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by Ardyan Pradana Putra

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The Effect of Different Washing Treatment against Protein Quantity and Agglutination Level in Bloodstain for Forensic Identification

Ardyan Pradana Putra[1], Hartono Kahar[2] and Ahmad Yudianto[1,3]
[1]Master of Forensic Science School Postgraduate School, Universitas Airlangga, Surabaya, Indonesia
[2]Department Clinical Pathology Faculty of Medicine Universitas Airlangga Surabaya
[3]Department Forensic Medicine and Medicolegal, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia

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Abstract: Forensic examination of bloodstain provides important information to uncover inquired offense because blood is easily scattered in almost all forms of violence, and its biological state has specific properties in each person. In reality, perpetrators often attempt to conceal blood stained evidences to block revelation during investigation by disposing, burying, burning & washing. A number of stained evidences account but worn clothes during the incident are usually treated in either of the form, and when washed, chemicals such as detergents and bleach solution (NaClO) are used. The purpose of this study was to investigate the effect of washing on protein quantity and agglutination level on bloodstain in clothes for forensic identification. This is a laboratory experimental study, in which 32 samples of bloodstained clothes were given different treatments: tap water only, detergents and bleach solution with a control without washing. From the total of 32 samples, 16 samples were measured for protein quantity using UV spectrophotometer and 16 samples for agglutination level. The results were then analyzed using statistical parametric One Way Anova with significance level of 0.05. The result of statistical test obtained (p value <0.05) showing that there was a difference between the mean of protein quantity and agglutination level on bloodstain sample in each treatment. This research concluded that there is an effect on blood protein quantity and agglutination level due to washing type used (tap water, detergent, and bleach solution) by its decreasing trend supported by leaching and protein denaturation behavior.

1 INTRODUCTION

Along with the development of science and technology in the field of molecular biology, in efforts to solve case, especially the criminal, the law enforcers i.e., the police, prosecutors, judges and legal counsel would require assistance from the experts. In accordance with their respective fields, the experts will examine the evidence (corpus delicti) scientifically so that the tragedy can illustrate the case, and at the end, to determine a bright spot about a crime act (Darmayani, 2011).

According to the data from Badan Pusat Statistik (2016), crime rates in Indonesia fluctuate during the period of 2013-2015. Of the many cases of crime, murder case tops the list, followed by crime against physical (violence). As per report of the Bureau of Development and Operations of the National

'MABES POLRI', the total number of crime incidents in 2013 was 342,084 cases which increased to 352,936 cases in 2015. Meanwhile, the risk of crime rate per 100,000 population is estimated to be 140 people by 2015.

In cases of murder or violence, it is very common to find evidence related to a crime both left at a crime scene and attached to the body of the perpetrator or victim. Physical evidence of body material can be bloodstain, sperm, tissue, hair etc. (Puspitaati et al, 2016). Forensic examination of bloodstain provides important information to uncover inquired offense because blood is easily scattered in almost all forms of violence, and its biological state has specific properties in each person (Idries, 2013). Serological examination of bloodstain can be done quickly and inexpensively with a variety of methods using preference of Absorption Elution Technique (Hoediyanto, 2012).

In reality, perpetrators often attempt to conceal blood stained evidences to block revelation during investigation by disposing, burying, burning &

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washing. A number of stained evidences account but worn clothes during the incident are usually treated in either of the form, and when washed, chemicals such as detergents and bleach solution (NaClO) are used (Yudianto, 2013). Detergent & sodium hypochlorite are some chemical examples and household cleaning agents used as cleanser because they contain surfactants, which is capable to clean stains of dirt and blood spots, so they can dissolve in water (Marcelisa, 2015). Based on the problem, phenomena, and gap described above, it is important to know the effect of different washing treatment towards protein quantity and agglutination level on bloodstained clothes for forensic identification.

2 METHODOLOGY

2.1 Research Design

This was a laboratory experimental study, posttest only control group design. In which 32 samples of bloodstain on cloth were given different treatments which are without washing (control), washing using tap water, detergents and bleach solution. From the total of 32 samples, 16 samples were measured for protein quantity using UV spectrophotometer and 16 samples for agglutination level. The result of protein quantity measurement was then analyzed using parametric One Way Anova with the requirement of normal and homogeneous data, while the agglutination level data was analyzed using nonparametric Kruskal Wallis statistic test. This research was conducted at Human Genetic Laboratory, Institute of Tropical Disease, Universitas Airlangga, Surabaya.

2.2 Materials and Method

2.2.1 Materials

Blood with blood group A (Rh+), cloth (cotton), Detergent (active ingredient 19% Anionic Surfactant), stain/bleach cleaner (5.25% NaClO), tap water & washing machine.

2.2.2 Reagent

Ether Alcohol, Anti A, tap water, 2% Red Blood Cell Suspension, distilled water, Guanidine HCL, Trizole reagent, Chloroform, Ethanol, Isopropanol, 1% SDS and 0.9% NaCl.

2.3 Research Procedures

2.3.1 Collection of Sample

Blood samples were obtained from a volunteer who had blood type A (Rh+) and stored in an EDTA vacutainer tube. Then, blood spots were made on cotton clothes by way of blood drops as much as 300µ in each spotting. The number of blood spots on each shirt was 2 spots of blood, 1 spot of blood for the examination of protein level and the other one for the measurement of level of agglutination. The total number of blood spots formed was 32 spots.

2.3.2 Washing of Stains

The clothes were washed using a washing machine (LG Inventer ®). The washing process was done separately based on the shirt label. A stained labeled shirt was not washed, for control. B labeled shirt that has blood spots washed in a 10 liters of tap water without detergent, C labeled clothes soaked in 10 liters washing machine that has been added 30 grams of powder detergent, while D labeled clothes were soaked in a 10 liters of tap water which has added 60 ml stain cleaning solution (5.25% NaClO) then washed with the washing machine with automatic setting. Once the washing process is completed, it was rinsed with tap water for 1 time. Then all clothes were allowed to dry by hanging at room temperature.

2.3.3 Sample Preparation

Each shirt labeled A, B, C & D that has dried after washing process is cut into small pieces on the area where the blood stains are marked. They were then inserted into the test tube based on the label to read protein content and the level of agglutination.

2.3.4 Examination of Protein Quality

The estimate of protein quantity in blood spots were measured using a Spectrophotometer with a wavelength of 280 nm, using an absorbance ratio of 280/260 to determine the correction factor present in a table. The level of protein was determined by the following formula: protein content (mg / ml) = A280 x correction factor x dilution. Finally, the protein was isolated using Trizole reagent, Chloroform, Ethanol, Isopropanol, SDS 1% & Guanidine HCL.

2.3.5 Examination of Agglutination Level

Agglutination level examination was done by soaking the pieces of cloth contained blood spots into the reaction tube containing reagent anti A. Then incubated at 4oC for at least 16 hours. After the immersion, the anti-A reagent in the test tube was removed and replaced with 0.9% NaCl. Washing was performed with NaCl 0.9% 5 times and centrifuged at 1000 rpm for 1 minute to remove the rest of the reagent. After the last centrifuge, 0.9% NaCl was added and warmed for 15 minutes at a temperature of 56oC. After the warming, 2% of blood suspense was added and incubated at 4oC for 2 hours then shaken strongly. After the procedure, the level of agglutination was recorded.

3 RESULT AND DISCUSSION

3.1 Bloodstain Protein Quantity Under Varied Washing Treatment

32 formed bloodstains samples on 16 pieces of cotton clothes spotted with blood drops of 300µ each and washed under treatments without washing, washing using only tap water, detergents, and bleach solution gave results on protein quantity and agglutination level as follows;

Protein content measurement using spectrophotometer at UV 280 nm wavelength by absorbance ratio of 280/260 determined the correction factor that exist in a table. These levels of protein were determined by the following formula: protein content (mg/ml) = A280 x correction factor x dilution.

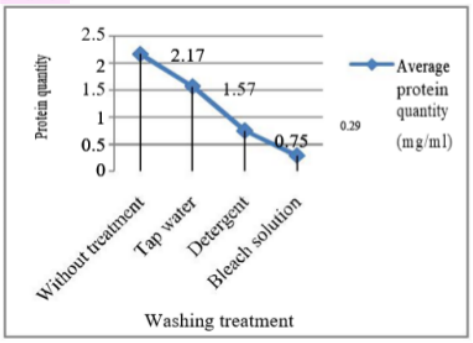

Figure 1. Graph of protein content in blood spots with different treatment

Blood protein levels shown in Figure 1 showed a decrease in the mean difference of protein levels in various groups, i.e. in the untreated group and the tap water washed group. The decrease of protein content was at 0.6 mg / ml. The group without treatment and the detergent washed clothes was at 1.42 mg / ml; and the group without treatment with bleach (NaClO) washed group was at 1.88 mg / ml.

Statistically; detergent leaching effect on protein content, Anova parametric analysis responded normally. According to the test, all data obtained results at p value> 0.05, ie p value for protein content based on the difference of treatment of (0.150-0.95), this stated a normal distribution of studied data. P value obtained, measured > 0.05. This study variance assume homogeneous data. The data in this study have qualified because of normal distribution and homogeny, then statistical analysis parametric Anova (One Way Anova) followed.

Table 1: One Way Anova parametric analysis of washing effect on protein content

Anova analysis	ΣSquare of protein quantity	df	Mean Square of protein quantity	F-ratio	p
Between Groups	8.417	3	2.806	10.316	.001
Within Groups	3.263	12	.272		
Total	11.680	15			

The result of statistical analysis of parametric One Way Anova in Table 1 obtained the value of p (sig.) = 0.001 because the p value (sig.) <0.05. Then by this test, H0 is rejected and H1 is accepted which means to effect of washing type on protein content at spotting blood with UV spectrophotometer method.

Bloodstained samples under different treatments as resulted in findings portray significant information on the effect of washing as concealment of evidence in forensic inquiries. Referring to blood protein quantity shown in Figure 1, a decreased in the mean of protein levels is observed in various groups. The summary is given by: the group without washing, the average protein content of 2.17 mg / ml is presented, tap water at 1.57 mg / ml, washing group detergent at 0.75 mg / ml, and washing group using bleach (NaClO) at 0.29 mg / ml.

Interpretation of the findings of this study showed that washing using bleach (NaClO) was more likely to decrease protein levels compared to detergents and tap water. The observed differences

of protein group without treatment with washing group using bleach (NaClO) were 1.88 mg / ml. The group without treatment, washing group using detergent of 1.42 mg / ml, the group without treatment, and washing group using tap water of 0.6 mg / ml.

Decreased protein levels from the total blood spots occurred due to washing and occurrence of protein denaturation is due to exposure to detergent chemicals and stain cleaning fluid. Denaturation of proteins is the loss of higher structural properties by disruption of hydrogen bonds and other secondary forces required by the molecule. Factors that can cause protein denaturation include temperature, pH, physical factor (mechanical), and addition of chemicals. Changes in temperature and pH can disrupt the structure of the protein and cause loss of function. The pH of the detergent alone > 10 also certainly cause protein denaturation (Silverthorn, 2014).

Washing the sticking blood stains on clothes requires three types of energy. The three energies are: chemical energy supplied by detergent, thermal energy provided by warm or hot water, and mechanical energy derived from washing machine or hand. The role of mechanical energy derived from the movement of the washing machine causes erasion of all blood spots sticking to the fabric. All of these three energies must work in synergy so that the washing process can be effective (Ophardt, 2013).

If leaching uses only water, the quantity of protein content remaining is more than washing using detergent because the detergent contains chemical composition as one of its ingredients is responsible for protein structural destruction. Similar to Sopiah Nida (2015), the function of surfactant in detergent is also to reduce surface tension so as to increase the wetting power of the water, which in return wet and erode the bloodstain, loosen and remove the dirt and suspend the loose dirt (Sopida Nida, 2015). Biological detergents contain enzymes designed to hydrolyze protein molecules, and therefore, are potential to further lower blood stains and protein levels (Oldfield, 2017).

Tap water will not be able to remove blood spots that have spread into the fabric fibers. In such cases, detergent is used to remove stains spotted blood. Structurally, protein remain active at 30oC-48oC, above it to 50oC the biological structure will be subjected to destruction hence denaturation process occurs (Silverthorn, 2014). The water temperature detergent added will usually increase. Therefore, high temperature washing can remove traces of blood to a greater extent than cold or lowered temperatures (Oldfield, 2017).

Similar to previous experiments conducted by C. Oldfield et. al. (2017), the interpretation of the research findings showed that detergent and NaClO are able to remove some blood traces. This finding may be useful for investigations of offenses that suspect the use of detergent and NaClO in concealing the bloodstain evidence by washing method, making it potentially useful to test suspected items and/or perform additional analysis on evident suspected material.

Research conducted by K.A. Harris in 2006 support this study by deriving that all of the cleaning agents, bleach has the most damaging effect on the quality of DNA profile obtained in blood spots. Therefore, from the citation above; it is learnt that the quantity measure of DNA in biological substances treated with bleach decreases over time due to degradation – congruent to the quality above. This condition is not visible on the substrate being cleaned with soap, detergent, and / or non-chlorine disinfectant.

3.2 Bloodstain Agglutination at Varied Washing Treatment

The result of measurement of blood group agglutination level by using Absorption Elution method of bloodstain on cloth in group without treatment, washing group using tap water, washing group using detergent and washing group using bleach (NaClO) can be seen in table 2 below:

Table 2: Agglutination Rate Data on Blood Spot on Cloth

Treatment	Sample Number	Aglutination level
Without treatment	A-A.1	+4
	A-A.2	+4
	A-A.3	+4
	A-A.4	+4
Washing using tap water	A-B.1	+2
	A-B.2	+2
	A-B.3	+2
	A-B.4	+3
Washing using detergent	A-C.1	+1
	A-C.2	+1
	A-C.3	Negative
	A-C.4	Negative
Washing using Bleach solution (NaClO)	A-D.1	Negative
	A-D.2	+1
	A-D.3	+1
	A-D.4	Negative

Based on the result of blood group agglutination level by using Absorption Elution method on the blood spots in cloth, the change of agglutination level (tend to decrease) in the group without treatment, washing using tap water, washing with detergent, and washing with bleach (NaClO). In the washing with detergent and washing with bleach (NaClO) group, there were 4 samples with negative agglutination results.

However, to test whether there is a detergent leaching effect on agglutination rates or not, a nonparametric statistical analysis by Kruskal Wallis was performed. The test resulted Kruskal Wallis' nonparametric statistical analysis, which is portrayed in Table 3.

Table 3. Kruskal Wallis Test for bloodstain agglutination level

	Agglutination Level
Chi-Square	13.551
Df	3
Asymp. Sig.	.004

a. Kruskal Wallis Test
b. Grouping Variable: Treatments

In Table 3, p value (Asymp Sig.) = 0.004, since p value <0.05, then H0 is rejected, and H1 is accepted. It means that there is a detergent leaching effect on the agglutination level in ABO blood group determination on blood spots with absorption elution method.

The data on blood group agglutination level in the sample group without treatment were obtained +4 by 100%. The group of water-washed samples obtained +3 by 25% and +2 by 75%. The group using detergent obtained +1 by 50% and negative by 50%. The group using bleach (NaClO) obtained +1 by 50% and negative by 50%.

Agglutination rate data in the Table 2 tended to decrease in the trend of untreated group, wash using tap water group, wash using detergent group, and wash using bleach (NaClO) group. Thus, the protein content of a bloodstain is directly proportional to the level of agglutination. If the protein level decreases, the agglutination rate also decreases.

As studded in the protein quantity above, the tending effect was also contributed by the impacting energy that directly or indirectly affects molecular structure of protein. The force energy: chemical energy, thermal energy, and mechanical energy are counted several inclusive factors (Ophardt, 2013). The other agglutinating factors (antibody-antigen reaction), apart from those generated by friction due to washing, are as discussed by Whitlock (2010). they are antigen-antibody specificity complex pairing, noncovalent bond, lock and key physical mechanism, antigens and antibodies concentration under prozone phenomenon, temperature, time, pH (7.2-7.4) plus the surface net negative charge - zeta potential surrounding red blood cells.

3.3 Blood Protein Quantity and Agglutination Level Effect

From the 16 bloodstain pairs (300µ each) made on 16 cotton pieces of cloth, one pair was assessed for agglutination and the other pair for protein content. The 32 blood drops (16 pairs) in total treated under similar treatment gave corresponded reaction according to the category of assessment. The findings were obtained from the two factors analyzed which was built on the potential impact. Discussing the factors in aggregation, it conveys a meaningful attribute of combined factors in ascertaining the extent.

Referring to the 4 different types of treatment used for both agglutination or protein content: without washing (which referred as control), average protein content was measured at 2.17 mg / ml with +4 agglutination level at 100%. The control reaction (without the use of treatment) portrays a set benchmark of a 100% positive reaction to agglutination with maximum protein content. Washing using only tap water (treatment) present a decreased amount of protein content to 1.57 mg / ml with agglutination level of +3 by 25% and +2 by 75%. Detergent washing treatment resulted into a continued lowering effect of protein content to 0.57 mg/ml when referred to control content. The corresponding drops agglutinatinated a level of +1 and negative by 50%/50%. Washing by bleach solution gave a protein content of 0.29 mg / ml at agglutination level of +1 by 50% and negatively too.

In this study, the differences in washing blood spots proved to affect protein content and agglutination levels. There was a significant decrease in the average level of protein in blood spots washed using tap water, detergent and bleach. Either washed using detergent and washed with bleach, both give equal chance of either obtaining agglutination at +1 or not obtaining at all (negative agglutination reaction). The probability to agglutination is counted and vice versa with the content of protein (Refer Figure 1 and Table 2). Likewise, various factors that affect the level of protein in bloodstain will also affect the results of agglutination examination level in bloodstain.

Thereby, the aggregation of findings analyzed from protein concentration and blood agglutination showed a paralleling and complementing dependency resulted by washing treatment.

This study is based in blood tissue cellular content. Conceptually, protein content and agglutination level determine the behavior and state of the blood cellular composition. Focusing on red blood cell (erythrocytes), the contained protein integrates with the cell wall, which is antigenic. The composition of the red blood cell membrane is 49% protein, 43% lipids and 8% carbohydrates. This variation in protein in erythrocytes, which results in blood, is divided into several groups called blood groups (Silverthorn, 2014).

Thus, each blood group has its own antigen on the surface of the red blood cell membrane. Blood type antigens consist of carbohydrates and proteins. They together form glycoproteins and all are attached to various components in the red blood cell membrane. Protein is one of the macromolecules that make up more than half of the cell (Silverthorn, 2014). Hence, the amount of protein contained directly affect agglutinin reaction. Thus, the more damaging and destruction to cellular protein, the more it responds to weakened agglutination in order to complicate in ascertaining and determination of the forensic inquiry.

4 CONCLUSIONS

Forensic examination of bloodstain provides important information to uncover inquired offenses because blood is easily scattered in almost all forms of violence. In this study, the difference of washing treatment on bloodstain proved to affect protein content and agglutination levels. There was a gradual significant decrease in the average of protein content and level of agglutination in blood spots washed using tap water only, detergent, and bleach. The decrease in the average of protein content and the level of agglutination in this study was caused by various factors, including mechanical factors derived from washing machines, chemical factors provided by detergents and bleach liquids, and thermal factors provided by water with a detergent mixture. In addition, the temperature factor, time, pH, and antigens-antibodies concentration also affect the decrease in the average protein content and the level of agglutination in blood spots. In general, a decreased protein content and agglutination level is by protein denaturation and destruction due to washing treatment and exposure to detergent chemicals and stain cleaning fluids.

Hence, amount of protein contained is directly affected by agglutinin reaction. Thus, the more damaging and destruction to cellular protein, the more it responds to weakened agglutination in order to complicate in ascertaining and determination of the forensic inquiry. The study opens up a new approach to blood identification even after washed with detergent, which could prove useful in solving important forensic criminal cases

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