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Research Article

DNA Degradation of Bloodstaines on Cotton Fabric Caused By Different Washing Procedures

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Abstract

The process of DNA degradation in biological material is not well understood. Bloodstains on washed clothing are disturbed by washing procedures, sometimes transferred to other fabrics, often with latent bloodstains and usually with significantly degraded DNA. The samples (cotton fabric with bloodstains) are divided into six main groups, depending on the method of washing with regards to water temperature (95, 60 and 30oC) and the use of detergent. After completing the washing process, samples were stored for a certain period of time (1 day to 6 months) and subsequently analyzed. Analyses were performed using standard protocols and commercial kits to measure the remaining DNA quantity (concentration) and DNA degradation index in the processed samples. Our results revealed that the high washing temperature (60 and 95oC) and the application of detergent have a synergic action on DNA degradation, while at 30oC this effect is absent. Furthermore, the effect of detergent on accelerated DNA degradation is observed about a month after the washing. This delayed effect of detergent has no explanation in current literature data. In order to obtain optimal results from the bloodstains, we made the recommendation that the period from crime event and attempted cleaning by a perpetrator to the laboratory analysis should be less than one month.

Keywords: Bloodstain; Detergent; DNA analysis; DNA degradation; Forensic science; Washing procedures

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Introduction

Physical evidences are very important in criminal investigations. During trials, eyewitness testimony often accounts as unreliable or biased, hence physical evidence becomes crucial for a conviction of a perpetrator. Crime scene investigators identify items of interest at a crime scene, and usually, items such as bloodstained clothing and footwear are submitted to a forensic laboratory for further testing [1-3].

DNA analysts in forensic laboratories are engaged in analyzing and sampling bloodstains from bloodstained items. One of the most important issues in understanding results of DNA analyses is the interpretation of the dynamic of DNA degradation in biological traces. Process of DNA degradation in biological material, including the biological forensic evidence, is not well understood. There are a few factors identified to be connected with the process of DNA degradation. The results of recent research support the assumption that degradation of DNA occurs randomly across the genome, and there is no evidence of existing regions with increased or decreased "affinity for degradation" [4].

One of the common challenges for DNA analysts and bloodstain pattern analysts are examinations of washed bloodstained clothing. Bloodstains on washed clothing are disturbed by washing procedures, sometimes transferred to other fabrics that were initially bloodstain-free, often with latent bloodstains and usually with significantly degraded DNA [5-15]. Degradation process of DNA was connected to some factors, such as temperature, sunlight, UV light, humidity and microorganisms [4, 12, 16], but still there is a gap between known scientific facts and genuine process of DNA degradation. We performed experiments to investigate the effects of temperature during washing, in combination with application of washing powder, on DNA degradation.

Materials and Methods

Blood

Blood samples were collected during autopsies conducted at our Institute. The inclusion criteria for blood sampling were normal blood test results and coagulation status that were within the reference range, just before the person deceased.

Textile

Samples are prepared from 100% cotton fabric with a density of 140 g/m^2. The fabric was cut to achieve sample size of 5x5 cm and total of 360 samples were made. The remaining 5 kg of fabric was used to simulate machine wash under real conditions.

Labeling of samples

Samples are divided into six main groups, depending on the water temperature and the use of detergent (Table 1):

H+	machine wash at 95oC using Ariel detergent (recommended by the manufacturer: 150 ml detergent); wash cycle duration: 2 hours 28 minutes; centrifuge: 1200 rpm
H-	machine wash at 95oC in water without detergent; wash cycle duration: 2 hours 28 minutes; centrifuge: 1200 rpm
V+	machine wash at 60oC using Ariel detergent (recommended by the manufacturer: 150 ml detergent); wash cycle duration: 2 hours 18 minutes; centrifuge: 1200 rpm
V-	machine wash at 60oC in water without detergent; wash cycle duration: 2 hours 18 minutes; centrifuge: 1200 rpm
P+	machine simulated hand washing at 30oC using Ariel detergent (recommended by the manufacturer: 50 ml detergent); duration of washing cycle: 34 minutes; centrifuge: 400 rpm - simulates manual fabric squeezing
P-	machine simulated hand washing at 30oC in detergent-free water; duration of washing cycle: 34 minutes; centrifuge: 400 rpm - simulates manual fabric squeezing

Table 1: Groups of samples based on the water temperature and the use of detergent.

Five subgroups were formed within each group, depending on the interval lapsed from the moment of washing to the start of the sample analysis (1 day, 15 days, 30 days, 3 months and 6 months).

Sample preparation

Volumes of 100 µl of blood were deposited on each fabric sample and the blood was allowed to passively soak within fabric before any further treatment. Samples with bloodstains were suspended on previously prepared stands until they were completely dry, before any further treatment.

Washing machine

Samsung Model, Type WF80F5E0W2W/AD. The washing machine was selected to have simulated manual washing program, in addition to classic high-temperature intensive washing programs.

Detergent

Ariel Washing Powder, Procter & Gamble: composed of 5-15% active ionic and <5% non-ionic surfactant-detergent, phosphates, water softener (zeoliths and polycarboxylates), enzymes, optical brighteners and perfumes) was chosen based on recent research showing that Ariel is the most effective detergent for removing bloodstains [5].

DNA extraction

DNA was extracted according to the standard laboratory protocol, as suggested by the commercial kit supplier. A commercial kit *QIAGEN QIAamp DNA Mini Kit (250)* was used to extract the DNA material. The isolates were stored in a dedicated deep-freezer at -80°C.

Quantification and degradation index of DNA

DNA quantity (concentration) and degradation index of DNA extracts were measured using a *7500 Real-Time PCR ABI* apparatus and a commercial *Quantifiler HP* quantification kit, while the results were read using a dedicated computer program *HID Real-Time PCR Analysis Software v1.2 Degradation Index*.

Statistical processing of results

Data are presented in the form of arithmetic mean ± standard deviations. Data analysis was performed in *SPSS 20.0* software package. The average values of the test groups were compared by ANOVA test or Kruskal Wallis test depending on the distribution of data. Analysis of average values in repeated measurements was performed using ANOVA for repeated measurements. The hypothesis was tested with a significance threshold of $p<0.05$.

Results

Taking into account all samples, regardless of washing temperature and use of detergent, the highest DNA degradation index was measured in samples analyzed six months after washing and the lowest at the point of one day after washing (Figure 1). The DNA degradation index was found to increase through subgroups over time from washing bloodstained fabric to start of the analysis ($p<0.001$). Post hoc analysis showed that there is a significant difference between all measuring points in time (1 day, 15 days, 30 days, 3 months and 6 months).

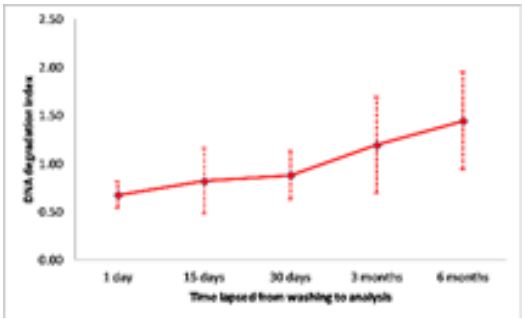

Figure 1: DNA degradation index with respect to the time lapsed from washing bloodstained fabric until start of analysis.

There is a statistically significant difference between all measuring points in time. At all three washing temperatures (95, 60 and 30°C), regardless of detergent use, we observe a similar pattern of DNA degradation progression (Table 2). The lowest DNA degradation index values were measured at day one after washing at all three temperatures, while at the 15th day we measured an increase in degradation index at 95 and 60°C, and, seemingly, a decrease at 30°C. Degradation index 30 days after washing at 95 and 60°C seemingly decreased in values compared to the previously measured point (15 days), while the value of DNA degradation index 30 days after washing at 30°C increased in value compared to the previous measuring point. At the measurement point of 3 and 6 months after washing we observed increased values of DNA degradation index for all three washing conditions.

Statistically significant difference in DNA degradation index values, with respect to time interval between washing and analysis, was found at all three washing temperatures ($p<0.001$ for all) and between all measuring points in time, except between measuring points at 15 and 30 days in group samples washed at 95 and 60°C, as well as between measuring points at 1 and 15 days in group samples washed at 30°C (Table 2). A seeming decrease in degradation index between day 1 and the 30th day after washing at 30°C, as well as a seeming decrease between day 1 and the 15th day after washing at 95 and 60°C, are within the range of randomly events and do not qualify as real phenomenon.

DNA degradation index	Time lapsed from washing to analysis start					p-value
	1 day	15 days	30 days	3 months	6 months	
Total	0,67 ± 0,14	0,82 ± 0,34	0,88 ± 0,25	1,19 ± 0,50	1,44 ± 0,51	<0,001
95 degrees C	0.69 ± 0.23	0.91 ± 0.22	0.86 ± 0.22	1.2 ± 0.33	1.62 ± 0.41	<0,001
60 degrees C	0.68 ± 0.09	1.04 ± 0.4	0.94 ± 0.35	1.5 ± 0.75	1.58 ± 0.66	<0,001
30 degrees C	0.61 ± 0.05	0.54 ± 0.11	0.83 ± 0.13	0.93 ± 0.12	1.13 ± 0.18	<0,001
without detergent	0.65 ± 0.17	0.78 ± 0.3	0.85 ± 0.22	0.95 ± 0.15	1.16 ± 0.29	<0,001
with detergent	0.7 ± 0.09	0.85 ± 0.38	0.91 ± 0.28	1.43 ± 0.6	1.71 ± 0.53	<0,001

Table 2: DNA degradation index in total samples summary at given measurement points in time with respect to washing procedures, including temperature and use of detergent. The significant difference in DNA degradation index was found between all measurement points in time, except between analysis after 15 and 30 days in group samples washed at 95 and 60oC, and between analysis after 1 and 15 days in group samples washed at 30oC.

Regardless of washing temperature, in the group of samples washed with the detergent the average DNA degradation index value was 1.17 ± 0.58, while in the group without detergent the average degradation index value was 0.89 ± 0.29. The results show a significant difference in the DNA degradation index ($p<0.001$) with respect to the application of the detergent when washing bloodstain fabric. The DNA degradation index at different time points between the washing and the beginning of analysis were significantly different in both groups (Table 2), with the use of detergent while washing ($p<0.001$) and without detergent while washing ($p<0.001$).

Regardless of washing temperature, in both groups of samples washed with or without detergent DNA degradation index values has the similar, increasing pattern, however following different dynamics, with respect to the time lapsed from washing to analysis (Figure 2). The DNA degradation index at day one after washing was almost identical in both the detergent-washed and the detergent-free samples. Values were similar for up to 30 days, but at the measuring point of three and six months a greater jump in values was observed in the group that used the detergent. The significant difference in DNA degradation indices, when compared between detergent-washed and detergent-free samples, were found in samples washed 3 and 6 months before the start of DNA analysis ($p<0.001$).

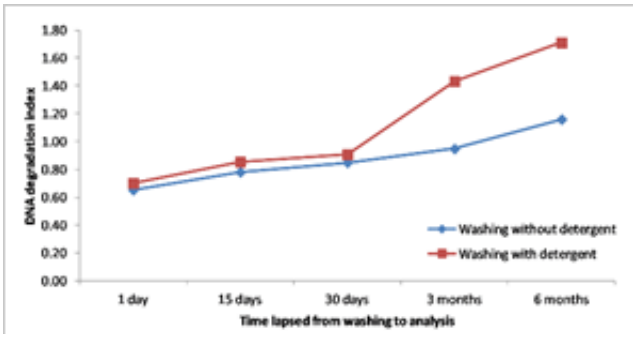

Figure 2: The significant difference in DNA degradation indices.

DNA degradation index with respect to time lapsed from washing bloodstained fabric to the start of analysis (DNA extraction) and with respect to the use of detergent. There is no observable or statistically significant difference of DNA degradation index on the measurement point 1 day, 15 days and 30 days after washing, in both the detergent-washed and the detergent-free samples. Note the significant increasing of DNA degradation index values in samples washed 3 and 6 months before analysis start

Regardless of detergent use, the seemingly highest DNA degradation index was measured in group samples washed at 60°C, followed by the group washed at 95°C, and the lowest degradation index was measured in the group of samples washed at 30°C (Table 3). Post hoc analysis confirmed a significant difference in the DNA degradation index with respect to washing temperature between the following groups: 95°C vs. 30°C ($p<0.001$) and 60°C vs. 30°C ($p=0.001$), but there was no significant difference between groups of samples washed at 95°C and 60°C. Samples washed at 95°C with detergent had significantly higher values of DNA degradation index than samples washed at 95°C without detergent ($p=0.012$). Samples washed at 60°C with detergent had significantly higher DNA degradation index values compared to samples washed at 60°C without detergent ($p<0.001$). In group samples washed at 30°C, there was no significant difference in DNA degradation index with respect to detergent application ($p=0.760$).

DNA degradation index	Total	Washing		p value
		with detergent	without detergent	
95 degrees C	1,10 ± 0,441	1,23 ± 0,49	0,98 ± 0,34	0,012
60 degrees C	1,14 ± 0,601	1,42 ± 0,71	0,86 ± 0,24	<0,001
30 degrees C	0,84 ± 0,25	0,85 ± 0,25	0,84 ± 0,26	0,760

Table 3: DNA degradation index in respect of washing temperature program and use of detergent. 1 – $p<0.001$ vs. washing at 30 degrees C. p value – represent statistically significant difference in DNA degradation between samples washed with detergent and washed without detergent at different washing procedures temperature.

Analysis of variance showed that there was a significant difference in the DNA degradation index both with respect to washing temperature ($F=11,886$, $p<0.001$, partial $N=0.071$) and with respect to detergent application ($F=19,957$, $p<0.001$, partial $N=0.060$). Post hoc analysis showed that there was a significant difference between washing at 95°C and washing at 30°C ($p<0.001$), as well as between washing at 60°C and washing at 30°C ($p<0.001$) (Table 2). In addition, it was shown that there was an interaction between washing temperature and detergent application ($F=3,716$, $p=0.025$, partial $N=0.023$). The interaction that exists is best seen in the graph below (Figure 3), where the lines representing the wash at 95°C and the wash at 60°C intersect, while the line representing the washing at water temperature of 30°C is completely flat and set aside.

DNA degradation index with respect to the washing temperature and the use of detergent. Note that the lines representing the washing procedures at 95°C and 60°C intersect, while the line representing the washing at water temperature of 30°C is completely set aside and almost horizontal. Correlation between lines presents the interaction that exists between washing temperature and use of detergent to DNA degradation

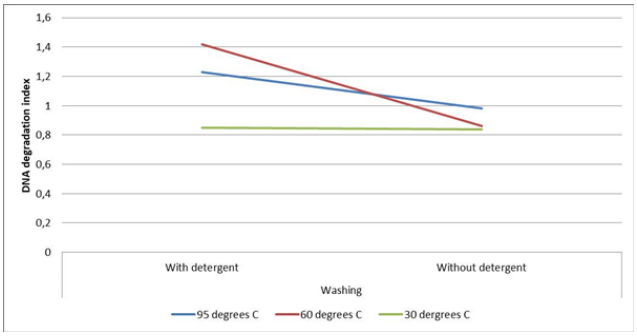

Figure 3: The DNA degradation index both with respect to washing temperature.

Discussion

According to literature data [5,6,8,10,14,15], single conventional washing cycle of bloodstained cotton fabric, regardless of bloodstain type, washing temperature, and detergent application, is insufficient to completely remove visible and invisible traces of blood. Our research was focused on degradation process of DNA material in bloodstains remaining on cotton fabric after washing.

Our research showed that the DNA degradation index in overall sample set, regardless of washing temperature and use of detergent, increases significantly with the time interval between washing of the bloodstained cotton fabric and the DNA analysis. The highest DNA degradation index values were measured in the sample group that had been analyzed six months after washing. Analysis of the DNA degradation index in the samples showed that washing temperatures at 95 and 60°C cause a significantly higher degree of DNA degradation than washing the bloodstained cotton fabric at 30°C. Those results could be interpreted by well-known data in literature that the time and also higher temperatures are increasing degradation of DNA molecules [4,12,16], depending on the length of action of the temperature.

A significant difference was found in the DNA degradation index of the blood that remained on fabric between detergent-washed and detergent-free samples at 95 and 60°C, while at 30°C this pattern is absent, implying the synergistic action of high temperature (60 and 95°C) and detergent on DNA degradation. (Figure 3) graphically demonstrates the dependence of the DNA degradation index and the washing temperature in addition to the detergent application during washing. Green horizontal line at 30°C represents the absence of influence of detergent on DNA degradation index at the given temperature. On the contrary, blue and red lines represent the increase of the DNA degradation index after the detergent application at two given temperatures, 95 and 60°C respectively. Our results revealed that effect of high washing temperature and detergent on DNA degradation is amplified by simultaneous act and interaction of those two factors.

Soaps and detergents are known to facilitate the extraction of DNA materials from samples in real cases [17], but it is not precisely established whether and how they affect the degradation of the DNA itself. In the current experiment, it was assumed that the detergent causes the DNA material to be "exposed" and hence faster decay with time. Statistical processing of results revealed that the effect of detergent application on the degree of DNA degradation was manifested somewhere between one and three months after washing bloodstained fabric (see Figure 3), with the trend extending to the point of six months after washing. Our research revealed that the effect of detergent application during washing on DNA degradation takes about a month after the washing to begin. This delayed effect of detergent has no explanation in current literature data.

Considering that our results are showing that a washing of bloodstained cotton fabric accelerate the process of DNA degradation in the remaining amount of blood on fabric, it is advisable to examine items of interest in DNA laboratories in the shortest possible time after those items are collected by police at the crime scene to ensure the optimal recovery of biological evidence. Moreover, since it has been proven that the application of detergent significantly accelerates the DNA degradation process after a period of more than one month after washing, in real cases the actual period from a critical event and an attempt of a perpetrator to cover up traces to the beginning of the laboratory analysis should be within the timeframe of one month.

Conclusion

Our research revealed that washing temperatures of 95°C and 60°C cause faster DNA degradation of remained blood on cotton fabric after washing, compared to washing procedures at 30°C. The use of detergent during the washing process of bloodstained cotton fabric further increases DNA degradation in time of the remained blood on the fabric after washing, for washing temperatures at 95°C and 60°C. Contrary to those results, use of detergent while washing of bloodstained cotton fabric at 30°C does not accelerate degradation process of DNA. Our results pointed out that detergent application accelerates degradation of DNA, if laundering process was performed at 95 or 60°C, but not at 30°C. The effect of detergent to DNA degradation is most evident between 1 and 3 months after washing procedure. Higher washing temperatures (95 and 60°C) and detergent application during washing bloodstained cotton fabric show a synergistic effect to accelerated DNA degradation process on blood remaining on cotton fabric after washing.

Declaration

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest.

Ethics approval

Authors confirms that the study was approved by the Ethic committee of the Institute of forensic medicine Nis and certify that the study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Availability of data and material

The datasets generated during the current study are available from the corresponding author on reasonable request.

Code availability

Not applicable.

Authors contributions

Conceptualization: Ivan Stojanović, Aleksandra Stefanović, Goran Ilić; Methodology: Ivan Stojanović, Aleksandra Stefanović, Goran Ilić; Formal analysis and investigation: Ivan Stojanović, Aleksandra Stefanović; Writing - original draft preparation: Ivan Stojanović; Writing - review and editing: Ivan Stojanović, Aleksandra Stefanović; Resources: Ivan Stojanović, Aleksandra Stefanović, Goran Ilić; Supervision: Ivan Stojanović, Aleksandra Stefanović, Goran Ilić.

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