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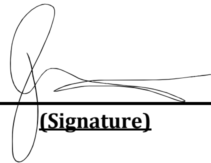
University of New Haven
Honors Program

2019-2020 Honors Thesis

Evaluation of DNA from Blood and Saliva Overtime

Janine Smalling

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



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12/10/2019

Evaluation of DNA from Blood and Saliva Overtime

Dr. Claire Glynn
Janine Smalling

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Abstract:

In this research, we will examine the quantity of DNA over time. In John M. Butlers, "Fundamentals of Forensic DNA Typing", he charts out the average amount of DNA extracted from different bodily fluid samples. Examples include blood, semen, saliva, urine, etc. Though valuable, the values presented are in the cases of a fresh extraction of the sample. It is important to note that in most cases when DNA is being extracted from crime scene evidence, the samples are not fresh and have been degraded to some extent. On a crime scene, time is only one of the factors affecting the degradation of the samples; temperature, pH, and quantity of the sample are other factors that affect the quantity and quality of the DNA extracted. The purpose of the research is to evaluate how time alone can affect the amount of DNA received from samples of bodily fluid, specifically blood and saliva. Understanding the relationship between time and the quantity of DNA present can give investigators knowledge of how long a sample has been deposited at a crime scene. Results showed that though there was not a steady decline in the amount of DNA yielded from fresh samples to 4-month samples, the study shows that quantifiable amounts of DNA can still be recovered despite being under unfavorable conditions. Time is a factor of the quality of DNA present in a sample, but there is not an absolute relationship between time and DNA yield according to this study.

Introduction:

When investigators arrive at a crime scene, if there are any fluids present, crime scene technicians collect the evidence and take it to the crime lab to analyze if it is a bodily fluid or not and whether DNA can be isolated from the samples. Sometimes, there is no telling how long the fluids have been there encountering environmental conditions that are not favorable for the preservation of DNA. Therefore, the timeline of when bodily fluids are deposited onto a scene vs. collection by crime scene technicians is important to understand to estimate if DNA from a crime scene can be retrieved and how long that DNA has been there. From the collection, technicians have to be careful about how they handle the evidence to ensure it is still viable for DNA isolation. When collecting evidence that may contain DNA, it must be dried completely before it can be stored in a breathable paper bag. Without proper collection procedures, DNA could degrade further in transit to the crime lab. This experiment focuses on how DNA in bodily fluids has degraded on its own, not from crime scene collection.

This study was designed to determine the relationship between time and quantified DNA of bodily fluids under controlled conditions. The results focus on the quantity not the quality of DNA. Studies like this can help future investigators understand how long a sample has been on a scene and to be able to create a timeline to help solve cases. Also, it emphasizes how important it is to collect possible DNA evidence correctly because if it has already degraded on its own, we do not want any further degradation during the collection process which could negatively affect the quality of and quantity of DNA in processes down the line.

Literature Review:

In John M. Butler's book, "Advanced Topics In DNA Typing: Methodology", he explains how DNA can be degraded through enzymatic and/or chemical processes. The DNA can undergo cellular nucleases as well as bacteria, fungus, and insects when an organism dies. The quality of DNA can also be affected by ultraviolet irradiation, heat, oxygen which can cleave DNA molecules. (Butler 2012). It is understood how temperature and other environmental conditions can affect DNA, but very little on time alone. However, there was research done by palaeogeneticists on the DNA in the leg bones of extinct birds. They determined that the half-life of DNA was 521 years; meaning that half the bonds between the nucleotides in the backbone of DNA were broken. The bones were about 600-8000 years old and kept at a temperature of 13.1 degrees Celsius (Kaplan 2012). Though they were able to obtain DNA from these samples, this DNA was collected from the bones of animals; there remains the question of how time affects DNA from liquid samples such as blood and saliva from humans. In a study conducted by Laura Johnson and James Ferris, they used single-cell gel electrophoresis to evaluate nuclear DNA fragmentation to evaluate postmortem cell death processes. Their goal was to see if they could improve the methods of determining the postmortem interval in homicide cases. They evaluated the degradation in DNA using the single-cell gel electrophoresis and determined that there was a positive correlation between DNA fragmentation and an increased post mortem interval. Therefore, the degradation of DNA present in postmortem samples can be used and analyzed as one of the methods to determine the time of death (Johnson 2002). This research is important because determining the time of death is essential in a forensic science investigation. It gives investigators a time as to when the crime must've taken place. Post mortem interval has an 8 hour

window time estimate and having that extra step of looking at the degradation of DNA could specify that estimate.

The effect of storage temperature on DNA has been previously studied. In one study, scientists extracted DNA from the animal tissues of albino mice to determine what the best storage temperature would be to get this highest DNA yield. The tissue samples were stored for one week at different temperatures of -20°C, 4°C, 25°C, and 40°C. Using the Chelex method of extraction, they found that the quantity of DNA extracted from the liver samples was greatest when stored under -20°C (Al-Griw et. al 2017). This is not surprising as the optimal temperature for storing DNA samples is from -4 degrees to about -20°C even up to -80°C. This study is important because the storage of samples and DNA is imperative to the quantity and quality of DNA retrieved from the sample which would affect further DNA qualification. In this study, the temperature will be kept constant in a temperature-controlled environment to focus on the effects of time on the degradation of DNA. In another similar study, bloodstains were stored at room temperature, 4 °C, −20 °C, and −80 °C for 20 years. Researchers also found that the DNA from bloodstains stored at 4 °C or room temperature were severely degraded compared to the other samples. The research also offers that blood should be stored as bloodstains and not samples because blood stains are better for the detection of blood-specific mRNAs (Hara et. al. 2016). I expect to see similar results in my study as we will be holding the samples at room temperature. I also wonder how it would affect the experiment when these samples are stored as pure samples and not as bloodstains. The DNA will have no substrate to cling to and it might affect the further degradation of the samples.

The purpose of the extraction process of DNA is to rid the sample of any inhibitors that would prevent DNA from being analyzed in any downstream processes. There are four well-

known methods of extraction that are each used depending on the sample type and which method would give the best yield of DNA. In one study, scientists aimed to discover what DNA extraction method was best to tackle the problems samples can contain. They subjected samples to different environmental conditions such as indoors, buried in different types of soils; also, stains were put on denim, cotton, and lycra. Indigo dyes in denim are known inhibitors of the PCR process that follow extraction and quantification. Small samples were collected after 1, 3, and 7 days. Extraction methods used were Chelex 100, QIAamp DNA Instigator Kit, DNA IQ System Kit, and Forensic DNA Extraction System. The samples that were extracted with Chlex and the DNA IQ System Kit had lower quantities of DNA than those extracted with the QIAamp DNA Instigator Kit and Forensic DNA extraction System (Bogas et al. 2011). The amount of DNA present is important to understand so that the correct amount of DNA in the next step, PCR, can be used to determine an accurate profile. The timeline for this experiment was short, comparing samples between 3 and 4 days, while my experiment will have larger and more timestamps to discover the effect of time. In our experiment, we will be using the DNA Investigator Kit.

As mentioned before, the timeline for DNA really depends on the conditions it is in. In a previous study mentioned, scientists were able to extract DNA from the bones of extinct birds. Similarly, researchers were able to sequence DNA fragments from 7,000-year-old human skeletons. These skeletons were recovered in the Cantabrian mountain range near Spain which is at about 1500 meters altitude. As a result of the cold winters with very low temperatures, the DNA in the bones was able to be preserved (Gonzalez 2012). The temperature of the environment allowed enough of the DNA to be preserved so that the researchers were able to analyze the genome of the skeletons. Even though an extensive amount of time has passed, the

temperature of the environment was very helpful in preserving the DNA. Most times in criminal cases, the temperature cannot be "set" for the preservation of DNA. Therefore, the more time has passed, the more likely the DNA will fragment and degrade if the environmental conditions are not ideal.

Preliminary Hypothesis:

If we deposit samples of blood and saliva onto glass plates and store it at room temperature with no sunlight, the more time that has passed, the less DNA will be present in the sample. Since these samples are not being stored under favorable conditions, the DNA will degrade and therefore affect the quantity of DNA retrieved from the samples the more time passes. The expected chart should be a negative slope showing that as more time passes the less DNA yielded.

Materials and Methods:

Blood and Saliva Collection

Following signed informed consent forms and approval from the Institutional Review Board (IRB) at the University of New Haven, venous blood and saliva were collected from volunteers. Blood donations were collected and stored inside sterile vacutainer EDTA vials stored in a refrigerator at 4 ºC.

Sample Prep

For this experiment, seven-time points were used to show the degradation of DNA over time. Extractions will be performed when the samples are fresh and after 48 hours, 2 weeks, 1 month, 2 months, 3 months, and 4 months. To prep, 200 microliters of sterile blood and saliva, in

triplicate, were places onto glass microscope slides with date deposited and future extraction date labeled onto glass (Figure 1). Blood and saliva samples were put onto separate glass slides. Slides were put stored in a dark cabinet in a room with a controlled temperature, around 72 ° F until it was time for samples to be extracted. The timeline for sample prep and extraction is shown in table 1.

Figure 1: 2 month and 3-month blood and saliva samples

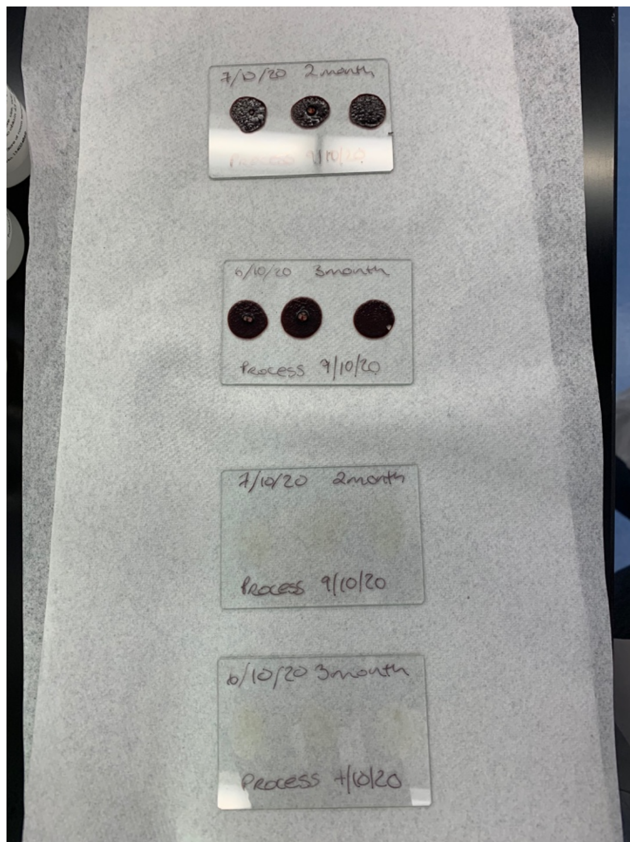


Table 1: Sample Prep and Extraction Dates

Sample	***Sample Deposit***	***Sample Extraction***
Fresh	September 25, 2020	September 25, 2020
48 hour	September 29, 2020	October 1, 2020
2 week	September 11, 2020	September 25, 2020
1 month	September 11, 2020	October 12, 2020
2 month	July 10, 2020	September 11, 2020
3 month	June 10, 2020	September 11, 2020
4 month	June 10, 2020	October 12, 2020

- Extraction dates are not exact due to limited student access to DNA lab

Extraction

To perform the extraction of samples, the DNA Investigator Kit will be used. Samples will be extracted following the procedure for the Investigator Kit can be found in the QIAamp DNA Investigator Handbook. All reagents needed are included inside the Kit. Before each extraction, the lab station and all tools were sterilized with 70% reagent ethanol. The station was cleaned when switching between blood and saliva samples. Microcentrifuge tubes were labeled 1-12 for each round of extraction.

For each sample, a fresh cotton swab was soaked in water and used to wipe up the blood/saliva samples. The cotton is cut off with a sharp sterilized razor blade and put into 1.5 mL Eppendorf tubes. Once all samples were in tubes, 400 uL of water, 20 uL of proteinase k, 400 uL of buffer AL was added, and using a pipet tip, the cotton swab was mashed to ensure that the

cotton swab was exposed to the solution. Each tube was mixed by pulse-vortexing for ten seconds and then incubated at 56 degrees Celsius for 10 minutes. After incubation, a spin basket was used to drain all of the liquid out of the cotton swab and separate the liquid. This was done by carefully using sterile forceps to take the cotton swab out of the tube and placing it into the spin basket. The basket was then put back into the tube and then the tube was closed. All tubes were then centrifuged at full speed for 1 minute to drain the liquid out of the cotton and into the tube. After ensuring all of the solutions were out of the cotton swab, the spin basket can be discarded into biohazard waste. After adding 400 uL of ethanol, a Qiagen spin column with a clean catch tube was used to filter all of the solutions through 700 uL at a time. Once all of the liquid has passed through the column after centrifuging at 8,000 rpm, the flow-through was discarded. Finally, after going through two rounds of wash buffers and centrifuging, using buffer AE, the DNA was extracted after being incubated again at 56° C in the thermomixer. The full detailed procedure can be found in the QIAamp DNA Investigator Handbook. After extraction, all samples were stored in a freezer at about -20° C to preserve samples until the quantification step.

Nanodrop

To quantify the DNA in the samples, we used the Nanodrop OneC UV-Vis Spectrophotometer. Data pulled from this instrument tells us how much total DNA is in our samples. This includes human DNA and non-human specific DNA like bacteria. First, our samples were taken out from the freezer and allowed to thaw out. On the monitor, the dsDNA (double-stranded DNA) function is selected for measurement. Before samples were measured, the instrument was calibrated with a blank measurement using sterile H2O. Each sample was

vortexed and then 1.1ul was pipetted out and put onto the pedestal of the Nanodrop. After a few seconds, the monitor displayed 3 values; ng/uL, A260/A280, and A260/A230 ratios. Values for each sample can be seen in Table 2. The average of all of the samples done in triplicate can be seen in Table 3.

Human Quantification

The human DNA from all samples was quantified using the Quantifier Human Kit and Quantstudio 5 instrument. First known DNA concentration (ng/uL) standards were prepared using standard dilution series. These standards are created with the Quantifiler Human DNA Standard $T_{10}E_{0.1}$ buffer which includes 10mM Tris-HCl, 0.1 mM Na_2EDTA, and the optional 20 ug/mL glycogen. Next, to prepare the reactions, the Quantifiler Human Primer mix was thawed, vortexed, and centrifuged. Next, the Quantifiler PCR Reaction mix was swirled, and then the required volume was added to a polypropylene tube. The required volume of the Human primer mix was also added to the same tube and then the tube was vortexed and then centrifuges briefly. To the 96-well reaction plate, 23 uL of this PCR mix was added to each well. Finally, to finish the reaction solutions, 2 uL of samples, standards, or controls were added to the appropriate wells and the reaction plate was sealed with the optical adhesive cover. The entire plate was centrifuged at 3000 rpm for 20 seconds in a tabletop centrifuge to remove any bubbles. The plate was then positioned in the instrument so that the A1 well is in the upper-left-hand corner and then the plate was run by the Quantstudio 5. Results from the instrument are displayed in Table 4.

Results:

DNA was extracted from 42 samples of blood and saliva using the QIAamp DNA Investigator Kit following the instructions from the handbook. The DNA extracted was then quantified for total DNA using the Nanodrop One UV-Vis Spectrophotometer and for human-specific DNA using the Quantifier Human Kit, Quantstudio 5. Each timeslot was extracted in triplicates and the measurements were averaged together.

Table 2: All sample data from Nanodrop One

Sample Type	ng/uL	A260/A280	A260/A230
4monthbloodA	8.5	4.05	0.05
4monthbloodB	9.0	4.06	0.05
4monthbloodC	9.0	4.28	0.06
4monthsalivaA	14.3	2.87	0.09
4monthsalivaB	17.1	2.76	0.10
4monthsalivaC	16.7	2.66	0.10
3monthbloodA	9.2	5.02	0.06
3monthbloodB	10.4	4.24	0.07
3monthbloodC	9.5	4.14	0.06
3monthsalivaA	16.3	2.46	0.10
3monthsalivaB	13.2	2.74	0.08
3monthsalivaC	17.1	2.60	0.10
2monthbloodA	10.6	2.61	0.07
2monthbloodB	11.9	4.43	0.06
2monthbloodC	10.4	3.79	0.07
2monthsalivaA	12.5	2.96	0.08

2monthsalivaB	13.2	2.76	0.08
2monthsalivaC	17.6	2.54	0.11
1monthbloodA	8.8	3.29	0.06
1monthbloodB	9.7	3.43	0.06
1monthbloodC	9.9	3.22	0.06
1monthsalivaA	28.0	2.37	0.17
1monthsalivaB	45.0	2.20	0.25
1monthsalivaC	34.5	2.23	0.20
2weekbloodA	9.6	3.23	0.06
2weekbloodB	9.8	3.59	0.06
2weekbloodC	11.3	2.59	0.07
2weeksalivaA	17.7	2.75	0.11
2weeksalivaB	16.3	3.03	0.10
2weeksalivaC	13.3	3.39	0.08
48hrbloodA	3.8	-0.59	0.02
48hrbloodB	6.0	-2.67	0.04
48hrbloodC	8.2	-5.42	0.05
48hrsalivaA	12.9	3.39	0.08
48hrsalivaB	11.6	3.47	0.07
48hrsalivaC	10.0	3.79	0.06
freshbloodA	11.1	3.79	0.07
freshbloodB	8.5	4.28	0.05
freshbloodC	10.8	3.65	0.07

freshsalivaA	16.8	2.37	0.10
freshsalivaB	13.5	3.27	0.08
freshsalivaC	16.8	2.72	0.10

Table 3: Average sample data from Nanodrop

Sample Type	***ng/uL***	***A260/A280***	***A260/A230***
4monthblood	8.8	4.13	0.05
4monthsaliva	16.0	2.76	0.10
3monthblood	9.7	4.47	0.06
3monthsaliva	15.5	2.60	0.09
2monthblood	11.0	3.61	0.07
2monthsaliva	14.3	2.73	0.09
1monthblood	9.5	3.31	0.06
1monthsaliva	35.8	2.27	0.21
2weekblood	10.2	3.14	0.06
2weeksaliva	15.8	3.06	0.10
48hrblood	6.0	-2.89	0.04
48hrsaliva	11.5	3.64	0.07
Freshblood	10.1	3.91	0.06
Freshsaliva	15.7	2.79	0.09

Figure 2: Nanodrop Quantified DNA

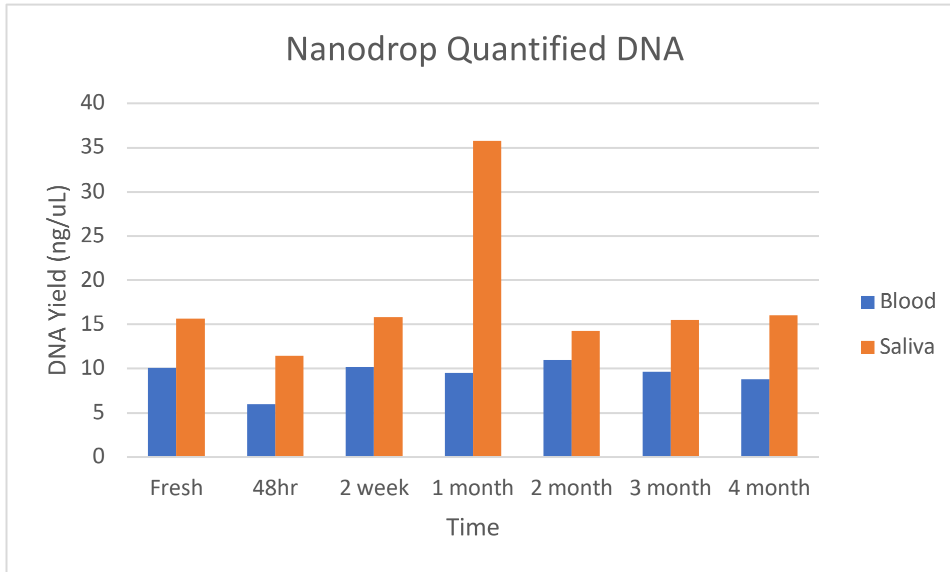


Table 4: Human Quantified DNA (ng/uL) for Standards and Samples

Sample Type	Ng/uL
Standard 1	50
Standard 2	16.7
Standard 3	5.56
Standard 4	1.85
Standard 5	0.62
Standard 6	0.21
Standard 7	0.068
Standard 8	0.023
4monthbloodA	2.944409
4monthbloodB	0.796566

4monthbloodC	1.73007
4monthsalivaA	2.974064
4monthsalivaB	0.610716
4monthsalivaC	3.218217
3monthbloodA	2.212715
3monthbloodB	2.678626
3monthbloodC	2.698258
3monthsalivaA	2.141042
3monthsalivaB	1.212839
3monthsalivaC	1.984146
2monthbloodA	2.871573
2monthbloodB	2.531519
2monthbloodC	2.902149
2monthsalivaA	1.237397
2monthsalivaB	1.06618
2monthsalivaC	1.608513
1monthbloodA	1.639173
1monthbloodB	2.746883
1monthbloodC	1.339745
1monthsalivaA	8.780309
1monthsalivaB	2.660499
1monthsalivaC	2.450003
2weekbloodA	0.867724

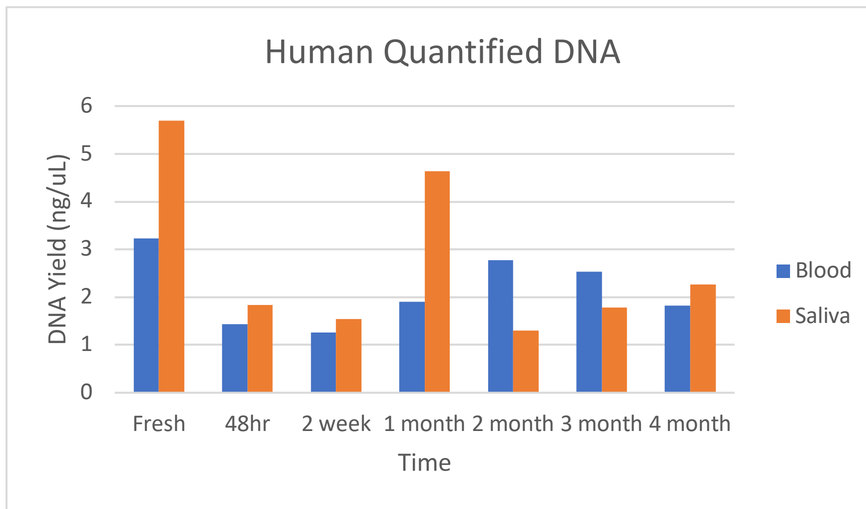
2weekbloodB	1.380661
2weekbloodC	1.538515
2weeksalivaA	1.594263
2weeksalivaB	1.534687
2weeksalivaC	1.494033
48hrbloodA	1.506039
48hrbloodB	0.499715
48hrbloodC	2.306225
48hrsalivaA	2.316119
48hrsalivaB	1.877956
48hrsalivaC	1.339494
freshbloodA	4.31383
freshbloodB	2.507519
freshbloodC	2.869704
freshsalivaA	3.127157
freshsalivaB	3.34571
freshsalivaC	10.59661

Table 5: Average DNA Yield for Samples (ng/uL)

Sample Type	***ng/uL***
4monthblood	1.82
4monthsaliva	2.27
3monthblood	2.53
3monthsaliva	1.78
2monthblood	2.77
2monthsaliva	1.30
1monthblood	1.91
1monthsaliva	4.63
2weekblood	1.26
2weeksaliva	1.54
48hrblood	1.44
48hrsaliva	1.84
Freshblood	3.23
Freshsaliva	5.69

*rounded to hundredths place

Figure 3: Human Quantified DNA



Overall, the amount of DNA retrieved from the Nanodrop was inconsistent with expected results. For example, 4-month samples had more DNA than the 48-hour samples. Also, the DNA yielded from the Nanodrop shows that saliva overall had the most DNA retrieved for each time point. DNA yielded from the Quantifier is also shows that saliva had more DNA present than the blood in most of the time points which can be seen in figure 3. However, human DNA yielded from the Quantifier Human Kit still does not represent the expected results from our hypothesis. The DNA yielded does not show a correlation to time, however, data proves that DNA can be extracted and quantified after long periods of time.

Discussion

Compared to the original hypothesis, the results from the experiment were not expected. Not only is DNA difficult to isolate, but it becomes even more difficult if it isn't stored under the correct conditions. In this experiment, we took away ample temperature and time by having samples sit in a dark cabinet at room temperature. The results from the Nanodrop One

instrument, which quantifies all DNA in the sample, shows a mix of quantities that do not show a trend. Samples that were expected to have a low DNA yield, had a higher yield and vice versa. This however is most likely due to the other DNA present in the sample such as bacterial DNA. Along with the DNA yield in ng/uL from the Nanodrop data, are the A260/A280 and A260/A230 ratios. These ratios represent the purity of the double-stranded DNA. A value of about 1.8 for the A260/A280 ratio and 2.0 for the A260/A230 is pure for DNA. If values are significantly lower, then it would indicate that there are contaminants present in the sample that absorb at either 280 or 230 nanometers. The A260/A280 ratios in this study are all larger than 1.8 except for the 48-hour blood samples (Table 3). Therefore, no contaminants were absorbed at 280 nanometers. However, all of the A260/230 ratios are significantly lower than the favorable 2.0 value which indicates contaminants being detected at 230 nanometers. The 48-hour blood samples had contaminants detected at 280 nanometers level as well as the lowest values at 230 nanometers showing contamination. This proves that the samples most likely had other kinds of DNA present which is why the DNA yield from the Nanodrop was high and inconsistent with expected values.

Regarding the human DNA that was quantified from the Quantifier Human Kit, there still was not the expected negative correlation from the amount of human DNA recovered from fresh samples to the samples taken after 4 months. However, there is a significant difference in the amount of human DNA recovered from the 4-month samples directly compared to the fresh samples which are expected. The 1-month saliva sample in both the Nanodrop and Quantifier DNA yield data is larger than expected and inconsistent with the rest of the data. This is due to one of the replicate saliva samples having a large DNA yield which drove the average higher than the other two samples' values.

Limitations

Certain limitations were present which could have led to the results present. First, all samples were not prepared with the same saliva samples. The 4-month, 3 month, and 2-month samples were prepared by the principal investigator, Dr. Claire Glynn with her own saliva while the rest of the time point samples were prepared by the co-principle investigator, Janine Smalling using her own saliva samples. Second, when extracting the DNA samples, the microscope slides that the samples were stored on were never wiped clean especially on the blood samples. A max of 3 cotton swabs was used to wipe up the blood on the plate, but there was still diluted blood and water left on the plate. Also, during extractions, due to the blood being dry and flaky, some flakes would not break apart and fly onto the bench not being picked up by the wet cotton swab. Blood ended up either stuck on the stick of the cotton swab, the plate, or even the razor blade used to separate the cotton from the stick. Lastly, extraction dates were not exact to the time that they were deposited. This is due to the limitations of the co-principle investigator's access to the DNA lab over the weekends.

The novel virus COVID-19, had a major impact on our study. The original time points to be used were 6 month, 4 months, 2 months, 4 weeks, 2 weeks, 48hr and fresh. However, because we had to move out mid semester in March, we were unable to get samples seeded. In addition, it also limited the number of samples we worked with. In this study, we only focused on two bodily fluids, blood and saliva, where there are more we could have included. Lastly, this study is limited because it only focuses on one type of environment; cool and dry. If we had more time and access to the lab, we could have worked with different kinds of environments to expand the research.

Conclusion

Overall, though the results from the experiment were not as clear as expected, the fact that there were results is the overall message from this experiment. The idea for this experiment came from the idea that in the field, investigators and scientists struggle when collecting DNA and understanding when evidence was planted and how much time has passed since then. Therefore, this experiment was designed to give a glimpse into how time alone can affect how much DNA is present in the sample. In the future, an experiment based on how DNA can be affected by environmental factors can also be included to give a realistic comparison to the conditions of DNA in crime scenes. In addition, going the extra step of retrieving the DNA profile, from the samples could give information on how the quality of DNA is affected.

The hypothesis for this experiment came from the knowledge that if DNA is not stored correctly, it is susceptible to degradation. Therefore, as time goes on without the correct storage conditions, DNA should degrade which should have been present in this experiment. However, even though it was not a steady decline, being able to quantify any human DNA at all from the samples is a testament to how resilient DNA really is and how far science has come. DNA plays a major role in solving many cases and it is usually the deciding factor in court. The underlying significance of this experiment is that we were able to isolate and quantify enough DNA to get a DNA profile which is all that matters when it comes to forensic science casework.

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