

Identifikasi Percikan Darah Kucing (*Felis catus*) pada Kain Katun Setelah Masa Pengerinan Menggunakan Reagen Leucomalachite Green Dan Takayama

Identification of Cat (*Felis catus*) Blood Splatter on Cotton Fabric After Periods of Drying Using Leucomalachite Green and Takayama Reagent

Vanessa Ann Charles¹, Tita Damayanti Lestari², Djoko Legowo³, Ismudiono², Nove Hidajati⁴, Retno Sri Wahyuni⁵

¹Student, ²Department of Reproduction, ³Department of Pathology, ⁴Department of Biochemistry, ⁵Department of Clinical Pathology, Faculty of Veterinary Medicine Universitas Airlangga

Corresponding author: vanessa.ann.charles-2015@fkh.unair.ac.id

ABSTRACT

Blood-stain or blood splatter analysis when used properly can assist in establishing a chain of events linked to violent crimes (Bevel and Gardner, 2008). The methods used in detecting blood splatters in the field are chemical methods. Leucomalachite green is a colorimetric test which is used to test the presence of blood (Castro and Coyle, 2008). Takayama reagent is a confirmatory test for blood (Strassman, 1922). The aim of this research is to detect the blood splatter on cotton fabric after it has been dried for 1 day, 3 days and 5 days using Leucomalachite green and Takayama reagent. Cotton fabric was specifically chosen for this experiment with 3 different periods of drying. The unstained cotton fabric was cut into squares, and a blood sample was splattered on each piece. The fabrics splattered with blood were then dried for 1 day, 3 days and 5 days. The blood splatter was then tested using Leucomalachite green and Takayama reagent, and the results were noted afterwards. For the control, red food dye was dried for 1 day then tested with Leucomalachite green and Takayama reagent. The image results of the Leucomalachite green test are analyzed using ImageJ software 1.8.0_112 where the red, green and blue pixels are converted to grayscale. The image results of the Takayama test are graded based on the number and pattern of crystal. In conclusion, Leucomalachite green and Takayama reagent are able to detect cat blood splatter on the cotton fabric. Leucomalachite green produced a higher intensity/ darker colour as a result of an older sample, and the lower intensity/ lighter colour as a result of a fresher sample of the Leucomalachite green test. Takayama reagent produced a densely packed pattern of crystals as a result of an older sample, and the loosely packed pattern of crystals as a result of a fresher sample of the Takayama test.

Keywords: blood splatter identification, Leucomalachite green, Takayama reagent, drying periods

Received: 02-01-2021

Revised: 03-03-2021

Accepted: 16-05-2021

INTRODUCTION

The lack of evidence to support a claim weakens a case making the person feel that reporting the crime is insignificant and futile, therefore introducing blood splatter identification techniques as a common knowledge at a crime scene helps provide evidence to

authorities. Blood-stain or blood splatter analysis when used property can assist in establishing a chain of events linked to violent crimes (Bevel and Gardner, 2008). The methods used in detecting blood splatters in the field are chemical methods. Blood splatter testing in

crimes related to animals can provide more evidence as well as uphold and strengthen the legal justice system.

In the Undang-Undang Republik Indonesia, Article 302 of the penal code states that anyone guilty of the maltreatment towards animals shall receive an imprisonment not more than 6 months or a fine Of 5,000,000 Indonesia Rupiahs (UU RI 41, 2014).

The animal welfare act 2015 of Malaysia defines animal cruelty as causing an animal: harm; discomfort; deliberate neglect; torture; kill; or extracting any parts of a live animal due to superstitious beliefs. Any violation of this act allows a fine with a maximum of 100,000 Malaysian Ringgit or imprisonment for a maximum of 3 years (Animal Welfare Act, 2015).

The subdued laws of these countries makes animal cruelty cases seem insignificant in comparison with human crimes to the culprit as well as a number of people. Bevel and Gardner (2008) states that the function of blood pattern analysis is that through the examination of the physical nature of the blood splatters, forensic analysts are able to identify information specific to the occurrence of events during the incident. Blood splatter can be divided into 3 basic groups; passive stain, transfer stain, and projected or impact stains (Bevel and Gardner, 2008).

The presumptive methods used to detect blood splatter in human crime scenes are commonly done using Luminol, Leucomalachite green, Phenolphthalein, Ortholidine and Tetramethylbenzidine. Leucomalachite green is a colorimetric test which is used to test the presence of blood (Castro and Coyle, 2008). Leucomalachite green is a colourless solution that turns bluish-green when haemoglobin breaks down the hydrogen peroxide through its peroxidase-like activity (Colotelo, 2009).

The confirmatory method is used to confirm that the sample tested is blood.

Takayama reagent is a commonly used method. The reagent works by testing a sample with a pyridine and glucose reagent. In the presence of blood, the reagent (pyridine and glucose) reacts with the heme group in blood and produces hemochromogen crystals (Strassman, 1922).

METHODS

Preparation of Leucomalachite green

Mix: Leucomalachite green 0.25g, Glacial acetic acid 100ml, Distilled water 150ml. Measure the materials needed. Prepare a beaker and slowly add each material, mixing well using a glass rod. Once the reagent is prepared, pour the reagent into a glass bottle filled with 5g of zinc to preserve the reagent for storage.

Preparation of Takayama reagent

Mix: Saturated aqueous glucose solution, 10ml 10% NaOH, 10ml Pyridine, 10ml Deionized water, 20ml (Vitriani et al., 2015). Measure the materials needed. Prepare a beaker and slowly add each material, mixing well using a glass rod. Once the reagent is prepared, pour the reagent into a glass bottle for storage.

Collection of blood sample

Blood is collected from the cephalic vein using a 22 size needle attached to a 3ml syringe. IACUC (2014) stated the total amount of blood than can be taken from a cat is 10 % of its total circulating blood volume. The volume of blood taken is 2.5ml. The puncture wound is then properly treated and the blood is flowed in EDTA coated vacuutainers to prevent coagulation and stored until use.

Making the blood splatter

Micropipettes are used to drop 50 μ l of blood on the cotton fabric (Isukapatla et al., 2016). The fabric is divided into 4 groups, each group having specific drying periods (1 day, 3 days, 5 days and

control). Each group contains 8 pieces of fabric.

Testing stain with Leucomalachite green

The test is done by dropping the reagent onto the blood stain on the cotton fabric. An immediate colour change determines that the reagent has oxidised and produces a false positive. The reagent which remains colourless is then followed by 3% hydrogen peroxide (Bevel and Gardner, 2008). This is done on each stain. Observe and note the colour changes. A colour shift from colourless to a bluish green will occur. The colour shift is captured and scored using ImageJ software 1.8.0_112. ImageJ software 1.8.0_112 converts the colour shift into mean gray value by analysing the red, blue and green components of the resulting colour shift and converting the pixels to grayscale using the formula $V(\text{gray}) = 0.299 (R) + 0.587 (G) + 0.114 (B)$.

Testing stain with Takayama reagent

A glass slide is prepared, 1 drop of saline is added. The fabric will be scraped using a sterile blade and the scrapings will be dropped onto the slide (Castro and Doyle, 2013). One drop of the reagent is added and covered with a cover glass. The slide is heated using a bunsen burner for 10 seconds and left to cool. The slide is placed under the microscope to detect and analyse the formation of pink crystals (Veeraghavan and Lukose, 2010, and Vitriani *et al.*, 2015).

Experiment design

Cotton fabric was specifically chosen for this experiment with 3 different periods of drying. The blood sample was collected from a healthy cat and stored in an EDTA coated vacuutainer. The cotton fabric was cut into squares of 5cm x 5cm, and a blood sample was splattered on each piece.

The fabrics splattered with blood were then dried for 1 day, 3 days and 5 days. The blood splatter was then tested using Leucomalachite green and Takayama reagent, and the results were noted afterwards. For the control, red food dye was tested with Leucomalachite green and Takayama reagent.

RESULT

















Experimental group D5 shows the lowest value of brightness for the Leucomalachite green test. The lowest value of brightness concludes treatment group D5 has the highest colour intensity compared to treatment group D1 and D3. Experimental group control shows the highest value of brightness for the Leucomalachite green test. The highest value of brightness was obtained due to Leucomalachite green test having a negative result in the control group. The results show that there is a significant difference ($p < 0.05$) in the mean gray value between control group and treatment group D1, D3 and D5; no significant difference ($p > 0.05$) between treatment group D1 and D3; and a significant difference between treatment group D5 with D1 and D3. The results of the blood splatter identification using Takayama test is shown in Table 4.3 with a grading scale inspired by a research done by Stewart *et al.* (2018). The results of the Takayama test show there is a significant difference ($p < 0.05$) between control group and treatment group D1, group D3 and group D5. However, there is no significant ($p > 0.05$) difference between treatment group D1, D3 and D5.

DISCUSSION

Leucomalachite green test results

Leucomalachite green test was conducted on the fabric sample. The test was applied then 3% hydrogen peroxide was added. Positive results concludes the presence of haemoglobin which is interpreted through the colour shift of

Table 1. Image results of colour shift when tested with leucomalachite green test

Group	I	II	III	IV
C				
D1				
D3				
D5				

Control: splatter made with chilli red food dye; D1: splatter made with cat blood and dried for 1 day; D3: splatter made with cat blood and dried for 3 days; D5: splatter made with cat blood and dried for 5 days.

Table 2. Mean gray value among treatment groups of Leucomalachite green test

Treatment	Label	Gray value	Mean
	Conversion of pixels to grayscale		
C	$0.299R + 0.597G + 0.114B$	741.084	185.271 ^a
D1	$0.299R + 0.597G + 0.114B$	590.082	147.521 ^b
D3	$0.299R + 0.597G + 0.114B$	569.892	142.473 ^b
D5	$0.299R + 0.597G + 0.114B$	454.780	113.695 ^c

















Control: splattered with red food dye and dried for 1 day; D1: splattered with cat blood and dried for 1 day; D3: splattered with cat blood and dried for 3 days; D5: splattered with cat blood and dried for 5 days; Label R: red, Label G: green; Label B: blue; Each treatment group was left to dry then tested with Leucomalachite green; Different alphabetical superscripts (a, b, c) in the same column represents a significant difference ($p < 0.05$).

Table 3. Mean ± SD haemochromogen crystal pattern grade among treatment groups of the Takayama test results

Treatment	Mean ± SD
C	0.00 ± 0.000 ^a
D1	2.25 ± 0.500 ^b
D3	2.25 ± 0.500 ^b
D5	2.50 ± 0.577 ^b

Control: splattered with food dye and dried for 1 day; D1: splattered with cat blood and dried for 1 day; D3: splattered with cat blood and dried for 3 days; D5: splattered with cat blood and dried for 5 days; Different alphabetical superscripts (a, b) in the same column represents a significant difference ($p < 0.05$).

Table 4. Image results of samples tested with Takayama test

Group	Replication			
	V	VI	VII	VIII
C				
D1				
D3				
D5				

C: control; D1: 1 day drying period; D3: 3 days drying period; D5: 5 days drying period.

Table 5. Results of Takayama test by numerical scale

Group	Replication			
	V	VI	VII	VIII
C	0	0	0	0
D1	3	2	2	2
D3	2	2	3	2
D5	2	2	3	3

Grading scale:

0: Negative for appearance of haemochromogen crystals.

1: Positive for haemochromogen crystals (<10 crystals).

2: Positive for >10 haemochromogen crystals, crystals are spread out.

3: Positive for > 10 haemochromogen crystals, crystals are densely packed.

the reagent from colourless to a bluish-green colour. Leucomalachite test results were scored using ImageJ software 1.8.0_112. The RGB plugin of the software calculates the mean gray value within the selection area. The mean is calculated by converting each pixel to grayscale using the formula $v = (\text{red} + \text{green} + \text{blue}) / 3$ or $v = 0.299 (R) + 0.587 (G) + 0.114 (B)$. A higher intensity of colour produces a lower mean gray value and vice versa.

Comparative analysis of each group

Control group was splattered with Koepoe Koepoe brand chilli red food dye to mimic real blood. Due to the negative results, the fabric remains unstained therefore producing a high mean gray value. Group D1, D3 and D5 produced positive results for all the samples. Group D5 produced the highest intensity of colour producing the lowest mean gray value compared to group D1 and D3.

Takayama reagent test results.

Takayama reagent test is done on a slide and the results have to be viewed under a microscope. A slide is prepared

with a drop of saline to allow the blood in the sample scrapings to be evenly distributed on the slide. The fabric sample is scraped and the scrapings are dropped onto the slide. Takayama reagent is added and heat is applied. The results are viewed 24 hours later under a microscope with a magnification of 400x. Formation of pink feather like crystals interpret a positive result. The results are photographed and graded by: the number of crystals, and the pattern formation of crystals (Stewart *et al.* 2018).

Comparative analysis of each group

Control group was splattered with Koepoe Koepoe chilli red food dye. Control group produced negative results for all samples. The results of this test confirms there is no haemoglobin or substances that may produce false positives in the food dye. Group D1- V produced the most dense pattern of crystals (grade 3) compared to D1-VI, D1-VII and D1-VIII (grade 2). Group D3-VII produced the most dense pattern of crystals (grade 3) compared to D3-V, D3-VI and D3-VIII (grade 2). Group D5-VII and D5-VIII produced the most dense

pattern of crystals (grade 3) compared to D5-V and D5-VI (grade 2). Among all the group samples group D5 produced the highest grade. The pattern of formation of crystals vary amongst each sample, some producing densely packed crystals while some produced spread out crystals. The varying pattern formations are due to the varying concentrations of blood present in the scrapings. The amount of scrapings cannot be fixed for each test.

Collection and storage of blood samples

The blood was collected from a Persian cat, which weighed 3.5kg with average health parameters. Blood with a volume of 2.5ml was collected from the cephalic vein and the puncture area was treated properly. The blood was then stored in EDTA coated vacuutainers to be transported from the Veterinary Hospital to the Clinical Pathology Laboratory. Vandewoestyne *et al.* (2015) found that EDTA does not affect the morphology or characteristics of the blood.

CONCLUSION

Leucomalachite green and Takayama reagent are able to detect cat blood splatter on the cotton fabric. Leucomalachite green produced a higher intensity/ darker colour as a result of an older sample, and the lower intensity/ lighter colour as a result of a fresher sample of the Leucomalachite green test. Takayama reagent produced a densely packed pattern of crystals as a result of an older sample, and the loosely packed pattern of crystals as a result of a fresher sample of the Takayama test.

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