3)	+	–
4	1	+	-	+ (2)	-	In addition to proper HBB61 and HBA197 products also non-specific products were formed.
5	1	+	-	+ (1)	-	Only HBB 61 product was obtained
6	2	+	-	+ (1)	+	Only HBB 61 product was obtained. DNA profile was of partial character
7	1	+	N.B.*	-	-	The reason may be too small quantity of blood or its absence

Key: „+"– positive result of test; „-" – negative result of test; „N.B.*" – sample was not examined due to insufficient quantity of material. In „RNA profile" column in brackets numbers of determined markers in multiplex reaction were given.

Discussion

Blood is one of the most often analysed biological traces found at crime scenes. Quite frequently, stains are small and the examined has to choose whether preliminary tests identifying the source of the trace should be undertaken at the risk of losing too much of the material, or immediately proceed to DNA profiling. Elaboration of a method that would enable identifying the type of biological material with minimum loss of material is of great importance in today's forensic science. The majority of the latest techniques proposed for that purpose involves RNA analyses and in particular analyses of mRNA expression patterns that are characterised with tissue specificity.

Basing on literature two mRNA molecules were selected: HBB – β-haemoglobin and HBA1 – α-haemoglobin 1 (Haas et al., 2011a; Haas, Hanson, Kratzer, Bär, Ballantyne, 2011b; Kohlmeier, Schneider, 2012; Nussbaumer, Gharehbagi-Scnell, Korschineck, 2006; Park et al., 2013), which are characterised by a high level of expression in blood. Obtained results confirm applicability of HBB and HBA1 markers for detection of human blood. However, the occurrence of potential individual differences in levels of expression of HBB and HBA1 transcripts may influence the results of tests based on mRNA profiling. According to the assumptions the analysis of amplification products melting curves for selected haemoglobin mRNA fragments would have enabled definite confirmation of blood presence in a trace in question. Both for beta haemoglobin and alpha haemoglobin starters were designed in such a way so as the amplified fragments were placed in the locations of exon bonds. This aimed at excluding the risk of DNA amplification, which might have led to false positive results. In designing starters also SNP polymorphisms occurring in the area of their bonding to an appropriate place on the matrix. The eventually created test for identification of human blood enables simultaneous analyses of melting curves for three fragments of various lengths: HBB61, HBA197 and HBB503, and additionally the reference gene: mRNA β-actin. Due to the fact that HBB61 was the shortest of the obtained amplicons, it had been expected to amplify with highest efficiency but it turned out to be the poorest multiplied fragment in the multiplex reaction. A likely reason for poorer amplification of this fragment was starter's sequence itself. Its both ends are partially complementary and thus have a tendency to create a buckle structure. This explains the necessity of using higher concentrations of those starters in a multiplex reaction.

Setting up of appropriate conditions during the reaction was of crucial importance for the entire analytical process. The applied method of detecting fluorescence using a non-specific dye does not require designing a complex probe, which, decreases the cost of analysis but imposes the need for precise optimisation of the method. SYBR Green as a non-specific detector may bond not only with expected product but also with dimer starters or other non-specific products of reaction, which makes interpretation difficult and falsifies the results. Therefore, an analysis of melting curves enabling confirmation of specific amplicon presence was conducted. In the process of designing

the multiplex reaction three mRNA fragments differing in lengths and Tm values by at least 4˚C (two respective fragments for HBB markers of 61 bp and 503 bp lengths and Tm values equal approx. 80,5˚C and 85,3˚C) and additionally the product of amplification of β-actin reference gene of 301 bp length and Tm value of approx. 86,8˚C. The melting points of first three amplicons differed to such a degree that their identification based on melting curves obtained from the multiplex reaction. The characteristic Tm value for the referential gene differed by approx. 1,5˚C from the value characteristic for the product of 503 bp length. This difference turned out insufficient to definitely confirm the presence of β-actin in the multiplex in case when blood is the analysed material. At the same time it should be acknowledged that it is formed in a monoplex reaction, in which the matrix originates from human blood and in a multiplex reaction in case of analysing RNA not originating from blood. Therefore it was assumed that that failure to visualise the β-actin marker was not important when in the multiplex test all haemoglobin markers were detected, however it constituted a certain positive control of the reaction. The presence of exclusively this amplicon proves that the examined trace has human origin, while lack of any product may indicate high degradation of matrix, presence of inhibitors that block the reaction or non-human origin of the analysed material. In order to confirm usefulness of HBB61, HBA197 and HBB503 markers for identification of human blood in forensic DNA analysis several validation experiments ought to be conducted on various human origin biological material such as epidermis, excrements and secretions.

Among four multiplex markers proposed for multiplex reaction HBA197 was amplified not only in case of human blood but blood of animals selected for analyses: *Bos Taurus, Sus scrofa f. domestica, Canis lupus familiaris* and *Gallus gallus domesticus.* The reason for that was a high level of homology between human alpha haemoglobin gene and animal genes. Due to the same length in bp and too small differences in Tm value of products obtained from human blood and blood originating from the animals used in the experiment, HBA197 fragment cannot be considered a marker specific for human blood. Three remaining markers (HBB61, HBB503 and β-actin) were multiplied only from human biological material. The results indicate a possibility of using the created multiplex reaction not only for confirming the presence of human blood but also for differentiating between human and animal blood. Formation of only one product, HBA197 indicates the presence of animal blood in the material in question. However, detecting the presence of all three haemoglobin markers gives certainty that analysed material contains human blood. In order to make a more precise evaluation of species specificity carrying out laboratory analyses of blood coming from other animal species is necessary.

In the hereby work, In the hereby work, various quantities of blood poured directly onto cotton fabric (10 µl, 1 µl, 0,1 µl and 0,01 µl, respectively) were used to determine method sensitivity. Difference in Ct values between samples coming from various persons are noticeable and they may be a consequence, among others, of the influence of death causing factors or degradation of RNA, which occurred between the time of death and the time of sampling, and even result from a difference in efficiency of RNA extraction from individual samples. Possibly, they can also derive from varying levels of haemoglobin mRNA in blood. In order to determine a sensitivity threshold it was assumed that that the result of the test is positive when in the examined sample all blood markers are detected in at least two repetitions. With such an assumption, identification would be possible also from 0,1 µl blood sample. Too large quantity of used material also affects the results. This may be a consequence of the amplification being inhibited by excessive matrix concentration or the presence of haem, which has not been eliminated during RNA extraction. The range of sensitivity of mRNA profiling with use of HBB marker cited in literature falls between 0,1-0,01 µl of blood (Haas et al., 2011a, 2011b; Kohlmeier, Schneider, 2012; Lindenbergh et al, 2012). Differences in sensitivity may result primarily from varying efficiencies of applied extraction and amplification methods. The fact that other researchers used fresh blood from healthy volunteer donors (Haas et al., 2011b) may be not without a significance. Moreover, the criterion they adopted for determining reaction sensitivity limit is the possibility of detection a product in at least one repetition, while in the hereby work the criterion of at least two repetitions is applied. The high sensitivity of mRNA profiling in combination with the possibility of simultaneous RNA/DNA extraction created enormous potential of using this method in routine work of forensic DNA laboratories.

In order to test a possibility of using the elaborated test in analysis of other than fresh bloodstains, results were analysed with use of materials stored for 0, 7, 14, 21 and 28 days. For every time point it was possible to amplify all the markers, which indicates relative RNA stability at least within one month. It has been reported that RNA is stable in blood stains stored for 16, 23 and even 28 years (Zubakov et al., 2009; Kohlmeier, Schneider, 2012; Lindenbergh et al., 2012). For assessment of RNA stability additional research is needed because it depends not only on the passage of time but also on other external factors, such as humidity, UV radiation intensity or varying temperature. Stains examined within the hereby project had been stored in a dry place and had not been exposed to sunlight, so there wasn't a significant degradation of genetic material. Haas et al. (2011s) analysed blood samples that had been exposed to the influence of environmental factors, such as rain, high temperature

or sunlight with an additional evaluation of the effect of donor's sex. The most destructive factors for RNA were rain and high temperature, however, the possibility of identification depended on the type of used mRNA markers. Similarly as in case of researching the influence of time, the most stable blood markers turned out to be HBB and HBAA1 detected in every examined sample (Haas et al., 2011a).

Biological traces recovered at a crime scene may be found on various surfaces, such as wood, paper, footwear or garments. Due to differing absorbance of the substrates and possible occurrence of inhibitors, which may be transferred to the extract during RNA isolation one might make a supposition that type of background may have an influence on the results of the identification test. Within the hereby work in order to check the type of background on the analysis RNA matrices originating from stains made on denim and Whatman blotting paper were used. In both cases in all the samples the presence of relevant haemoglobin markers was successfully confirmed, which indicates that those particular substrates do not influence the reaction of RNA profiling created within this project. However, in order to fully evaluate admissibility of the elaborated test for identification of blood on evidential items it is necessary to test a larger number of various surfaces. It is also important to check whether the type of background may influence not only the course of the reaction but also the pace of degradation of biological material the stain is made of.

Suitability of the elaborated test for mRNA profiling was verified on authentic biological traces. The Authors selected 22 samples, for which the non-specific screening test based on detecting haem oxidation properties gave positive results, however, in only 3 cases presence of human blood was confirmed with a specific test (group 2 in Table no. 4). For 21 analysed traces DNA profile was obtained and a few cases it was of poor quality. In 17 samples all blood specific mRNA markers were detected and in 4 samples a partial mRNA profile was obtained, thus the presence of blood in those traces was definitely confirmed. By means of validation experiments it was determined that the sensitivity limit of HemCheck test is at the level of 10 000 x dilution of blood, which is an equivalent of approx. 0.01 µl of blood. Therefore the above results suggest that in the real biological casework analysed within the project mRNA markers were more stable than haemoglobin detected with conventionally used tests. In spite of the fact that taking into consideration the unpredictable character of casework traces as compared to artificially created samples used for validation it cannot be fully excluded that the reason for the negative result of the test on a plate combined with the positive result of mRNA profiling there are differences in sensitivity or robustness of both methods. Analysis of melting curves for multiplex reactions reflecting the presence of individual specific mRNA markers and their quantitative relationship, as well as presence of potential non-specific PCR products, in combination with results of other blood detection tests and DNA profiling enables more detailed concluding on the origin and state of preservation of a given sample. For example, the analysis of one of the traces (group 4 in table no. 4) demonstrated presence of two specific mRNA products and additional non-specific fragments, which may indicate a significant degree of trace degradation, possibly presence of RNA of other living organisms, e.g. bacteria. Similarly, degradation of biological material in traces can be indicated by amplification of only the shortest HBB61 fragment or its amplification in much larger abundance than other, longer fragments, like, for example, in three samples in category 1 and in the samples from categories 5 and 6, particularly that in case of those traces also lack of or a partial DNA profile. Moreover, by analysing the melting curves for one of the samples qualified in group 1, a significantly higher increase of the product of 503 bp as compared to the remaining shorter fragments. The peak of HBB503 marker was also wider than other peaks. A probable explanation for the multiplex melting curves described above is a much more efficient amplification of β-actin marker, as compared with blood markers and overlapping of the melting curves for the product of the reference gene and HBB503, which may be a proof for presence a mixture of blood with another human biological material. This hypothesis is strengthened by the fact a mixed DNA profile was obtained for this trace.

Conclusions

Identification of human blood based on analysis of the level of haemoglobin genes expression is undoubtedly a promising method, which already finds application in forensic practice. Among the advantages of this method one should emphasise first of all its high sensitivity, specificity and possibility of simultaneous RNA/DNA extraction, which is particularly important when DNA analysis is the priority and there is a limited quantity of material. Authors' own research and the data quoted from literature of the subject prove that both transcripts of alpha haemoglobin and beta haemoglobin are unquestioned blood markers. The test based on profiling of HBB61, HBB503 and HBA197 markers still requires a lot of research work. After completion of a broad spectrum of validation experiments, and particularly determining the specificity of markers for various body fluids, analysing a larger number of real casework biological traces and precise indication of the optimal quantity of use RNA, as well as examining the individual differences in gene expression levels routine it will be possible to use the test for examination of most stains that may contain blood. In addition to that, the result of the test may have a predictive value as regards the quality of genetic material, as well as

presence of body fluid mixtures. At present, the test based on analysis of the selected mRNA markers may constitute a complementary method to immunological and immunochromatographic tests available on the market.

Source of figures and tables: author

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