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Investigating the Use of SALIgAE® to Identify Expirated Blood on Various Material

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A thesis presented in partial fulfillment of the requirements of the Undergraduate
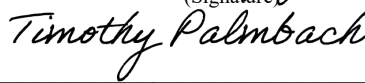Honors Program at the University of New Haven.

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Abstract

Expirated bloodstains and impact bloodstains have very similar physical characteristics. The main way Blood Pattern Analysts identify bloodstains in based on their physical characteristics. This can lead to difficulty distinguishing between expirated blood and impact spatter. Expirated blood can be identified through the presence of air bubbles, however these are often only present on non-porous surfaces. However, if expirated bloodstains can be identified through the presence of saliva, this can provide an alternative way to identify expirated bloodstains. This study examined if SALIgAE® can be accurately used to identify the presence of saliva in blood and thus, be a way to identify expirated bloodstains. Dilutions of saliva to water, saliva to blood, as well as reconstructed expirated stains were created. Expirated stains on white butcher paper as well as five different substrates were produced. The substrates consisted of, carpet, white T-shirt, denim, black T-shirt, and tile. SALIgAE® was visually examined for a color change. SALIgAE® was also analyzed with a UV-VIS spectrophotometer to obtain quantitative data. The results showed that SALIgAE® can identify the presence saliva in dilutions of saliva to blood as well as expirated bloodstains. The spectrophotometer allowed the concentration of salivary α amylase to be determined. This study proves that SALIgAE® can be used to accurately identify expirated bloodstains as well as be analyzed by the spectrophotometer to obtain the concentration of salivary α amylase in the sample.

1 Introduction:

1.1 Blood Patter Analysis

Blood Pattern Analysis (BPA) is a widely used technique in the field of Forensic Science, and has been around since the 1800's. It uses Math, Physics, and Biology, to examine the size, shape, distribution and location of the bloodstains to determine how a pattern was produced [1]. There are three main classifications of bloodstains; passive, transfer, and impact/projection spatter [1]. Passive pattern includes stains that are typically a result of gravity acting on an injury. A transfer pattern is produced when a bloody object comes in contact with a clean object and results in a transfer of blood onto the clean object. Impact/projection patterns occur when blood from an injury is projected through the air resulting in blood spatter droplets. Blood Pattern Analysis looks at the characteristics of the blood to determine the type of pattern that was produced. Once the pattern type is determined, it can then be put into context with other information about the crime scene to aid in reconstruction. Due to BPA being used in reconstruction it is highly important that the analysist identify the correct type of pattern. If the analyst fails to do so, the reconstruction of the events at the scene could be affected. One type of pattern that is commonly seen at crime scenes is impact spatter, specifically high velocity. This type of spatter is typically produced by a gunshot or other high speed objects. The resulting pattern is made up of very fine blood droplets. Impact spatter is typically not difficult to identify. However, other patterns can look very similar to it, making the identification of the pattern more difficult. Expired bloodstains look very similar to impact spatter and often leads to issues properly identifying the spatter, and subsequent scene reconstruction. Expired blood is produced when there is an internal

injury, which causes blood to mix with air from the lungs which is then expelled through the mouth or nose [1]. This results in very fine blood droplets being produced as the blood is exited through the mouth due to the pressure from coughing. The resulting pattern looks very similar to impact spatter. Both result in a collection of fine blood droplets. Expired blood is typically identified through the presence of air bubbles and beaded stains [2]. Air bubbles are produced when the blood mixes with air when being coughed out. Beaded stains occur when saliva or mucus link blood droplets together to create a beaded effect. These indicators of expired blood are readily seen on nonporous surfaces, however, are hard to see and may not be present on porous surfaces. Currently, there are no unique methods to discriminating expired bloodstains from impact spatter on stain shape alone [2]. This makes expired blood hard to identify on items such as clothing when air bubbles are not present. This is due to the air bubbles sinking into the porous fabric and not being visible. One study done to determine the reliability of blood pattern analysis assessments, found that on porous surfaces the error rate was 16% for determining expired bloodstains and analysts were prepared to give an unambiguous classification for expired blood only 24% of the time [3]. Compared to analysts giving an unambiguous classification for cast off patterns 51% of the time. This shows that expired blood is very hard to identify on porous surfaces and can be easily miss identified across the field, leading to an issue within Forensics.
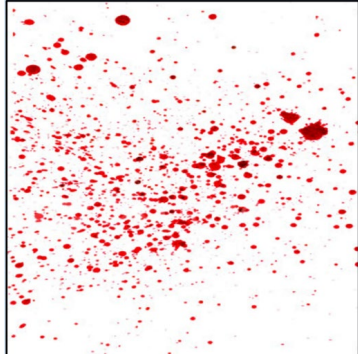
Figure 1. Impact Spatter
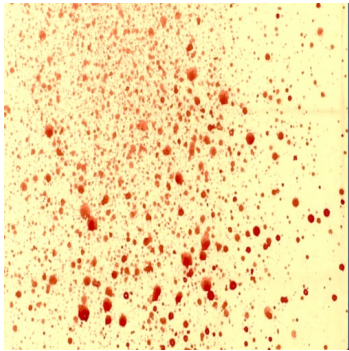
Figure 2. Expirated Blood

1.2   Saliva

This is an issue within the field of Forensics because while blood is commonly found at crime scenes, saliva has been reported as the most common source of DNA in volume crime [4]. The ability to identify expirated stains is important to help determine the events that may have occurred at the crime scene as well as a potential DNA source. Saliva is produced and secreted in the parotid glands, located in the back of the mouth, in front of the ears. Saliva is comprised of mostly water as well as enzymes, electrolytes, and proteins. The main enzyme found in saliva is salivary alpha (α)-amylase. It is an enzyme that hydrolysis α 1-4 glycosidic bonds which breaks down carbohydrates for digestion. Salivary α amylase can also be found in semen, sweat, and breast milk.

However, the concentration of salivary α amylase found in these substances is distinguishably lower than the levels found in saliva. There are also many isoenzymes of salivary α amylase, the most common being pancreatic alpha amylase. This is produced in the human pancreas at levels comparable to salivary α amylase levels. This similarity between the pancreatic and salivary α amylase can lead to issues when determining if a substance is saliva. Other animals salivary α amylase can also interfere in the determination of human saliva, as well as plants that are capable of breaking the glycosidic bonds.

1.2.1 Saliva Identification Methods

This is because the most widely used tests for determining the presence of human saliva look at the presence or activity of salivary α amylase in the substance. In addition to testing a substance for saliva, a possible saliva stain can be identified through Alternate Lighting. An Alternative Lighting Source (ALS) can be used to look at a piece of evidence under different wavelengths and filters to see the fluorescence of different substances. Under certain ultraviolet wavelengths and filters, saliva is known to weakly fluoresce. One study found that the optimal wavelength for the fluorescence of saliva was at 470 nm excitation with a 555 nm interference filter [5]. Using these parameters, saliva weakly fluoresced, have the most concentrated fluorescence at the outer ring of the stain. While using ALS is a way to identify possible saliva stains, other substances such as semen also fluoresce under ALS. The two substances have different fluorescent characteristics, however other tests need to be done to further identify the stain as saliva. The most common tests for saliva in the Forensics field is; Phadebas, Starch Iodine, RSID-saliva, and SALIgAE®. The Phadebas test uses a water insoluble starch covalently

linked to a blue dye. In the presence of salivary α amylase the starch is broken down,

releasing the blue dye into the solution[6]. Phadebas test comes in two testing methods,

one is a tube test, while the other is a paper test. The tube test is a lengthier process,

which requires an incubation period and the need to be centrifuged. The tube test,

however, can be rated on a scale of color change intensity that corresponds to the level of

salivary α amylase activity in the solution. The paper test is a faster test that is comprised

of a sheet of paper preloaded with the reagents. It is then pressed onto the evidence

containing the suspected saliva stain and left for a period of time. If there is any blue

color change on the sheet when it is removed from the evidence, it is indicative of saliva.

Starch Iodine test is done by letting a sample of suspected saliva incubate with a starch

solution and adding iodine to the solution. The addition of iodine creates a purple/blue

color. If salivary α amylase is present in the solution, it will break down the starch,

causing the color to disappear from the solution [6]. Therefore, the purple color is

indicative of no saliva being present, and the disappearance of color indicates saliva is

present. RSID-saliva is an immunochromatographic assay the looks for the presence of

salivary α amylase rather that the activity of it. It does this by utilizing two monoclonal

anti-human salivary α amylase antibodies [7]. In the presence of salivary α amylase, they

will bind to from an antigen-antibody complex at a specific point on the strip that will

then turn red, indicative a positive result. If there is no salivary α amylase the test has

other anti-antibodies that will bind to antibodies further up the strip, turning it red. This

acts as a control, indicative a negative result but ensuing the test worked properly.

SALIgAE® is a vial test where, in the presence of saliva, salivary α amylase will go

through a patented reaction turning the clear solution yellow. When no saliva is present,

the reaction won't occur and the solution will remain clear, indicating a negative result. It has a time limit of 10 minutes for the color change to occur. If any color change occurs after the 10 minutes, it should be considered a negative result. The test can also be performed two ways. One is to take a cutting or section of swab and put directly into the vial. The second way is to take the sample and let it soak in distilled water for a period of time and then take that solution and pipette it into the vial[8].

1.3   Benefits of SALIgAE®

While all of these tests have different limitations, all of them by definition are considered presumptive tests because they all interact with another form of salivary α amylase. Phadebas and starch iodine tests are the older of the four tests. A study was done to compare Phadebas and Starch iodine to SALIgAE® and it found that they were both less sensitive than SALIgAE® [8]. However, all three test had some form of positive reaction when tested with Guinea pig and rat saliva [6]. In addition to Phadebas and Starch iodine tests being less sensitive than SALIgAE®, they both take considerable time. They both require an incubation period and require the addition of reagents. This means that these tests can only be done in a laboratory setting. Whereas, SALIgAE® requires only the vial and the suspected saliva substance and no other reagents, with results ready in 10 minutes. The ease of SALIgAE® enables it to have the ability to be used in the lab and in the field if needed.

When blood was mixed with saliva and used to test all three saliva tests for their ability to detect saliva in expired blood, only SALIgAE® was successful [6]. This is because blood interfered with the ability to determine the color change of the Phadebas and Starch iodine test. SALIgAE® also indicates the presence of saliva through a color

change. The way the test reacts with saliva, also allows the saliva to be diluted until most of the color from blood is gone, if need be. The diluted solution is then added into the vial and allows for the color change to be seen without interference. This makes SALIgAE® a better choice for identification of expired blood over Phadebas and Starch Iodine tests.

One of the more recent tests for the identification of saliva besides SALIgAE® is RSID-saliva. While SALIgAE® is a colorimetric test and RSID-saliva is an immunochromatographic test, they are still comparable. When a study was done comparing the two tests, it found that both tests have a similar sensitivity level. However, it was determined that RSID-saliva is more specific than SALIgAE®. When both were tested against eight different animal saliva, RSID-saliva was positive for only one animal saliva. Whereas, SALIgAE® tested positive for all but one animal saliva [9]. Both were tested against pancreatic amylase and both tested positive. Due to the animal saliva results, RSID-saliva is more specific than SALIgAE®. Both were tested with mixtures of blood and saliva and both found to have no interference from the blood. While RSID-saliva is more specific than SALIgAE® and found to have no interference with blood, SALIgAE® is still preferred to be used. This is because SALIgAE® is a colorimetric test and thus can have qualitative and quantitative data. There is a RSID reader system that can automatically document and determine the results of a RSID-saliva test. This is done by inserting the test strip into the instrument. The instrument then reads the pixel density of the two test strip regions and compares them to a pre-determined calibration data to then give a positive or negative result [10]. However, at this time the reader cannot quantitatively give data on the concentration of salivary α amylase present in the sample.

Due to this, SALIgAE® is the preferred saliva test to use for research in expirated

bloodstains because it can give both qualitative and quantitative data on the presence of

saliva.

1.4   UV-VIS Spectrophotometer

SALIgAE® can give both qualitative and quantitative data when used in

conjunction with a UV-VIS spectrophotometer. A UV-VIS spectrophotometer uses

ultraviolet visible wavelengths to determine the percent transmittance of the wavelengths

through a cuvette with the SALIgAE® solution. The percent transmittance can then be

used to calculate the absorbance of the sample. Using the absorbance, the concentration

of the substance, which is the yellow generated by SALIgAE®, can be calculated [11].

As long as amount of SALIgAE® reagent is larger than the amount of amylase, the

concertation of the yellow product is equal to the concentration of amylase [11]. Using a

UV-VIS spectrophotometer also allows for detection of the presence of yellow color

change in SALIgAE® better than examining it visually. A study determined that the peak

wavelength for SALIgAE® after the addition of saliva is 403 nm. If SALIgAE® is used

with a very diluted or degraded suspected saliva sample, the instrument can be used to

determine if a color change occurred, even if it is not visible to the naked eye. If there is a

color change in the sample, then there should be a corresponding peak at 403 nm. This

can help in identifying a color change when it is very weak and hard to see with the

naked eye. In addition to this, if there are other peaks besides the one corresponding to

salivary α amylase, it can mean that the sample is mixed or does not contain saliva.

When using a UV-VIS spectrophotometer, it can give valuable quantitative data on the

concentration of salivary α amylase in the sample, along with aiding in determining color

change in very diluted or degraded samples. This makes SALIgAE® test in conjunction with a spectrophotometer very valuable in examining expired bloodstains. Using the UV-VIS spectrophotometer can aid in the determination of color change. It can also help with color change determination if there is still some blood color in the sample. For the purposes of this research, the NanoDrop One^c instrument was used. The NanoDrop One^c is a UV-VIS spectrophotometer designed for micro-volume analysis [12]. It is used in this research as opposed to the NanoDrop One because the NanoDrop One^c includes a microvolume sample reader to measure microvolumes, dilute samples, and colorimetric assays at lower detection limits [12]. Due to this it makes it an ideal instrument to use for analyzing SALIgAE® tests.

The overall aim of this study is to qualitatively and quantitatively analyze expired bloodstains for saliva detection using SALIgAE® in conjunction with the NanoDrop One^c. As previously stated, SALIgAE® was chosen to be used in this study due to its ability to be quantitatively analyzed, even though other tests such as RSID-saliva has higher sensitivity and specificity. The NanoDrop One^c was chosen to be used in the research due its low detection limit and microvolume sample reader designed to measure diluted and colorimetric samples. Expired blood was chosen as the research topic due to its difficulty being identified. Specifically, on porous surfaces where air bubbles are typically not present, making it hard to differentiate between impact spatter. It is hoped that SALIgAE® can be used as a way to identify expired bloodstains and help analysts be more definitive when identifying bloodstain patterns. If this can be accomplished, then it can help determine the events of a crime more accurately and will result in a more accurate reconstruction of the crime.

2 Material and Methods:

2.1 Sample collection

Upon approval by the Institutional Review Board, venous blood was collected from the faculty advisor or the research student, with informed consent. Blood collection was performed by a trained phlebotomist. Blood was properly persevered and stored in EDTC vacutainer tubes at 4°c until used. Saliva was also taken from the faculty advisor or the research student. Saliva was taken at least an hour after eating or drinking and collected in sterile falcon tubes.

2.2 Methods

2.2.1 SALIgAE® Saliva Identification Test

SALIgAE® was used to test for the presence of saliva using standard protocol issued by the manufacturer (Abacus Diagnostics) [13]. The protocol was performed by placing the sample into a glass vial and then mixing it gently. The results of the SALIgAE® test were read after 10 minutes. A yellow color change indicates a positive result, while no color change indicates a negative result.

2.2.2 Saliva: Water/ Blood Mixtures*

Saliva and deionized water, as well as saliva and blood, were first mixed together to make a dilution series. For both sets of dilutions the following ratios were used; 1:1, 1:5, 1:10, 1:10,000, and 1:100,000. SALIgAE® was then tested on each of the dilutions according to the manufactures protocols.

2.2.3 Expirated bloodstains on butcher paper*

Expirated stains were then created using standard reconstruction techniques in a laboratory environment. This involved placing 1 mL of the participants own blood in their mouth and coughing it out onto the receiving surface. The receiving surface was white butcher paper placed in front of the participant on both the horizontal and vertical planes. The papers were approximately 12 inches in front of the participant. A total of three expirated bloodstains were created on separate days. After the creation of the stains, a grid was then laid out over the stains. Five sections were then picked from each overall expirated bloodstain to be sampled. A total of 42 individual stains within the overall expirated stains were selected to be tested with SALIgAE®. These individual stains were sampled by paper cutting. Upon the completion of the SALIgAE® tests, each vial was examined for a positive yellow color change.

2.2.4 Expirated Stains on Various Material

Expirated bloodstains were reconstructed using standard techniques as previously explained. These stains were created on both porous and non-porous surfaces. The porous surfaces included; carpet, denim, White T-shirt, and Black T-shirt. The non-porous surface included was ceramic tile. All of the expirated stains were created on the same day. The stains were created in a laboratory environment. The substrates were placed spproximately 12 inches from the participants mouth. The white T-shirt, black T-shirt, carpet, and ceramic tile, were on the vertical plane when the expirated stains were created. The denim was on the horizontal plane when the expirated stain was created.

This required the participant to lean over 12 inches above the denim to create the stain. After the creation of the expired stains, they were photographed and documented for the overall characteristic of the stain. After documentation, 10 stains were collected and tested with SALIgAE® from different parts of the stain. Negative and positive control were also tested to ensure SALIgAE® was working properly. Upon completion of the SALIgAE® tests, each vial was examined for a positive yellow color change.

2.2.4.1   Carpet Sampling

Ten samples from the carpet were taken to be tested with SALIgAE®. Five samples were taken by cutting and five samples were taken by swabbing. The samples were taken from all over the bloodstain. The cuttings were about 1 cm^2 and were directly placed into the SALIgAE® vial. The swabbing's were taken using standard procedure. This included moistening a sterile swab with deionized water. The swab was then rotated around the bloodstain. A scalpel was then used to cut the swab and half of the swab was placed into the SALIgAE® vial.

2.2.4.2   White T-shirt Sampling

Ten samples from the white T-shirt were taken to be tested with SALIgAE®. All of the samples were taken by cutting. The samples were about 1 cm^2 and taken from all over the expired stain. The cuttings were placed directly into the SALIgAE® vial.

2.2.4.3   Denim Sampling

Ten samples from the denim were taken to be tested with SALIgAE®. All of the samples were taken by cutting. The cuttings were about 1 cm^2 and taken from all over the expirated bloodstain. The cuttings were placed directly into the SALIgAE® vial.

2.2.4.4   Black T-shirt sampling

Ten samples from the Black T-shirt were taken to be tested with SALIgAE®. All of the samples were taken by cutting. The cuttings were approximately 1 cm^2 and taken from all over the expirated bloodstain. The cuttings were placed directly into the SALIgAE® vial.

2.2.4.5   Ceramic Tile Sampling

Ten samples from the tile were taken to be tested with SALIgAE®. All of the samples were taken by extraction. This was done by first swabbing the individual bloodstains using standard technique previously exlpained. The whole swab was then placed into a 2 mL microcentrifuge tube containing 200 µl of deionized water. The swabs were then left to sit in the tube for five minutes. A spin basket was then placed into the microcentrifuge tube and the swab was placed into the spin basket. The tubes were then centrifuged in a ThermoFisher mini microcentrifuge, for two minutes. The whole 200 µl solution was then pipetted into the SALIgAE® vial.

2.2.5   Quantitative Analysis Using NanoDrop One^c UV-VIS Spectrophotometer

The NanoDrop One^c is a spectrophotometer that was used to quantitively

analyze the color change of the SALIgAE® tests. The absorbance was used to determine the concentration of salivary α amylase in each sample. This was done by placing 2 µl of SALIgAE® solution onto the lower pedestal and having it analyzed at the wavelength of 403 nm. The absorption of the SALIgAE® solution was used in conjunction with the general equation for the quantitative analysis of SALIgAE®, found on the back of the SALIgAE® manufactures instruction sheet. The visual test was used for qualitative analysis, while the NanoDrop One[c] was used for quantitative analysis.

indicates that the part was performed previously by another research student.

3   Results:

3.1   Saliva: Deionized water/Blood Mixtures

Positive SALIgAE® results were obtained with dilutions of both saliva: water and saliva:blood from 1:1- 1:1,000. Negative SALIgAE® results were obtained with dilutions of saliva:water and saliva:blood from 1:10,000 – 1:1,000,000. The sensitivity of both saliva:water and saliva:blood using SALIgAE® were determined to be 1: 1,000 using visual analysis alone.

The quantitative analysis for salivary amylase within saliva:water dilutions had absorbance values ranging from 1.28-10.00. This correlated to salivary α amylase concentrations ranging from 0.12 µg/mL – 1.33µg/mL. Quantitative analysis of

saliva:blood dilutions had salivary α amylase concentrations ranging from 0.24 -2.03

μg/mL.

**Table 1.** Saliva:blood color change and Salivary α amylase concentration.

| Saliva:Blood and Saliva:Water Dilutions	Visible Color Change	Concentration of Salivary Amylase in Saliva:Blood (μg/mL)
1:1	Yes	2.03
1:10	Yes	1.78
1:100	Yes	1.72
1:1,000	Yes	1.20
1:10,000	No	0.27
1:100,000	No	0.19
1:1,000,000	No	0.24

3.2   Expired Stains on Bucher Paper

From each of the three reconstructed expired stains, 42 individual stains were

tested with SALIgAE® and then analyzed with the NanoDrop One[c]. Stain one had a total

of eight stains test positive for the presence for saliva. Stain two had nine stains test

positive for saliva. Stain three had 23 stains test positive for saliva. The positive stains

were then analyzed with the NanoDrop One[c] and had absorbances range from 0.08- 1.05.

This correlated to salivary alpha amylase concentrations ranging from 0.00- 0.28 μg/mL.

The stains that tested positive for saliva also had visible color change occur with

SALIgAE®.

Table 2. Color change of SALIgAE® and concentration of salivary amylase of sampled expired stains.

	Stain 1	Stain 2	Stain 3
Number of stains tested	42	42	42
Positive Color Change	8	9	23
Salivary Amylase concentration (μg/mL)	0.00-0.09	0.00-0.15	0.00-0.28
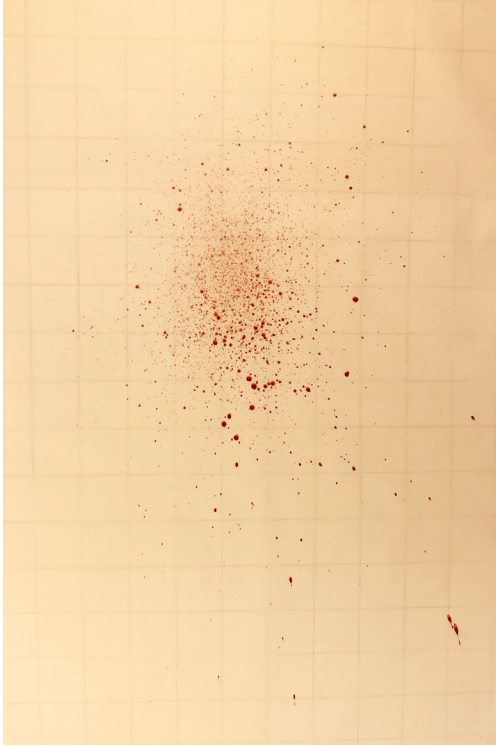
Figure 3. Expirated Bloodstain 1.
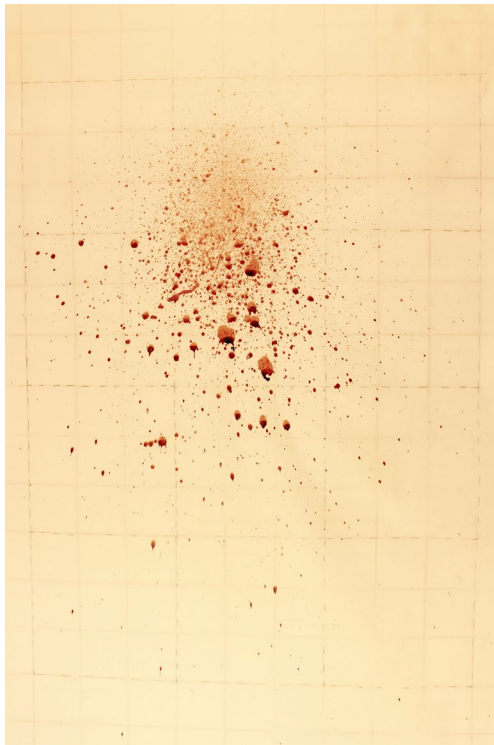
Figure 4. Expirated Bloodstain 2.
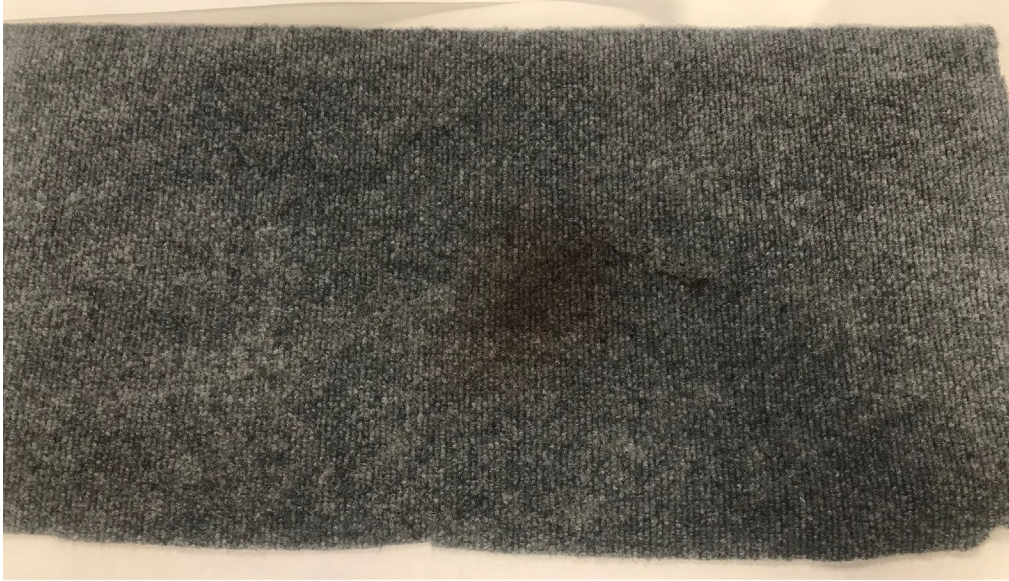
Figure 5.Expirated Bloodstain 3.

3.3   Expirated Stains on Various Material

3.3.1   Substrate A, Carpet

      The reconstructed expirated stain was focused on the middle right of the carpet. The main part of the stain was an oval shape. It consisted of a fine mist of blood droplets. There were individual blood droplets going towards the right of the main stain. Ten individual stains were sampled and were tested with SALIgAE®. All ten of these stains tested positive for the presence of saliva. This was determined by a visible yellow color change of the SALIgAE® within ten minutes. The SALIgAE® tests were then analyzed with the NanoDrop One[c]. The absorbances obtained ranged from 18.51 - 26.87. This correlated to salivary α amylase concentrations ranging from 52.47 – 76.73 µg/mL.

Figure 6. Substrate A, Carpet Expirated BloodStain.
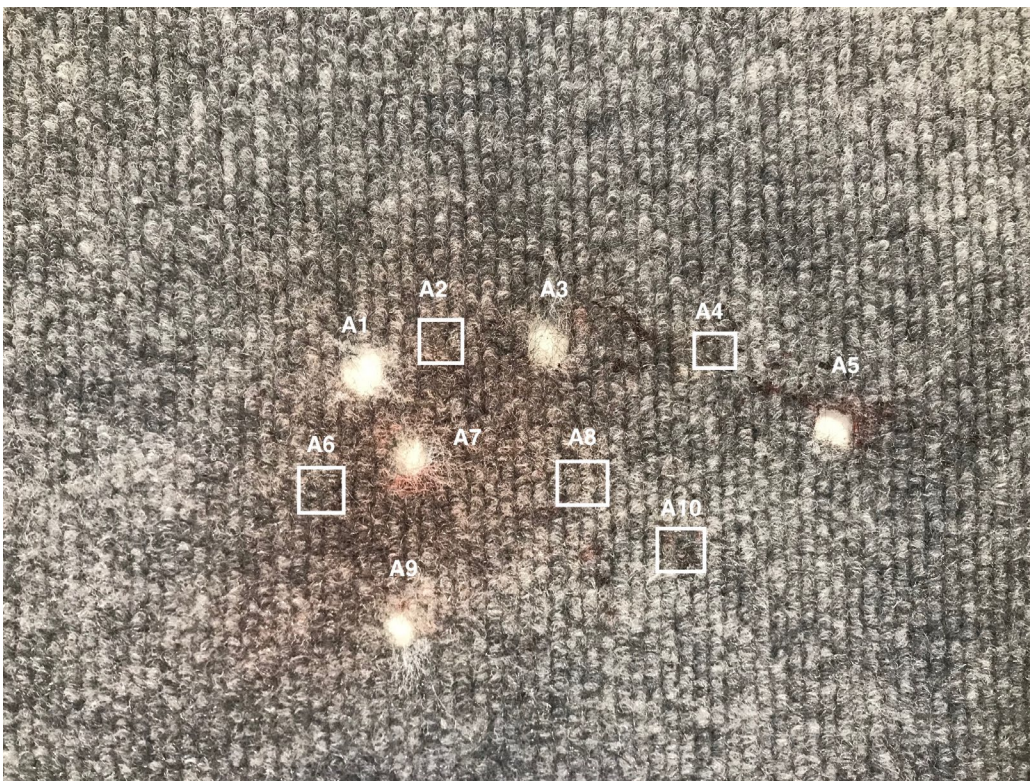
Figure 7. Substrate A, Sampled Bloodstains.

Table 3. Concentration of Amylase and Color Change of SALIgAE® for Substrate A.

SALIgAE® vial	Concentration of Salivary α Amylase (µg/mL)	SALIgAE® Color Change
A1	65.07	Yes
A2	60.23	Yes
A3	76.73	Yes
A4	59.82	Yes
A5	68.52	Yes
A6	56.31	Yes
A7	64.5	Yes
A8	52.47	Yes
A9	62.11	Yes
A10	55.7	Yes

3.3.2 Substrate B, white T-shirt

The main section of the expirated stain was located on the middle upper portion, or chest area, of the T-shirt. There were blood droplets traveling to the left side of the shirt. The stains traveled down the majority of the shirt, towards the bottom. The expirated stain consisted of fine blood droplets. Most of these blood droplets could be seen individually. The bloodstains were about 0.5 cm or less. Ten individual stains were samples and tested with SALIgAE®. All ten of these stains tested positive for the presence of saliva. All of the SALIgAE® vials exhibited a visual yellow color change. The SALIgAE® tests were then analyzed with the NanoDrop One[c]. The absorbances obtained ranged from 4.11 – 22.08. This correlated to salivary α amylase concentrations ranging from 10.73 – 62.84 µg/mL.

Figure 8. Substrate B Expirated Bloodstain.

Figure 9. Substrate B, Sampled Bloodstains.

Table 4. Concentration of Amylase and Color Change of SALIgAE® for Substrate B.

SALIgAE® vial	Concentration of Salivary α Amylase (μg/mL)	SALIgAE® color change
B1	29.9	Yes
B2	58.11	Yes
B3	62.84	Yes
B4	36.19	Yes
B5	60.64	Yes
B6	18.5	Yes
B7	23.58	Yes
B8	43.09	Yes
B9	22.04	Yes
B10	10.73	Yes

3.3.3   Substrate C, Denim

The expirated stain was mostly located on the middle, thigh portion, of the pants. A few stains were located farther up the pants going towards the waist. Some larger blood droplets were located on the inner seam, while smaller blood droplets were located closer to the outer seam. Some beading could also be seen between the larger bloodstains on the left leg of the pants. The size of the blood droplets ranged from 0.5 cm – 3 cm in length and 0.5 cm – 1 cm in width. Ten individual bloodstains were sampled and tested with SALIgAE®. All ten of the stains tested positive for the presence of saliva. All ten of the SALIgAE® vials exhibited a yellow color change. The SALIgAE® tests were then analyzed by the NanoDrop One[c]. The absorbances obtained ranged from 4.95 – 25.06. This correlated to salivary α amylase concentrations from 13.17 – 71.48 µg/mL.

Figure 10. Substrate C, Expirated Bloodstain.

Figure 11. Substrate C, Sampled Bloodstains.

Table 5. Concentration of Amylase and Color Change of SALIgAE® for Substrate C.

SALIgAE® vial	Concentration of Salivary α Amylase (µg/mL)	SALIgAE® color change
C1	13.17	Yes
C2	59.33	Yes
C3	18.73	Yes
C4	40.54	Yes
C5	38.22	Yes
C6	32.13	Yes
C7	19.72	Yes
C8	16.03	Yes
C9	17.72	Yes
C10	71.48	Yes

3.3.4 Substrate D, Black T-Shirt

The expired bloodstain was mainly located on the right upper portion, or chest area, of the shirt. The bloodstains appeared as a fine mist of individual blood droplets. Due to the black color of the shirt, bloodstains appeared red/brown and were more visible when oblique lighting was used. Ten individual stains were sampled and tested with SALIgAE®. All ten of the stains tested positive for the presence of saliva. All the SALIgAE® vials exhibited a yellow color change. The SALIgAE® tests were then analyzed with the NanoDrop One[c]. The absorbances obtained ranged from 16.35 – 23.77. These absorbances correlated to salivary α amylase concentrations ranging from 46.22 – 67.74 µg/mL.

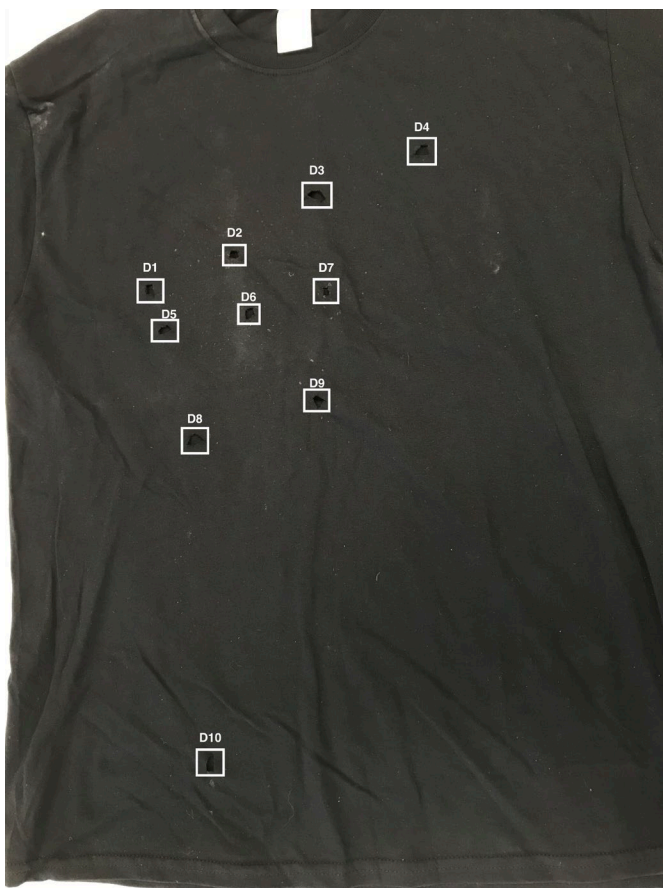*Figure 12. Substrate D, Expirated Bloodstain.*

Figure 13. Substrate D, Sampled Bloodstains.

Table 6. Concentration of Amylase and Color Change of SALIgAE® for Substrate D.

SALIgAE® vial	Concentration of Salivary α Amylase (µg/mL)	SALIgAE® color change
D1	46.22	Yes
D2	63.65	Yes
D3	64.87	Yes
D4	63.19	Yes
D5	50.08	Yes
D6	52.69	Yes
D7	60.17	Yes
D8	58.98	Yes
D9	67.74	Yes
D10	63.88	Yes

3.3.5   Substrate E, Tile

The expirated bloodstain had stains all over the tile. The stain consisted of both a fine mist of individual blood droplets as well as drip stains. The drip stains were

due to the expirated bloodstain being created while the tile was in a vertical position. The drip stains were focused on the middle of the tile. The individual stains were throughout the tile. Air bubbles could be seen in both the drip stains and finer stains. There was a wide range of bloodstains on the tile. The larger stains were about 0.5 cm by 0.75 cm. Smaller bloodstains were approximately 0.1 cm by 0.3 cm or smaller. Ten individual bloodstains were sampled and tested with SALIgAE®. All ten of the tests were positive for the presence of saliva. The SALIgAE® tests were then analyzed with the NanoDrop One[c]. The absorbances obtained ranged from 1.86 – 17.81. This correlated to salivary α amylase concentrations ranging from 5.6 – 67.22 μg/mL.

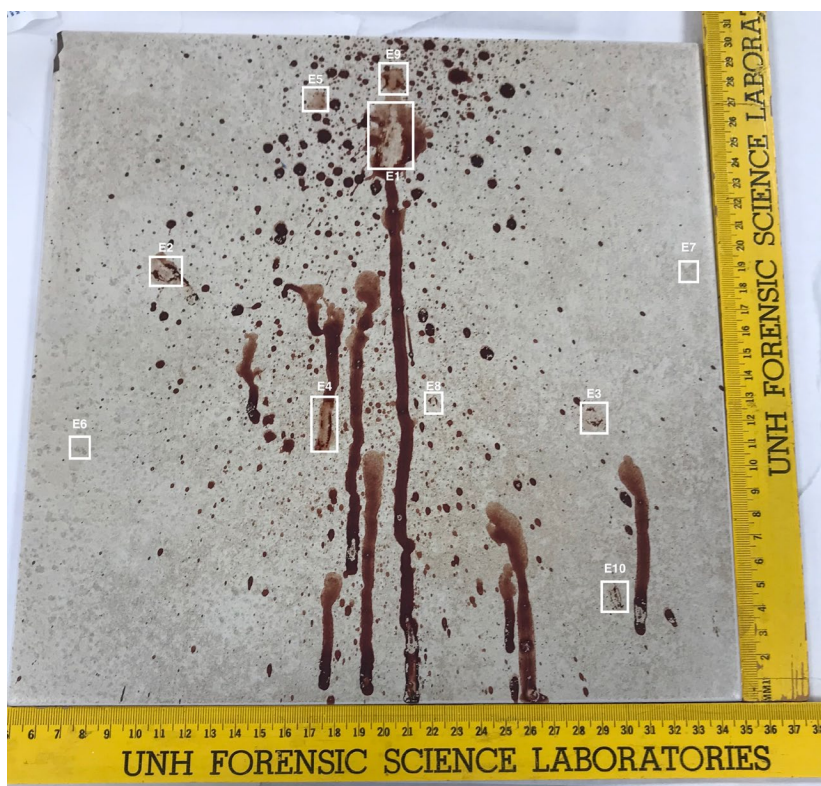*Figure 14. Substrate E Expirated Bloodstain.*

Figure 15. Substrate E, Sampled Bloodstains.

Table 7. Concentration of Amylase and Color Change of SALIgAE® for Substrate E.

SALIgAE® vial	Concentration of Salivary α Amylase (ug/mL)	SALIgAE® color change
E1	65.33	Yes
E2	65.13	Yes
E3	62.13	Yes
E4	67.22	Yes
E5	54.05	Yes
E6	9.04	Yes
E7	5.6	Yes
E8	39.52	Yes
E9	29.01	Yes
E10	38.13	Yes

4   Discussion:

The current main way to identify expired blood is through physical characteristics. This study proposed a new way to identify expired stains through the use of SALIgAE® in conjunction with the NanoDrop One[c]. The sensitivity of SALIgAE® to identify the presence of saliva in ddH_2O and venous blood dilutions was 1:1,000. This sensitivity aligns with other reported sensitivities of SALIgAE®. In the dilution series it was demonstrated that in a controlled dilution, SALIgAE® could detect saliva in the presence of blood. By demonstrating that both saliva dilutions have the same sensitivity, it shows that venous blood did not affect the sensitivity of SALIgAE®. This is important in proving that SALIgAE® can be an accurate way of identifying expired bloodstains. Part 3.2 of the study was to see if SALIgAE® could still be used as an accurate method to identify saliva in the presence of blood in reconstructed expired stains. A total of 42 samples were taken from each expired stain. A number of positive stains were found within each bloodstain. Part 3.3 of the study, took this a step further. On all materials tested, a number of positive stains were found. This provides evidence that SALIgAE® can be used to identify expired bloodstains on both porous and non-porous surfaces. This shows that SALIgAE® can be used at a crime scene regardless of the surface the expired stain is located on. These results show that SALIgAE® is an accurate way of determining if a stain is expired blood by identifying the presence of saliva. This validated the hypothesis posed at the beginning of the study; expired blood stains can be accurately identified with SALIgAE®, regardless of the presence of air bubbles.

However, based on the results, SALIgAE® can only accurately identify saliva if the bloodstain is sampled properly.

SALIgAE® had positive results in all of the reconstructed stains from part 3.2. However, the number of positive results in each stain varied. The first stain had eight positive stains, the second had nine positive results, and the third stain had 23. A total of 42 stains were tested in each stain, showing that there is variability between each stain as well as variability within each stain from different regions of the stain. In part 3.3, ten bloodstains were tested with SALIgAE® from each material. All ten bloodstains from each material tested positive for the presence of saliva. The variability of positive stains both within and among part 3.2 and 3.3 shows that saliva levels vary.

The variability within each stain is due to how expirated blood is created. When blood comes into the mouth from an internal injury it is only in the mouth for a short period of time before it is expelled. During this time is when the blood mixes with saliva. However, primarily only the surface area, or outside portion of the blood will interact with saliva in the mouth. This is because saliva is on the surface of the mouth, i.e. tongue and inner cheeks. When blood comes into your mouth, only the outer surfaces of the blood will interact with the walls of the mouth and pick up saliva. The inner area of the blood will not come into contact with the walls of the mouth. When the blood is then expirated from the mouth the outer surface of the blood with the saliva will land on the closest surface in no discriminative way compared to the inner portion of the blood. This means that the outer surface of the blood with the saliva, will not automatically make up the outer portion of the subsequent stain. Many studies have tried to determine the formation of expirated patterns, studying how the blood leaves the mouth using high

speed videos [2],[14]. However, they were unable to determine how the sequence of blood leaving the mouth forms the stain. This is why it is important to sample many areas in a suspected expirated stain. In part 3.2, on stain one, only eight samples tested positive for saliva, while in stain three, 23 samples tested positive. The levels of salivary α amylase also varied in each expirated stain. In part 3.3, while all samples from each material tested positive for saliva, the levels of salivary α amylase varied. This shows that each expirated stain is unique and picks up different amounts of saliva each time upon its exit from the mouth. Subsequently, each individual bloodstain within the expirated stain will have a different amount of saliva. Thus, in order to accurately determine if a stain is expirated, it is important to take multiple samples from the inner, middle, and outer portion of the stain. This ensures that the scientist will likely sample blood that has come into contact with saliva in the mouth.

The concentration of salivary α amylase was also found to be varied in each sample. For part 3.2, stain one had 0.00-0.09μg/mL, stain two had 0.00-0.15μg/mL, and stain three had 0.00-0.28μg/mL. For part 3.3, substrate A ranged from 52.47 – 76.73 μg/mL. Substrate B ranged from 10.73 – 62.84 μg/mL. Substrate C ranged from 13.17 – 71.48 μg/mL. Substrate D ranged from 46.22 – 67.74 μg/mL. Substrate E ranged from 5.6 – 67.22 μg/mL. This shows that while SALIgAE® can accurately identify the presence of saliva, the concentration of saliva was different in each sample. This is due to the levels of salivary α amylase constantly changing in the body. The levels change throughout the day and can be affected by; time of day, food consumptions, water intake, stress, age of person, and other environmental factors[15] [16] [17]. For part 3.2, each stain was created on separate days so some variation in salivary α amylase concentration is to be

expected. For part 3.3, all the expirated stains were made on the same day, one after the other. However, since the study was done in a lab setting, variable such as food and water intake should be minimal. However, there is a significant difference in the levels of saliva in part 3.2 compared to part 3.3. This is most likely due to the expirated bloodstains being created by different people. There is great variability between levels of saliva in different people thus, it is not unexpected that the concentrations of salivary α amylase determined was significantly different in part 3.2 and 3.3. The amount of time the blood was allowed to sit in the mouth before being expirated may also account for the different concentrations of salivary α amylase between part 3.2 and 3.3.

The difference in salivary α amylase concentration is important because the concentration of salivary amylase is directly related to the severity of the color change of SALIgAE®. In some of the samples taken, the color change of SALIgAE® could not readily be seen or was obscured by the color of blood. This is why the use of the NanoDrop one^c can be beneficial to use in conjunction of SALIgAE®. When the color change of SALIgAE® is hard to see visually, the use of the NanoDrop One^c to confirm the presence and to determine the concentration of salivary α amylase in the sample can be very helpful. This study proves that the NanoDrop One^c can be used to obtain quantitative data of SALIgAE®. This allows the scientist to get quantitative data to go along with the qualitative color change, which aids in the determination in whether a stain is expirated blood or not.

The results of this study shows that SALIgAE® can accurately identify expirated blood through the presence of saliva. In addition, the NanoDrop One^c can be used on the SALIgAE® tests to determine the concentration of salivary α amylase. This technique is

particularly beneficial to suspected expired bloodstains on non-porous surfaces such as clothing, where air bubbles or beaded stains are usually not present. However in order to obtain accurate results, samples need to be taken from different areas of the suspected bloodstain.

5   Conclusion:

Overall, the use of SALIgAE® in conjunction with the NanoDrop One^c to identify expirated stains can be very beneficial to BPA and the field of forensics in general. This will cause less confusion between expirated and impact spatter stains, resulting in a more accurate reconstruction of events. By using SALIgAE® to identify stains, which is already widely used in the field, there is no need to verify that SALIgAE® itself works, only that it works with samples mixed with blood, which this study has proven. In addition, SALIgAE® has no effect on STR typing[8]. This is beneficial because if there is only the suspected bloodstain as a means of obtaining DNA, SALIgAE® can still be used. SALIgAE® can be used to determine if the stain is expired blood and the same sample can then be used to obtain DNA. Thus, the most information can be obtained and can lead to giving more insight to aid in reconstruction.

However, more studies need to be done to determine the limitations of this technique. This study did not look into how the age of the expired bloodstains affects the ability of SALIgAE® to identify the presence of saliva. Due to this, the technique using SALIgAE® should only be used to identify bloodstains at new crime scenes and not in cold cases. Regardless, the results of this study prove SALIgAE® is an accurate way to

identify expired bloodstains. SALIgAE® is user friendly and based on the results of the study, can begin to be used in the field in the immediate future to determine whether a suspected stain is expirated blood.

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